DOCTOR OF PHILOSOPHY

Albumin in tears

Gunilla Runstrom

2013

Aston University

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ALBUMIN IN TEARS

Gunilla Kristin Runström

Doctor of Philosophy

ASTON UNIVERSITY

April 2013

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Albumin in Tears

Gunilla Kristin Runström, Doctor in Philosophy 2013

Albumin is not endogenous to the tear film and is present as a product of plasma leakage. It is used as a diagnostic marker of ocular insult and inflammation. Tear albumin is, however, poorly understood, with large variations in reported concentrations between studies. There is also no authoritative information on whether its presence in tears is responsive or part of an adaptive reaction.

The presented research aimed to resolve the disparities in published tear albumin concentrations and investigate the role of albumin in the tear film. Collation and evaluation of the available literature identified collection method, stimulus, assay technique, and disease state as factors able to influence quoted tear albumin to different extents. Difference in sampling technique exhibited the largest variations in mean tear albumin concentrations. Review of the literature also highlighted that little systematic investigations of the daily cycle of tear albumin levels, and subject-to-subject-variation, had been carried out. In order to remedy this shortcoming, variations in tear albumin concentration were investigated in 13 subjects throughout the waking day. Results identified a time period where albumin levels are relatively stable (2-6 hours post-waking). This was designated a suitable baseline for the determinations of tear albumin concentrations and subject-to-subject comparisons. Significantly, a previously unrecognised progressive increase in albumin concentration during the latter part of the day was also identified in the population. This increase suggests that albumin may play a more active and dynamic role in the ocular environment than is commonly perceived. To facilitate the collection of additional tear albumin data, tear sampling and point-of-care analysis in contact lens clinics were investigated. Two instruments were evaluated and were found to be suitable for the analysis of tear albumin in commercial institutions.

Collectively, the described research has provided new insight into tear albumin and a strong foundation for further studies.

Key phrases: tear sampling; point-of-care analysis; diurnal; vascular permeability; biomarker

TO MY FAMILY

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GLOSSARY

Aphakic: Lacking the normal crystalline lens due to disease or surgical removal.

Blepharitis: Inflammation of the eyelids, which causes them to become red and swollen.

Bulbar injection: One or more blood vessels in the bulbar conjunctiva are red and visible.

Conjunctivitis: Inflammation of the conjunctiva.

Creatinine: A breakdown product of muscle metabolism, which is created at a fairly constant rate and filtered out of the blood by the kidneys.

Hyperemia: Increase of blood flow to a tissue or body site.

Keratoconjunctivitis sicca: Also known as dry eye syndrome and is a multifactorial disease that affects the tear film production and/or stability.

Keratoconus: A degenerative disorder of the eye where the structure of the cornea is progressively altered to form a more conical shape.

Keratomalacia: An eye disorder that leads to a dry cornea. It is characterised by degradation of corneal epithelium and subsequent ulceration. One of the major causes is vitamin A deficiency.

Myeloma cell lines: A cancer cell line selected for its ability to grow in tissue culture and lack of antibody synthesis. Can be fused with an antigen producing B-cell to produce monoclonal antibodies.

Ocular pemphigoid: Autoimmune condition that can effect the eye by causing inflammation of the conjunctiva.

Oncotic pressure: A form of osmotic pressure exerted by plasma proteins on the capillary wall.

Phakic: An eye possessing its crystalline lens or an intraocular lens implant.

Sjögren's syndrome: An autoimmune disorder where the body's immune system attacks the glands that secrete fluids, such as the lacrimal gland and the saliva glands.

Von Ebners gland: Exocrine glands found in the mouth.

CHAPTER 1
INTRODUCTION

1.1 Introduction

Human serum albumin is an important plasma protein. It is present in higher concentrations than any other plasma protein and performs a vast array of different functions. Albumin has also been identified in tears, as a product of plasma leakage through the blood-tear barrier that separates the blood vessels from the ocular environment. Increased levels of albumin in the tear film have been reported in ocular disease or insult (Sen and Sarin, 1986a; Gupta *et al*, 1988; Versura *et al*, 2010) and during problematic contact lens wear (Lundh *et al*, 1984; Nichols and Green-Church, 2009). Ocular insults that lead to inflammation and activation of the immune response produce gaps between cells in the blood-tear endothelium. This promotes plasma leakage and may be responsible for some of the reported albumin influx. Albumin has, however, also been detected in atopic subjects and it has been impossible to verify whether albumin leakage is passive or if albumin is purposely recruited. Additionally, the in situ effects of increases in tear albumin concentration have yet to be determined. A possible scenario is that albumin, and potentially other serum products, are recruited into the tear film as part of an active process to create a dynamic adaptable ocular environment. The determination of albumin concentrations in the tear film could provide information on the integrity of the blood-tear barrier and on how a subject is able to withstand deviations from the ocular norm, such as contact lens wear. There exist presently no comprehensive evaluation of albumin concentrations in the tear film. The aim of the research presented in this thesis was therefore to provide a foundation for the investigation of whether albumin in the tear film is responsive and recruited, or passively leaked.

The objectives were fourfold: firstly, it was vital to characterise and develop methods suitable for sampling and determining albumin concentration in tears. Secondly, it was important to compile and critically assess the conflicting data presented in the literature, where albumin concentrations vary significantly between studies. Thirdly, it was imperative to identify factors that significantly impact the measurement of albumin concentrations in tears. This information is necessary for the determination of tear albumin baseline concentration and investigation of subject-to-subject variation. Fourthly, a more commercial aspect of albumin analysis was investigated. This involved the development and evaluation of existing urinary point-of-care analysis equipment for the measurement of tear albumin concentrations.

This introductory chapter provides background information for the conducted research. The sections discuss the anterior surface of the eye, with focus on the tear film function and its

constituents. Human serum albumin is also evaluated with respect to physical characteristics and functions, the defined plasma role and the less defined presence in tears.

1.2 The anterior surface of the eye

The anterior surface of the eye is compromised of the anterior portions of the cornea, the conjunctiva and the tear film. The cornea and the conjunctiva (Fig 1.1) will be described briefly, whilst a more detailed overview of the tear film will be given. The structure and function of the tear film is integral to the understanding and characterisation of tear albumin.

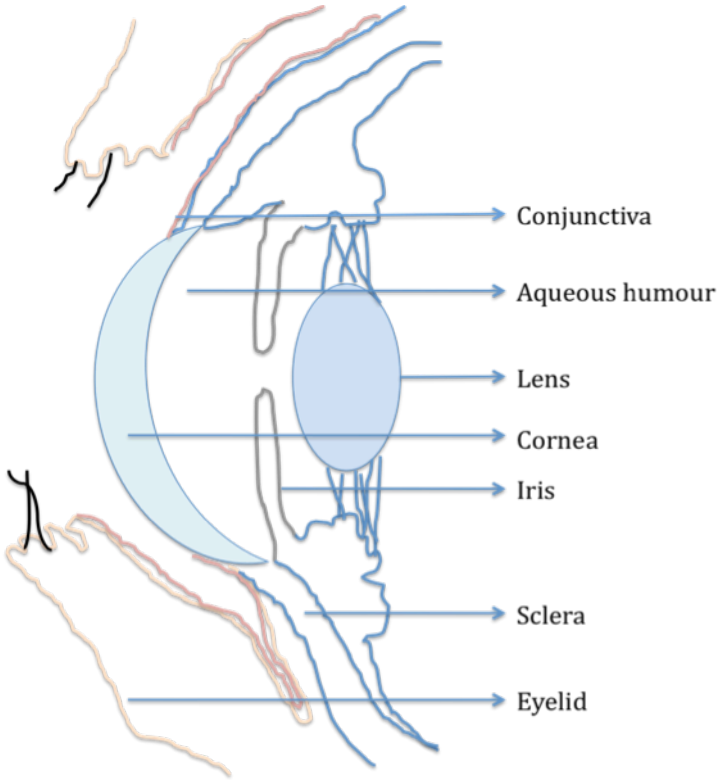


Fig 1.1 Cross-section of the anterior part of the eye showing the cornea, conjunctiva and their positions.

1.2.1 The cornea

The cornea is an avascular tissue covering the iris, pupil and anterior chamber. Lack of blood vessels provides the transparency necessary for the optical clarity of the cornea. Nutritional metabolites and oxygen are acquired via diffusion through the tear film and the anterior chamber. The cornea is responsible for 65-75 % of the refractive power of the eye. Conditions affecting refraction, such as myopia, presbyopia and astigmatism arise from the abnormal

curvature of the cornea. The cornea consists of five distinct layers. These are described below and pictured in Fig 1.2.

- Corneal epithelium
- Bowman's layer
- Corneal stroma
- Descemet's membrane
- Corneal endothelium

Fig 1.2. Histology of the cornea showing the different layers. (New York University, www.med.nyu.edu)

The outermost layer, the corneal epithelium, provides a smooth refractive and protective barrier towards foreign material. Corneal epithelium represents roughly one tenth of the cornea and is penetrated by nerve endings providing sensitivity and pain upon touch and/or insult. The subsequent Bowman's layer is a transparent sheet of tissue consisting of collagen. Whilst quick to repair, damage to bowman's layer can result in scar tissue affecting vision. The corneal stroma represents the remaining nine tenths of the corneal thickness and its major constituents are water (78%) and collagen (16%). Descemet's membrane is another strong thin collagen layer providing an additional barrier towards injuries and infection. Endothelial cells provide the collagen in this layer and Descemet's membrane is easily repaired after injury. The innermost layer, the corneal endothelium, is also the thinnest and performs the vital function of keeping the fluid balance in the stroma. Damage to endothelial cells can lead to corneal oedema and blindness, as the cells cannot be replaced once destroyed.

1.2.2 The conjunctiva

The conjunctiva is a thin transparent vascularised mucous membrane consisting of three parts (palpebral, bulbar and fornix conjunctiva) respectively lining the eyelids, covering the sclera and forming the junction between the two. Epithelial cells of the conjunctiva can be divided into 5 cell types (I-V) where only type I, the goblet cell, is well characterised. The rest of the cell types are described as stratisfied squamous cells. The goblet cells secrete mucins onto the ocular surface and are most concentrated by the fornices, compared to the bulbar and palpebral sites. Alongside this role, the conjunctiva also secretes significant amounts of water and electrolytes into the tear film. By the selective re-absorption of these components and other tear metabolites, the conjunctiva fulfils a regulatory function.

1.3 The tear film

1.3.1 Structure and function

The pre-corneal tear film is integral to the health of the eye and serves many key roles for ocular health. These have been aptly described by Milder (1987):

- The constant flow of tears and the blinking action flushes debris and potential pathogens out and away from the ocular surface.
- Tear fluid contains anti-microbial components and transports the oxygen and nutrients required by the cornea.
- The tear film reduces the friction of the eye-lid on the cornea and keeps the blinking action smooth and comfortable. Tear fluid also aids refraction by providing an even surface that appears smoother to incoming light.

Historically, the tear film has been described as a three-layered structure (Fig 1.3) consisting of an outer lipid layer, an aqueous layer in the middle, and a mucin layer closest to the cornea (Wolff, 1946, 1954). A more complex six-layer structure has since been suggested taking into account the internal structures of the lipid layer and the mucin layer (Tiffany, 1988). The thickness of the tear film has been under dispute with several reported conflicting values. Minimum and maximum values (3µm vs. 40µm) have both been produced using interferometry (King-Smith *et al,* 2000; Prydal *et al* 1992; Prydal and Campbell, 1992) and show great disparity. A compilation of thickness data made by Bron *et al*, (2004), showed that the most frequently reported values were within the range of 6-11µm. The relative sizes of the individual tear film layers are represented in Fig 1.3., where the aqueous layer is the dominant entity with an

estimated thickness of 7 µm. The interference between the aqueous layer and the mucin layer is diffuse and is better described as a gradation of mucin with a decrease in concentration towards the lipid layer. This diffuse transition makes it difficult to estimate the thickness of the mucin layer, as there are no defined reference points of where the aqueous layer begins.

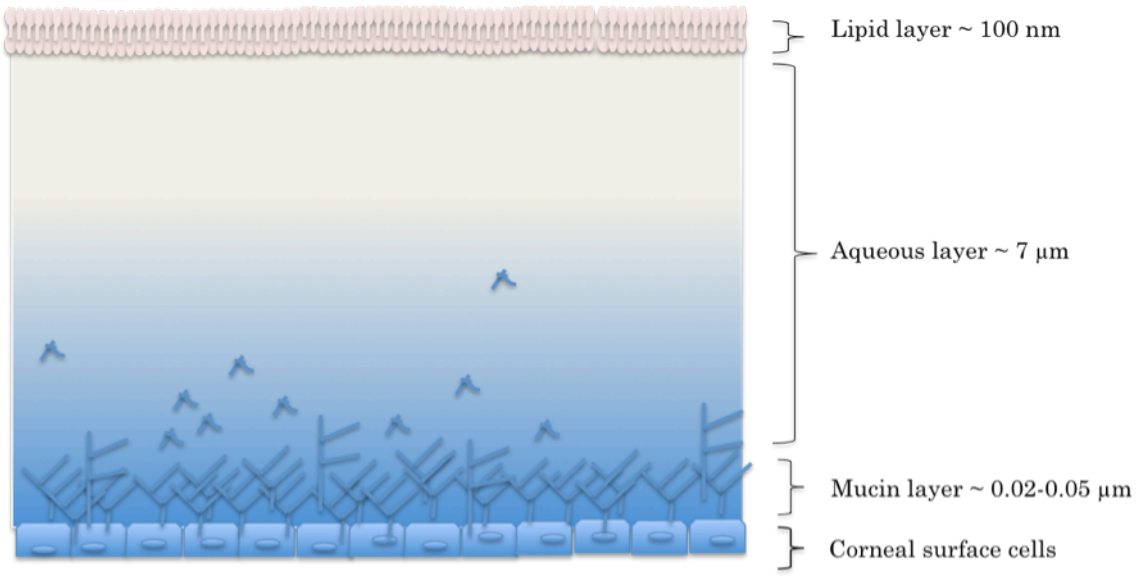


Fig 1.3. Diagram representing the structure of the tear film. Adapted from Craig, (2002).

1.3.2 The lipid layer

The lipid layer is composed of a mixture of polar and non-polar lipids, which are secreted primarily by the meibomian glands (Bron and Tiffany, 1998) and released during blinking. The Meibomian glands are tubulo-acinar holocrine glands located in the tarsal plates in the upper eyelids (30-40 glands) and lower eyelids (20-30 glands) (Wolff, 1954) (Fig 1.4). Non-polar lipids, such as wax esters, cholesterol and cholesterol esters, make up 60-70% of the lipid composition. The remainder consists of polar phospholipids and glycolipids, as well as minor amounts of alcohols, free fatty acids, mono- and di-glycerides and neutral fats (Nicolaides *et al*, 1981). The three most prevalent free fatty acids in meibomian gland secretions, or meibum, are palmitic, stearic and oleic acids. Reported concentrations of the individual meibum components are varied, and have been shown to differ significantly between studies (Wojtowicz *et al*, 2009). Similarly, meibum composition has been shown to vary between individuals, with respect to cholesterol and unsaturated fatty acid esters (Shine and McCulley, 1991). Both these observations add to the contentions regarding the lipid composition of the tear film, which is still a large research area of interest.

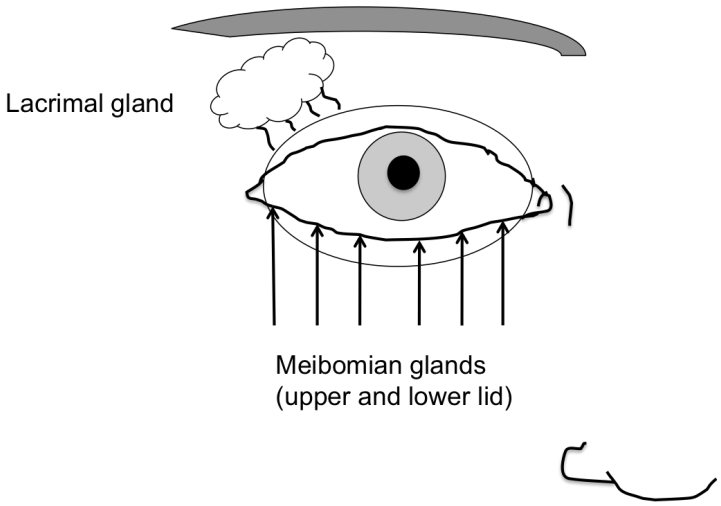


Fig 1.4. The position of the lacrimal gland and the meibomian glands.

The internal structure of the lipid layer has been described as a complex structure, exhibiting a non-polar phase at the air interface, and a polar phase at the aqueous layer interface (Fig 1.5), (McCulley and Shine, 2002). A review on the lipid layer thickness by Bron *et al* (2004) showed that early measurements reported 100 nm and more recent studies reported lesser numbers between 13-70 nm. The difference between these values may be related to technique, as the former data is based on specular microscopy and the latter derived from reflectometry measurements.

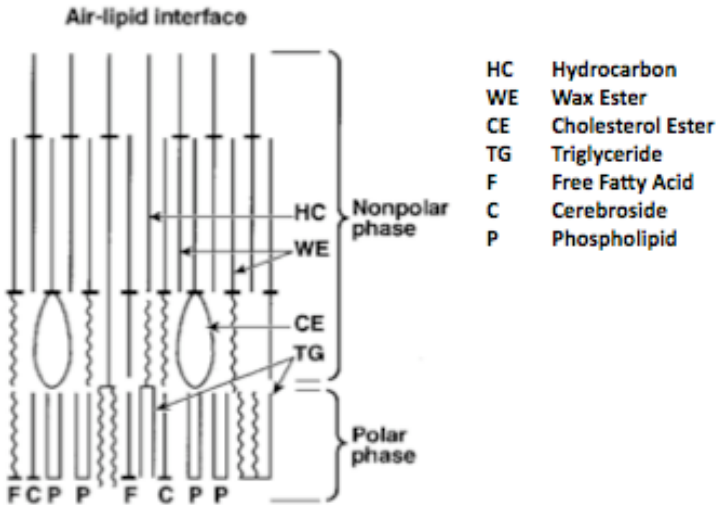


Fig 1.5. Diagram showing the division and interaction between the polar and the non-polar components of the lipid layer. Adapted from McCulley and Shine, (2002)

The lipid layer has important functions, both in the lid margin reservoirs, where it prevents tear over-spill and resists sebum contamination, and as the outermost barrier of the tear film. One of the most important functions of the lipid layer is as a lubricant for the eyelids when blinking.

During the blinking action, the lipid layer can conveniently be thought of as a concertina being compressed and stretched out when it is played (Bron *et al*, 2004). During eye closure, the lipid layer is compressed and rests between the lid edges during eye closure. When the eye opens again, it exhibits rapid spreading to cover the conjunctiva and promote stability of the tear film. The lipids protect against evaporation of the aqueous portion of the tear film and provide a smooth optical surface for the cornea. In the case of a non-confluent lipid layer or the absence of a lipid layer, evaporation of the aqueous tear has been shown to increase four-fold (Craig and Tomlinson, 1997). The same study also found a relationship between the lipid layer thickness and tear break up time, which emphasises the important link between a healthy lipid layer and ocular disorders such as dry eye. The lipid layer also provides a protective physical barrier against foreign particles and contributes towards the immunological defence with some anti-microbial activity.

1.3.3 The aqueous layer

1.3.3.1 The lacrimal gland

The lacrimal gland is responsible for the main secretion of the aqueous component of tears (water, proteins, glyco-proteins and electrolytes) and is situated in the lacrimal gland fossa, under the superolateral orbital rim (Fig 1.4). There are also the accessory glands of Krause (20-40 glands situated in the superior conjunctival fornix) and of Wolfring (along the superior tarsal border in the upper eyelid), which contribute to the aqueous secretions (Milder, 1987). New aqueous fluid from the lacrimal gland is expelled during every blink to refresh the tear film. The production of tear fluid is under central nervous system control and the lacrimal gland can be stimulated by ocular irritation or emotional experiences to increase tear flow (Milder, 1987). Normal tear flow has been estimated with the help of fluorescein instillations, to be 1.2 µl/min (Mischima *et al*, 1966). Tear volume was similarly estimated to have a value around 7µl where 3 µl is thought to be present in the marginal strip of the eye, forming the tear meniscus, which leaves 3-4 µl to cover the conjunctiva and a further 1 µl to cover the cornea. Tears are drained through the upper and lower eyelid puncta. The physical forces provided by the eyelids during blinking aid the drainage and presses tear fluid into the puncta (Doane, 1984).

Electrolytes determined in the tear film include: sodium, potassium, calcium, magnesium, chloride and bicarbonate ions. These are responsible for the tear film osmolarity (Bothelo, 1964). Tear film composition has been shown to vary between individuals and can be further altered depending on ocular health and disease. The identification of tear protein biomarkers is therefore an important tool in investigating ocular disorders. 491 proteins have been identified

to date in the tear film using various analytical techniques (De Souza *et al,* 2006). Lacrimal gland proteins are secreted by one of two pathways: regulated secretion or constitutive secretion. In the constitutive secretion pathway, proteins are secreted directly after being assembled whereas in the regulated pathway they are stored until sensory stimulus promotes release (Lucarelli *et al* 2003). The regulated pathway is the predominant protein secretion mechanism. Plasma derived proteins also occur in the tear film and these originate from serum leakage through the ocular blood-tear barrier. Levels of plasma proteins in the tear film are therefore varied, as they are dependent on the integrity of the blood-tear barrier. Albumin is the main protein originating from plasma leakage.

1.3.3.2 Proteins present in the tear film at high concentrations

The five most concentrated proteins in the tear film are: lysozyme, lactoferrin, lipocalin, secretory immunoglobulin A (sIgA) and albumin. Albumin will be discussed in more detail at the end of this chapter, as it is the main focus of this thesis. Other substances identified in the tear film but present to a lesser extent include: complement factors, transferrin, matrix metalloproteinases, histamine, prostaglandins and other immunoglobulins such as IgG, IgE and IgM.

1.3.3.2.1 Lysozyme

Lysozyme is a 14.6 kDa positively charged protein that originates from the lacrimal gland and is secreted simultaneously with lactoferrin and lipocalin. It is one of the most concentrated proteins in the tear film, making up almost a third of the total protein. Reported concentrations vary between 1.0 and 3.1 mg/ml (Fullard and Snyder, 1990). Lysozyme is also present in saliva, serum, nasal secretions and urine. Alexander Fleming, who detected the presence of a protein with antiseptic and antibacterial qualities, first reported lysozyme in tears in 1922. It is the most important antibacterial enzyme in tears and functions by hydrolysing the ß-1,4-glycosidic bond in the polysaccharide walls of microorganisms. It has also been suggested to be present at the tear film surface, mixing and adsorbing to the lipid layer, where it potentially fulfils a stabilising function (Mudgil *et al,* 2006). Several studies have reported a reduction in lysozyme concentration during ocular disorders, and concentrations have also been shown to decrease with age. Some variation over the day has been reported (Sen and Sarin, 1986b; Horwitz *et al*, 1974) although earlier reports found no diurnal variation (Pietsch and Pearlman, 1973). No significant differences have been found for samples obtained over the course of a year (Sen and Sarin, 1986b).

Lysozyme has been implicated in contact lens complications due to its heavy deposits on conventional hydrogels during wear (Bilbaut *et al*, 1984; Lin *et al,* 1991). Interactions are likely to be due to electrostatic attraction between the positively charged lysozyme and conventional anionic lens materials (Soltys-Robitaille *et al*, 2001). More attention to the nature and consequence of the deposits are needed to establish whether deposited or denatured lysozyme on the contact lens is a significant factor of complications and discomfort or not.

1.3.3.2.2 Lactoferrin

Lactoferrin is a positively charged 82 kDa protein produced and secreted by the lacrimal gland. It was first described by Masson *et al* in 1966 and tear concentrations have been reported ranging between 0.63-2.9 mg/ml with an average of 1.42 mg/ml (Flanagan *et al*, 2009). Bovine ocular surface epithelial cells have been shown to independently produce lactoferrin (Santagati *et al*, 2005) and the presence of lactoferrin precursors in mouse meibomian glands suggest that these might also be involved in secretion (Tsai *et al*, 2006), although, this is yet to be determined in human subjects.

Lactoferrin is an important part of the ocular defense mechanism where it has been shown to have both antimicrobial and anti-inflammatory properties. It has a high affinity for iron and, through binding, depletes the tear film of this component required for bacterial growth (Kiljstra (1990). Consequently lactoferrin is able to inhibit the growth of several bacterial species damaging to ocular health including *Escheria coli., Staphylococcus spp*. and *Pseudomonas spp*. (Flanagan *et al*, 2009). The positive charge of lactoferrin also attracts it to negatively charged surfaces of bacteria, where it interacts with the lipopolysaccharide components of the cell membranes and increases the permeability, with damaging effects for the bacteria. Recent data have suggested the presence of an up-regulation of lactoferrin secretion in females compared to males (Ananthi *et al*, 2011; Mii *et al*, 1992).

1.3.3.2.3 Lipocalin

Tear lipocalin is a 17.4 kDa protein that has previously been referred to in literature as a von Ebner's gland protein (Schmale *et al*, 1990) and tear specific pre-albumin (Bonavida *et al*, 1969). It is the second most concentrated protein in the tear film and approximately 15-33 % of the total protein consists of lipocalin under normal ocular conditions (Redl, 2000). Lipocalin is a lacrimal gland protein, which is secreted accompanied by lysozyme and lactoferrin. There is strong evidence that lipocalin interacts with both lysozyme and lactoferrin, based on electromagnetic resonance spectroscopy, and possibly works in cohort with these proteins

(Gasymov *et al*, 1999). The same experiment showed no interaction between lipocalin and the focus of this thesis, albumin.

The main function of lipocalin is as a carrier protein able to bind a broad range of ligands. It can bind and transport vitamin E, vitamin A and steroid hormones but most significantly ocular lipids. Lipocalin has been shown to have a protective function by binding and removing ocular lipids attached to dehydrated or other abnormal corneal surfaces exposed through ocular disorder (Glasgow *et al*, 2010). Lipocalin can also integrate with the meibomian lipids at the air-lipid interface through a very complex mechanism to potentially promote its integrity and retard evaporation (Millar *et al*, 2009). Other functions of lipocalin include contribution to the viscosity of tears and reducing surface tension of the tear film. It is also the primary endonuclease in tears. Alongside lactoferrin, lipocalin has also been found to be up-regulated in female tears compared to male (Ananthi *et al*, 2011).

1.3.3.2.4 Secretory immunoglobulin A

Immunoglobulins are glycoproteins produced by B-lymphocytes and are part of the adaptive immune system. They are present in all types of bodily fluids from serum, saliva, urine and breast milk to tears. Secretory immunoglobulin A (sIgA) is the main immunoglobulin in tears. Other immunoglobulins sometimes present in the tear film, although in much smaller concentrations, are IgG and IgE, IgD and IgM. IgG is serum derived and IgE will rarely be detectable outside of allergic responses. Secretory Immunoglobin A has a molecular weight of 385 kDa, as it is the dimeric form of IgA (160 kDa), and is essential for immunity at mucosal surfaces. Whilst lactoferrin, lipocalin and lysozyme are part of the regulated pathway, sIgA is part of the constitutive secretory pathway of the lacrimal gland and is continuously secreted into the tear film (Lucarelli *et al*, 2003). Localised production of sIgA in conjunctival cells has also been verified (Knop *et al* 2008). As part of the adaptive immune system, sIgA has a protective role against disturbance of the corneal surface by foreign pathogens and also has reported antiviral properties (Mazanec *et al*, 1993). A possible SigA mediated excretory pathway has been suggested (Mazanec *et al*, 1993; Robinson *et al,* 2001) where sIgA clears antibody/antigen complexes from the tear film.

Reported sIgA concentrations are varied and range between 71 μg/ml and 2.4 mg/ml in the open eye tear film (Sullivan, 1994). Concentrations of sIgA are dramatically increased during prolonged eye closure from 0.85 mg/ml to 8.40 mg/ml (Sack *et al*, 1992). The closed eye is described as a static environment with ongoing sub-clinical inflammation suggesting a possible vital protective role of sIgA during the night. In contrast to the closed eye environment, sIgA

concentrations have shown to be reduced in keratoconus. and during contact lens wear (Balasubramanian *et al*, 2012; Willcox and Lan, 1999; Pearce *et al*, 1999). Although Stapleton *et al* (1998) found no difference in sIgA concentrations between samples taken from contact lens wearers and non lens-wearing baseline samples.

1.3.4 The mucin layer

Mucins are extensively glycosylated protein cores with varying oligosaccaride side chain lengths. This has given rise to a large heterogeneity in mucin molecular mass from 500–40,000 kDa. The different types of mucins are collectively known as MUCs and 19 types of mucin protein cores have been identified in humans, numbered in order of discovery.

The mucin component of the tear film can be described as a two-layer model of membrane bound and gel forming mucins (Lemp *et al*, 1992; Gipson *et al,* 2004). MUC1, MUC3A, MUC3B, MUC4 and MUC 16 are membrane bound mucins attached to the stratisfied squamous cells of the conjunctival and corneal epithelia. This membrane-attached layer is covered in soluble gel forming mucins (MUC2, MUC5AC, MUC5B, MUC6, MUC7 and MUC9). Membrane bound mucins can also be found in soluble form in the tear film, most likely because of detachment by epithelial shedding (Dartt, 2004). The majority of mucin in the tear film is secreted by the conjunctival goblet cells and the stratisfied squamous cells of the cornea and conjunctiva. Origins of MUC7 and MUC5B are more uncertain and have been identified in human lacrimal tissue but not in tear samples (Jumblatt, *et al,* 2003; Spurr-Michaud *et al*, 2007).

The ocular surface mucin layer is a prolific area of research, especially with respect to ocular disorders and contact lens wear. Mucin is a vital defence barrier of the eye and fulfils several complex functions. Because of the extensive glycosylation, mucins are highly resistant to proteolytic degradation and have a hydrophilic nature, able to retain water on the ocular surface. The retaining of water is an important barrier against desiccation and the mucin also anchors, and facilitates even spread of the aqueous tear film to the ocular surface (Tiffany, 1990). The mucin layer provides a smooth lubricating surface protecting the corneal and conjunctival surfaces from kinetic friction damage by blinking. MUC5AC is the largest gel forming protein and has an anionic nature. Because of this it moves easily across the ocular surface and performs a scavenger function by the trapping and the removal of cellular debris and pathogens. Mucins have also been suggested to be partially responsible for the surface tension of tears. Millar *et al* (2006) was, however, only able to find surface activity of mucin when mixed with meibomian gland lipids, suggesting a supportive role in stabilising the lipid layer rather than a direct influence on surface tension.

Mucin concentration is regulated by mucin production, mucin release, and mucin cell proliferation (Dartt, 2002), where secretion is under parasympathetic nervous control. Overproduction or deficiency of mucin is associated with ocular disorders including dry eye (Lemp *et al,* 1971; Danjo *et al,* 1998) and Sjögren's Syndrome (Pflugfelder *et al,* 1997; Argueso *et al*, 2002). Alterations in mucin concentration with contact lens wear are still a contentious issue, where changes have been noted in some reports (Herraras *et al*, 2004; Pisella *et al*, 2001) but not in others (Hori *et al*, 2006). Differences in study populations, lens wear times, and biochemical analysis methods have contributed to the difficulties in finding an answer.

1.4 Human serum albumin

1.4.1 Albumin structure

Human serum albumin is a structurally simple protein made up solely of amino acids. It is one of the few plasma proteins without any carbohydrate groups, prosthetic groups or other additives. Human serum albumin is composed of 585 amino acids in total, compared to 583 and 584 in the corresponding bovine (BSA) and rat albumin versions (Peters, 1996). The molecular weight of albumin is 66 kDa, which makes it slightly smaller than lactoferrin.

Crystallisation experiments have shown that the tertiary structure of human serum albumin in the solid crystal state is heart shaped (Carter and He, 1992) with a length of 80 Å and a width of 30 Å. (Fig 1.6)

Fig. 1.6. Heart shaped structure of albumin in the solid state (Carter and He, 1992)

However, these experiments have only been performed on fatty acid free albumin and it is known that the binding of long chain fatty acid ligands (C_{16}-C_{20}) alters the shape of the molecule, making it more compact and more resistant to heat denaturation and proteolytic degradation.

(Peters, 1996). The shape of albumin in solution might therefore be slightly altered in comparison. An ellipsoid, or cigar shaped, structure in solution has long been the general concensus based on theoretical viscosity models using BSA (Squire *et al,* 1968; Bendedouch and Chen 1983). This view has however lately been challenged by Ferrer *et al* (2001) who studied the motions of a dye-bovine albumin complex in solution using phosphorescence. The results from that experiment, and experiments on BSA and albumin using bead modelling, suggested that BSA and albumin in solution in all likelihood would appear rigid and very similar to the heart shaped structure.

The albumin molecule itself is made up of nine loops grouped into three homogenous domains (Fig 1.7) (Brown, 1976; Carter and He, 1992). Loops 1-2, 4-5, 7-8, are the longer loops and these are labelled AI, AII and AIII. Conversely loops 3, 6, 9 are labelled BI, BII and BIII. Two longer loops separated by a shorter loop constitutes a domain, labelled I, II or III. These domains are similar in structure but appear to have vastly different ligand binding properties.

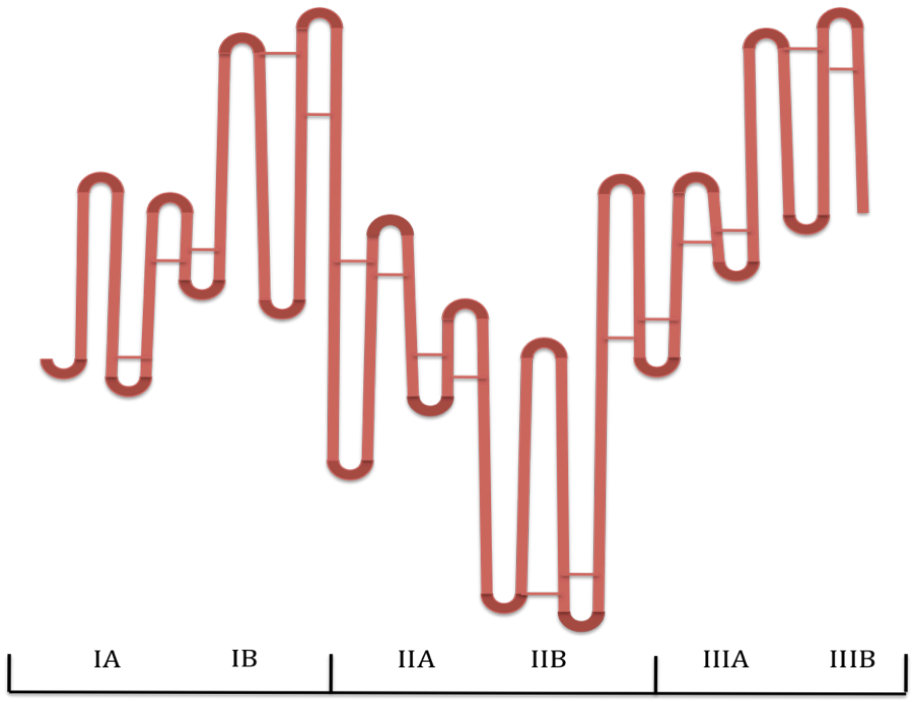


Fig 1.7. The loops and domains of the albumin molecule, showing the 17 di-sulphide bridges. Adapted from (Peters, 1996)

Albumin contains 35 half-cystins, which form 17 disulphide bridges (Dugaiczyk, 1982) (Fig 1.7). This disulphide-bonding pattern is a prominent structural feature of the albumin molecule as the positioning of the cystins enables short-range coupling, which leads to both flexibility and increased resistance to extreme conditions. Both these characteristics are defining for the functions of the albumin molecule. Albumin also has a high total charge (185 ions per molecule

at pH 7) due to a large number of ionised residues, which aid solubility (Peters, 1996). Because a high proportion of the amino acids are acidic (i.e. aspartate and glutamate), albumin has a negative net charge at physiological pH.

Due to the structural similarities of human serum albumin to bovine serum albumin, the latter is commonly used as a cheaper substitute for the human version in research. This is perhaps favourable in terms of cost and potentially useful as a tool for the early stages of designing a model or method where a large amount of waste is to be expected. The proteins are however not identical and it is not advisable to ascribe results using BSA to be directly comparable to human serum albumin. Use of BSA as a substitute for human serum albumin is more prominent in older reports, when the human version was less accessible.

1.4.2 Albumin metabolism

Albumin is synthesised in the liver daily and is not stored (Redman, 1968). The plasma concentration of albumin lies in the range of 35-50 g/L and between 1 fifth and 1 third of hepatocytes are designated to albumin production with an average synthesis of 9-25 g per day. Total albumin in the body amounts to around 250 -300 g for a 70 kg adult. Albumin is present to some degree in every bodily fluid, although the extent varies significantly between sites. Ca 42% is present in the plasma compartment, some is tissue-bound and the rest is situated in the extravascular compartment (Nicholson *et al*, 2000). Both these compartments are normally in a state of dynamic equilibrium. The rate of albumin production is controlled by changes in the colloid osmotic pressure, osmolality of the extravascular liver space and substances such as insulin (De Feo *et al*, 1991) and hormones (Johnson *et al*, 1991; John and Miller, 1969). Acute phase trauma, burns or infections can impede albumin production at the transcriptional level by slowing down the rate of mRNA transcription from the gene (Cairo *et al*, 1982; Liao *et al*, 1986). Similarly, direct deprivation of amino acids results in depression of albumin synthesis (Pain *et al*, 1978; Sakuma *et al*, 1987). Albumin catabolism is less understood than albumin synthesis but they generally operate at similar rates in healthy subjects and keep albumin concentrations stable. Degradation of albumin (on average 14 g/day) occurs in most organs of the body. The liver is only responsible for 15% of the breakdown whereas muscle and skin degrade 40 - 60 % (Nicholson *et al*, 2000; Yedgar *et al*, 1983). The rest is broken down by the kidneys or lost to the gastrointestinal tract through the stomach wall. As mentioned in section 1.3.1, binding of long chain fatty acids on albumin appear to have a protective function preventing degradation. Albumin half-life is long (>20 days), compared to other plasma proteins. This longevity is utilised by drug manufacturers, who use albumin as a carrier to improve the pharmacokinetic profile of circulating drug components (Krantz, 2008).

The albumin structure is very resiliant towards denaturation. Exposed to temperature changes, albumin shows some denaturation at 65°C but still retains most of the secondary structure. Above 70°C severe structural changes take place and albumin exhibits clear mass spectral differences compared to the native form (Kempson *et al*, 2010). Similar to temperature, drastic changes in pH are able to alter albumin. However, even significant structural changes of the albumin molecule can be reversed due to the flexibility and ease of reformation of the di-sulphide bridges. Prolonged circulation of albumin increases the opportunity for modifications that do not denature albumin but affect function and conformation. Non-enzymatic glycosylation (the addition of glucose, or derivatives, to free amino acid groups) affect certain ligand binding functions negatively, in particular the binding of bilirubin and LCFA (Shaklai *et al* 1984). This is also evident to a greater extent in subject suffering from diabetes mellitus, where extent of glycosylation can increase two- to three-fold (Bourdon *et al*, 1991). Similar to the effects of glycosylation, mild oxidative stress brings about conformational changes in albumin and affects its ligand binding. Oxidative stress also indicates increased surface hydrophobicity of albumin, which results in a slight loss in solubility in high salt solutions (Meucci *et al* 1991).

1.4.3 Functions of albumin in serum

Functions of albumin in serum have been extensively researched in the literature but will be dealt with briefly as they are not the main focus of this thesis. The primary functions are as follows:

- Regulating oncotic pressure
- Affecting coagulation
- Providing anti-oxidant effects
- Functioning as a transport molecule

1.4.3.1 Oncotic pressure

Oncotic pressure (the osmotic pressure exerted by colloidal blood proteins) is vital for maintenance of fluid balance in tissues and to stop osmotic flow e.g. preventing blood pressure to force water through capillaries and into tissue. Proteins in the blood counteract this flow by attracting water into the circulatory system. Albumin accounts for around 50% of total plasma protein but contributes up to 80% of the normal oncotic pressure in capillaries at 25 mmHg. This is due partially to its high concentration in plasma and high molecular weight, which makes

it unable to diffuse across the semi-permeable membrane separating the blood from the lymph. The other contributor is the negative charge of albumin, which attracts positively charged solute particles and keeps them in the intravascular compartment (the Gibbs-Donnan effect). E.g. albumin attracts positive sodium ions, which in turn brings water with them, and changes the colloid oncotic pressure. The effect of changes in albumin concentration, and resultant changes in oncotic pressure, in critical illness is a much-debated issue and was recently reviewed by Nicholson *et al* (2000). Low serum albumin in the critically ill is associated with negative outcome and increased mortality. Conversely, the apparent correction of this using intravenous injection of albumin is not necessarily correlated with an improvement in condition or preferential to other colloidal therapies. Results are unclear as to why this adjustment of albumin concentration does not live up to its theoretical promise. Furthermore, humans who are suffering from genetically related analbuminemia (plasma concentration of less than 1g/L) present with some pathological features, but the condition is not directly fatal (Peters, 1996). Potentially, prolonged albumin deprivation allows the body to adapt accordingly whereas sudden fall in concentrations have more severe consequences, which are difficult to resolve.

1.4.3.2 Coagulation effects

Albumin appears to exhibit heparin-like anti-coagulant activity on blood clotting and enhances the inhibition of factor Xa by anti-thrombin III. It also inhibits the aggregation of blood platelets (Jorgensen and Stoffersen, 1979, 1980). This is particularly valuable with respect to implanted biomaterials. The laws of thermodynamics show that interfaces will adapt to reach a system with as little interfacial energy as possible. For a blood vessel replacement, high surface energy amongst other unwanted properties may result in unwanted clotting and eventually plaque formation and blockage. By attracting albumin to the surface, the formation of clotting and other detrimental interactions are bypassed. For a solid implant the best scenario for this kind of interaction results in a thin fibrous encapsulation produced by the adsorption of other proteins, primarily albumin, shielding it from further interaction (Hench and Jones, 2005). Goncalves *et al*, (2009) tried to increase the beneficial albumin binding by attaching C-18 ligands to poly-hydroxyethylmethacrylate. The aim of the ligands was to attract albumin by mimicking the long chain fatty acid (C_{16}-C_{20}) binding site of albumin. The amount of poly-hydroxyethylmethacrylate-albumin binding increased at high concentrations of C-18 (> 5%). However, when albumin was present in competition with other proteins, high concentrations of C-18 were found not to have a significant effect on binding.

1.4.3.3 Anti-oxidant

Albumin has been implicated in more than 70% of the scavenging of oxygen free radicals that occurs in serum (Bourdon and Blache, 2001). The free reduced cysteine residue (Cys34) on albumin is a potent scavenger of oxidising agents (Gutteridge, 1986). Amongst other functions Cys34 enables the removal of the powerful oxidant hypochlorous acid, which is a by-product of enzymes released by activated phagocytes and neutrophils (Weiss, 1989; Winterbourn *et al*, 2000). Albumin also performs a protective anti-oxidant function by binding detrimental plasma components and removing them from circulation. Plasma copper and iron ions in the free form are liable to interact with hydrogen peroxide leading to the formation of damaging hydroxyl radicals. When bound to albumin, products created by the metal ion interactions (if they interact at all) are diverted towards albumin and less likely to affect more important tissue target (Roche *et al*, 2008). Albumin also provides indirect protection by gathering bilirubin, which has some anti-oxidant properties, from sites of haemoglobin breakdown. Bilirubin bound to albumin has been shown to be an inhibitor of lipid peroxidation (Neuzil and Stocker 1993, 1994) and thus albumin might also play a role in protecting polyunsaturated fatty acids from oxidation.

1.4.3.4 Transport molecule

Albumin is able to bind and transport a multitude of different ligands with high affinity. This is facilitated by the three homologous domains and the added flexibility of the molecule by the disulfide bonds. The ligands with the highest affinity were described by Peters (1996) as being " hydrophobic organic anions of medium size, 100-600 Da - long chain fatty acids, hematin and bilirubin". This versatility to bind a large variety of entities with high affinity, in conjunction with the large concentration of albumin in plasma, has led to a lot of interest in the drug binding properties of albumin by pharmaceutical companies (Fasano *et al*, 2005). Table 1 lists some high affinity endogenous albumin ligands as well as some common pharmaceutical agents.

Table 1. Albumin ligands and their relative association constants.

Ligand	Association constant	Reference
Endogenous		
Long chain fatty acids	(1-69) x 10^7	Richieri *et al* 1993
Bilirubin	9.5 x 10^7	Brodersen, 1980
Hematin	1.1 x 10^8	Adams and Berman, 1980
Copper (II)	1.5 x 10^{16}	Masouka *et a*l, 1993
Zinc (II)	3.4 x 10^7	Masouka *et al*, 1993
Pharmaceutical		
Salicylate	1.9 x 10^5	Kragh-Hansen, 1988
Benzylpenicillin	1.2 x 10^3	Joos and Hall, 1969
Diazepam	3.8 x 10^5	Kragh-Hansen, 1991
Ibuprofen	2.7 x 10^6	Kragh-Hansen, 1981

It is estimated that more than 99.9% of all 'free' long chain fatty acids (C_{16}-C_{20}) in serum are transported on albumin molecules. Statistically with long chain fatty acid concentrations in plasma reaching 1mM and circulating albumin levels reaching only 0.6mM, each albumin molecule would carry 1-2 LCFA molecules. Ligand binding alters the structure of the albumin molecule and can in the instance of fatty acid binding lead to increased stability and better resistance towards degradation (Peters, 1996). Medium-chain fatty acids (C_6-C_{14}) have less affinity for the albumin binding sites, although binding might occur at long chain fatty acid sites if medium chain fatty acids are present in excess in the plasma. As these medium chain fatty acids are rarely present outside cells this has little practical influence (Peters, 1996). Six sites for long chain fatty acid binding have been detected on albumin but only three have been properly characterised. These are the three strongest ones and they each lie in different domains. The binding of very long-chain saturated fatty acids (>C_{20}) has been demonstrated on bovine serum albumin (Choi *et al*, 2002) and was shown to decrease in affinity with increase in chain length. The transport of fatty acids is vital for the functions of a variety of tissue cells: adipocytes for storage, Leydig cells for hormonal production and myocytes for energy production.

1.5 Albumin in tears

1.5.1 Albumin in the pre-corneal tear film and anterior eye

Albumin is not an indigenous lacrimal gland protein and is present in tears as a result of vascular leakage from serum through the blood-tear barrier. Due to its high concentration in plasma and relatively low molecular weight (66 kDa) it can easily transverse the blood-tear capillary layer and is an effective marker for vascular leakage and the integrity of the blood-tear barrier. Whilst albumin in serum has been extensively researched, tear albumin levels remain poorly defined. The available data are not completely understood and a distinct lack of authoritative information concerning the presence and role of albumin in tears is noticeable. It is well recognised that tear albumin concentrations vary significantly from study to study compared to other major lacrimal gland proteins such as lysozyme and lactoferrin. This variability makes it difficult to establish a range even in asymptomatic subjects, where minimum and maximum determined values are 8.41 μg/ml and 1830 μg/ml respectively (Fullard and Tucker, 1991; Farris, 1985). The evaluation and characterisation of published tear albumin concentrations was key for this research. Literature tear albumin concentrations and factors for variations are therefore extensively discussed in chapter 3.

1.5.2 Albumin in the cornea

Albumin is present in the cornea, although reports differ with respect to abundance and location. Studies carried out in rabbits suggest that albumin originates from peripheral blood vessels and diffuses towards the center of the cornea, exhibiting a concentration gradient of albumin (Maurice and Watson, 1965; Wiig, 1990). In mouse and bovine corneas, albumin is present almost exclusively in the stroma with localised staining in cells next to the Bowman's and Descemet's membranes (Nees *et al*, 2003). Gong *et a*l (1997), using similar techniques, also reported albumin staining in both bovine and human stroma but found a difference between the anterior and the posterior portion; the anterior one third of the stroma staining more intensely.

1.5.3 Albumin transport across the blood tear barrier

Mechanisms of transcapillary transport of albumin across the blood-tear barrier have yet to be determined and may be a combination of receptor-mediated pathways and other pathways including: intercellular junctions, vesicular transcytosis and transendothelial channels. In plasma, transport of albumin into the extravascular compartment is across capillaries. Historically, trans-capillary transport has been attributed to the four Starling forces (osmotic

and hydraulic forces) but newer research into fluid movement across compartments in the lymphatic system suggest more complex pathways (Levick, 1991). Binding of albumin to endothelial layers are consistent with those binding patterns seen in a receptor-ligand interaction. Four kinds of receptors have been identified for the possible transport of albumin across endothelial transcytosis and endocytosis: SPARC, gp60 (otherwise known as albondin), gp30 and gp18. gp30 and gp18 have been shown to have a preferential binding to albumin that is not in its native form, suggesting a scavenging function for the removal of altered albumin species (Antohe *et al*, 1991; Schnitzer and Oh, 1994). Albondin is a 60kDa endothelial cell surface protein. It has been shown to have specific interaction with albumin and to facilitate transendothelial transport in cell culture. The introduction of antibodies raised against albondin had a negative impact on endothelial binding of bovine serum albumin and inhibited movement (Schnitzer and Oh, 1994). The albondin antibodies also recognised SPARC suggesting common domains between the two receptors. This was not found with gp18 and gp30. Schnitzer and Oh also investigated effect of albondin on transcapillary transport. Un-labelled BSA competing with labeled BSA for albondin receptors revealed a 50% dependence on concentration and suggests alternative transportation pathways for BSA are also in place. This is significant for the study of albumin in tears as the presence of albumin is dependant on transcapillary movement. The characterization of albumin and its role is made more difficult by lack of definitive transport mechanisms into the tear film. Other available transport mechanisms apart from receptor-mediated are via: vesicular transcytosis, intercellular junctions and transendothelial channels, as mentioned above.

Albumin distribution in serum between the intra-vascular and extra-vascular compartments is altered during disease states. This is attributed to increased permeability in capillary leakage. Nicholson *et al* (2000) compiled identified mediators of capillary leakage in serum in a review of albumin in critical illness.

These are:

- Endotoxin from gram-negative bacteria
- Cytokines –TNF-alpha and IL-6
- Arachidonic acid metabolites – leukotrienes and prostaglandins
- Complement components C3a and C5a
- Other vasoactive peptides – bradykinin and histamine
- Chemokines – macrophage inflammatory protein 1alpha

These identified mediators may also be responsible for the leakiness of the blood-tear barrier, and may have a role in the control and augmentation of albumin concentrations in the tear film.

Although the precise modality of albumin leakage into the tear film is not yet understood, it is recognised that ocular disorders such as giant papillary conjunctivitis or dry eye, amongst others, result in increased albumin concentrations. Contact lens wear has also been implicated in increased albumin concentrations in the tear film (Lundh et al, 1984; Choy et al, 2004).

1.5.4 Albumin in the closed eye tear film

The closed eye tear film became a research area of growing interest in the early 1990s, as researchers and clinicians were trying to determine factors that have an impact on contact lens wear. The safety of overnight wear of contact lenses was a disputed issue, as there was little information on risk factors associated with lens wear during prolonged eye closure. Previous studies had shown that overnight eye closure is associated with corneal swelling and vasodilation (Smelser and Ozanics, 1952; Holden *et al,* 1983*).* Increased risk of infection, ulcers and corneal hypoxia were therefore major concerns (Weissman *et al*, 1984; Poggio *et al,* 1989; Holden and Mertz, 1984). Studies on the closed eye showed that the tear film composition was significantly different from the open eye state (Sack *et al*, 1992; Tan *et al*, 1993; Sack *et al,* 1993; Choy *et al*, 2004). The seminal study by Sack *et al*, (1992) compared tear samples collected immediately upon waking (closed eye tears) with samples taken during the daytime (open eye tears). They found that total protein concentration in the closed eye tears was double the concentration in the open eye (9.0 mg/ml to 18 mg/ml) and that SigA concentrations were increased by tenfold (0.85 mg/ml to 8.40 mg/ml) in the closed eye tear film. More significantly for the present study, albumin concentrations exhibited a twenty-fold increase from 0.06 mg/ml in the open eye to 1.10mg/ml in the closed eye tear film. Complement factors and polymorphonuclear neutrophils were also increased in the closed eye (Sack *et al*, 1992; Wilson *et al,* 1989). These entities, together with increased albumin, are classical diagnostic signs of an acute inflammatory process. As a result, Sack *et al,* (1992) ascribed some of the marked rise of albumin concentration in closed eye tears to this induced "sub-clinical inflammation". Concentrations of other major tear proteins, such as lactoferrin, lipocalin and lysozyme, remained static between the open and the closed eye tear film. Consequently, the closed eye tear film was identified as an albumin and SigA rich fluid, whose composition potentially could be critical in protecting the closed eye from pathogens (Sack *et al,* 1992). Research by Tan *et al,* (1993) showed that the albumin increase during eye closure was progressive and only became statistically significant after five hours of sleep. Relatively little is known about the underlying mechanisms and the effects of this compositionally different fluid to the open eye tear film. The presently conducted research focuses on the open eye tear state. Nevertheless the observations made by Sack and colleagues are important for albumin studies and albumin concentrations in relationship to the closed eye tear film are discussed further in chapters 3 and 4.

1.6 Summary of albumin in the tear film

Components of the pre-corneal tear film and the ocular surface are part of a tightly regulated environment with sophisticated response mechanisms. A significant amount of albumin influx from plasma is reported during ocular disorders, and insults, and has also been linked to contact lens wear. Despite this increase in concentration during ocular events, albumin in the tear film is still poorly understood. The measurement of albumin has seldom been the primary target in previous reports and therefore factors that influence concentrations have remained uncharacterised. Published tear albumin concentrations have been derived from symptomatic and asymptomatic subjects using a variety of sampling and collection techniques, and show large differences between studies. Mode and logistics of tear albumin increase are also undetermined and there is no authoritative information on the subject.

Significantly, the implications of albumin leakage into tears have been even less investigated. Albumin performs a series of vital functions in plasma (section 1.3.3), and it is not unfeasible that it has the potential to execute similar roles in the tear film. Research has shown that ocular application of human albumin serum has a beneficial effect on detrimental conditions such as corneal ulcers and dry eye disorders (Shimmura *et al*, 2003; Unterlauft *et al*, 2009). These beneficial effects suggest that influx of albumin in the tear film might have a protective role and could be part of the eye's response mechanism to ocular insult. Alternatively, albumin in the tear film is a result of non-specific plasma leakage. Potential implications of albumin in the tear film can be summarised in two statements:

1. Albumin is a protein, which passively leaks into the tear film during ocular distress and is best utilised as a marker of vascular leakage.

2. Albumin is a part of ocular homeostasis and is recruited (or is a marker for recruitment of other products) from serum to deal with an ongoing ocular event.

The determination and characterisation of tear albumin concentrations are therefore of interest, as they may help to identify whether albumin is passively leaked or beneficial in nature and recruited. Tear albumin concentrations may potentially be a valuable biomarker and a predictor of a subject's ocular response to insult. Subjects exhibiting higher or lower concentrations of tear albumin may be better or worse at accommodating deviations from the normal stable tear film. The identification of subject-to-subject variation is therefore key in understanding the role of albumin.

1.7 Aims

The overall aim of the research is to try to gain a greater understanding of the role and nature of tear albumin and of factors that affect tear albumin concentrations**.** It is clear that in order to do this, the previously uncharacterised published tear albumin data needs to be collated. Factors that affect tear albumin needs to be taken into account to interpret the significance of reported concentrations and to provide a foundation for further work. The choice of sampling and analysis technique for the determination of tear albumin is also of great importance. Another aim is therefore to create and evaluate an in-house enzyme-linked immunosorbent assay for tear albumin analysis.

Having characterised the tear albumin literature, a second aim of the work is to investigate the possible occurrence of systematic variation in tear albumin. Although, a great effort has been put into characterising the closed eye, diurnal variation of tear albumin has not been studied - despite the fact that variation of albumin has been identified at other body sites**.**

A major driving force for the present investigation is that tear albumin levels may produce some information into the responses of the eye to insult. Therefore tear albumin may be a predictive factor in the determination of contact lens wear success in a subject (50% of potential contact lens-wearers drop out for unknown reasons). It was intended from the outset to investigate the possibilities of adapting existing point of care techniques, currently used to measure urinary albumin, for tear analysis in the contact lens clinic. The potential benefits of this are:

- Means of gaining additional data related to the investigation into albumin as a biomarker
- The aforementioned value of tear albumin, as a predictive factor for contact lens wear potential or as a marker for adverse events

CHAPTER 2
MATERIALS AND DEVELOPMENT OF METHODOLOGY

2.1 Introduction

This chapter details the techniques and methods used for albumin analysis and related tear film analysis. Where necessary, further explanations and details of deviation from method protocols are provided in the relevant results chapters.

2.2 Methods for tear collection

Tears were collected from all subjects in line with the ethical guidelines of Aston University. No signs of ocular disease or discomfort were found during any of the sampling periods and contact lens wear was restricted with respect to the relevant studies. This research project used two different techniques to evaluate albumin concentration in tears:

- Microcapillary pipette with positive displacement
- Schirmer strip

The use of both microcapillary pipettes and Schirmer strips to collect tears for albumin analysis has been previously reported in the literature (Stuchell, 1984; Farris, 1985; Fullard and Tucker, 1990). Whilst Schirmer strip is considered a more invasive technique out of the two, the use of it was necessary to evaluate external factors that affect albumin concentration. The use of Schirmer strip for tear protein analysis is also beneficial in subjects with low tear volume, where microcapillary collection is more difficult. Effects of tear sampling technique on measured albumin concentration are discussed in detail in chapter 3.

2.3 Microcapillary sampling

Microcapillary sampling was the main technique employed for the collection of tear samples in the research presented in this thesis. This method of collection uses narrow-bore glass microcapillaries and was carried out in line with protocols described by Bjerrum and Prause (1994), Fullard and Snyder (1990), and Mann and Tighe (2007). Capillaries have a volume range of 1-10µl and are provided with a separate stainless steel plunger wire used to express the tear sample. The use of the plunger to express the tear fluid eliminates the need for an extraction step, which is often needed for absorbable collection techniques. Gradation on the capillary makes it simple to assess the volume collected, depending on the accuracy and precision

necessary. With patience, 2-5 µl of tear sample can be achieved from subjects without inducing increased tear flow. This un-stimulated tear represents the basal tear environment. It should be noted, however, that in a number of subjects, even the most minimally invasive sampling technique was able to cause excessive tearing. To illustrate this in the thesis, collected tear samples are labelled as basal, medium (some stimulation of tear flow) and stimulated tears. Effect of tear flow stimulation on tear albumin concentration is discussed in chapters 3 and 5. The reported disadvantages of the capillary methods, disregarding the training required, are two-fold: previous stimulation is sometimes necessary to collect enough fluid, especially from dry eye or other symptomatic subjects, and the method can be slow and erratic (Esmaeelpour *et al,* 2008).

2.3.1 Materials for microcapillary sampling

- Microcapillaries (Sigma Aldrich, UK)
- Wire plungers (sold together with microcapillaries)
- Microcentrifuge tubes (650 µl)

2.3.2 Microcapillary sampling protocol

Subjects were asked to sit down for the duration of the sampling and to face the researcher performing the sampling with their eyes looking up towards the ceiling and away from the capillary. The capillary was then placed at the outer canthus of the eye (Fig 2.1) to collect the tear fluid by capillary action.

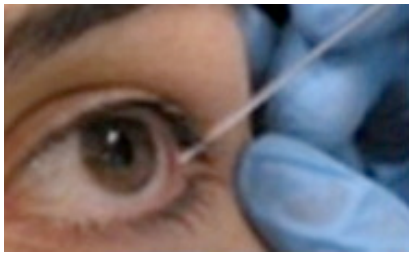


Fig 2.1. Showing the position of the microcapillary during sampling. Adapted from Soria *et al*, (2013).

Care was taken not to touch the conjunctiva with the capillary as that might produce stimulation and reflex tearing. The eye of collection (right or left) was also noted. After sufficient volume had been collected, samples were expelled into microcentrifuge tubes (650 µl), using the steel wire plunger. Samples were then stored at – 80 °C until time of analysis.

2.4 Schirmer strip sampling

The German ophthalmologist Otto Schirmer first described the Schirmer strip method in 1903, which uses filter paper strips inserted into the eye, to measure tear flow. Schirmer strips (Whatman No 41 filter paper) are 35 mm long and 5 mm wide. The top section of the strip has a rounded edge to reduce discomfort for the subject being sampled. Assessment of the degree of wetting is simplified by the imprint of a ruler onto the filter paper, which divides the strip into 1 mm sections (0-35 mm in total). Optometrists and ophthalmologists presently use the technique as an aid in the identification of dry eye. There is an indication of dry eye if ≤5mm of the graded paper strips have been wetted in 5 minutes. Despite its original purpose of measuring tear flow, the Schirmer strip has since also been used extensively to collect tears for use in research.

2.4.1 Materials for Schirmer strip sampling

- Schirmer strips (TEARFLO™, Sigma Pharmaceuticals)
- Eppendorf microcentrifuge tubes (650µl and 1.7ml)
- Micropipettor and disposable pipette tips
- Mini centrifuge
- Tweezers

2.4.2 Schirmer strip sampling protocol

The rounded end of the Schirmer strip was bent to form a lip over the lower eyelid, which keeps the strip in place during sampling. Subjects were asked to face the researcher and look up. The filter paper strip was then placed in the lower conjunctival sac, one third of the palpebral width from the temporal canthus, to absorb the tear fluid (Fig 2.2.). Subjects were told to blink normally or close their eyes during sampling and also to report if any significant discomfort was experienced. Wetting length and time of wetting were recorded during the experiments. The Schirmer strip was removed with tweezers after 5 minutes or when the full 35 mm of the strip had been wetted. Tweezers were used to avoid contamination of the Schirmer strip by excessive handling. If a subject wet the full strip before 5 minutes, the time at which full wetting occurred was recorded. The Schirmer strip was then subjected to extraction as discussed below.

Fig 2.2. Schirmer strip sampling (Legarreta Eye Center and MedNet Technologies, Inc).

2.4.3 Schirmer strip extraction protocol

The method for Schirmer strip extraction described below was designed by the Biomaterials Research Group. Other protocols of Schirmer strip extraction exist and are discussed more thoroughly in section 3.6. The removed Schirmer strip was placed in a microcentrifuge tube (650 µl), which had a hole punctured in the bottom. This 650 µl tube was then positioned in a larger microcentrifuge tube (1.7 ml) and centrifuged for 5 minutes at 10, 000 rpm (Fig 2.3).

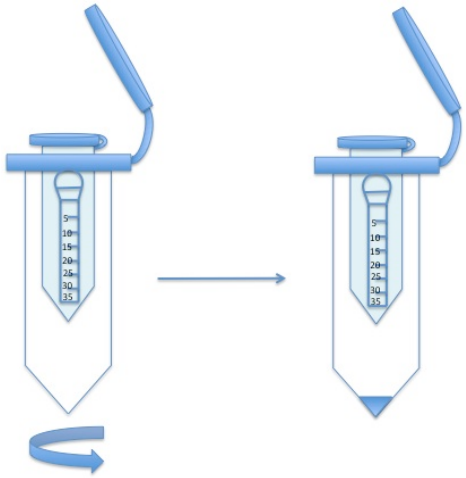


Fig 2.3. Centrifugation protocol to collect the Schirmer strip

The resultant sample fluid was collected in the larger microcentrifuge tube and stored at -80°C until time of analysis. In the events of a subject producing a wetting length of less than 14 mm, an addition of 4µl of deionised water was made to the Schirmer strip prior to centrifugation. This was necessary to ensure adequate sample fluid for analysis.

2.5 Methods for the analysis of tear albumin

Protein analysis of individual tear samples is dependent upon the availability of systems able to quantify proteins in small sample volumes (2-5 µl). Sensitivity is also a key issue, especially in terms of albumin analysis, where reported concentrations are in the µg/ml range. Tear quantification in the present study was performed using enzyme-linked immunosorbent assay (ELISA) and two point-of-care kits (POC): the HemoCue® 201 albumin analyser (Hemocue AB) and the Afinion™ AS100 Analyser (Axis-Shield).

2.6. Enzyme-linked immunosorbent assay

A review on clinical application of tear proteomics identified ELISA as the most commonly used assay method for the quantification of tear proteins (Wu *et al*, 2007). The method has four features, which makes it advantageous for a wide range of applications (Crowther, 2009):

- It is versatile with many different combinations of systems available and easy separation of bound and unbound reactants is possible.
- It is simple to use with the ability to do a high capacity of samples whilst remaining relatively cheap.
- It is sensitive due to the enzyme-catalyst amplification.
- It is quantifiable, either by eye or by spectrophotometry.

One disadvantage of using ELISA is that commercially available kits are relatively expensive compared to the individual components. Preliminary investigations for the research project described in this thesis identified the need for analysis of a substantial amount of tear samples. The issue of material and method costs was therefore important to discuss as relying on commercial ELISA kits could become very expensive. Consequently, an aim for this research was to construct a laboratory based ELISA to keep the costs low. The procedure of constructing a sandwich based ELISA protocol for the determination of albumin, upon which a considerable amount of time was expended, is detailed in appendix A. The present chapter provides the materials and buffers used in the development of the assay. Ultimately, this laboratory based human albumin ELISA was unable to provide acceptable accuracy and precision for the project and a commercial assay was chosen for the analysis of tear albumin. Nevertheless the time spent on ELISA development provided valuable insight into the assay components and the technique itself.

2.6.1 Principle of the ELISA assay

ELISA is a colorimetric analytical technique based on the specific interaction between antibodies and their antigens. An antigen is any entity able to elicit an antibody-mediated immune response. The ELISA procedure involves the stepwise addition and reaction of reagents to a solid phase-bound substance. Four ELISA systems are available: direct, indirect, sandwich and competition. Due to the small working volume in tear sample analysis it was found that sandwich ELISA was the most suitable technique. In sandwich ELISA, capture antibodies specific to the antigen of interest are coated onto wells in a microtiter plate. Samples are then added to the wells and the capture antibodies identify and bind any antigen present in sample solution. The antigen is then retained in the system for quantification.

A key feature is the addition of another antibody, which is enzyme-conjugated, and also recognises and binds to the antigen. This antibody is known as the detection antibody. The enzyme bound to the detection antibody catalyses a subsequently added reagent and this produces a quantifiable change in colour. The colour intensity corresponds to the enzyme-linked antibody, which in turn corresponds to antigen concentration in the sample, and this is how the method has received its name: enzyme-linked immunosorbent assay. Benefits of the sandwich ELISA are: high specificity and the conservation of antigen sample volume, as antibodies are the entity needed in excess. Although components of the ELISA assay had been previously described, publication of the complete ELISA procedure can be attributed to two papers: Engwall and Perlmann (1971) and Van Weemen and Schuurs (1971). Several sandwich assay varieties exist but this project focused on the most common procedure which utilises a horseradish peroxidase (HRP) - 3,3',5,5'-tetramethylbenzidine (TMB) system. The assay method is exemplified in Fig 2.4

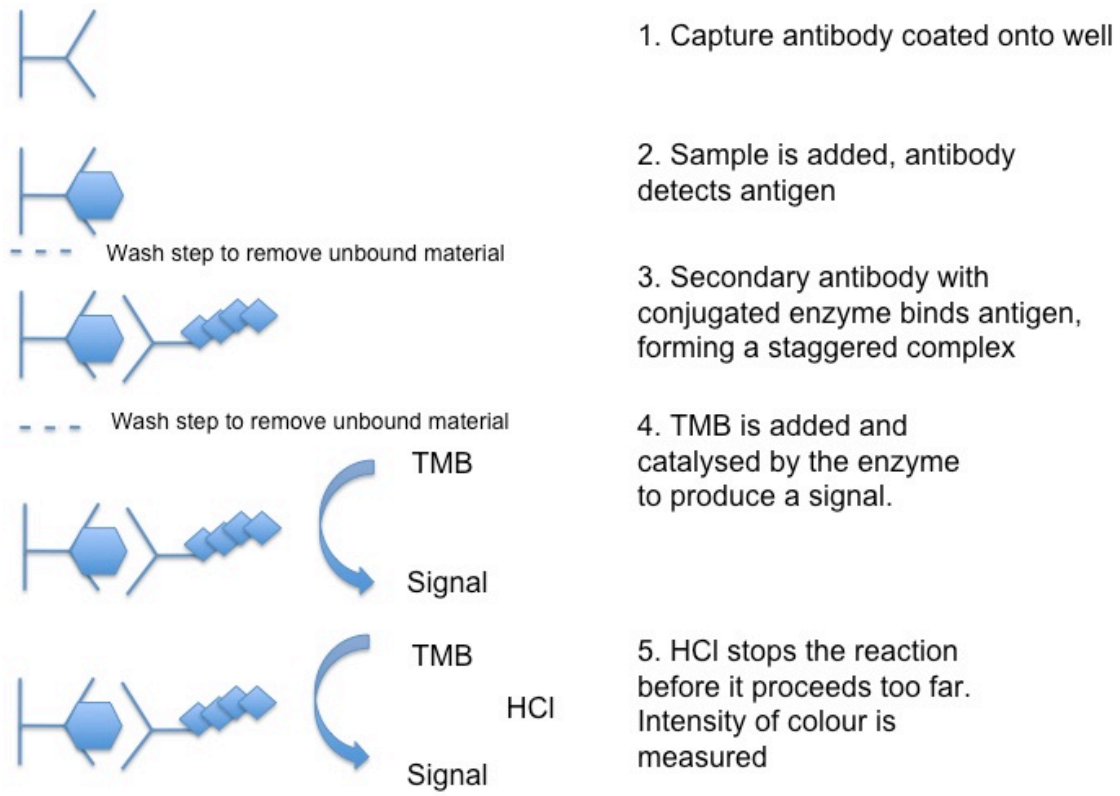


Fig 2.4. Example protocol of a sandwich ELISA

Further explanation of the steps illustrated in Fig 2.4 is given below:

1. Specific antibodies, raised against the antigen of interest, are coated onto the bottom of wells in microtiter plates at set concentrations. Standard plates are conventionally made of polystyrene and have 96 wells, although other materials and sizes are available. Well volume is normally between 300-400 µl. After incubation with the coating antibody, a blocking agent is added to the wells to adsorb to empty sites. The role of the blocking agent is to limit interactions with the plate material and other assay components.

2. Pre-diluted sample fluid is added to wells in duplicate. The coated "capture" antibodies will form a strong specific interaction with any antigen present in the sample added to the well. This is a normally a 1: 1 interaction making the system very precise. After the incubation with the sample solution, the plates are washed with a surfactant solution. This removes all unbound material.

3. A solution containing HRP enzyme-labeled antibodies, which are also specific to the antigen, is then added. This secondary antibody binds the antigen and the structure formed is known as the sandwich complex. The plate is incubated to allow the interaction between the secondary

antibodies and the antigen to proceed. Subsequently all unbound matter is washed away; ensuring that only antibody-antigen complexes remain in the wells.

4. The chromogenic substance, TMB is added to the wells. The conjugated enzyme attached to the secondary antibody catalyses the TMB and the reaction gives rise to the quantifiable change in colour. The incubation during this step takes place in the dark to prevent colour amplification by sunlight.

5. The catalysis reaction is stopped by the addition of a weak acid solution e.g. 0.5 M hydrochloric acid (HCl). The intensity of colour is measured by spectrophotometry at 450nm. Concentration of antigen in the sample is directly correlated to concentration of bound enzyme-antibody and this in turn is directly correlated to the colorimetric change. A set of standards with known antigen concentrations is included on the plate and these constitute the standard curve from which the sample concentration is determined.

2.6.2 Specific component information

The sections below discuss the components of the ELISA system in more detail with specific attention to the conditions necessary for a working sandwich ELISA protocol.

2.6.2.1 Immunoglobulins

Immunoglobulins, or antibodies, are the key part of the acquired immune response, which is the selective intervention of the body against a specific foreign invader. Antibodies are proteins secreted by plasma B cells with tailored functions towards the identification and eradication of extracellular pathogens. The structures and generation of antibodies, as well as the applications of the antigen-antibody interaction for immunoassay use are well explained by Price and Newman (1997).

The basic antibody molecule is Y-shaped with four polypeptide chains; two identical light chains and two identical heavy chains (Fig 2.5.).

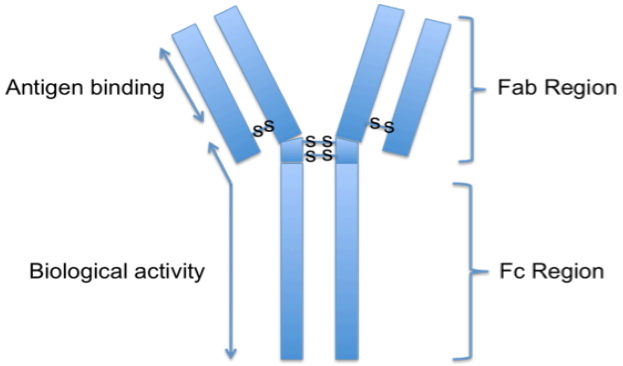


Fig 2.5. Representation of an antibody molecule.

The antigen binding terminal of both light and heavy chains contain variable regions with independently folded amino acids units that create highly specific paratopes. The differences in these amino acid units determine the antibody complementarity towards antigens. The antibody proteins contain inter- and intra-chain disulphide bonds and renders some flexibility from the disulphide bridge hinge region. The shorter Fab-regions express the variable domains that determine the antibody specificity whilst the Fc region determines the class of the immunoglobulin. There are 5 classes of immunoglobulins: IgG, IgA, IgE, IgM and IgD (Table 2).

Table 2. Immunoglobulin classes and characteristics

	Molecular mass (kDa)	**Structure**	**Normal Concentration in serum (mg/ml)**	**Function**
IgG	150	Y shape	10	Primary antibody in the blood. Secondary antibody after IgM detection but with much stronger response.
IgA	160	Y shape or Dimeric	2	Mucosal and secretory immunity.
IgM	950	Pentameric	1	Initial antibody detecting foreign entities. Efficient at complement fixation.
IgD	190	Y shape	0.04	Poorly understood. Possibly a regulator of the immune system.
IgE	190	Y shape	0.0003 (increases significantly during allergic response)	Allergic responses by activating mast cells and basophiles. Also targets parasitic invasions.

The antibodies are raised in a host animal by an injection of the desired protein antigen into the bloodstream. The B-cell lymphocytes in the animal, often a sheep or a rabbit, produce specific antibodies to the injected foreign protein. These antibodies are then harvested. IgG is the most commonly used ELISA antibody, because of its strong response to the presence of an antigen. This results in a higher serum concentration, or a higher yield, compared to the other antibodies.

Antibodies and can be either monoclonal or polyclonal. Monoclonal antibodies are raised from a single B-cell lymphocyte and this generates identical proteins capable of detecting structural alterations as small as singular amino acid changes. The specificity of monoclonal antibodies has made the discovery of several isoforms of proteins possible. Disadvantages of monoclonal antibodies are that they are usually provided by the fusion of B-cells with myeloma lines at some cost. Polyclonal antibodies on the other hand originate from many B-cells and it is comparatively inexpensive to produce high affinity species in large quantities (Albert, 1984). However the antibodies are heterogeneous and able to bind more than one epitope on the antigen. There is also a possibility of cross-reaction between similar antibodies, especially if originating from the same species. It is therefore recommended to use different host animals for polyclonal capture and detection antibodies.

Moonoclonal antibodies are usually derived from murine spleen cell lines fused with murine myeloma cells (Kuus-Reichel *et al*, 1994; Fernandez and Moller, 1991; Shan *et al*, 1994). Other animals, such as hamsters, rabbits or rats, are sometimes used but often lack the availability of species-specific fusion cell lines. Mice also have many inbred strains so can usually provoke aggressive immune response upon immunisation in at least one strain, which results in efficient antibody harvesting. Rabbits, chickens, sheep and goats are useful for harvesting polyclonal antisera, where goats and sheep are particularly valuable for larger scale manufacture in terms of volume of serum available (Price and Newman 1997).

2.6.2.2 Solid-phase microtiter plate materials

Common plate materials are polyvinyl chloride for a more flexible plate and polystyrene for rigid plates. Although available in several sizes, most often a 96 well (8 x 12 well format) microtiter plate is used. Wells are available in flat-bottomed or round-bottomed styles but the latter is less suitable for spectrophotometric readings. Well volumes also range but are normally around 300-400 µl to accommodate for the addition of TMB and stop solution and to ensure that there is enough space for adequate washing. Observed difficulties are temperature gradients across the plates due to thermal insulation properties and concentration drifts across the plates due to pipetting time (Crowther, 2009). Microtiter plates are also the solid phase

material with the lowest antibody binding capacity. Reaction kinetics can however be sped up by incubation at 37°C and by vibration, which is most easily achieved by placing the plates on shakers.

2.6.2.3 Coating agents

Typically ELISA solutions are made up in phosphate buffered saline (PBS), which resembles bodily fluid conditions and is complementary to most proteins. Commercially they often contain sodium azide, which prolongs the potential storage life of the solution. In the coating procedure, the capture antibody passively adsorbs to the plastic wells by hydrophobic interactions. The aim of the coating buffer is to aid this interaction, which can be done by increasing the available hydrophobic residues displayed by the protein (Crowther, 2009). Plate-Protein interaction is dependent on the net-charge of the protein and partial denaturation. Changes in pH alters the balance and exposes more hydrophobic regions. A pH value a couple of units above the isoelectric point of the protein is a useful starting point. It is important that no other proteinacious material that can compete with the capture antibody is present in the buffer. Typical example of a coating buffer is given in Table 3.

Table 3. Carbonate coating buffer

Reagent	**Amount**
Sodium carbonate	5.3g
Sodium bicarbonate	4.2g
Sodium azide	1g
Distilled water	900 ml
Actions:	
• Adjust pH to 9.6	
• Adjust volume to 1L with distilled H2O	

2.6.2.4 Blocking agents

Sufficient blocking of the plate material is imperative in order to keep the sensitive reaction between antibody and antigen specific without interference. For example, non-specific binding of the detection antibodies to the plate material renders the results useless. Equally detrimental is a reaction between the detection antibody and the blocking agent. A good blocking agent must therefore be capable of saturation of any empty site of the coated plate whilst remaining passive in any further reactions taking place. The quality of the blocking buffer also determines

the level of background noise of the plate. This is reflected in the blank absorption value. A high blank OD reduces the sensitivity of the assay and the ability to detect and differentiate between low concentration samples.

Common blocking agents are: bovine serum albumin, foetal calf serum, casein and non-fat dry milk. Added to this list are synthetic blockers and carbohydrates. Polyvinyl alcohol and Ficoll® (a high molecular weight sucrose polymer) have been proven effective when there is risk of cross-reaction with the protein based alternatives (Huber *et al*, 2009). The inclusion of a gentle surfactant such as Tween 20 may also be beneficial to reduce possible interactions between the blocking protein and the capture antibody. A typical blocking solution is described in Table 4.

Table 4. Typical example of a blocking solution

Reagent	**Amount**
Phosphate buffered saline solution	500 ml
Sodium azide solution (10%)	1ml
Bovine serum albumin	5 g

2.6.2.5 Sample and conjugate components

The conjugate bears its name from the conjugation of the detection antibody to an enzyme. Horseradish peroxidase is the most widely used enzyme in ELISA procedures. Other options include alkaline phosphatase, ß-galactosidase and urease (Crowther, 2009). Detection antibodies must be carefully chosen to avoid cross-reaction with capture antibodies and/or blocking buffer. The purpose of the conjugate diluent is to inhibit passive absorption to the solid phase surface but enable an immunological reaction with the capture-body antigen complex. This can be done in two ways:

- Include another protein at high concentration to compete for solid phase binding.
- Include a detergent at low concentration, i.e. a blocking agent.

Sample and conjugate diluents also often contain a tris-buffered system as tris is considered a very effective inhibitor of further protein modifications. A typical sample and conjugate diluent is described in Table 5.

Table 5. A typical ELISA sample and conjugate diluent

Reagent	Amount
Trizma base (tris-HCl)	2.42g
Sodium chloride	8.77g
Sodium azide	1g
Distilled water	1 L
Actions	
• Adjust pH to 7.6 using 1M HCL	

2.6.2.6 Washing solutions

The aim of washing the plate is to remove unbound material from the wells without disturbing the attached antibodies. Due to the covalent nature of the bond between the solid phase and the antibody, some de-sorption can occur during the assay time. Vigorous washing could potentially lose some desorbed material, although this has not been reported as a common problem (Crowther, 2009). Washing solutions are therefore usually buffered to maintain isotonicity and keep optimal conditions for the formation of antibody-antigen complexes. Flooding the assay wells with solution can sometimes be sufficient for removal of unbound material although the inclusion of surfactants is also common; normally Tween 20 or Triton X. A disadvantage of surfactants is the generation of foam, which traps air and results in uneven distribution of solution. Potential denaturation of the antigen is another problem reported. A typical washing solution is described in Table 6.

Table 6. A typical ELISA washing solution

Reagent	Amount
Phosphate buffered saline solution	4L
Sodium azide solution (10%)	8ml
Tween-20	2ml

2.6.2.7 Substrate

ELISA substrates are chromogenic substances that are catalysed by the enzyme conjugated to the detection antibody. The TMB-HRP system was the only system used in this research. TMB functions by being an effective electron donor for the reduction of hydrogen peroxide on the conjugated horseradish peroxidase (Fig 2.6). The oxidation of TMB into the diimine form results

in a blue-green colour, measurable at a wavelength of 650 nm. Colour intensity is directly related to the quantity of enzyme-conjugated antibodies and subsequently directly related to antigen concentration.

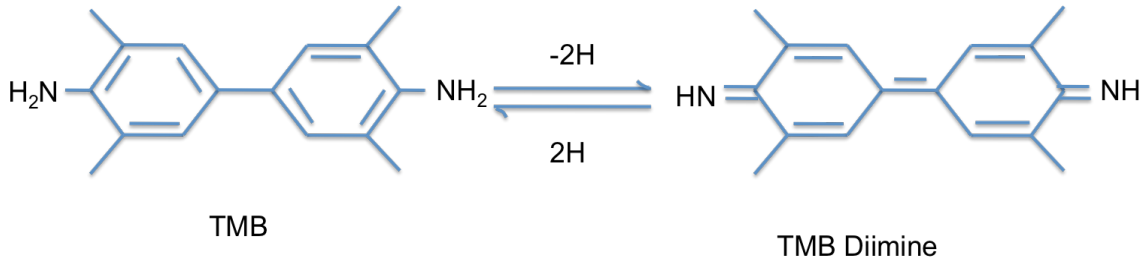


Fig 2.6. Reaction scheme of the oxidation of TMB to its blue coloured TMB diimine counterpart.

TMB is sensitive to light sources and fluorescent lighting resulting in degradation so any incubation must be carried out in the dark. The TMB is conventionally supplied as a ready to use solution or as solid discs to be dissolved.

2.6.2.8 Stop solution

The stop solution is conventionally a weak hydrochloric or sulphuric acid solution that halts the TMB catalysis and development of colour to prevent saturation. In the TMB-HRP system, this also alters the colour from blue to yellow, which can be analysed at 450 nm as opposed to the previous 650nm.

2.6.3. Materials used in ELISA development

- 8 channel pipettor with adjustable volume 100-300µl (Appleton Woods)
- Adjustable pipettors to cover volumes between 1-1000µl (Appleton Woods)
- Disposable pipette tips (Fischer Scientific)
- Solution container 60 ml (Appleton Woods)
- Microplates and microplate lids
    - Corning half area 96 well plate, well volume of 205µl (Sigma Aldrich).
    - Sero-wel, flatbottomed 96 well plate, well volume of 400µl (Appleton Woods).
    - Costar, flatbottomed 96 well plate, well volume of 360 µl (Fischer Scientific International).
- Moist box
- Paper towels

- Glass ware (volumetric flasks, beakers)
- pH meter
- Magnetic stirrer
- Balance
- Microplate reader (Molecular Devices, CA, USA)

2.6.4 Immunochemicals used in ELISA development

- Polyclonal goat anti-human albumin capture antibody (Sigma Aldrich)
- Polyclonal rabbit anti-human albumin antibody (Sigma Aldrich)
- Polyclonal goat anti-rabbit IgG horseradish peroxidase (Sigma Aldrich)
- Polyclonal rabbit anti-goat IgG horseradish peroxidase (Sigma Aldrich)
- Monoclonal mouse anti-human albumin capture antibody (Abcam)
- Polyclonal sheep anti-human albumin antibody horseradish peroxidase-conjugated (Abcam)
- Human serum albumin, Bovine serum albumin (Sigma Aldrich)

2.6.5 Buffers used in ELISA development

- Phosphate buffered saline
- Tris-buffered saline
- Wash buffer
- Conjugate solution/secondary antibody diluent
- Coat buffer
- Block buffer 1
- Block buffer 2
- TMB solution

Buffers for the ELISA protocol were prepared as detailed below. All buffer material was sourced from Sigma Aldrich (UK) unless otherwise stated. Stability of the solutions was varied due to the lack of sodium azide. Because of this, buffers were assessed visually for turbidity or contamination before use and were used fresh when possible.

2.6.5.1 Buffer preparation

Solution recipes for the preparation of buffers used in the ELISA development are described below in tables 7-13. The preparation of a TMB solution was more complicated than the other buffers and is described in more detail.

Table 7. Phosphate buffered saline

Reagent	**Amount**
Sodium chloride	8.0g
Monobasic potassium phosphate	0.2g
Dibasic sodium phosphate	1.15g
Potassium chloride	0.2g
Distilled water	900ml

Actions

- Adjust pH to 7.4 by dropwise addition of 1M NaOH or 1M HCl
- Adjust volume to 1L with distilled water

Table 8. Sodium carbonate/bicarbonate coating buffer

Reagent	**Amount**
Sodium carbonate	0.210g
Sodium bicarbonate	0.265g
Distilled water	50ml

Actions

- Adjust to pH 9.6 with the drop wise addition of 1M HCl

Table 9. Block buffer 1

Reagent	**Amount**	**Source**
Non-fat dry skimmed milk	3g	Tesco
PBS	100 ml	

Table 10. Block buffer 2

Reagent	Amount	Source
Stabilguard® Choice	5 ml	Surmodics, Inc
Distilled water	5 ml	

Table 11.Trisbuffered saline (TBS) sample diluent

Reagent	Amount
Trizma base (Tris-HCl)	2.42g
Sodium chloride	8.77g
Distilled water	1L
Actions	
• Adjust the pH to 7.6 with the drop wise addition of 1M HCl	

Table 12. Wash buffer

Reagent	Amount
Tween 20	1ml
PBS	1000ml

Table 13. Conjugate solution and secondary antibody diluent

Reagent	Amount
Tween 20	0.5ml
TBS	1000ml

TMB solution preparation initially required the preparation of a 0.1M citric acid solution (1.92 g citric acid in 100ml deionised water) and a 0.2M dibasic sodium phosphate solution (2.84g dibasic sodium phosphate in 100ml deionised water). These were combined to formulate a 0.05M phosphate citrate buffer by the addition of 25.7 ml 0.2M dibasic sodium phosphate to 24.3ml 0.1M citric acid and 50ml of deionised water. The pH was adjusted to pH 5 with 1M HCl if necessary. Two TMB substrate tables were dissolved in 2ml dimethylsulphoxide and this solution was added to 18ml of 0.05M phosphate citrate buffer. Immediately before use on the

ELISA assay, 2ml of fresh 30% hydrogen peroxide solution was added per 10 ml of substrate buffer.

2.6.6 Spectrophotometry instrumentation for ELISA analysis

The Spectramax M2 (Molecular Devices, California) was used for the measurement of sample optical density for ELISA analysis. It is a monochromator-based microplate reader (Fig 2.7), which is able to measure absorbance and fluorescence in various standard microplate sizes as well as in a cuvette. Absorbance wavelengths can be set between 200nm and 1000 nm, excitation wavelengths between 250nm and 850nm and emission wavelengths between 360nm and 850nm. Absorbance was read on ELISA plates at 450 nm according to commercial specifications.

Fig 2.7. The Spectramax M2 instrument used for ELISA analysis.

Sample absorbance, or optical density, is the quantification of the amount of light passing through solution compared to the amount of light available in the system. This includes the absorbance of the sample solution and any light scattering brought on by turbidity. In the absence of turbidity, optical density is equal to the sample absorbance calculated according to the Beer-Lambert equation below:

$$A = log\ (I_o/I)$$

Where, I_o is the incident light and I is the transmitted light of the sample. All samples analysed in this research were visually assessed to be absent of turbidity.

Sample data were analysed using the SoftMax Pro software, which is compatible with the SpectraMax M2. This allowed the application of the Path-check function, which further reduced the error-margins. Beer-Lamberts law states that absorbance is proportional to the distance that light travels through a sample. The distance for microplate wells is dependent on the resulting sample volume in the well and leaves room for discrepancy by careless pipetting. The Pathcheck function normalises all samples to a 1cm path length and therefore eliminates such factors. The sample and standard information was entered into the SoftMax Pro software, prior to analysis, and assay specifications were set to determine the endpoint OD at 450nm with Pathcheck turned on. The microplate was then placed in the microplate drawer and the OD was measured. The standard curve was altered to a 4-parameter configuration and the r^2 value was noted. If this was below 98 the standard curve was considered to be defective and the results could not be used. Resultant sample values were read from the curve and presented in ng/ml by the SoftMax Pro software. Final calculations to µg/ml were done manually taking the prior dilution factor into consideration. This was done according to the equation below:

*µg/ml concentration = (ng/ml concentration / 1000) * Dilution factor used.*

2.7 Immunology Consultants Laboratory ELISA

A commercial ELISA kit was chosen to perform the quantitative analysis of tear albumin needed; the E-80 AL Human Albumin ELISA kit from Immunology Consultants Laboratory (Oregon, USA). The sandwich ELISA kit has a measuring range of 6.25-200 ng/ml and requires 2.5 hours to run, including 100 mins of incubation time. The microtitre plate is a standard 96 well polystyrene plate with 12x8 removable micro well strips.

2.7.1 Materials for running the Immunology Consultants Laboratory ELISA

- 8 channel pipettor with adjustable volume 100-300µl (Appleton Woods)
- Adjustable pipettors to cover volumes between 1-1000µl (Appleton Woods)
- Disposable pipette tips (Fischer Scientific)
- Solution container 60 ml (Appleton Woods)
- Glass ware (volumetric flasks, beakers)
- Microplate reader (Molecular Devices, CA, USA)

2.7.2 Immunology Consultants Laboratory ELISA reagents

The commercial assay components were predominantly ready-to-use. All components were stored at 4°C and brought up to room temperature (>20°C) before analysis. The 96 well microtiter plate (coated with affinity purified polyclonal goat anti-human albumin antibodies), the TMB and the stop solution (0.3M sulphuric acid) were provided ready-to-use. Sample diluent was supplied as a 5X concentrate and diluted in deionised water (16 ml concentrate to 64 ml water) to achieve the final 1X solution. The wash buffer was supplied as a 20X concentrate and diluted 1/19 with deionised water (20 ml of concentrate to 380 ml of water) to reach the final 1X concentration. The solutions detailed above could be made up in advance but the enzyme-antibody conjugate and the human albumin calibrator could only be prepared a couple of hours before analysis because of lesser stability. The conjugate was supplied as un-diluted horseradish-peroxidase anti-albumin conjugate. The required dilution for one 8 well test strip was 10 µl of conjugate to 990 µl of sample diluent and this was multiplied to fit the total number of strips used. Standards for calibration were supplied as lyophilised human albumin. This was dissolved in 1ml of deionised or distilled water and then diluted to the necessary concentrations according to the protocol below (Table 14).

Table 14. Dilution protocol for the preparation of Immunology Consultants Laboratory ELISA albumin standards.

Standard	**Concentration (ng/ml)**	**Volume added to 1 x Diluent**	**Volume of 1X Diluent**
A	6255	10 µl Human alb calibrator	990 µl
6	200	22 µl standard A	666 µl
5	100	300 µl standard 6	300 µl
4	50	300 µl standard 5	300 µl
3	25	300 µl standard 4	300 µl
2	12.5	300 µl standard 3	300 µl
1	6.25	300 µl standard 2	300 µl
Blank	0		500 µl

2.7.3 Immunology Consultants Laboratory ELISA running protocol

- Tear samples were diluted 1:200 in sample diluent when enough tear fluid was available, otherwise, nearest possible dilution was used. The deviation was recorded so that the proper dilution factor was applied during analysis of the data.

- The standards and the samples were pipetted in duplicate into pre-designated wells on the microplate at a volume of 100µl / well. The plate was then covered with a plate lid and incubated for 60 minutes at room temperature

- The contents of wells were then emptied down the sink and the plate was banged dry against paper towels until no more wet spots appeared. The wash solution was subsequently pipetted into all wells at 300µl per well and the solution was emptied down the sink and banged dry. This was repeated three times for a total of four washes.

- 100µl of enzyme conjugated antibody solution was then pipetted into each well and the plate was covered and incubated for 30 minutes at room temperature. After the incubation, another wash cycle as the one previously described took place.

- 100 µl of TMB was then pipetted into each well and the plate was covered and incubated in the dark at room temperature for ten minutes.

- Lastly, 100µl of HCl stop solution was pipetted into each well and the absorbance was read on the Spectramax M2 at 450 nm.

2.7.4 Assessment of Immunology Consultants Laboratory ELISA

Although the lowest standard has a value of 6.25 ng/ml, OD comparison with the blank revealed the potential to identify even lower albumin concentrations by extrapolation of the curve. The actual sensitivity threshold was determined experimentally to be 1 ng/ml by additional serial dilution of standard 6, which leaves plenty of room for determination of low concentration albumin samples. Seven standard curves run on different days were also compared for inter-assay homogeneity (Fig 2.8), showing some disparity in terms of OD but consistency in shape.

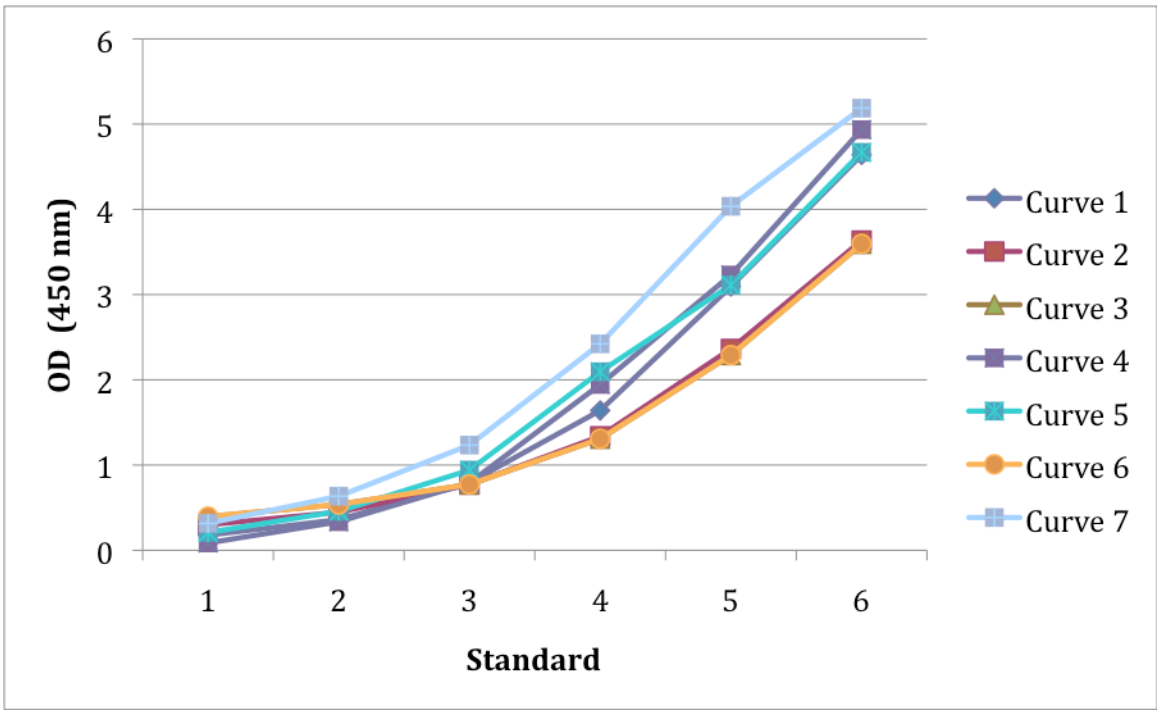


Fig 2.8. Comparison of standard curves from the Immunology Consultants Laboratory ELISA kits.

ICL Duplicate precision was well below 10% CV for the large majority of test samples assayed and the few high % CVs present were presumed to be results of human error. Samples that produced high %CVs were re-assayed at a later date and precision was then found to be satisfactory. Inter-assay precision and accuracy was found to be excellent, even with low concentration samples assayed two weeks apart (5.3 µg/ml, 5.13 µg/ml). The small difference in concentration can be attributed to loss through protein adhesion to the storage eppendorf or assay condition differences. Double dilutions of tear samples also provided accurate values. Although tear albumin concentrations differ significantly between studies the sensitivity and range of the commercial assay ensure that samples as low in concentration as 1µg/ml can be determined with confidence. This is sufficient for the detection of albumin in reflex tears, which suffers increased dilution with lacrimal gland fluid (detailed in chapter 3). Assayed tear samples also diluted in a linear manner confirming repeatability and accuracy in albumin determination. This supports the notion of utilising several different dilution schedules to account for differences in volume available without affecting the results.

The level of colour formation, and the resultant OD, is decided by the extent of the enzymatic reaction and the ambient temperature will affect the final OD value. A higher ambient temperature will speed up the reaction time and a higher OD will develop before the reaction is stopped. It is therefore reasonable to assume that some of the discrepancy between the curves in Fig 2.8 could be related back to temperature differences experienced in the laboratory. Batch to batch variation of assay components could also infer some differences between standard

curves. All albumin concentration results have been read off the standard curve present on the same plate as the samples. Any difference in colour development between plates will not affect the results as they are related back to their specific curve, which has been individually scrutinised every run. The ELISA technique is a viable option for the analysis of human albumin concentrations in tear samples and the ICL sandwich ELISA was found to be a suitable instrument for further albumin analysis.

2.8 Point-of-care technology (POC) for the measurement of tear albumin

Two point-of-care kits were evaluated for the determination of tear albumin in the research presented in this thesis: the HemoCue® Albumin 201 Analyser and the Afinion™ AS100 Analyser. Both instruments are designed for the quantitative measurement of albumin in urine. The intended use is for the detection of microalbuminuria, which is a specific marker for kidney dysfunction and also signifies elevated risks of vascular disorders and increased mortality (Viberti *et al*, 1982; Weir, 2007). Both instruments are compared and contrasted in detail with respect to tear albumin analysis in chapter 6 and thus only a very brief description of the instruments and the respective analysis methodology is provided in the sections below.

2.8.1. Principle of the Hemocue® Albumin201 Analyser assay (HemoCue AB, 2010)

The Hemocue® Albumin 201 analyser is fully automated and quantifies albumin concentration by turbidity measurements. The sample fluid is absorbed into disposable cuvettes that contain polyclonal rabbit anti-human albumin antibody. Any albumin present in the sample forms an aggregate with the antibody in the cuvette. The cuvette is then inserted into the HemoCue Analyser and the extent of aggregation is measured. This measurement is compared to a reference curve, derived from the standardised serum protein reference material CRM 470, and the resultant albumin concentration is calculated. The sensitivity of the aggregation reaction is enhanced by the incorporation of inert polymer particles into the cuvettes. These particles enlarge the complex formation and amplify the reaction so that lower levels can be detected. In total, the analysis time takes 1.5 min. The sample volume required for analysis is 18 µl and the assay range is 5-150 µg/ml. In addition to the HemoCue Analyser and the HemoCue cuvettes, the kit manufacturers supply two control solutions (AlbuTrol) to assess the upper and lower regions of the measuring range.

2.8.1.1. Materials for tear analysis by the HemoCue® Albumin 201 Analyser

- HemoCue® Albumin201 Analyser
- HemoCue albumin cuvettes containing polyclonal rabbit anti-human albumin antibody
- AlbuTrol control solution I (23 ± 10 µg/ml)
- AlbuTrol control solution II (76 ± 23 µg/ml)
- Parafilm
- Micropipettor (20 µl)
- Disposable plastic pipette tips
- Paper tissues

2.8.1.2. Running protocol for the HemoCue® Albumin 201 Analyser

- 20 µl of sample fluid was pipetted onto a small square of parafilm. Although the minimum specified volume is 18 µl it was found that any air bubbles present in the cuvette would ruin the analysis. The higher amount of 20 µl was therefore used as a precaution against this.

- The sample fluid was absorbed by the HemoCue albumin cuvette containing polyclonal rabbit anti-human albumin antibody. The sides of the cuvette was wiped with paper tissues to remove excess fluid and the cuvette was then placed into the albumin analyser.

- The analyser measured the light absorbance of the sample at 610 nm and the calculated amount of albumin was displayed in mg/L.

2.8.2 Principle of the Afinion™ AS100 Analyser assay (Kvam *et al,* 2007)

The Afinion™ AS100 Analyser is an automated instrument that uses chemical and mechanical assay methods to perform multiple analyses in individual test cartridges. All-in-one reagent cartridges are supplied specific for the entity to be analysed and are labelled with a barcode, which is scanned by the analyser to determine the assay procedure. The present research used the Afinion™ ACR cartridges, designed for the determination of albumin, creatinine and the albumin/creatinine ratio in human urine. The volume requirement of the ACR cartridge is 3 µl and the measuring range is 5-200 µg/ml. Two control solutions are also supplied to test the accuracy of the system. The analysis is performed using a solid phase immunochemical assay contained in the cartridges. A detachable microcapillary is used to absorb the sample and is then replaced into the cartridge, where the sample is soaked through a membrane coated with

monoclonal antibodies. The membrane is then sequentially soaked with conjugate solution containing ultra-small gold particles and lastly a washing solution. Simultaneously, the sample is analysed for creatinine using enzymatic colorimetry. The resulting colour intensity of the membrane is measured using a digital camera.

2.8.2.1 Materials for tear analysis by the Afinion™ AS100 Analyser

- Afinion™ AS100 Analyser
- Afinion™ ACR cartridge
- Control solution 1 (9.9 – 15.8 µg/ml)
- Control solution 2 (67.6 – 101.4 µg/ml)
- Parafilm
- Micropipettor (4µl)
- Disposable plastic pipette tips

2.8.2.2 Running protocol for the Afinion™ AS100 Analyser

- 4 µl of sample fluid was pipetted onto a small square of parafilm.
- The detachable microcapillary was removed from the cartridge and used to collect the sample fluid by capillary action.
- The microcapillary was then returned to the cartridge and the cartridge was transferred into the Afinion™ AS100 analyser.
- After 3.5 minutes results were displayed on the analyser in mg/L.

CHAPTER 3
INTERPRETATION OF TEAR-BORN ALBUMIN DATA: THE INTERRELATIONSHIP OF SAMPLING TECHNIQUE, ASSAY TECHNIQUE, AND OCULAR DISORDERS OR INSULT

3.1 Aim

The aim of the research presented in this chapter was to critically assess the disparities in published tear albumin concentrations and to use that information to design subsequent studies. Objectives of the research were therefore to establish what factors affect the determination of a true albumin baseline scenario and what conditions have an added impact on the resultant sample value. Because of the scattered nature of reported tear albumin concentrations and lack of previous collation and analysis of the data, this was a very significant task.

3.2 Introduction

Blood-borne albumin has been extensively researched but albumin in tears has received less focused attention. Reported tear albumin concentrations have not been evaluated collectively and the role and function of albumin in the tear film remains unclear. Early published tear albumin concentrations were obtained using less sensitive, or semi-quantitative techniques, (Berman *et al*, 1973; Janssen and Van Bijsterveld, 1986) and subsequently, are less accurate than results obtained from modern techniques, such as ELISA, high-pressure liquid chromatography and mass spectrometry. The heightened sensitivity provided by modern analytical techniques have made it more attainable to accurately determine albumin concentrations and consequently to investigate the purpose of albumin in tears. However, the investigation of albumin also requires the understanding of natural variations and external factors that affect concentrations. Several conditions have been identified to be related to higher albumin concentrations. These include: ocular disorders (Sen and Sarin, 1986b), contact lens wear (Lundh *et al*, 1984) and the closed eye scenario (Sack *et al*, 1992). It is intuitively predictable that conditions affecting the integrity of the blood tear barrier would result in increased plasma influx but the specific links between external factors and albumin concentrations are still unclear. Sampling materials, in particular the Schirmer strip, have also been correlated to an increase in serum protein concentrations (Stuchell *et al*, 1984) although the level of impact has yet to be fully determined. The most recent review relating to tear-borne albumin focuses mainly on contact lens deposition phenomena (Luensmann and Jones, 2008). Consequently, albumin levels in tears have remained poorly defined, as the available data are not completely understood. Available

information on reported albumin levels in tears has been assembled here in an attempt to summarise the modest amount of information that exist regarding this major plasma protein in tears. Factors affecting albumin concentrations in tears have been identified and compiled in order to assess the conflicting nature of the published albumin concentration data. These variables will be dealt with individually in the chapter and are:

- Tear flow rate (including externally stimulated induction of reflex tears, e.g. use of onion, ammonia vapour and yawning)
- Time of day (closed eye, overnight tear film)
- Method of collection (e.g. microcapillary, Schirmer strip)
- Ocular insult or disorder (e.g. dry eye, corneal ulcer and contact lens wear)
- Assay technique (e.g. immunodiffusion techniques, ELISA and SDS-PAGE)

The implications of individual determinations of albumin tear concentrations are difficult to determine unless the factors that affect them are fully comprehended and taken into account. The Interrelationship between the different variables e.g. sampling technique, ocular health and contact lens wear is also of importance as influences on albumin concentration variation in the tear film may be multifactorial.

3.3 Effect of tear flow rate on measured tear albumin concentration

3.3.1 Introduction

Tear volume and tear protein composition are dependant on the interaction between the autonomic nervous system and the endocrine system alongside localised effects by cytokines. The eye can exhibit different states during tear collection, which produce distinguishable differences in the qualities of the sampled tear fluid. States are characterised by the effect they have on tear flow rate and tear protein composition and can be divided into:

- Basal tears
- Reflex or stimulated tears
- Emotional or psychogenic tears

Basal tears are defined as the normal tear production required to replenish the continuous turnover of tear fluid. Tear flow rates close to the innate tear production of 1-2 µl/min are therefore representative of basal tears. Reflex tears demonstrate increased volume from the lacrimal gland, often due to physical stimuli such as ocular irritation. The increased volume

provides flooding of the system and removal of potential pathogens. Reflex tear flow rates cited in literature vary but tend to lie within 20-50 µl/min (Berta, 1986; Fullard and Tucker, 1991; Sitaramamma *et al,* 1998a). Emotional stimulus induces excessive lacrimation, similar to physical stimulus, but exhibits significant differences in protein composition (Frey *et al,* 1981).

3.3.2 Effect of tear flow rate on measured total protein concentration

The aim of this section is to describe how total tear protein and other tear components are affected by alterations in tear state and tear flow rate. These data will subsequently be compared to the variations in tear albumin concentrations with flow rate and tear state. Table 15 shows a cross-section of reported total tear protein concentrations collected in the three tear states. Only studies that used atopic subjects and microcapillary tubes to collect samples were included in Table 15 to keep sampling technique consistent across the comparisons.

Table 15. Total protein concentration in atopic normal subjects collected by microcapillary, distinguishing between basal, stimulated and emotional tears. (n/s = not stated).

Type of tear	Total Protein composition (mg/ml)	Population (n=)	Reference
Basal	11.7 ± 2.5	11	Fukuda *et al*, 1996
	7.3	10	Fullard and Tucker, 1991
	9.0 ± 6.6	6	Sack *et al*, 1992
	7.14 ± 2.22	16	Markoulli *et al,* 2011
	7.01 ± 1.8	8	Green-Church *et al*, 2008
	4.18 ± 1.92	11	Glasson *et al*, 2006
	7.37 ± 1.54	101	Gachon *et al*, 1982
	11.3 ± 2.7	11	Baleriola-Lucas *et al*,1997
Reflex (Stimulated)			
Nasal applicator/dilute ammonium hydroxide vapour	5.0 - 3.9	10	Fullard and Tucker, 1991
Nasal applicator	5.8 ± 1.0	7	Fukuda *et al,* 1996
Physically holding eye open	6.0 ± 2.5	6	Sack *et al*, 1992
Nasal stimulation cotton bud	6.01 ± 2.11	16	Markoulli *et al,* 2011
Ammonia vapour	4.6 - 6.9	5	Coyle *et al*, 1989
Cloroacetophenone or onion vapour	5.47	61	Frey *et al,* 1981
Emotional	6.50	42	Frey *et al*, 1981

Concentrations for the different tear states are consistent across studies, especially in the stimulated eye. This makes it reasonable to assume that they represent the true environment. Based on the studies represented in Table 15, mean total protein in basal tears was calculated to be 8.1 mg/ml. The mean total protein concentration in reflex tears was 5.6 mg/ml. Stimulated reflex tears are consistently lower in total protein concentration as the increased volume leads to overall dilution of the proteins present in the basal state (Fullard and Snyder 1990). A clear comprehensive picture of the normal healthy eye might therefore not be available during reflex tearing as smaller levels of proteins could be diluted into a range where they are undetectable.

The stimulation of reflex tears can be brought about by a variety of procedures that increase tear flow, and can be chemical, physical or physiological, as illustrated in Table 15. It is important to recognise that tear proteins are not uniformly affected by external stimulation. Lactoferrin, lipocalin and lysozyme maintain a constant proportion of total tear proteins during reflex tearing (Fullard and Snyder, 1990). SIgA on the other hand is significantly reduced from 15.5±3.2 % to 9.5±3.5 % of total tear protein during reflex lacrimation (Sitaramamma *et al* 1998a). The different dilution pattern of sIgA to the other three major lacrimal gland produced tear components is due to the different secretion pathways mentioned in chapter 1 (section 1.3.3.1).

Emotional tears also have decreased total tear protein content (6.50 mg/ml) compared to basal tears (8.1 mg/ml) but not as low as externally stimulated tears (5.6 mg/ml). This could be a reflection of the limited studies on emotional lacrimation or alternatively, the psychogenic response might not be as strong as direct physical insult. A lower tear flow compared to reflex tearing by physical stimulus would lead to less flooding of the system and the marginally higher tear protein concentrations reported in emotional tears.

3.3.3 Effect of tear flow rate on measured albumin concentration

In the same way as total protein, serum albumin is detected in higher concentrations in low flow rate tear samples and lower concentrations in stimulated tears. One study reported a decrease from 750µg/ml at ~1µl/min to 250µg/ml at a flow rate of 20µl/min, following increased nasal stimulation with 80% ethanol (Berta, 1986). Similar experiments using nasal stimulus, although with dilute ammonia vapour, also found a decrease in albumin concentration with increasing flow rate (Fullard and Tucker, 1991). A significant decrease from 42.0µg/ml in non-stimulated basal tears to 3.0µg/ml, collected at a flow rate of 50µl/min, was observed. As opposed to the total tear protein data exhibited in Table 15, tear albumin concentrations measured in basal and reflex tearing are not consistent between studies. This is apparent by comparing the data from Berta (1986) and Fullard and Tucker (1991), who determined albumin concentrations in basal tears to be 250 µg/ml and 42 µg/ml, respectively. Assay methodology and tear collection technique are further variables, which affect tear albumin concentration and these are discussed in subsequent sections.

Interestingly the study done by Frey *et al* (1981) on emotional tears (Table 15) also measured the albumin fraction of total tear protein. The mean concentration of total protein in emotional tears was 6.50 mg/ml compared to the irritant induced reflex tear concentration of 5.47 mg/ml. This represents a difference in total protein of 16% between the two states. The albumin

fraction was however 24% higher in emotional tears than in irritant induced reflex tears. Further research into these two states with respect to albumin would be beneficial as the studies are very limited.

3.3.4 Summary of effect of tear flow rate on measured albumin concentration

Even though further study is necessary, it is clear that albumin concentrations in the tear film, and its relative fraction of total tear protein, are able to vary over large ranges in response to tear flow stimulus. The full effect of tear flow stimulation is difficult to de-convolute from other variables, such as tear collection and assay methodology. This is apparent when comparing measured tear albumin concentration collected under both basal and reflex tearing conditions. Some doubts exist about whether there is such a thing as a true basal tear or whether all tear fluid has some fluid resulting from the reflex lacrimation pathways (Jordan and Baum 1980). To avoid confusion, basal tear in this thesis denotes tears, which were visibly un-stimulated during the time of collection and have a flow rate comparable to that reported by Mischima (1966) (1.2 µg/min). Other tears will be described as reflex tears, although details, regarding stimulation are given to distinguish between modes of collection. Tears that exhibit some degree of increased stimulus but not reaching the flow rate of 20-50 µl/minute are described as medium flow rate tear samples.

3.4 Effect of prolonged eye closure on measured tear albumin concentration

3.4.1 Introduction

The closed eye environment is a unique state with vastly different tear composition compared to the open eye and more research is required to fully understand its implications. It can be described as exhibiting the opposite attributes of stimulated tear flow. The closed eye tear film is characterised by decreased or stagnant tear flow, due to lack of blinking and lack of refreshment of tear fluid during sleep (Sack *et al*, 1992). Total protein concentrations determined in the closed eye are 18.0±7.4mg/ml and 15±3mg/ml (Sack *et al*, 1992; Sitaramamma *et al*, 1998a). The significant increase in total protein concentration during the closed eye could be attributed to the stagnant tear film environment with limited tear component turnover and drainage. It could also be representative of a beneficial active ocular defence mechanism involving the up-regulation of sIgA, complement proteins and albumin.

3.4.2 Effect of prolonged eye closure on measured tear albumin concentration

Despite the research interest in closed eye tear fluid, quantitative information of albumin concentration in this environment is scarce. Sitaramamma *et al* (1998a) used HPLC to analyse SigA and albumin in the closed eye tear fluid but results were only presented as a combined fraction percentage of total protein. Albumin concentrations measured at hourly intervals throughout the sleep cycle have been shown to increase progressively and to reach statistical significance after 5 hours of sleeping (Tan *et al,* 1993). A figure in the paper by Tan *et al* (1993) showed albumin concentrations between 800 and 1000 μg/ml at this point of statistical significance but no definite value was given. Only two studies have been identified that provided quantified albumin concentrations in the closed eye tear fluid. These are presented in Table 16.

Table 16. Albumin concentrations in the closed eye sampled by microcapillary.

Closed eye (μg/ml) (*Range where given)*	Population	Time of day stated	Analysis method	Reference
200 *(150 - 580)*	8	Y	SDS-PAGE	Choy *et al*, 2004
1100 ± 760	4	Y	Immunofixation, Immunodiffusion	Sack *et al,* 1992

The magnitude of albumin increase in the closed eye is significantly different between the two studies. Research done by Sitaramamma *et al* (1998a) on total tear protein in the closed eye reported a decrease from 15 mg/ml at first time of eye opening to 12, 9, 6 and eventually 4 mg/ml with consecutive collections over seven minutes. Thus, collecting the sample a couple of minutes after eye opening could result in the decreased levels reported by Choy *et al* (2004) compared to Sack *et al* (1992). Both studies have however detailed that sampling was conducted as soon as possible after waking, making acclimatisation a less likely cause for the disparities seen in concentration. Disparities in the reported closed eye concentrations in Table 16 are therefore most likely due to differences in analysis method and/or population as the same tear collection method was employed.

3.4.3 Summary of effect of prolonged eye closure on albumin concentration

The closed eye is characterised by its increase in albumin and other components such as sIgA, plasmin, vitronectin and pro-inflammatory mediators. The magnitude of albumin increase in the

closed eye tear film varies between reports but it appears to increase progressively during prolonged eye closure. Tear fluid sampled immediately post-waking represents the closed eye tear sample and exhibits increased albumin levels, compared to the open eye state.

3.5 Effect of tear collection technique on measured tear albumin concentration

3.5.1 Introduction

Difference in sampling technique has been implied to contribute to variation in albumin concentration (Stuchell, 1984; Fullard and Snyder, 1990; Esmaeelpour, 2008). Protocols and materials available for tear sampling are varied and include both absorptive and non-absorptive techniques. Most common absorptive techniques are: Schirmer strips, filter paper discs, sponges and polyester wicks. Non-absorptive techniques are microcapillaries and glass pipettes. Schirmer strip, filter paper and wick techniques collect tear fluid by direct contact with the eye. Filter paper disks are placed in the tear fluid between the lower eyelid and the conjunctiva whereas Schirmer strips or wicks are inserted between the lower eyelid and the conjunctiva (section 2.4.2). Microcapillary, glass pipettes and sponge techniques, on the other hand, collect tear fluid by capillary action or absorption from the marginal tear strip. Samples are taken from the lateral canthus of the eye and contact with the conjunctiva is avoided (section 2.3.2).

Effect of sampling technique on tear proteins can be related back to two factors: stimulation of tear flow and invasiveness. Stimulation of tear flow has already been discussed in section 3.3.2. What is meant by invasiveness is how much the technique irritates the eye and provides physical stimulus. Irritation of the conjunctiva leads to stimulation of the blood-tear barrier and transudation of plasma proteins into the tear fluid. The increase of plasma proteins levels in tears upon ocular irritation is a well-known concept, which has been shown repeatedly in studies (Josephson and Lockwood, 1964; Stuchell, 1984; Fullard, and Snyder 1990; Fullard and Tucker, 1991; Green-Church, 2008). Invasiveness can therefore lead to misrepresentation of the levels of proteins found in the normal healthy eye. Degree of invasiveness is of particular importance for albumin sampling and requires careful control.

3.5.2. Effect of tear collection technique on measured tear albumin concentrations

Reported tear albumin concentrations vary considerably between studies and also between sampling techniques used. Table 17 shows the range of albumin concentration in the literature obtained from normal asymptomatic controls as a function of collection tool and stimulus. Concentrations are listed in increasing order of magnitude irrespective of sampling technique.

Table 17. Albumin concentration in asymptomatic subjects as a result of collection technique and stimulus

Concentration mean (µg/ml) ***(Range where given)***	**Collection method**	**Stimulus**	**Size of study population (n =)**	**Time of sampling recorded? (Y/N)**	**Analysis method**	**Reference**
8.41 ± 1.12 - 3.00 ± 0.61	Micropipette	Sneeze reflex (with increasing stimulus)	10	N	SE-HPLC, ELISA	Fullard and Tucker, 1991
10.18 ± 2.95	Microcapillary	Contact lens wear	4	N	ELISA	Balasubramanian et al, 2012
12.1± 5.5 *(1.6-24.0)*	Microcapillary	Strong light	76	N	Immunodiffusion	Gupta et al, 1988
12.2 ± 3.8	Micropipette	Sneeze reflex	30	N	SE-HPLC, ELISA	Fullard and Snyder, 1990.
16 ± 15	Microcapillary	Eye flush method	19 eyes	Y	SDS-PAGE	Ng et al 2000a
18 ± 60 *(0-290)*	Microcapillary	Ammonium hydroxide vapour	12	N	Immunochemical methods	Stuchell et al, 1984
20 ± 10	Microcapillary	Physically holding eye open	4	Y	Gel electrophoresis	Sack et al, 1992
20 *(10-50)*	Microcapillary	Yawning	8	Y	SDS-PAGE	Choy et al, 2004
23 ± 15	Microcapillary	Yawning	11 eyes	Y	SDS-PAGE	Ng et al, 2000a
59.2 ± 54.0	Microcapillary	Contact lens wear	16	Y	ELISA	Baleriola-Lucas et al, 1997
4.4 -125	Cellulose sponge	Organic gases and vapours	62	Y	Microelectroimmunoassay	Thygesen et al, 1987
380 ± 640 *(330-1480)*	Periopaper	N/A	57	N	Electro-immunochemical methods	Farris, 1985
600	Schirmer strip	Saline drops	19	N	Radioimmunoassay	Proud et al, 1990
600 *(100-4200)*	Filter paper discs	N/A	40	N	Radial immunodiffusion, SDS-PAGE	Van Bijsterveld and Janssen, 1981
660 ± 460	Schirmer strip	N/A	12	N	SDS-PAGE, Maldi-TOF	Li et al, 2010
750 -250	Microcapillary	80% Ethanol (with increased stimulus)	60	N	SDS-PAGE	Berta, 1986
800 *(200-6600)*	Filter paper discs	N/A	30	N	Radial immunodiffusion	Van Bijsterveld and Janssen, 1986
900 *(100-15800)*	Filter paper discs	N/A	23	N	Radial immunodiffusion, SDS-PAGE	Janssen and Van Bijsterveld 1986
980 (10-4200)	Periopaper	N/A	31	Y	Rocket electrophoresis	Farris, 1986
1000 *(<100-3000)*	Filter paper discs	N/A	11	N	Radial immunodiffusion	Janssen and Van Bijsterveld, 1983
1240 ± 1420 *(0-4500)*	Schirmer strip	N/A	12	N	Immunochemical methods	Stuchell et al, 1984
1510 *(10-4550)*	Schirmer strip	N/A	31	Y	Rocket electrophoresis	Farris et al, 1986
1830 ± 2440 *(860-3320)*	Schirmer strip	N/A	57	N	Electro-immunochemical methods	Farris, 1985

In general, the higher albumin levels were determined in conjunction with Schirmer strip collection and the lowest detected albumin concentrations were collected by microcapillary. It should be noted that results derived from filter paper disc sampling all originate from the same research group (Janssen and Van Bijsterveld), which could bring some bias to the results. Mean albumin concentration reported by stimulated microcapillary collection is 86.2 µg/ml compared to 954.5 µg/ml with filter paper techniques. Here it is evident that the harsher collection tools such as filter paper discs and Schirmer strips cause irritation and present the highest concentration detected. Schirmer strip tear protein profiles have been found to contain more cellular products (Green-Church *et al*, 2008) and more low molecular mass peaks than the profiles of subjects sampled by microcapillary (Grus *et al* 2005). Similarly, samples taken by microcapillary result in a lower concentration of endogenous antibodies compared to Schirmer strip samples (Remington *et al*, 2009). These results further support that the insertion of filter paper into the eyelid margin is able to disrupt the ocular epithelium, leading to increased availability of albumin and cellular components. Schirmer strip tear sampling has long been reported as an invasive uncomfortable experience. Even back in 1982 it was suggested that surgical sponges or filter paper should be avoided for the collection of tears, particularly when investigating albumin where higher values will occur due to irritation (Gachon *et al*., 1982).

Lower albumin concentrations reported in the literature (Table 17) were almost invariably collected by microcapillary under stimulus. The highest stimulated microcapillary concentration recorded was by Berta (1986) at 750 µg/ml. This significantly different albumin concentration compared to other microcapillary results may originate from a number of causes, the most likely being the mode of stimulus used. The presentation of 80% ethanol into the nasal passage is potentially enough irritation to disturb the blood-tear barrier and result in increased serum leakage. Prolonged reflex tearing has also been shown to produce an increase in albumin and other plasma protein levels (Fukuda *et al,* 1996). The assay technique used by Berta is replicated in a number of other studies detecting much lower albumin levels and is therefore unlikely to be a dominant factor. Excluding the possibly erroneous results of the study by Berta (1986), which used harsh stimulation techniques, the mean stimulated microcapillary concentration is reduced even further to 19.9 µg/ml. Compared to 19.9 µg/ml, concentrations sampled by Schirmer strip and filter paper discs represent an almost 50 times increase in reported albumin levels reported in normal subjects. The vast differences seen in albumin concentration between Schirmer strip and microcapillary techniques are however unlikely to be due to the dilution of protein by stimulus in microcapillary pipette samples. This is exemplified in Table 18, which shows albumin concentrations in normal asymptomatic tears sampled by microcapillary without external stimulation. The concentrations are tabulated in order of increasing magnitude.

Table 18. Albumin concentration in non-stimulated tears from normal healthy control subjects collected by the non-invasive microcapillary pipette technique.

Concentration mean (μg/ml) *(Range where given)*	Size of study population (n=)	Time of sampling recorded? (Y/N)	Analysis method	Reference
10.3 ± 4.6 *(1.4-21.6)*	53	N	Microelectroimmunoassay	Prause, 1983
10.3 ± 9.2	n/s	Y	Microelectroimmunoassay	Thygesen, *et al* 1987
11.85 ± 5.97	50	N	Immunodiffusion	Sen and Sarin, 1986a
14.60 ± 8.60	11	N	ELISA	Balasubramanian *et al,* 2012
20.50	24	N	Immunodiffusion	Zavaro *et al,* 1980
21 ± 28	30	Y	SDS-PAGE	Ng et al, 2000a
~ 40	n/s	N	n/s	Bron and Mengher, 1989
42.0 ± 11.7	30	N	SE-HPLC, ELISA	Fullard and Snyder, 1990
42.0 ± 4.7	10	N	SE-HPLC, ELISA	Fullard and Tucker, 1991
45.1 ± 32.5	11	Y	ELISA	Baleriola-Lucas *et al*, 1997
54	88	N	SDS-PAGE, Behring laser nephelometer	Gachon *et al*, 1982
60± 20	4	Y	Immunofixation, Immunodiffusion	Sack *et al,* 1992
10-200	4	N	Immunodiffusion	Berman *et al*, 1973

n/s = not stated

Albumin concentrations in Table 18, collected by microcapillary only, display a considerably narrower range of concentrations obtained between different studies (10.3-200 μg/ml) compared to Table 17 (8.4–1830 μg/ml). Mean calculated albumin levels in Table 18 are 29.4 μg/ml. Collection by microcapillary pipette evidently exerts least direct influence on relative protein concentration in resultant tear samples. The compiled concentrations in Table 18 represent the available literature of albumin tear collections that can be described as basal tear fluid. Interestingly, this evaluation of concentrations in normal asymptomatic tears sampled by microcapillary shows that a level of variation is still apparent even when sampling is kept consistent.

3.5.3 Summary of effect of tear collection technique on measured tear albumin concentration

Choice of sampling technique has a profound impact on the measured tear albumin concentrations. More invasive tear collection methods result in plasma influx and significantly increased tear albumin concentrations. Collection by microcapillary produced the lowest albumin concentrations and less difference between studies compared to harsher filter paper techniques. Stimulation of tear flow by sampling technique reduced tear albumin concentrations as discussed in section 3.3.3. The combined effects of stimulation/irritation, which directly affect

albumin concentration, and of stimulation, which increases tear flow are difficult to de-convolute and add to the complexity of assessing albumin concentrations.

3.6. Effect of sampling material on measured tear albumin concentrations

3.6.1 Introduction

Some sampling techniques (Schirmer strips, filter paper disks, wicks and sponges) require extraction, as they collect tears by the absorption of fluid onto a solid substrate. When using these methods, the extraction efficiency of the tear fluid must be taken into account in addition to any direct material influences on the tear film. Several different extraction possibilities exist, which produces further variables for tear sampling studies and makes comparisons of protein concentration more complex. Table 19 shows the available extraction protocols reported in the literature.

A closer investigation of these protocols revealed a common feature between studies, of lack of attention to extraction methodology design. An example is the group of Shoji *et al,* (2003), who investigated eosinophil cation protein concentrations, sampled by Schirmer strips, in subjects with Sjögren's syndrome or allergic conjunctivitis. Only the first 5 mm of the Schirmer strip was used for analysis and was incubated in phosphate buffered saline surfactant solution for three hours at room temperature. Shoji *et al*, stated that the recovery efficiency of this elution procedure was above 80% with respect to eosinophil cation protein but there are no details on how this was measured apart from unreferenced preliminary studies.

Table 19. Extraction protocols for the determination of tear proteins sampled by absorptive tear collection techniques.

Extraction Protocol	**References**
Phosphate buffered saline with and without surfactancts	Janssen and Van Bijsterveld (1981, 1983, 1985, 1986) Grus *et al,* (2005) Shoji *et al*, (2003) Zhou *et al,* (2008) Kramann *et al*, (2011) Denisin *et al*, (2012)
Sodium chloride buffer with surfactant	Van der Meid,(2011)
Ammonium bicarbonate buffer with acetone precipitation	Green-Church *et al*, (2008) Lema *et al,* (2010) Li *et al*, (2010)
Sodium barbitol buffer	Prause (1979)
TMED-acetic acid buffer	Stuchell *et al* (1984) Farris (1985, 1986)
Ethylebediaminetetracetic acid (EDTA)	Proud et al, 1990
Centrifugation	Thyegesen *et al,* (1987) Tuft and Dart, (1989) Jones *et al*, (1997) Afonso *et al*, (1999) Solomon *et al*, (2001) Koo *et al*, (2005) Remington *et al*, (2009) Kim et al, (2012)

Several other studies showed distinct neglect to comment on the reasoning behind the protocol chosen and the extraction efficiency with respect to the investigated protein. Discussions of any error or variation that the protocol could add to the results were also deficient. Research studies that did report investigations of material influence are discussed below with their relative approaches.

3.6.2 In vitro spike and recovery

In vitro spike and recovery of proteins from the substrate provides the extraction efficiency for the specific protein of interest. Van der Meid (2011) assessed cytokines and matrix

metalloproteinases (MMPs) in Schirmer strips from clinical studies. Samples were eluted in a 0.5 M sodium chloride 0.5% Tween 20 buffer and efficiency of protein recovery was determined on in-vitro spiked Schirmer strips. A known concentration of sample was added to the Schirmer strip and then compared to the eluted concentration. Results showed that cytokines on average have an extraction efficiency of 62.3% and MMPs an average of 79.6% under this protocol.

3.6.3 Comparison with microcapillary sampling

Another widespread method of assessing extraction protocols is to compare electrophoretic profiles between the extracted sample and a microcapillary sample (Tuft and Dart, 1989; Jones *et al,* 1997; Grus, *et al* 2005; Green-Church *et al*, 2008; Remington *et al*, 2009). Whilst this approach does not provide quantitative information of protein recovery it can assess if inherent ratios between tear proteins remain similar between the two techniques. Studies done by Tuft and Dart (1989) and Jones *et al*, (1997) showed comparable concentrations between centrifuged sponge samples and microcapillary samples. The entitites studied were IgE (Tuft and Dart) and lactoferrin and ephithelial growth factor (Jones *et al*). The groups of Green-Church, Remington and Grus all found differences between tear protein profiles with Schirmer strip and microcapillary sampling techniques. The major lacrimal gland proteins remained similar, but Schirmer strip samples contained more cellular products, endogenous antibodies and low molecular weight entities compared to microcapillaries.

3.6.4 Volume recovery

Centrifugation, with or without added buffer, is the dominating technique for the extraction of sponge materials but has also been used for Schirmer strip analysis. Collection efficiency of sponges by centrifugation is often calculated by volume recovery. Studies by Esmaeelpour *et al*, (2008) and Lopez-Cisternas *et al,* (2005), attempting to develop new absorptive tear collection materials, compared a variety of substrates. Their results are shown in Table 20 and concern volume recovery rather than specific protein recovery. References are denoted by author initial.

Table 20. Comparison of collection efficiency by centrifugation in various sponge materials.

	Cellulose acetate rod	Poly urethane	Poly vinyl acetyl	Polyester	Cellulose sponge
Collection efficiency (%) (Ref)	76 ± 1.5 (E)	88.6 (L-C)	58.2 (L-C)	82.2 ± 2 (E)	42 ± 31 (E) 53.4 (L-C)

Here it can be seen that significant differences exist between substrates and that no collection protocol was 100 % efficient even without taking specific protein adsorption into account.

3.6.5 Effect of sampling material on measured tear albumin concentration

Albumin concentrations determined by absorptive techniques are detailed in Table 17 and protocols used are shown in Table 19. Similar to other published tear protein investigations using absorbable techniques, extraction efficiency was often neglected in the description of methodology for tear albumin analysis. Extraction protocols used for the determination of tear albumin are described in the subsequent section, which, is followed by the only two studies that provide actual efficiency data for the extraction of tear albumin.

3.6.5.1 Extraction protocols for the determination of tear albumin sampled by absorptive techniques.

Li *et al,* (2010) used a combination of 100Mm ammonium bicarbonate elution buffer and centrifugation at 14, 000 g for 10 min to elute Schirmer strips from controls and subjects with allergic conjunctivitis. Sample volumes of the Schirmer strips were produced by in vitro additions of known concentration solution of 1mg/ml of BSA and differences in weights between dry and wetted strips. Differences between the two study groups were apparent in total protein concentration as well as albumin concentration. Stuchell *et al* (1984) and Farris (1985, 1986) also performed in vitro spiking of Schirmer strips to assess tear volume, although with a different study setup and elution protocol. Schirmer strips were incubated in TMED-acetic acid buffer (0.01M N,N,N'N tetra-methyl-1-2-deamoethane in 0.029 M acetic acid) at a pH of 5.0. Volume of elution buffer was measured to represent a 1:10 dilution of the calculated tear volume. The aim of the study by Stuchell *et al* was to compare Schirmer strip to microcapillary sampling and differences in concentrations of IgG, transferrin and albumin were found between the two techniques. Studies by Farris on the other hand investigated contact lens wear and keratoconjunctivitis sicca and found no difference in serum albumin compared to controls. Whilst the in vitro spiking of Schirmer strip and the resultant wetting length standards help quantify the volume collected, neither Li *et al*, Stuchell *et al* nor Farris focused on extraction efficiency.

Proud *et al*, (1990) used a different protocol for the elution of Schirmer strips to investigate the ocular allergic response. 300 µl of ethylenediaminetetracetic acid (EDTA), at a concentration of 40 mMol/L in saline, were added to the Schirmer strips prior to storage. Volume collected was

measured by comparison the weight of strips before and after sampling had been conducted. The extraction efficiency of the protocol was not detailed but the group were able to show differences between subjects receiving an allergic challenge (by allergens such as ragweed pollen and cat hair) and controls. Albumin concentrations sampled in the studies by Janssen and van Bijsterveld (1981, 1983, 1985, 1986) using filter paper discs, were extracted by a 1 in 4 dilution of tear fluid in phosphate buffered saline but extraction efficiency was not determined. Thygesen *et al* (1987) measured albumin in samples collected from cellulose sponges centrifuged at 2500 g for five minutes. They were able to identify increased serum albumin in study groups exposed to organic gases and vapours at fixed intervals, compared to controls. The extraction efficiency of the cellulose sponges was however not discussed.

3.6.5.2 Studies recording actual extraction efficiency of tear albumin from the Schirmer strip

An early study by Prause (1979) investigated extraction efficiency of albumin from spiked Schirmer strips. 10 µl of diluted albumin solution was applied to the notch of the Schirmer strip and allowed to diffuse for 1 minute. The filter paper strip was then cut up into pieces of 1 mm and placed into 500 µl of 0.002 sodium barbitol buffer at pH 8.6, after which it was centrifuged for 15 min at 500g. This was repeated for a total of 8 experiments and concentrations were assayed using immunoelectrophoresis. Elution of albumin using this protocol was shown to be incomplete and Prause recorded an average of 32 ng of residual albumin on the Schirmer strip.

Denisin *et al* (2012), recently made a more thorough investigation of diffusion based elution from Schirmer strips. Recovery of lysozyme, human serum albumin, sIgA, lactoferrin and MUC4, amongst others, was determined from spiked Schirmer strips. Five µl of protein solution with known concentration were added to 5mm^2 squares of Schirmer strip paper, which were then incubated with 10µl phosphate buffered saline for 3 hours. Concentrations of the recovered solution were tracked over time and it was found that maximum extraction was reached after 3 hours. Extraction efficiency was shown to be highly protein dependent and molecular weight and surface hydrophobicity were significant factors for retaining proteins on the Schirmer strip. Albumin was one of the proteins tested, which exhibited the best recovery efficiency where an average of 9.5% were lost to the Schirmer strip. The repeatability of the protocol was tested over a period of 12 months with 270 Schirmer strips spiked with BSA and was shown to be consistent.

3.6.6 Summary of effect of sampling material on measured tear albumin concentration

A large variety of extraction protocols exist for tear fluid sampled by absorptive techniques but no one protocol has been proven superior to the others. These investigations show that assessment of the extraction of absorptive material is something that is often ignored in study design. By keeping sampling technique and extraction constant across the study population, discriminations between protein concentrations in different study groups can however be made. It is unadvisable to provide quantitative data without recognising study limitations and possible effects of selective adsorption to the substrate. There is no one extraction protocol for the analysis of albumin concentrations sampled with absorptive techniques. Taking sampling material into account is important, as results using absorptive techniques could be erroneous due to insufficient removal of albumin adsorbed to the substrate surface (Prause, 1979; Denisin, 2012).

3.7 Effect of ocular health on measured tear albumin concentration

3.7.1 Introduction

Proteins in the body affect and constitute signalling, transport, structural and defence responses. The composition and dynamics of the tear film affects its stability and function and there is a large research focus on investigating levels of different tear proteins and how they differ in the disease state (Zhou *et al,* 2008). Ocular disorders are varied and include dry eye, Sjögren's syndrome, allergic reactions and ulcers. The identification of biomarkers for these conditions is an important field of research as disease causes and aetiologies aren't always straightforward to detect and diagnose. Some recognised tear biomarkers are IgE (for allergic reactions) and alpha-enolase and S100 A4 for dry eye disease (Allansmith *et al,* 1976; Dart *et al,* 1986; Zhou *et al*, 2008).

3.7.2 Effect of ocular health on measured tear albumin concentration

It is predictable that disorders that irritate the eye also affect the stability of the blood-tear barrier and result in influx of plasma proteins. Increase in albumin concentrations have consequently been linked to a variety of ocular conditions and disease states. Albumin concentrations in ocular disorders are described in Table 21. As in previous tables, concentrations in Table 21 are presented in increasing magnitude, with the exception of the first six references, which only detail an unquantifiable change compared to controls.

Table 21. The influence of stimuli and collection technique on perceived albumin levels.

Concentration mean or quoted value (µg/ml) ***(Range where given)***	**Condition**	**Collection Method**	**Size of study population (n =)**	**Time of sampling recorded? (Y/N)**	**Assay method**	**Reference**
Decrease *	Blepharitis	Polyester wick	19	N	2-D gel electrophoresis, ESI-Q-TOF MS/MS	Koo *et al.*, 2005
Increase	Primary Sjögrens syndrome	Microcapillary	49	N	SDS-Page	Bjerrum, 1997
Increase	Contact lens related dry eye	Microcapillary	11	Y	SDS-Page, Differential gel electrophoresis , nano-LC-MS/MS	Nichols and Green-Church, 2009
Increase	Evaporative dry eye	Micropipette	60	N	Gel electrophoresis, LC-MS	Versura *et al.*, 2010
Increase	Symptomatic soft contact lens wear	Microcapillary	17	N	SDS-Page	Maissa *et al.*, 2007
>3 fold increase	Keratoconus	Micropipette	12	N	2-D gel electrophoresis, LC-MS	Acera *et al.*, 2011
12.31± 7.50	Vernal conjunctivitis	Microcapillary	14	N	Immunodiffusion	Sen and Sarin, 1986
12.82 ± 5.26	Phylctenular keratoconjunctivitis	Microcapillary	10	N	Immunodiffusion	Sen and Sarin, 1986
13.00 ± 7.30	Trachoma II	Microcapillary	30	N	Immunodiffusion	Sen and Sarin, 1986
14.89 ± 6.05	Phylctenular conjunctivitis	Microcapillary	10	N	Immunodiffusion	Sen and Sarin, 1986
16.47 ± 9.66	Keratomalacia	Microcapillary	8	N	Immunodiffusion	Sen and Sarin, 1986
25.24 ±8.44	Superficial punc. keratitis	Microcapillary	6	N	Immunodiffusion	Sen and Sarin, 1986

63.25 ± 21.59	Corneal Ulcer (Hepatic)	Microcapillary	4	N	Immunodiffusion	Sen and Sarin, 1986
66.17 ± 21.47	Corneal Ulcer (Fungal)	Microcapillary	8	N	Immunodiffusion	Sen and Sarin, 1986
69.50 ± 22.48	Keratoconjunctivitis sicca	Microcapillary	6	N	Immunodiffusion	Sen and Sarin, 1986
70.53 ± 18.74	Neuroparalytic keratitis	Microcapillary	3	N	Immunodiffusion	Sen and Sarin, 1986
74.90 ± 21.75	Corneal Ulcer (Bacterial)	Microcapillary	20	N	Immunodiffusion	Sen and Sarin, 1986
85 *(25-630)*	Contact lens wear - various symptoms	Micropipette	30	N	Electrophoresis	Lundh *et al.*, 1984
85.6 ± 60.5 *(18.6-171.5)*	Acute adenovirus conjunctivitis (Mild)	Microcapillary with strong light	25	N	Electroimmunodiffusion	Gupta *et al.*, 1988
108.6 ± 41.3 *(63.4-211.1)*	Corneal Ulcer	Microcapillary	16	N	Micro-electroimmunoassay	Prause, 1983
164.98 ± 36.15	Alkali Burn	Microcapillary	12	N	Immunodiffusion	Sen and Sarin, 1986
170.05 ± 21.61	Allergic conjunctivitis	Microcapillary	6	N	Immunodiffusion	Sen and Sarin, 1986
179.22 ± 18.10	Acute mucopurulent conjunctivitis	Microcapillary	25	N	Immunodiffusion	Sen and Sarin, 1986
183.2 ± 14.2 *(141.5-206.8)*	Acute adenovirus conjunctivitis (Severe)	Microcapillary with strong light	38	N	Electroimmunodiffusion	Gupta *et al.*, 1988
10-1000	Corneal Ulcer	Microcapillary	7	N	Electroimmunodiffusion	Berman *et al.*, 1973
580 (0-1800)	Keratoconjunctivitis sicca	Periopaper	18	Y	Rocket electrophoresis	Farris *et al.*, 1986
590	Vernal conjunctivitis	Microcapillary	10	N	Immunodiffusion	Zavaro, 1980
~ 700	Keratoconjunctivitis sicca	n/s	n/s	N	n/s	Bron and Mengher, 1989
773	Follicular conjunctivitis	Microcapillary	9	N	Immunodiffusion	Zavaro, 1980
1430 ± 820	Allergic conjunctivitis	Schirmer Strip	21	N	SDS-Page, Maldi-TOF	Li et al., 2010

1760 (80-5770)	Keratoconjunctivitis sicca	Schirmer Strip	27	Y	Rocket electrophoresis	Farris et al., 1986
3700 (200-22600)	Keratoconjunctivitis sicca	Filter paper discs	25	N	Radial Immunodiffusion	Van Bijsterveld and Janssen, 1986
7300	Ocular provocation of allergy patients	Schirmer Strip	23	N	Radio Immunoassay	Proud et al., 1990
8300 (700-24700)	Keratoconjunctivitis sicca	Filter paper discs	20	N	Radial Immunodiffusion, SDS-PAGE	Janssen and Van Bijsterveld, 1986

* 2 HSA precursors found down regulated by 50%

Table 21 demonstrates that an increase in albumin levels is exhibited with ocular disorders such as dry eye (keratoconjunctivitis sicca), ocular allergy and contact lens-related problems, although it is unclear in these conditions whether leakage is causative or consequential. Apart from the paper of Sen and Sarin (1986a), which describes albumin concentrations in a large range of disorders, reports of albumin in ocular disorders and diseases are fairly limited in their scope. The majority of research focusing on albumin and ocular disorders identified in the literature concerned dry eye related syndromes and various forms of conjunctival and allergic reactions. Elevated albumin levels have not been effectively linked to any specific disease or ocular pathology. Thus albumin is not as yet a biomarker specifically used to monitor any individual ocular pathology. Bjerrum (1997) suggested that an albumin:lactoferrin ratio above 2:1 was indicative of primary Sjögren's disease, as this ratio was not seen for other connective tissue disorders. This has however not translated into clinical use and, as can be seen in Table 21, increase in albumin concentration is apparent in a range of disease states.

It is again apparent in Table 21 that the use of the less-than gentle Schirmer strip and filter paper techniques to collect tears results in higher albumin levels quoted. For example two individual papers, which investigated albumin concentration in the tears of patients with allergic conjunctivitis recorded mean values of 1430±820 mg/ml (Li *et al*, 2010) and the significantly less 170±21.61 mg/ml (Sen and Sarin, 1986a). Schirmer strip collection was used in the former study and microcapillary pipette was used to collect the tears in the latter. The discrepancy in albumin levels detected between the two studies is likely again to be that of the mode of collection. Irritation of the conjunctiva, seen in allergic and other conjunctivitis disorders, is associated with symptoms like itching, burning, red eye and inflammation. This type of reaction can vary in severity, possibly explaining the range of concentrations reported in and between studies of these disorders. Vernal conjunctivitis concentrations reported by Zavaro (1980) (590 µg/ml) and Sen and Sarin (1986b) (12.31±7.50 µg/ml) are however significantly different. Both studies sampled tears using microcapillary and analysed samples with immunodiffusion. Vernal conjunctivitis is characterized by massive infiltration of inflammatory cells (Leonardi *et al* 2008) leading to the expectation of increased plasma in the tear film and consequently higher albumin concentrations. Different population subsets and stage of disease during sampling might be attributed to some of the disparity but further investigation would be valuable. Concentrations reported by Sen and Sarin (1986b) fall within the range of normal values reported in atopic subjects in tables 17 and 18 on several occasions. This highlights the notion that "normal" albumin concentrations in the asymptomatic control and relative magnitude of increase in the disease state might be dependent on the individual subject to a high degree.

Albumin concentrations in Table 21 are shown to be significantly increased in keratoconjunctivitis sicca, or as its more commonly known, dry eye. Keratoconjunctivitis sicca is a complex syndrome with an aetiology that is not wholly understood. A good review of the subject is provided by Johnson and Murphy (2008). Causes of dry eye can be related back to deficiency in providing the aqueous tear portion or excessive evaporation. Tear ceruloplasmin, another diffused plasma protein, is increased in established dry eye but not in questionable dry eye or ocular pemphigoid (Mackie and Seal, 1984), which further supports multiple disease pathways able to affect extent of plasma leakage.

3.7.3 Summary of effect of ocular health on measured tear albumin concentration

Research and an understanding of disease aetiology is needed to establish whether the presence of albumin in ocular disorders is purely a marker of the extent of inflammation or if other factors are involved. Inconsistent tear collection and assay methodologies make cross-study comparisons difficult. Presently, the solitary diagnostic role of albumin in tears is as a marker of vascular permeability and plasma leakage. In general however, it is clear that the incidence of ocular disorder results in the influx of this plasma leakage marker, although there is no link to specific diseases.

3.8 Effect of contact lens wear on measured tear albumin concentration

3.8.1 Introduction

The contact lens is approximately ten times thicker than the aqueous layer of the tear film within which it sits (approximately 70 μm vs. 7μm). Interactions of the lens with the tear film are both dependent on the material and the individual tear film of the wearer. Despite rigorous material development strategies and care regimes to reduce contact lens disorders, a portion of the population are still unable to wear contact lenses for a prolonged period of time, or at all. Symptoms most often experienced are end of day discomfort and ocular dryness. It has been estimated that over 50% of contact lens-wearers suffer from contact lens related dry eye, which affects the stability and integrity of the tear film (Begley *et al*, 2000; Nichols *et al,* 2002; Nichols and Sinnott, 2006).

3.8.2 Effect of contact lens wear on measured tear albumin concentration

The current literature provides a very conflicting view on whether contact lens wear is causative of increased albumin levels or not. Studies can be split up into those assessing symptomatic and non-symptomatic wear:

3.8.2.1 Non-symptomatic wear

A study of albumin concentrations in neophytes by Carney, *et al* (1997) showed no significant difference at baseline compared to after one night, six nights or six months of extended contact lens wear. Similarly, subjects with long contact lens wearing experience exhibited no difference in concentration compared to non-wearers (59.2± 54.0 µg/ml vs. 45.1± 32.5 µg/ml)(Baleriola-Lucas *et al* 1997). Differences in the study by Baleriola-Lucas were however found if the contact lens-wearers were split up into groups with and without bacterial deposits on lenses. Albumin concentrations in subjects with bacterial deposits on lenses were 79.0 ± 60.1 µg/ml compared to 26.3 ± 12.1 µg/ml in the non-bacterial deposit group.

3.8.2.2. Symptomatic wear

Lundh *et al* (1984) found an increase in tear albumin in contact lens-wearers experiencing difficulties with wear - termed abnormal wearers. These abnormal wearers presented with an altered tear eletrophoresis profile, trouble when wearing lenses, hyperemia and/or bulbar injection. In total 30 subjects wearing different contact lens materials (42 soft hydroxyethyl methacrylate lens-wearers, 6 hard polymethyl methacrylate lens-wearers and 2 methacryl methyl siloxane lens-wearers) were analysed. Mean tear albumin concentrations were 85 µg/ml (25-630), where levels inferior to 50 µg/ml were termed normal by the authors. Concentrations above 50 µg/ml were found in 70% of the determined abnormal wearers. Farris (1985) on the other hand found no significant difference between control groups and phakic or aphakic contact lens-wearers using either daily or extended wear modalities. Interestingly, a significant difference was however found between albumin levels in the different control groups not wearing lenses. Sampling was conducted using periopaper (absorbent strips used for collecting gingival fluid) and the Schirmer strip, which might explain the larger concentrations of 380±640 µg/ml and 1830±2440 µg/ml reported in reflex and basal tears of these controls. Nichols and Green-Church (2009) were able to show significant increase of albumin in subjects with identified contact lens related dry eye (by questionnaire) compared to normal contact lens-wearers.

The over night tear film is associated with albumin increase and it appears that overnight contact lens wear amplifies this increase (Choy *et al* 2004). Post-sleep concentrations rose from 200 (150-580) µg/ml to 540 (330-1240) µg/ml with lens wear. Extended wear, which incorporate sleeping in the lenses, on the other hand does not appear to affect open eye tear albumin concentrations in asymptomatic subjects on the whole (Carney *et al* 1997; Baleriola-Lucas *et al* 1997).

3.8.3. Summary of effect of contact lens wear on measured tear albumin concentration

Non-symptomatic wearers of contact lenses have not exhibited any differences in albumin concentrations apart from groups with bacterial deposits on the lens. Studies investigating symptomatic contact lens wear on the other hand have reported some changes in albumin concentrations, although sampling, wear modalities and control variables are confounding factors. Dry eye is already linked to increase in albumin concentration and the wearing of contact lenses may potentiate or initiate dry eye symptoms in susceptible subjects. In this scenario, albumin increase may be linked to the presence of dry eye rather than contact lens wear itself.

3.9 Effect of analytical technique and study population on measured tear albumin concentration

3.9.1 Introduction

Choice of assay technique is limited by sample volume. Normal tear production rate is estimated to be between 1 and 2 µl/min and total tear volume has been estimated to be 7µl, whereof 3 µl is present in the meniscus of the eye (Mischima, 1966). Many analytical techniques require volumes collected to be of a certain size to be able to identify lower levels of proteins or changes in concentration. Current proteomic and chromatographic techniques such as ELISA and HPLC are able to analyse smaller samples (1-2 µl), as their sensitivity levels are in the range of ng/ml. Tear protein determinations made over 20 years ago were made with less sensitive techniques and are consequently less accurate. Comparisons between different studies therefore need to address which analytical techniques were used to obtain the results.

3.9.2 Effect of analytical technique and study population on measured tear albumin concentration

A variety of assays have been used over the years to ascertain albumin concentration in tears but no pattern of assay influence has emerged to suggest that the assay of investigation has a significant detrimental bearing on the outcome. Effect of assay methodology in this thesis was assessed using information from Table 18, which is illustrative of albumin concentrations without the confounding factors of invasive sampling and previous stimulus. Concentrations range from 10.3 μg/ml to 200 μg/ml, suggesting the presence of individual subject variation or variation in assay sensitivity. Immunodiffusion is only a semi-quantitative technique and, as represented by the range reported in four asymptomatic subjects (10-200 μg/ml) by Berman *et al*, (1973) obviously responsible for some assay related variability. Ranges produced by the more sensitive electrophoresis, HPLC and ELISA techniques are however more likely to describe subject related variability and the heterogeneity of the sample population. The increased precision with these techniques also allows improved comparability between results within and between studies. The majority of quantitative studies were unfortunately not carried out using ELISA or HPLC making it more difficult to draw definite conclusions. This literature assessment was also unable to provide discrimination between population subjects in terms of age and gender and simply differentiated between controls and symptomatic subjects. Whilst a minority of studies do report on age and gender specific concentrations, the data available are insufficient to establish a generalistic view on effects.

3.9.3 Summary of effect of analytical technique and study population on measured tear albumin concentration

Apart from the obvious fact that techniques and assays have improved considerably over the years allowing greater sensitivity, the choice of assay technique may not be as crucial as other factors in tear albumin studies. It should however be recognised that the accumulation of variability imposed by all the external factors (sampling technique, tear state, assay technique) will lead to increased standard deviation. Eventually the increased standard deviation will dilute the significance of the results obtained if factors are not accounted for. Variations that occur with sampling techniques, and potentially with assay methodology therefore masks any attempt to investigate normal person-to-person and individual within-day variation.

3.10 Factors affecting tear albumin concentrations: conclusion

This chapter was important for the identification of factors that affect albumin concentration in tears and their interrelationship. In order to investigate the role of albumin in tears (i.e. whether albumin is merely present as a marker for the leakiness of the blood-tear barrier or present as a response to ocular deviation from the normative state) confident albumin measurements must be possible. Any factors, irrespective of whether internal or external, that produce albumin rich or deficient environments and mask the true tear film concentrations need to be evaluated and controlled. Three key variables were identified during the investigation presented in this chapter. They are summarised below:

- Sampling technique is critical in the determination of albumin concentration: harsher techniques (e.g. Schirmer strip) stimulate concentrations that are significantly greater than when the less invasive microcapillary method is used.

- Insult to the eye and/or ongoing disease states are associated with an increase in albumin levels but a direct link between albumin levels and disease aetiology is lacking.

- Assay methodology can influence measured albumin concentrations but it is the least influential factor. Modern sophisticated techniques are highly sensitive and therefore this influence is less important than other factors.

Sampling technique appears to be the most influential variable where the trend of increased albumin concentration with filter paper techniques is apparent even during ocular discomfort and disease (Table 21.). This trend could in part be explained by the fact that certain disorders, such as keratoconjunctivitis sicca and Sjögren's Syndrome, are associated with a decrease in tear volume and flow rate and require a more invasive technique to collect adequate volume of tears. Nevertheless, filter paper techniques represented a mean of 48 times increase compared to microcapillary levels in asymptomatic subjects. The combined effects of stimulation/irritation (which directly affects albumin concentration), and of stimulation (which increases tear flow) are difficult to de-convolute and also add to the complexity of assessing albumin concentrations. On-going disease states and ocular insult are associated with an increase in albumin levels, but a specific link between albumin levels and disease aetiology and insult is lacking. The aforementioned sampling and analytical variability make this link all the more difficult to quantify. Reports of increase in tear albumin concentration during contact lens wear appeared to be limited to symptomatic subjects. Whether the increase is a result of subjects with poor compatibility to the lens itself, or a result of subjects with an underlying ocular condition being

potentiated by the presence of a contact lens, is difficult to determine. The establishment of subject-to-subject variation in the non contact lens-wearing state is needed to further evaluate the effects of contact lens wear on tear albumin concentrations.

Whilst assay technique was found to have an impact on albumin concentration, it was not on par with the vast discrepancies between microcapillary and filter paper sampling. Effect of extraction of tear albumin from absorbable sampling materials was only investigated in two different studies (Proud, 1990; Denisin *et al*, 2012). Although the extraction efficiency of tear albumin from Schirmer strips was shown to be high (>90%), residual protein will affect the accuracy of the result and is a minor variable.

The closed eye tear film is a unique environment and can't be categorised as a factor of variation in the same manner as assay methodology, tear sampling method, ocular health and contact lens wear. Measurements of albumin concentrations in closed eye tears are however affected by the factors of assay methodology (section 3.4.2) and contact lens wear (3.8.2.2). The interrelationship between factors is of importance for the determination of tear albumin in any tear state. Factors that have been identified to affect albumin concentration lead to increased variability and standard deviation in albumin studies. Lack of control of the identified factors of variability detracts from the possibility of determining individual inherent albumin concentrations and ultimately the viability of the use of albumin as a diagnostic marker. To better understand the nature and role of albumin in tears, it is imperative that parameters affecting albumin are evaluated and acted upon in further studies. Individual subject-related albumin concentration variation was presumed to be present in the reported research, but difficult to ascertain due to the variation in methodology of the studies presented.

CHAPTER 4
ALBUMIN IN TEARS: POPULATION AND DIURNAL VARIATION

4.1 Aim

No systematic research into diurnal variation of albumin in the waking tear film has been produced. Diurnal variations are already established in albumin at other mucosal surfaces and also in other blood and tear components. The determination of albumin concentrations throughout the day is necessary for further research into albumin and could also supply important additional information regarding the extent and onset of the albumin increase in the closed eye. Consequently the aim of the research carried out in this chapter was to investigate albumin concentrations during waking hours and to observe the transition to the overnight tear film. This was to assess if albumin levels were relatively stable (or demonstrated periods of stability) or if levels had diurnal variations, similar to the trends observed in other biological fluids.

4.2 Introduction

Chapter 3 introduced the fact that relatively little is known about human albumin levels in the tear film under normal ocular conditions. Three key variables were identified: sampling methodology, ocular health and analytical technique. Sampling and methodology factors only represent external sources of variation, however, and internal variations differentiating albumin concentrations in subjects are just as important. Unstimulated microcapillary collected tears from healthy subjects were observed to have a range of albumin concentrations between 10.3-200 μg/ml (section 3.5.2). Disregarding the influence of assay variability, the large differences between studies suggest the presence of individual variability between subjects. Acknowledgement of subject-to-subject variation is important in the design of future research studies, especially with regards to population selection. Differences between individuals could also clarify the role of albumin and its potential benefits in tears, or alternatively reveal individual susceptibilities towards ocular irregularities. In order to evaluate the possibility of innate albumin concentration differences between subjects or populations, all possible variations need to be excluded.

4.2.1 Diurnal variations of tear proteins

A review of data from the literature suggests that diurnal variation could have an affect on tear protein concentrations. Variations across the day of several tear components have been

observed. For example, matrix metalloproteinase 9, an enzyme important for tissue maintenance and degradation of proteins in the extracellular matrix, is significantly elevated in the morning compared to the rest of the day (Markoulli *et al,* 2012). Group IIa phospholipase A_2, an- antimicrobial protein with functions similar to lysozyme and lactoferrin, has also shown some diurnal variation. Concentrations in tears sampled with microcapillary increased between 8:00 hrs and 12:00 hrs and then decreased between 16:00 hrs and 20:00 hrs (Aho *et al*, 2003). An 18 month study of total antioxidant activity in tears, measuring low molecular weight antioxidants, also revealed a diurnal variation where concentrations peaked within 10 minutes of waking at 8:00 hrs and then decreased, reaching a minimal value at 12:00 hrs (Kowalski *et al,* 2009).

4.2.2 Diurnal variation of blood proteins

Diurnal variations of proteins in serum could potentially point towards similar variations when the proteins are present in tear fluid. For example, inflammatory cytokines in blood have circadian or diurnal rhythms. IL-1, IL-10, TNF-alpha and IL-12 in blood are linked to the diurnal rhythm of cortisol with low concentrations during the afternoon and high during the evening (Petrovsky *et al,* 1998). Similarly, cytokines sampled in tears (IL-1ß, IL-1, IL-10, IL-12p70, TNF-alpha) have been observed to have a moderate increase in concentration in the morning, followed by a slight decrease in the afternoon and finally a significant increase between 21:00 and 24:00 hrs (Uchino *et al*, 2006). Total serum protein has also exhibited a diurnal variation pattern (Seaman *et al,* 1965) with decrease in concentration during the night and early morning hours. Naturally, correlations cannot always be made between variations in plasma proteins and tear proteins but investigation of alterations in plasma may give some information about variations in tears.

4.2.3 Albumin in other biological fluids and mucosal surfaces

Diurnal variation has been shown to be a hugely influential factor on albumin levels detected in biological fluids and mucosal surfaces other than tears. In blood, serum albumin exhibits circadian variation where levels remain constant during the day (38-45 mg/ml) but decrease significantly at night-time to reach a low point between 02:00 hrs and 04:00 hrs (35-38 mg/ml) (Jubi*z et al* 1972). Fluids at mucosal surfaces such as nasal secretions also appear to be subject to diurnal variations. Passàli and Bellussi (1988) found a progressive increase in albumin levels in nasal secretions sampled by cotton wool swabs. A 25% increase in concentration from the 10:00 hrs baseline was established every three hours up until and including the last time point at 04:00 hrs. Two other studies, assessing nasal lavage fluid (Greiff *et al,* 1996; Zhang *et al,*

2009), found increased albumin levels in the morning and early hours of the night. However, concentrations remained constant during the daytime in their studies.

4.2.4 Diurnal variation of albumin in tears: the closed eye tear film

Little is known about diurnal variations in concentrations of tear albumin apart from a few studies investigating the closed eye tear film (Sack *et al*, 1992; Tan *et al* 1993). In the study of Sack *et al* (1992), tears were sampled from four subjects and assayed for albumin at 3 time points: 12:00, 17:00 and immediately post-waking. Previous research had found that prolonged eye closure led to initially dry eyes upon waking with a subsequent gush of tears increasing the available volume but also diluting the proteins present (Gilbard *et al*, 1988). Consequently subjects selected for the Sack study were screened with respect to their stimulation response and did not experience reflex tearing upon self-sampling. Subjects slept with microcapillaries next to their bed and instigated sampling immediately upon waking. When excessive reflex tearing occurred, the samples were rejected. The effect of reflex tearing was subsequently evaluated in the study, with induced lacrimation (by physically holding the eye open) on daytime tears, and was found to reduce albumin concentrations by two-thirds (from 60 μg/ml to 20 μg/ml). Keeping tear flow constant within subjects is therefore essential to be able to assess natural variations in concentration.

The study by Sack *et al*, (1992) found that albumin, total protein and sIgA were all significantly increased in the closed eye tear film compared to open eye tears collected at 12:00 and 17:00. Total protein concentrations were doubled and sIgA concentrations were increased by tenfold. Most significantly, albumin concentrations reached values of 1100 μg/ml, almost 20 times greater than the open eye value of 60 μg/ml. The rest of the major tear film proteins remained static. The closed eye tear is used as a representative of the overnight tear film environment. As tears don't leak from the eyelids during sleep, a change in tear film dynamics following prolonged eye closure is evident. The environment is described as a stagnant pool of fluid with restricted tear flow and significantly different composition to the open eye (Sack *et al,* 1992). Increases in inflammatory tear components, such as complement factors are also described (Sack *et al*, 1992), which indicates a state of hypoxic sub-clinical inflammation.

As mentioned in the introduction (section 1.3.3.2.4), sIgA is the predominant player in mucosal immunity and subject to a different transport mechanism to the other major tear film proteins. These two factors could explain the significant and specific increase in sIgA in the closed eye tear, as it may be vital for the protection of the ocular surfaces during this low fluid volume hypoxic state. Albumin movement, on the other hand, is not completely understood and the

mechanisms of albumin transport into the tear film are yet to be determined. Similarly, the role of albumin in tears and the consequence of increased albumin concentrations during prolonged eye closure are undetermined. Albumin could be a passive marker of an influx of inflammatory proteins from the plasma or provide a protective beneficial role akin to sIgA.

4.3 Methods

4.3.1 Study population and sampling

4.3.1.1 Study population

13 healthy subjects without any observed ocular disease or disorder were recruited within the department for this study. The population consisted of 6 male and 7 female subjects with a mean age of 30 (range: 23-46). No contact lenses were worn for the duration of the sampling period. In the case of subjects wearing contact lenses outside of the study, wear was stopped at least two days prior to the start of the study. The sleep patterns for all the subjects were normal, i.e. no subjects with 'unusual' sleep patterns or insomnia were included. Subjects were also asked to report any observations or discomfort during the sampling.

4.3.1.2 Tear collection

Sampling was conducted using microcapillary tubes as detailed in the materials and methods section. Chapter 3 showed that a relationship between tear flow stimulus and albumin concentrations exists and these variables were therefore monitored. Tear flow rate was determined for all samples using the microcapillary tube etching and samples were recorded as being basal, medium or reflex tears.

Initially tear samples were collected by an experienced researcher but in order to sample during out of office hours, a subset of the study population were taught to self-sample to collect these later time points. Self-sampling was done by standing in front of, or holding, a mirror and positioning the microcapillary pipette against the tear film in the lateral canthus, taking care not to touch the conjunctiva. A stainless steel wire plunger was then used to dispel the sample into a labelled microcentrifuge tube. Samples were stored in microcentrifuge tubes at -80°C until time of analysis but not longer than one week after collection. For the sampling time points taking place out of work hours, samples were stored in the fridge until transit to a -80°C freezer. Studies by Sitaramamma *et al* (1998b) detected no significant change in protein concentration after 1 week of storage at 4°C. Losses of albumin due to the handling and storage in

polypropylene microcentrifuge tubes have been estimated to be in the region of 10% (Denisin *et al*, 2012) and consequently results have to be interpreted with this error margin in mind. Adherence to the microcapillary tubes during sampling is also a factor of variation but likely to be more important for the analysis of positively charged proteins attracted to the negatively charged glass surface, rather than albumin.

4.3.2 Sampling schedule

Subjects were divided into two populations according to the sampling schedule in Table 22. This division was based on subject availability as well as level of experience and confidence in self-sampling by microcapillary.

Table 22. Sampling schedule for both populations examining diurnal variation of albumin. 106 samples are common to both groups.

	Subjects	Sampling Duration	Interval	Tear Samples Analysed	Number of Days
Group 1	13	10:30-16:30 hrs	2 hours	199	2-6
Group 2	5	Waking-Bedtime	2 hours	307	9-11

Group 1 (n=13, 7 female, 6 male) had tear samples collected at various time points over the working day for a minimum of 2 consecutive days to assess diurnal and subject-to-subject variation. 199 samples were analysed in this group.

Group 2 (n=5, 4 female, 1 male) comprised the subset of subjects trained to collect their own samples. Their first sample of the day was collected within 10 minutes of waking (before 8.00) and then a sample was collected every two hours until 22.30 in the evening for a number of consecutive days (3-5 depending on subject availability). Early assayed results suggested a change in albumin concentrations as the day progressed into evening. Consequently, Group 2 subjects were asked to extend the self tear sampling to include extra samples up to and including a pre-bed tear sample; the last two measurements being half an hour before bedtime and pre-retiring to bed (at >17 hours post-waking). This was done for six additional days. In total, 307 individual tear samples were analysed for this part of the study. The sampling time for each subject in Group 2 was clock-matched relating each subject's rising to sleep pattern to

time. This allowed for comparison between subjects with different sleep patterns and to relate the early risers and early to bed subjects with the late risers and late to bed subjects. The actual times of waking and retiring to bed did not vary between subjects with more than an hour and no subject woke up later than 08:00.

4.3.3 Tear sample dilution schedule

Tear samples were analysed using the Immunology Consultants Laboratory (ICL) ELISA as previously described in chapter 2. A subset of samples (morning and evening), reading at concentrations above the range of the assay, was subsequently re-assayed with a higher dilution (1:550, 1 µl tear fluid in 550 µl sample diluent).

4.4 Results

4.4.1 Albumin concentration in Group 1

Distinct variations in albumin concentration were seen in the study population between different subjects and in the population as a whole. The levels of albumin for individual subjects appeared to show minimum values and greatest stability during the time points 10:30 - 14:30 hrs. Concentrations measured over successive days consistently showed little variation in subjects over this four hour period. Fig. 4.1 shows the mean tear film albumin concentrations over successive days during these hours for the 13 subjects. Standard deviation identified the slight variability for each individual subject between sampling days, indicating the day to day variation. The degree of between-day variability differed between subjects but did not overshadow the wide subject-to-subject variation evident by the distinct distribution curve.

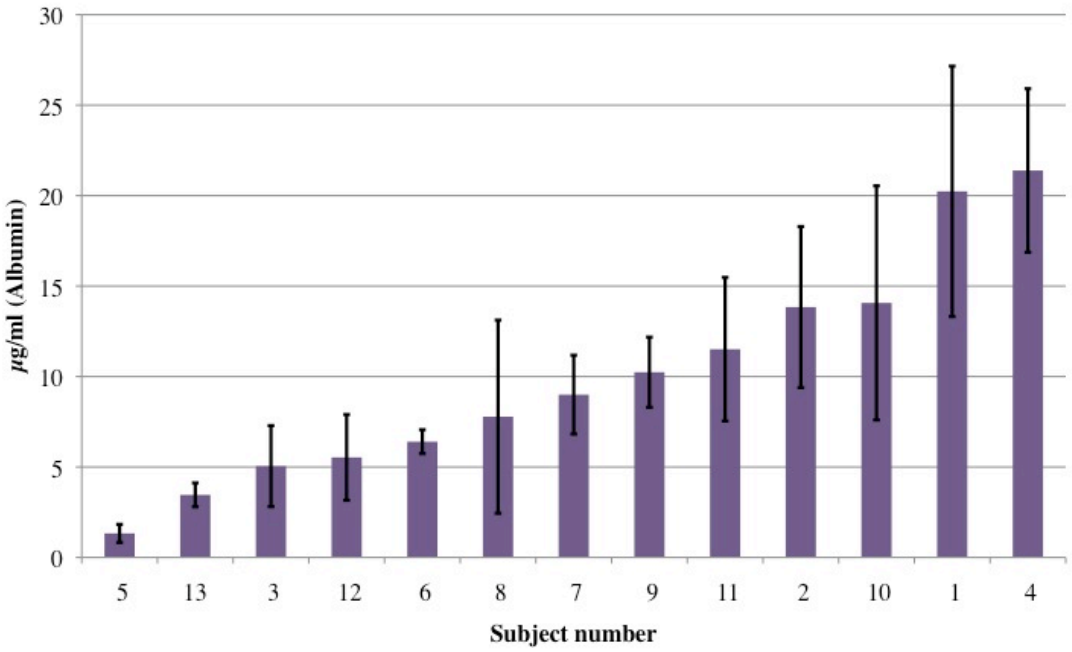


Figure. 4.1. Individual baseline albumin concentration for all subjects. The results are expressed as mean concentration from sampling times between 10:30 - 14:30 hrs for each individual over a period of 2-6 successive days.

The relative stability during this daytime period can also be illustrated by looking at the mean, minimum and maximum of albumin concentrations for all subjects at three individual time points (10:30, 12:30 and 14:30 hrs). These data presented in Fig. 4.2 show a small variation in mean albumin values (8.4 – 11.6 µg/ml), suggesting again that, as a population, this is a time suitable for determining albumin concentrations.

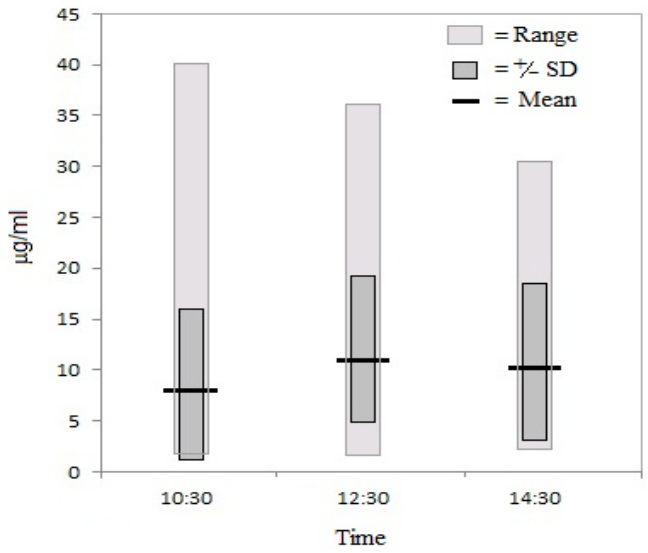


Figure 4.2. Mean 'stable' albumin concentration for the study population, between the hours of 10:30 – 14:30 hrs over 2-6 days. An average of 130 tear samples were taken during this time schedule.

Furthermore, variations between subjects at these time points indicate the existence of a range of indigenous albumin concentrations in a population. This information is important for albumin studies and potentially the study of other proteins that albumin may be a marker for. It is also valuable for the investigation of whether albumin is responsive or reactive in terms of its presence in the tear film as it might enable the distinguishing between different subject groups.

4.4.2. Evening rise in albumin concentration

Variation in albumin levels outside of the daytime period of relative stability was investigated in a subset of 5 subjects (Group 2). Samples were initially taken at two-hourly intervals for 3-5 successive days, which suggested a potential shift in albumin concentrations towards the later part of the day. The study was then extended by 6 days and sampling times were amended to include a sample 30 minutes before bed and a pre-retiring to bed sample. All sample data, within 10 minutes post-waking (designated 0 hours) to immediately before retiring to bed are displayed in Fig 4.3 in the form of a stacked graph.

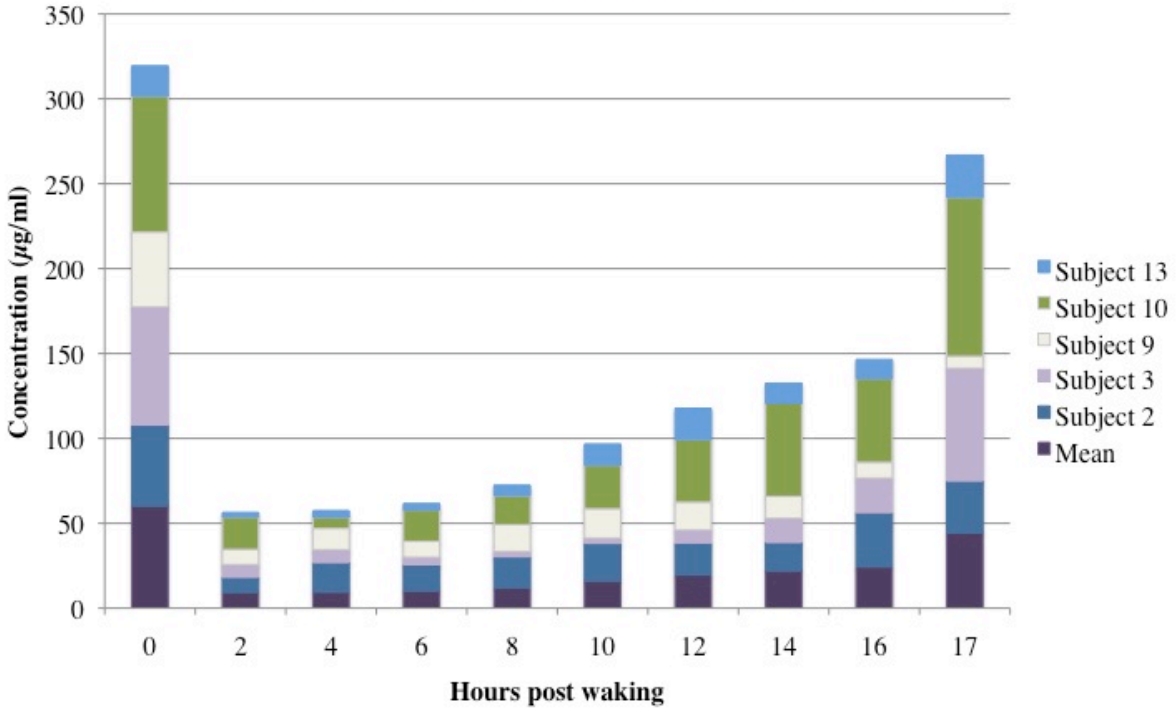


Figure 4.3. Diurnal albumin concentration variation (n=5); all sample time points including 10 minutes post-waking (0 hours) and pre-retiring to bed (17 hours).

The data collected from Group 2 revealed a previously unreported difference in albumin concentrations in subjects between daytime and evening, prior to bed, and show a clear pattern of variation within the waking day. These data illustrate that the levels recorded in Figures 4.1 and 4.2 (10:30-14:30 hrs) indeed marked the period where albumin concentrations were at

their lowest and most stable for all subjects. From this it was decided to designate these hours a useful baseline period for the purpose of albumin sampling.

All subjects in Group 2 exhibited the pattern of variation in concentration between the stable time period and the evening sampling times. The data indicates the presence of a consistent asymmetric diurnal variation during the hours of waking in the population. The asymmetric shift is illustrated by concentrations decreasing rapidly in the first two hours post-waking followed by the period of stability. Concentrations in the latter part of the day then exhibited a general progressive increase up to 16 hours post-waking as shown in Fig 4.3. The additional extended sampling time-points, illustrated at 17 hours post-waking, demonstrate an even more prominent albumin increase similar to post-waking concentrations (which are already established in the literature as being increased). Although all subjects showed an increase in the evening albumin levels, some disparity among the subjects existed. Two subjects (3 and 13) showed marked increase after 20:30 but the remaining three subjects showed an earlier progressive increase. Subject 9, interestingly, differed from the rest and did show increasing albumin concentrations from baseline but peaked at 12 hours post-waking and exhibited a small decrease pre-retiring to bed. Although the baseline levels of albumin varied among subjects, the data shows that the concentration of albumin was higher in samples collected in the evening (and upon waking) compared to samples collected during the daytime schedule. This difference in albumin concentrations between the daytime and the evening tear film was shown to be statistically significant ($P < 0.05$, paired student's t-test). The differing modes in which the established diurnal variations present itself merely highlights that albumin concentrations are, to a high degree, affected by individual subject variations in tear film response.

Although the rapid drop in levels following waking is to some extent predictable, the gradual and consistent observed rise in albumin levels that has been shown here to take place during the evening has not been previously characterised. Samples in Group 2 taken immediately post-waking and pre-retiring to bed were significantly higher in albumin than at any other time. These values appear to characterise a transition from and to levels observed in the overnight tear film (Sack et al, 1992)(Tan et al, 1993). To further illustrate the apparent shift from lower to higher albumin concentrations during the day, a frequency distribution of all sample data was calculated (Fig 4.4.).

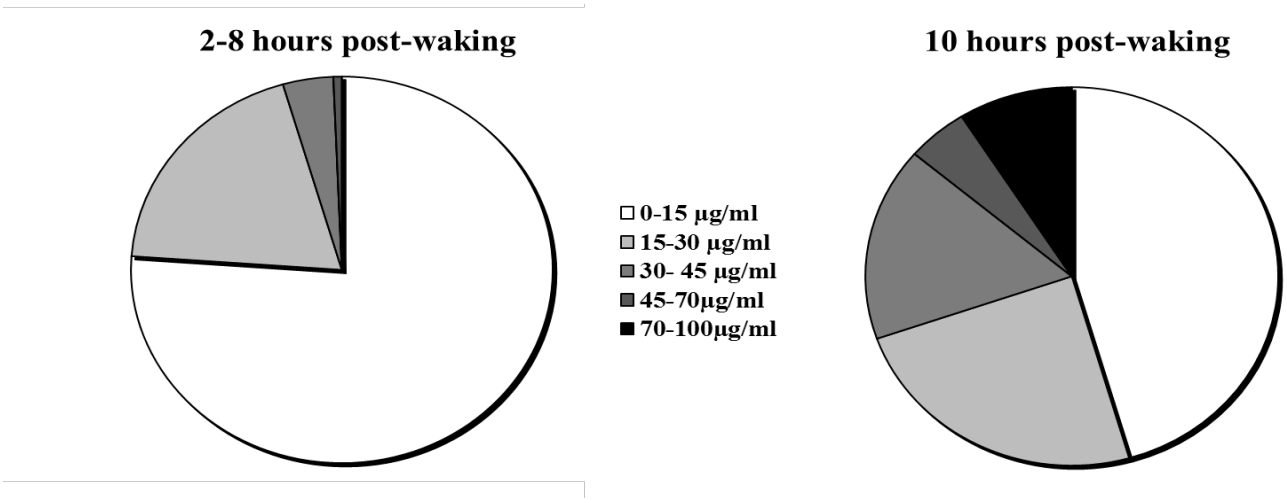


Figure 4.4. Frequency distribution of the occurrence of specific albumin concentrations in samples collected during the daytime (2-8 hours post-waking) and evening (10 hours post-waking).

This showed that 76% of tear film measurements during daytime hours presented levels of albumin below 15 µg/ml. Higher concentrations of albumin, on the other hand, occurred predominantly or exclusively in the evening tear film. These data imply that the evening tear film (post 10 hours waking) is a different environment to the daytime tear film and has a significantly altered protein composition with respects to albumin.

4.5 Discussion

A novel asymmetric pattern was identified in this study, which was evidenced by: a high post-waking albumin concentration; a rapid decrease leading to the lower 'stable' midday albumin levels; and finally a progressive increase in albumin levels towards the latter part of the day. This late-day increase may mark the early compositional changes associated with that of the closed eye overnight tear fluid, which has been shown to be a tear fluid rich in plasma proteins including a high albumin concentration (Sack *et al*, 1992; Tan *et al* 1993). Excluding the two extremes related to the overnight tear film (within 10 minutes post-waking and pre-retiring to bed, there is still a trend of increased albumin towards the end of the day (Fig 4.3). This pattern of progressive increase differed slightly in one subject (subject 9); the highest levels were detected after 12 hours post-waking with a slight dip immediately pre-retiring to bed. The variation in behaviour supports the notion that the tear film is not uniform between individuals and indicates that there exist many divergent subsets of people with varying tear composition. The observed end-of-day concentrations were between two and four times higher than their daytime tear film baseline stable levels. These variations observed go well beyond anything that

could be explained by tear flow variation. Subject-to-subject tear flow may differ but, as previously stated, each individual subject demonstrated the same response to the stimulus of microcapillary sampling throughout the sampling schedule.

The significant elevation in concentration observed in the current study, in the samples taken within 10 minutes post-waking accords with the trends observed in the literature, which demonstrate similarly high post-waking values. Two previous studies have assessed albumin variation in part. Ng *et al.* (2000b) investigated between-day variation over a period of three days (samples collected between the hours of 13:00 and 18:00 hrs) and found no statistical difference in either total tear protein content or major tear protein fractions, including albumin. Secondly a study by Sack *et al* (1992) assessing overnight variation, found no statistical difference in albumin concentration for the open eye state of the tear film (samples collected at 12:00 and 17:00 hrs) apart from elevated levels 10 minutes post-waking. Both studies present results, which correspond to the present findings in terms of the stability of albumin concentration in the tear film during the daytime under normal ocular conditions and the elevated post-waking albumin levels. The actual concentration of albumin in the post-waking samples investigated in this study is however somewhat lower than those levels reported in the literature. This can be explained by the fact that the levels reported in the literature correspond to tears collected immediately upon eye opening (in order to represent the closed eye environment), whereas the samples analysed here represent a more acclimatised sample taken in the region of 10 minutes post-waking.

The results presented in this chapter clearly demonstrate a pattern of diurnal variation during waking hours, which has not been previously identified. This should perhaps not be regarded as unusual as it is similar to the circadian rhythms of many mucosal surface fluids, which also experience plasma leakage, that have been explored in detail and show clear and defined changes in albumin concentration during the day (section 4.2.3). The assayed albumin levels in the present study were found to be most consistent at approximately two and six hours post-waking. This represented the most stable tear film in terms of albumin concentrations and was subsequently designated as baseline. Whilst concentration levels during these hours varied slightly within individual subjects between the different sampling days, they tended to fall within a narrow bracket (i.e. a subject displaying lower levels of albumin would do so consistently). The range of albumin levels exhibited at the designated baseline in the present population indicates that subjects appear to have indigenously lower or higher levels of leakage. These variations at the defined baseline suggest the existence of a range, or range of subsets, of individual albumin concentrations within a population. The underlying baseline albumin

conditions may be linked to the ability to adapt to new environments and to maintain ocular health.

The values given in the literature (Table 18) for albumin concentration in the uncompromised open eye are quoted anywhere between 10.3-200mg/ml in tears, although higher values are related to use of semi-quantitative measuring techniques. The mean albumin concentration in this study for all subjects was determined to be 10.2 µg/ml with a range of 1.1 to 40.0 µg/ml. However this mean is based on the time period where the levels were most consistent and would be much higher had all the diurnal variation data been included. The identified gaps in the literature filled by the present research are best illustrated by integrating the data obtained with existing literature concentrations. Table 23 provides an overview of the current study results in comparison to existing albumin concentration data sampled during the daytime. Only concentrations with a defined sampling time period were included, which left four studies carried out during the daytime and two closed eye studies. Also, as sampling technique is the most influential externally imposed factor that affects albumin concentrations, only samples collected by microcapillary without stimulus were chosen for comparison. The results gained from samples in the literature obtained by microcapillary encompass both subject-to-subject variation and the influences of assay technique and time of sampling (Table 23).

Table 23. Integration of the current study results with the available literature albumin concentrations sampled by un-stimulated microcapillary.

Time of Sampling	**Concentration µg/ml**	
(hours)	**Current Study**	**Literature**
10:30	9.4 ± 5.9	45.1 ± 32.5 (Baleriola-Lucas *et al* 1997)
12:30	9.6 ± 5.5	10.3 ± 9.2 (Thygesen *et al*, 1987)
14:30	10.2 ± 6.5	21 ± 28 (Ng *et al*, 2000a)
16:30	12.1 ± 6.9	60 ± 20 (Sack *et al,* 1992)
18:30	16.1 ± 8.8	No data
20:30	19.6 ± 10.7	No data
22:30	22.1 ± 18.4	No data
30 minutes pre-retiring	24.4 ± 16.5	No data
Pre-retiring to bed	44.5 ± 33.1	No data
Post-waking (closed eye)	No data	200 ± 580 (Choy *et al,* 2004)
		1100 ± 760 (Sack *et al*, 1992)
10 minutes post-waking.	60.3 ± 24.3	No data

Concentrations reported in the literature were collected between the hours of 9:30 and 17:00 hrs. The corresponding concentrations obtained in the current research (3.5 – 19 μg/ml) are within the range of literature concentrations minimum (1.1 μg/ml) and maximum (80 μg/ml). Apart from deviation related to assay methodology (only Baleriola-Lucas *et al,* 1997 used the same analysis method as the present research) it is reasonable to attribute concentration differences compared to the literature with variation in population, or more specifically subject-to-subject variation. Both the research presented in this chapter and the reported literature values exhibit standard deviation as large as the mean reported. This is identified as subject-to-subject variation in the current research and presumably a result of a similar effect in the reported literature concentrations. Subject-to-subject variation can clearly exert a significant influence on measured albumin concentrations. In this table, it is evident that only by measuring albumin concentrations in the same individuals over various time points within and between days can the effect of diurnal variation be distinguished from that of subject variation and they can be effectively determined. The majority of other reported studies, not included in this integrated table (tables 17, 18 and 21), do not state sampling times but are likely to have been restricted by opening hours in clinics and research departments. Consequently, albumin sampling will have been performed within this time frame, which fortunately falls within the time period when albumin concentration was found to be most stable in this study. Studies outside of this stable daytime period run the risk of adding a diurnal variation factor to the already long list of possible variations that can affect albumin concentration.

The evaluation of the literature in chapter 3 highlighted factors that affect the measurement of albumin concentration in tears. Similarly, the research by Sack *et al,* (1992) provided some useful information on the design of this type of investigation. In this study as many as possible of the factors identified in chapter 3 and by Sack *et al* were negated by the control of the careful selection of population. Our research group has extensive practice in tear sampling and self-sampling results were validated by comparison with tear samples taken by experienced researchers. Although different subjects responded in different ways to sampling stimulus, the behaviour in the assembled group was consistent throughout the day. Other important factors that were considered for the assessment of diurnal variation in albumin concentration are tear flow rate and tear volume. Menisci heights are indicative of tear volume and have shown significant increase immediately after waking, with subsequent stabilisation to normal levels within an hour, supporting the notion of an altered ocular environment during sleep (Shen *et al,* 2008). No difference however, was found in the menisci height throughout the day or in the evening just before bed, suggesting that altered tear volume is not responsible for the evening shift seen in albumin concentrations in this study. This is further supported by the measurement of tear evaporation by Tomlinson and Cedarstaff (1992). Evaporation was

measured at two-hourly intervals for 14 hours using resistance hygrometry and was lowest upon waking and then rises within two hours to consistent values for the rest of the sampling period. Similarly diurnal variation of tear osmolarity, measured between 8:00 and 17:00 hrs by TearLab®, has been shown not to be statistically significant (Oncel *et al*, 2011). In the present study, tear flow rate was carefully monitored for each subject using the grading on the microcapillary and correlating collection time with collection volume. While tearing response was different between subjects, tear flow rate did not deviate significantly within a subject between collections throughout the day. This, together with the literature data above, suggests that the diurnal variation shift in seen in the evening is a real phenomenon and not a result of altered lacrimation dynamics.

It is also important to remember the variety of functions performed by albumin in plasma (section 1.4.3). The versatility of albumin to bind a large variety of ligands with high affinity and its high concentration in plasma expands the possible consequences of the late evening albumin increase in tears. It might denote an albumin mediated ligand effect rather than being a reflection of albumin concentrations on their own. E.g. albumin could provide extra fatty acids to improve the stability of the lipid layer or bilirubin to increase antioxidant activity in the tear film. In addition to this, other plasma components able to move in similar ways to albumin need to be evaluated as albumin may only be a marker of effects by other components. The potential consequences of the altered ocular environment in the evening presented in this study cannot be properly ascertained until the specific roles of albumin, its ligands, and other plasma derived proteins which are also increased overnight have been determined.

4.6 Conclusion

A distinctive asymmetric pattern throughout the waking day was established, which encompassed individual subject-to-subject variation in absolute concentration levels. Importantly, this work highlights for the first time a consistent pattern of variation in albumin concentration throughout the day. The effect of vascular leakage on the ocular surfaces is at present poorly understood and it is unclear whether albumin levels rise simply as a consequence of external insult and vascular leakage, or as a means of protection of the ocular environment through the recruitment of plasma components. The fact that increases in albumin levels occur in conjunction with a number of ocular disorders has been widely established (chapter 3). Similarly, the closed eye environment has increased albumin and is described as exhibiting sub-clinical inflammation. In line with ocular disorders, this is a non-specific link. The research presented in this chapter shows increases in albumin in the seemingly healthy eye, prior to the eye closure that triggers the hypoxic state of the cornea and the change in tear fluid

state. This early increase suggests an active, rather than passive, role of albumin (or other proteins it may be a marker for) in the evening tear film. It is clear that the concentration of albumin in tears is not stable. It is subject to change in a manner that is unlike that of any other prominent tear protein.

CHAPTER 5
EVALUATION OF SCHIRMER STRIP AND MICROCAPILLARY SAMPLING FOR COMPATIBILITY WITH POINT-OF-CARE TECHNIQUES

5.1 Aim

The aim of the experiments presented in this chapter was to further compare and assess the two most commonly used and commercially available sampling techniques (Schirmer strip and microcapillary) and their impact on the collection of tear albumin. Ultimately, the purpose was to evaluate their suitability for use in conjunction with automated point-of-care analysis in contact lens clinics. Microcapillary sampling has little impact on the observed tear albumin concentrations and is comparatively non-invasive. Schirmer strip sampling is harsher but has other benefits and is useful for subjects with low tear volume, where microcapillary sampling is more difficult. The Schirmer strip also requires less training than microcapillary before use, and tear flow is easily assessed. Most importantly, the Schirmer strip is already an accepted and widespread technique routinely used in contact lens clinics and would therefore be highly suitable for point-of-care analysis. However, these benefits are reduced if the induced albumin increase overrides any possibility of discerning and discriminating between albumin concentrations in a population.

5.2 Introduction

The research presented in the previous chapters highlight the fact that albumin is still poorly understood in the tear film and that there is a need to gather additional data sets. Requirements for the undertaking of albumin research have also been identified. Important factors to consider are diurnal variations, consistency in sampling and assaying technique, and population selection. Contact lens clinics are ideal locations for recruiting larger populations for tear film research for several reasons. Follow up routines and regular eye checks for customers enable study subjects to be assessed over longer time periods without extra effort involved on the subject's behalf. Visual assessments of the tear film are also conducted during appointments, and can therefore be correlated with any subsequent albumin results. Furthermore, as shown in chapter 4, albumin concentrations are relatively stable during the daytime, which corresponds well with normal opening hours of clinics and research centres. The negative aspects of sampling in a commercial setting are the issues surrounding sample analysis and tear collection. Loss of protein, through degradation and adsorption to the sample vial, has been shown to be a factor during long periods of storage (Sitaramamma *et al* 1998b) and sample transit between contact lens clinics and laboratories will promote such degradation. Additionally there are few laboratories available for tear film protein analysis, which will increase the cost and the time

frame of obtaining the results. There will also still be a factor of variability imposed by the assay methods employed at different research laboratories. A solution to sample analysis problems might be to utilise mobile analysis devices that are able to assay the tear fluid sample in the clinic and provide the practitioner with the results within the duration of the appointment. For these reasons, it was decided to investigate point-of-care analysis as a solution to avoiding some of the sampling and assay related albumin variations. Point-of-care analysis is performed by small automated bench-top, or handheld, analysers and is usually fast (<10 minutes) and simple. It is already prevalent in the quantification of proteins in other biological fluids for diagnostic purposes e.g blood glucose in diabetes and urinary albumin in microalbuminuria (Warsinke *et al,* 2009). Further details on point-of-care analysis are provided in chapter 6.

Inconsistency in tear sampling protocol provides another source of variation between tear film studies. In terms of use in contact lens clinics, the choice of tear sample technique also carries ethical concerns. Microcapillary tear sampling represent the least invasive sampling tools but requires training and rigorous assessment. Schirmer strips on the other hand are already routinely used in clinics and would not pose any ethical issues if used for albumin sampling. The assessment of reported albumin concentrations in the literature showed a correlation between increased levels of albumin and filter paper based sampling techniques (section 3.5.2). Reported albumin concentrations sampled by Schirmer strip are almost 50 times greater compared to the less invasive microcapillary. Similarly, directly comparative studies revealed an increase in plasma products, including albumin, with Schirmer strip sampling (Stuchell *et al,* 1984) although these were not always quantitative (Grus *et al*, 2005)(Green-church *et al*, 2008). The literature, however, lacked detailed commentary on Schirmer strip wetting profiles and microcapillary stimulus, which require more in-depth analysis. It is therefore important to investigate the suitability of Schirmer strip sampling further.

5.2.1 Objectives for the evaluation of Schirmer strip sampling

The majority of assay methods used in the literature to quantify albumin concentrations by Schirmer strip were less sensitive compared to modern assays e.g. ELISA and HPLC (Table 17). Thus the first research objective was to assess Schirmer strip sampling with ELISA analysis to evaluate if the technique produces similar concentrations and good discrimination between subjects. A subset of sampling protocols in the literature has only assayed portions of the Schirmer strip rather than the strip as a whole (Shoji *et al,* 2003; Zhou *et al*, 2008; Grus *et al,* 2005). Therefore, the distribution of albumin in worn Schirmer strips was also assessed to examine the diffusion rates of albumin and the validity of selecting sections for analysis. The second objective of this investigation was to confirm and expand upon the discrepancies in

albumin levels found between sampling methodologies in the literature. This was done by comparing albumin levels in a population sampled by both microcapillary and Schirmer strip, paying special attention to individual response to sample stimulus.

5.2.2 Choice of extraction protocol for the evaluation of Schirmer strip sampling

Schirmer strip extraction was discussed in chapter 3 (section 3.6) and showed a large variation in protocols. Furthermore, albumin specific extraction data was even more scarce and in some parts conducted more than 20 years ago. Choice of extraction technique was therefore important for the analysis of albumin in worn Schirmer strips. A study carried out in the Aston University Biomaterials Research Unit, prior to the present research, necessitated the evaluation of Schirmer strip extraction protocols. The aim of the study was to design an extraction protocol for Schirmer strips obtained from a contact lens clinic. Ten different protocols and five different extraction buffers were assessed, covering phosphate buffers at a range of pHs and acetonitrile trifluoroacetic acid buffer. The experiments devised to compare extraction protocol were of a spike and recover type, where 5µl of microcapillary tear samples were added to Schirmer strips and allowed to diffuse. The wet portion of the strip was then dissected and placed in a 500µl microcentrifuge tube before it was subjected to the various extraction protocols. Electrophoretic protein profiles of the extracted sample and the original microcapillary sample were compared using the Agilent 2100 Bioanalyzer, as described by Mann and Tighe (2007), to evaluate the extraction protocols. Centrifugation without the addition of buffer solution was shown to be on par with, or preferential, to the other protocols. In this method, a hole is punctured in the bottom of the 500µl microcentrifuge tube containing the Schirmer strip sample. The 500µl microcentrifuge tube is then placed in a 1.5 ml microcentrifuge tube and centrifuged for 5 minutes at 10,000g, after which, the residual liquid in the larger tube is collected for analysis (section 2.4.3). Benefits of the described protocol are that it is: fast and cheap with a simple procedure and it exhibits a good protein profile in comparison to its respective microcapillary collected tear profile.

Schirmer strip samples in the present experimental research were therefore collected by this centrifugation protocol without the addition of elution buffer. This choice was made with several factors in mind. It was important to maintain a good correlation in protein content with microcapillary samples, while avoiding extraction mediums that could affect the subsequent ELISA analysis or future point-of-care systems. Another factor was the ease and simplicity of the protocol and the proven usefulness for extracting samples obtained in a clinical setting. An additional benefit was keeping sample techniques consistent within the departmental research. Information in the literature did not suggest that centrifugation was unadvisable compared to

other protocols (section 3.5) but it is fully recognised that the resultant sample value is not a complete representation of the albumin concentration sampled by the Schirmer strip. It should also be noted that the present study was conducted before the research of Denisin *et al,* (2012), described in section 3.5, was published and was thus unable to benefit from the results of that particular investigation.

5.3 Detailed concentration analysis along the sampling length of the Schirmer strip.

The aim of this experiment was to investigate the validity of choosing sections of Schirmer strips for analysis rather than extracting the whole strip. This was done by analysing albumin concentrations in worn Schirmer strips to assess albumin diffusion through the Schirmer strip.

5.3.1 Schirmer strip collection and preparation

Samples used for the assessment of diffusion of albumin along the filter paper were obtained from 9 worn Schirmer strips. Schirmer strips were obtained both from an off site clinical study population (6) and from one subject in the university research department (3). Schirmer strips from the clinical study were collected from healthy non contact lens-wearers and were supplied whole in eppendorf microcentrifuge tubes. Samples had been labelled with wetting lengths and eye of collection, and were stored at –80 °C until time of analysis. No ocular discomfort or abnormality was reported in the subjects during the sampling. It was impossible to control time of sampling in the clinical population as rigorously as in the samples collected in the university department. All samples were however collected during working hours (9:00-17:00 hrs).

Schirmer strips were cut up into portions, using scissors, and assayed in sections of 5 mm (0-5mm, 5-10mm, 10-15 mm etc.), including the bent section forming the lip over the lower eyelid. The whole Schirmer strip was dissected for samples from the clinical study, which had been stored, but only the visibly wetted sections were dissected from the subject from the research department. This dissection took place immediately after sampling, before the albumin had time to diffuse across the entire strip. The dissection protocol provided 7-9 samples for every Schirmer strip. Schirmer strips were characterised by wetting lengths and divided into two groups 17-21 mm and 35 mm, to distinguish between medium and full wetting of the strips. Schirmer strip sections from the clinical study population were weighed before analysis to assess inter- and intra-Schirmer strip weight differences and subsequent relationship to albumin concentrations. Dry unworn Schirmer strips were also manually dissected and weighed to assess the error associated with the procedure.

5.3.2 Sample analysis

Each Schirmer strip section piece was placed in a separate 650µl eppendorf and extracted by centrifugation as described in section 2.4.3. Additions of 4 µl of deionised water to the segments were also necessary in this part of the study to collect enough sample material. In total 66 samples were assayed. Samples were analysed by the Immunology Consultants Laboratory ELISA as described in chapter 2, following a 1:500 and a 1:1000 dilution protocol of the Schirmer strip samples.

5.3.3. Results

5.3.3.1 Manual dissection of unworn Schirmer strips

The error related to the manual dissection of the Schirmer strip was assessed by a dissection of sterile dry unworn Schirmer strips and comparison of segment weights. Table 24 shows the individual weights of the Schirmer strip sections as well as the sum. It also gives the standard deviation between the section weights, which illustrates the error margin of manual dissection.

Table 24. Dry sterile Schirmer strip sections and their corresponding weights.

Section	**Weight (mg)**		
	SS1	**SS2**	**SS3**
Bent lip	1.494	1.633	1.697
0-5 mm	1.847	1.915	2.067
5-10 mm	2.099	2.191	2.152
10-15 mm	2.260	2.053	2.040
15-20 mm	2.150	1.982	2.035
20-25 mm	1.989	2.041	1.798
25-30 mm	1.785	1.977	1.671
30-35 mm	1.829	2.052	1.829
Sum	15.453	15.844	15.289
SD	0.2442	0.1619	0.1841

There was consistency across the Schirmer strips in terms of total weight and section weight. The bent lip is rounded and has a smaller area compare to the other sections. Differences in weights between the bent lip and the other sections are therefore ascribed to this variation in size rather than an expression of error when cutting. Consecutive weighing of the same Schirmer strip section was also shown to be very consistent. Consequently, weight differences seen on worn Schirmer strips are unlikely to be a result of the dissection procedure.

5.3.3.2 The distribution of albumin concentration along worn Schirmer strips

The six Schirmer strips (SS1-SS6) obtained from the clinical study were also dissected and weighed prior to centrifugation extraction and analysis. The aim of this was to investigate fluid distribution in the strips and any potential effect of storage on diffusion. Two of the 6 strips were labelled with a final wetting length of 35 mm, during tear sampling, and the remaining four had a final wetting length of 20 mm. Evaporation was found to be a problem when measuring the weights of the Schirmer strips. When samples were placed on a microbalance, the numbers on the display decreased steadily without reaching a point of stability. The effect of evaporation has been previously reported in literature (Hypher *et al,* 1980) and is a known factor for volume variation when using the Schirmer strip. In order to achieve some results for the comparison between sections weights from different worn Schirmer strips, the initial microbalance reading for every strip was taken and is presented in Table 25.

Table 25. Weights of sections from worn Schirmer strips

Section	**Weight (mg)**					
	SS1 35mm	**SS2 35 mm**	**SS3 20mm**	**SS4 20 mm**	**SS5 20 mm**	**SS6 20mm**
Bent lip	3.150	1.553	1.476	1.714	1.606	2.388
0-5 mm	3.448	2.096	1.882	2.086	2.605	2.624
5-10 mm	4.111	2.258	1.970	2.413	2.663	2.702
10-15 mm	4.263	2.220	1.939	2.291	2.948	2.583
15-20 mm	4.119	2.386	1.961	2.526	2.837	2.143
20-25 mm	3.864	2.238	1.935	2.082	3.172	1.887
25-30 mm	3.112	2.240	1.236	2.250	2.776	1.945
30-35 mm	2.732	2.540	2.567	2.628	2.962	2.203
Sum	28.799	17.531	14.966	17.990	21.569	18.475
SD	0.5678	0.2893	0.3910	0.2902	0.4758	0.3127

There was large heterogeneity between the weights of the different Schirmer strips. Schirmer strip 1 (SS1) was more than twice as heavy as most of the other five strips. Intra-strip section weights were more consistent with a possible increase in weight at the bottom of the strip (SS2, SS3, SS4) but this was not true for the whole group. The weight distribution is otherwise fairly stable, which points towards even diffusion of tear fluid in the strip. There was no apparent correlation between wetting length and weight of strip sections or total strip weight. Albumin concentrations in the Schirmer strip sections were subsequently measured by ELISA and are shown in Table 26.

Table 26. Albumin concentrations in separate Schirmer strip sections.

	Concentration (µg/ml)								
Section	**SS1**	**SS2**	**SS3**	**SS4**	**SS5**	**SS6**	**SS7**	**SS8**	**SS9**
B-L	17.76	<6	83.74	>460	359.36	47.61	>460	>460	>R
0-5 mm	15.50	60.28	194.63	>460	>460	25.76	413.42	110.1	66.1
5-10 mm	19.16	73.00	190.62	>460	>460	37.03	202.70	105.5	67.2
10-15 mm	22.77	<6	>460	>460	452.09	136.62	160.40	46.53	53.8
15-20 mm	168.45	66.75	>460	146.26	216.43	>460	132.25	10.76	69.8
20-25 mm	>460	25.62	>460	188.10	143.98	>460	146.74	/	48.3
25-30 mm	415.31	<6	>460	192.23	<6	126.27	122.73	/	/
30-35 mm	435.51	31.92	>460	208.54	<6	71.3	/	/	/

It was impossible to produce the aggregate albumin concentration of the Schirmer strips as some samples had values outside the range of the ELISA assay. Samples were re-assayed in these circumstances but this was only possible to do twice due to limited sampling material. Consequently, a subset of samples are labelled >460 or <6 µg/ml (above or below range). The majority of the bent lip sections were above the range of the assay. This is not surprising as the presence of the Schirmer strip in direct contact of the epithelial lining of the lower eyelid is likely to produce irritation and produce increased albumin concentration. Previous research on Schirmer strip albumin sampling revealed a larger portion of epithelial cellular debris in samples taken by Schirmer strip compared to microcapillary sampling (Green-church *et al,* 2008).

In general the results in Table 26 show significant heterogeneity in albumin concentrations within the Schirmer strip. SS1 exhibits significantly more albumin in sections 25-30 mm and 30-35 mm compared to the other sections. This Schirmer strip was also significantly heavier than the other Schirmer strips (Table 25), which signifies large volumes of tear fluid. During sampling, this large flow of volume, or reflex tearing, may have diluted the albumin bound to the top of the strip and transferred it to the bottom sections. Similarly to SS1, SS3 exhibits increased concentrations in the bottom sections whereas SS4 shows the opposite profile. SS2, SS5, SS6 and SS7 show more randomised albumin concentrations although the samples outside of the range makes it harder to determine an exact profile. Here it is apparent that only assaying the first 5mm or 10mm of the Schirmer strip would produce results inconsistent with the profile obtained by assaying the whole strip. Only in SS8 and SS9, which were dissected before storage, can this approach be justified. On the other hand, SS7 was also dissected before storage and would not be suitable for analysing the first 10 mm only.

5.4. The Analysis of albumin concentrations in worn Schirmer strips to determine effective dilution factors.

9 additional Schirmer strip samples were obtained from the clinical study population. These samples were used to determine optimal Schirmer strip dilution factors for the subsequent ELISA comparison with microcapillary-sampled concentrations. The research conducted in section 5.3 showed that Schirmer strip concentrations can range significantly and effective dilutions are therefore important. The additional Schirmer strip samples also acted as a valuable inter-site comparison to concentrations obtained from subjects in the present study conducted at Aston University.

5.4.1 Methods

Worn Schirmer strips were centrifuged whole, as described in section 2.4.3 and resultant sample fluids were assayed on the Immunology Consultants Laboratory ELISA at dilutions of 1:1000, 1:1500 and 1:2000.

5.4.2 Results

Table 27 shows the resultant Schirmer strip albumin concentrations from three different dilution protocols.

Table 27. Albumin concentration in the Schirmer strip using different dilution factors.

ID	Dilution factors	Concentration (µg/ml)
1015 SK	1:1000	>R
	1:1500	304.2
	1:2000	296.1
1017 MS	1:1000	>R
	1:1500	261.5
	1:2000	233.2
1019 MC	1:1000	21.2
	1:1500	19.3
	1:2000	18.9
1020 CS	1:1000	153.4
	1:1500	159.7
	1:2000	155.2
1028 HU	1:1000	23.2
	1:1500	23.5
	1:2000	23.4

Here it is apparent that the dilution factors suitable for Schirmer strip analysis by ELISA are somewhere in the regions of 1:1000 to 1:2000. A dilution of 1:1500 was able to analyse concentrations from all five strips, ranging from 18.9-304.2 µg/ml. The repeatability of samples assayed under different dilutions confirm that the ELISA assay is precise in determining Schirmer strip albumin concentrations and that significant dilution does not effect the end value.

5.4.3. Albumin concentration and wetting length

Table 28 shows the wetting lengths and albumin concentrations in all 9 Schirmer strip samples obtained from the clinical study population. Samples are tabulated in increasing concentration.

Table 28. Schirmer strip-sampled albumin concentrations and corresponding wetting lengths in the clinical study population (n=9).

ID	Wetting length (mm)	Concentration (µg/ml)
1019 MC	35	19.80
1024GBL	35	21.44
1028 HU	35	23.40
1024 GBR	35	27.25
1016 SK	35	27.30
1015 RA	35	38.60
1020 CS	33	156.11
1015 SK	31	300.17
1017 MS	13	247.37

This population displays a high proportion of subjects wetting the full 35 mm of the Schirmer strip. The range between the subjects with 35 mm wetting is 19.80-38.6 µg/ml, however, which shows that discrimination between subjects is still possible. The albumin distribution in the whole subject population is 19.80-300 µg/ml. Unfortunately the clinical study did not record the wetting time for these events, which makes the data harder to assess.

5.5 Comparison between Schirmer strip and microcapillary sampling of tear albumin concentrations.

Albumin concentrations were determined in a population sampled by Schirmer strip and microcapillary. The purpose of this was to further investigate the discrepancies between the two techniques reported in the literature. Special attention was paid to Schirmer strip wetting profile and tear flow stimulus by sampling. Ultimately, the aim was to see whether it was possible to distinguish between albumin concentrations in individuals, or group of individuals, with Schirmer strip sampling and if results could be related back to microcapillary concentrations.

5.5.1 Methods

5.5.1.1 Tear collection and study population

Albumin levels were examined within a population of 10 subjects (5 male, 5 female, mean age 30 years). None of the subjects wore contact lenses during the sampling or had any external signs of ocular disorder. Tear samples were collected by an experienced researcher and all sampling was conducted in the same one-hour period (15:30-16:30) to avoid any time-related variables. Firstly, tear samples were collected from the subject's left eye using microcapillary and secondly from the subject's right eye using the Schirmer strip. A detailed description of sample collection protocols is given in chapter 2. 4µl of tears were collected by microcapillary from the left eye and samples were recorded as being reflex, basal or slightly stimulated (medium) based on tear flow rates. Post microcapillary sampling, a Schirmer strip was placed into the right eye and removed after 5 minutes. The wetting length at this time was recorded. If the strip was fully wetted prior to the allotted five minutes, the strip was removed at the time of full wetting and this time was recorded. In total 20 samples were collected from the population.

5.5.1.2 Tear sample processing and analysis

Collected microcapillary tear samples were expelled into individual 650 µl microcentrifuge tubes by a sterile wire plunger. The Schirmer strip fluid was collected by centrifugation of the whole strip in an eppendorf microcentrifuge tube, as described in section 2.4.3. It should be noted that 1 Schirmer strip sample (subject 3) had a wetting length below 14 mm. Previous studies in the research department have shown that this low wetting length necessitates an addition of 4 µl of deionised water to the Schirmer strip in the microcentrifuge tube before centrifugation to collect sufficient amount of fluid. Whilst this does have a small dilution effect, it

doesn't have a major effect on resultant concentration value, as can be seen in subsequent result sections where subject 3 still presented with the highest measurable amount of albumin.

Samples were analysed using the Immunology Consultants Laboratory ELISA. Microcapillary samples were diluted at 1:200, which ensured that the majority of samples were within range during assaying. Assayed Schirmer strips in the clinical population produced a large range of concentrations (section 5.4.3). Samples in the present experiment were therefore initially assayed at 1:1500, which appeared to place the majority of concentrations within the range of the assay.

5.5.2 Results

5.5.2.1 Population demographic and response to sampling technique

The sample collection data and population demographic is displayed in Table 29. This enables the comparison of Schirmer wetting length, wetting time and microcapillary tear sampling flow in the population.

Table 29. Population demographic and sample collection data of the parallel microcapillary and Schirmer strip sampling.

Subject	Gender	Age	Schirmer length (mm)	Wetting time (min, sec)	Microcapillary tear sampling flow
1	F	25	21	5	Medium
2	M	22	26	5	Reflex
3	F	29	13	5	Basal
4	F	23	33	5	Basal
5	M	26	17	5	Reflex
6	M	44	35	3 min 20 seconds	Reflex
7	F	24	21	5	Basal
8	M	25	35	1	Reflex
9	M	39	30	5	Reflex
10	F	40	35	39 seconds	Medium

The mean Schirmer wetting length for the population was 26.6±8.2 mm, which corresponds well with previously reported afternoon values of 25.9±10.6 mm in the literature for 51 subjects (Lira *et al,* 2011). This indicates that the population was normative in terms of Schirmer wetting length and distribution. Subjects 6, 8 and 10 gave the most rapid generation of Schirmer wetting. Half of the study population produced reflex tears when sampled with microcapillary tube. Subjects 1, 6, 7, 8 and 10 have been previously sampled as part of intra-departmental

research and have been well characterised in terms of lacrimation response. The remaining subjects produced reflex (three) and basal (two) tears in response to sampling. The distribution of reflex tearing in the population is either merely a representation of the normal state of the subjects in terms of stimulus response or a reflection of sampling neophytes. Nevertheless there is no reason to suggest that the study subjects were in any way abnormal and unsuitable as a representation for a wider population.

The results in Table 29 showed no apparent correlation between microcapillary tear flow and Schirmer strip wetting length in the population. This lack of relationship is further visualised in Fig 5.1. Microcapillary samples were divided into medium, reflex or basal tears for each subject and compared to the Schirmer wetting length.

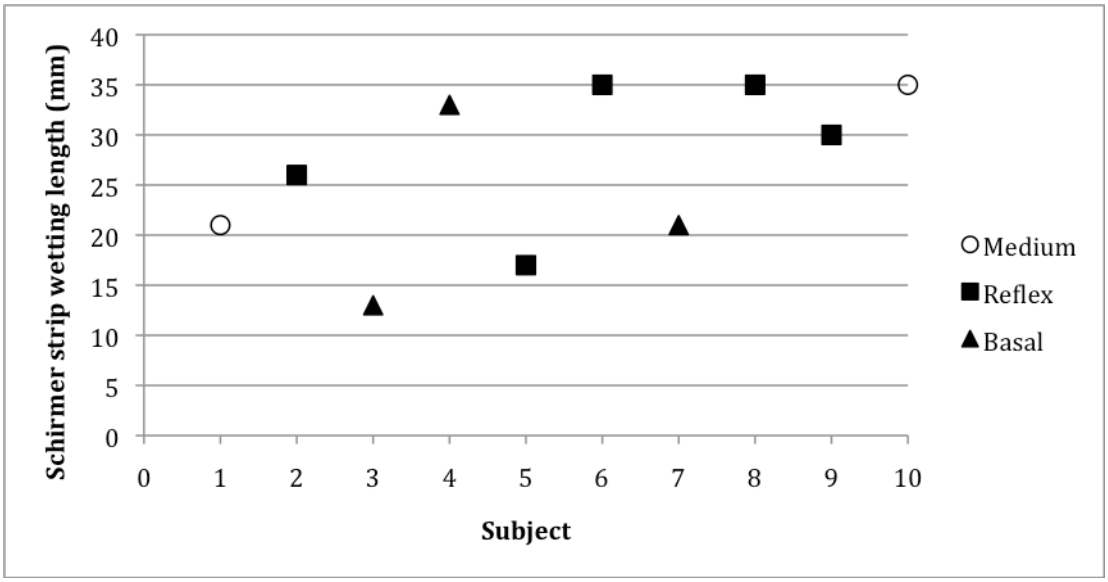


Fig 5.1. Comparison of Schirmer strip wetting length and response to microcapillary tear collection in subjects.

Reflex tearing during microcapillary sampling did not lead to the longest wetting length when sampled by Schirmer strip. Subjects with medium tear flow (neither basal nor obvious reflex tearing) when sampled by microcapillary represented 20 mm and 35 mm of the wetting length distribution.

5.5.2.2. Microcapillary and Schirmer strip-sampled albumin concentrations

Albumin concentrations in the population sampled by both techniques are shown in Table 30. Subjects were ranked in order of increasing concentration for microcapillary and Schirmer strip sampling respectively to investigate any correlations between the two.

Table 30. Microcapillary and Schirmer strip tear sampling concentrations (n=10)

Subject ID	**Microcapillary concentration (µg/ml)**	**Subject ID**	**Schirmer strip concentration (µg/ml)**	**Does ranking agree?**
8	0.75	8	3.0	Y
1	3.9	4	36.8	-
9	4.9	10	89.0	-
2	5.2	2	199.4	Y
5	7.1	7	>200	-
10	7.5	1	296.5	-
7	7.9	6	361.7	-
6	13.9	9	>400	-
4	16.3	5	>400	-
3	21.5	3	>400	Y
Mean	**8.9**		**~238.7**	

Microcapillary sampled albumin concentrations ranged between 21.5 µg/ml and 0.75 µg/ml with a mean concentration of 8.9 µg/ml. These values are slightly lower compared to the concentrations sampled by microcapillary in asymptomatic subjects in the literature (Table 18). Table 18 has a range of 10.3-200 µg/ml, although as previously discussed in chapter 3, the higher values could potentially be traced back to assay techniques. As half of the population in the present study has been sampled regularly by experienced researchers and is well characterised, it is reasonable to believe that the difference between results in the literature is related to a difference in study population and assay technique. In terms of the albumin distribution in the whole subject population (0.75->400 µg/ml) results are within a similar range to the data obtained in the clinical population (19.80-300 µg/ml) (shown in Table 28).

Four of the samples obtained by Schirmer strip sampling were out of range of the ELISA assay even with substantial dilution (Subjects 3,5,7,9) and a minimum value was only able to be determined. The calculated average value and range is therefore a useful description of the population but not completely accurate. Mean Schirmer strip albumin concentration in the present study population is 238.7 µg/ml, compared to the 954.5 µg/ml literature concentration quoted in Table 17, and the range is 3.0->400 µg/ml. The relative discrepancy between the reported literature values and the current study concentrations could be three-fold. Firstly the Schirmer strip concentrations in the present study only represent the minimum values detected

in half of the samples and true concentrations could be radically underestimated. Secondly the highest concentrations reported in the literature were obtained over 20 years ago with less sophisticated techniques, allowing some margin of error. Thirdly, the present albumin concentrations were collected through centrifugation rather than diffusion extraction, which could leave albumin residues on the Schirmer strip (Prause, 1979; Denisin *et al,* 2012).

The majority of microcapillary-sampled concentrations are assembled in a cluster between 5 and 10 μg/ml. Corresponding Schirmer strip concentrations however show a much more significant range between 90 and 400 μg/ml far exceeding the differentiation between microcapillary levels. Ranking concentrations obtained from subjects by both techniques (Table 30) showed that only three subjects exhibited a direct correlation in albumin concentration. Two of the three subjects (8 and 3) represented the second fastest and the slowest tear flow rates in the population when sampled by both techniques. The albumin concentrations in these cases are therefore likely to be a direct result of tear flow i.e. the most basal tear flow has the least diluted albumin and the highest reflex tear flow has the most diluted albumin.

There appears to exist subject-to-subject variation in the population that was not related to the separate sampling techniques. Figures 5.2a-5.2c attempt to provide some supporting evidence for this hypothesis. The variables have been chosen as variations in Schirmer flow rates rather than variations in the already established microcapillary technique for the comparisons made. Fig 5.2a compares albumin concentration in the subject population sampled by Schirmer strip and microcapillary without any discrimination in terms of sampling conditions. Fig 5.2b displays the same data as in Fig 5.2a but the subjects have been categorised with respects to wetting length and time. The three categories chosen were the subjects with normal wetting time (5 min), the subjects with rapid Schirmer wetting (<5 min) and the subjects with slow lacrimation and a resultant wetting length of <20 mm. Fig 5.2c shows the same subject data with the addition of circles around the subjects producing reflex tearing upon microcapillary sampling.

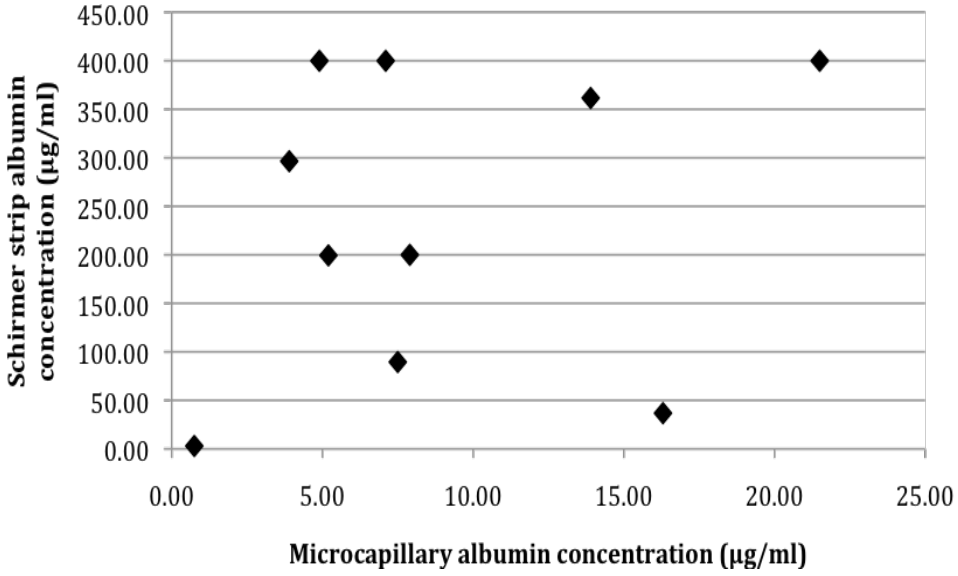


Fig 5.2a. Microcapillary albumin concentration in the ten subjects and the corresponding Schirmer strip concentration.

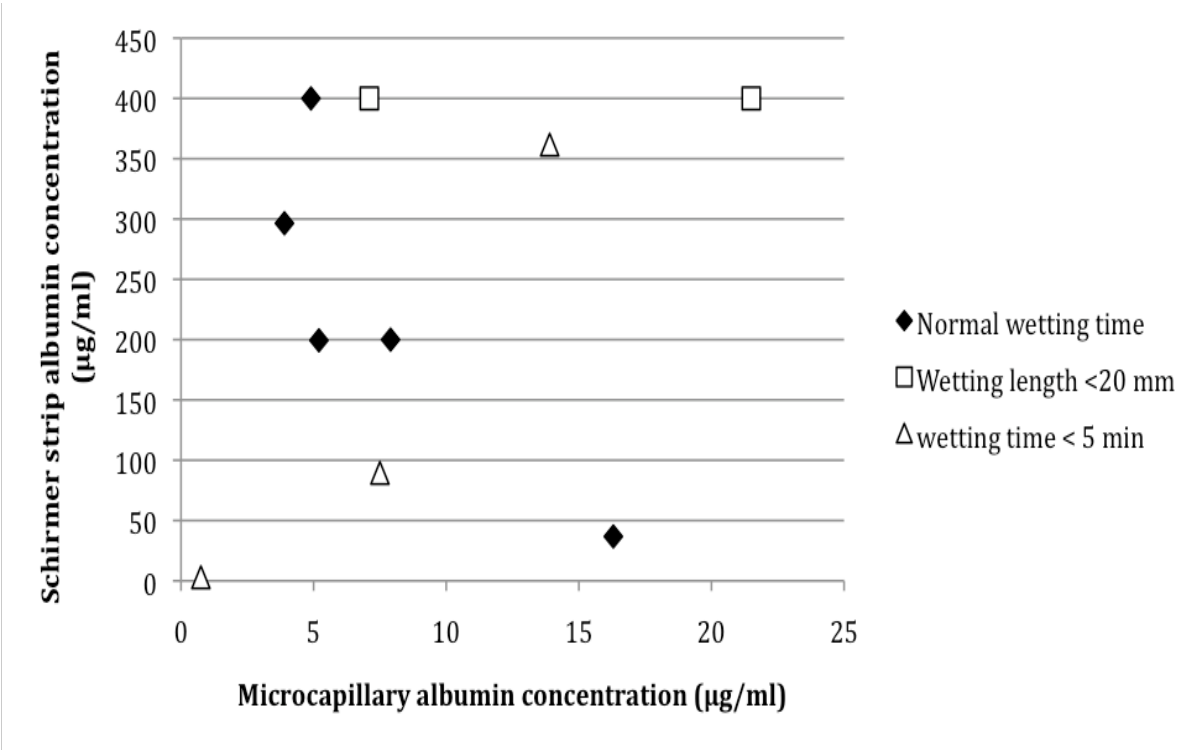


Fig 5.2b. Albumin microcapillary concentration and corresponding Schirmer strip concentration in subjects divided into three categories according to stimulus response.

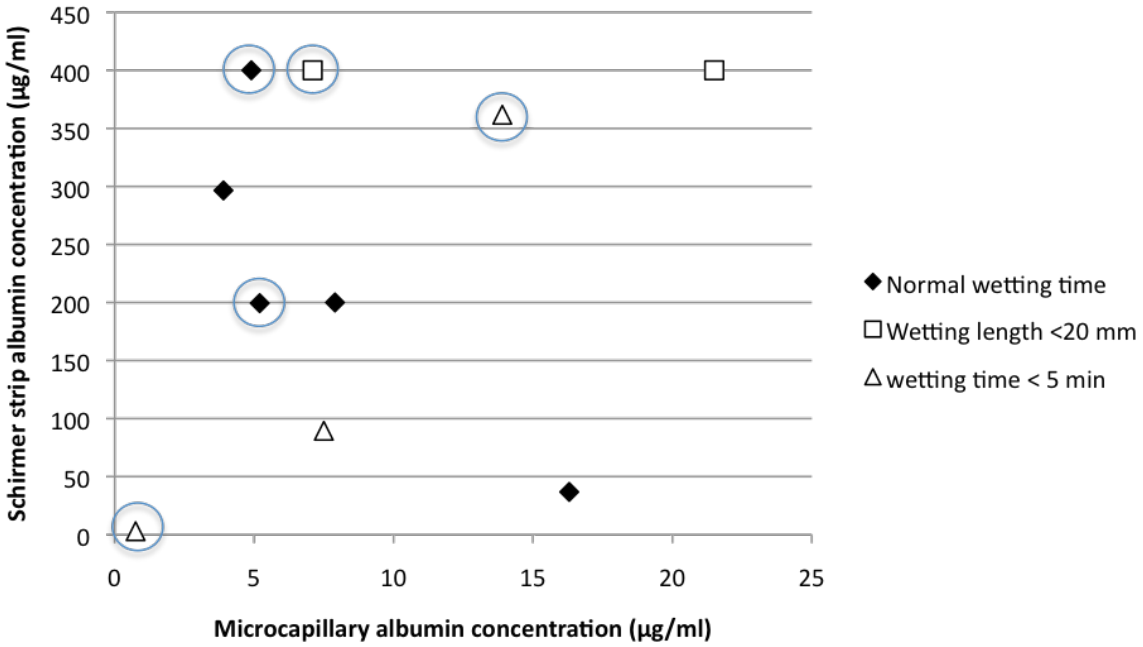


Fig 5.2c. Duplicate of data presented in Fig 5.2b with the addition of discrimination of reflex tearing subjects when sampled by microcapillary. These subject data points are identified with a blue circle.

Figure 5.2a displays no apparent relationship between microcapillary albumin concentration and Schirmer strip albumin concentration. When discriminations are made in terms of sampling technique stimulus (Fig. 5.2b-5.2c), the normal wetting subjects follow the trend of no correlation between sampling techniques. Subjects experiencing decreased lacrimation, or a wetting length below 20 mm, both presented with the highest measured Schirmer strip concentrations suggesting some relationship between wetting length and albumin concentration. Increased lacrimation in the two subjects with wetting times below 1 min appear to show a definite negative correlation between microcapillary and Schirmer strip concentration and stimulated tear flow. The third subject with a wetting time closer to the normal 5 minutes (3.26 min) appears to not support this trend inferring that only extreme cases of induced lacrimation affect albumin concentration in a predictable way. Fig. 5.2c confirms that whilst degree of lacrimation has an effect on albumin concentration it is impossible to infer Schirmer strip responses and concentrations in a subject from corresponding microcapillary responses and vice versa. There are however several problems in assessing the data as there were variations in flow rate present both with microcapillary sampling and variation in flow rate with the use of the Schirmer strip. Table 31 examines some of the more unusual subjects in terms of concentration and stimulation.

Table 31. Albumin concentration and sampling technique stimulus response in a few selected subjects.

Subject	Schirmer length (mm)	Wetting time (min)	Schirmer strip µg/ml	Microcapillary µg/ml	Tear sampling flow
3	13	5	>400	21.5	Basal
5	17	5	>400	7.1	Reflex
10	35	0.66	89.5	7.5	Medium

Subject 3, which had a basal tear sampling flow and the highest microcapillary-sampled albumin, also had the highest Schirmer strip concentration and the lowest wetting length. This is useful evidence that supports the view that flow rate is undoubtedly a factor in albumin sampling. Contrastingly subject 10 had the most rapid wetting time for Schirmer and a medium tear flow with a microcapillary value of 7.5 µg/ml and a Schirmer value of 89.5 µg/ml. This microcapillary concentration is slightly less than the average of 8.9 µg/ml but by no means at the lowest end. The Schirmer wetting rate was significantly faster in comparison to all the other subjects and consequently the resultant albumin concentrations would be expected to be much lower if flow rate was only a factor. Subject 5 complicates the results further in having the second lowest Schirmer wetting length but reflex tears when sampled by microcapillary. The resultant Schirmer strip value is among the highest (out of range of the assay) and the reflex microcapillary tear value on the other hand is at the lower end of average.

These data presented in Table 31 suggest that whilst degree of lacrimation does have an effect, stimulation by Schirmer strip does not necessarily equate to stimulation by microcapillary. The possible conclusion is the presence of an individual enhancement factor, able to override technique-induced stimulation effects on albumin concentration. The step-by-step analysis of subjects presented above leads to two apparent deductions regarding sampling and stimulus:

- Tear flow rate was contributory to albumin concentration differentiation between subjects but not an overriding factor.
- Individual subject susceptibility to tearing exists by either sampling technique but not necessarily both.

5.6 Discussion

This chapter aimed to further investigate the two most common tear sampling techniques, Schirmer strip and microcapillary, with respect to in-vivo and ex-vivo effects on the determination of tear albumin.

Diffusion of albumin concentration within worn Schirmer strips was determined to investigate the validity of analysing a selection of the strip rather than the whole. The tear fluid in worn Schirmer strips, stored in microcentrifuge tubes before assaying, appeared to reach equilibrium in terms of volume and weight distribution in the strip (Table 25). Assessment of albumin diffusion in the Schirmer strip, however, showed significant differences in concentration between sections of the strip (Table 26). Furthermore, larger volumes of fluid in the Schirmer strip appeared to potentially concentrate albumin at the bottom sections. These results suggest that it is not advisable to assay separate sections of the strip and the full wetted length should be assayed instead. Consequently, Schirmer strips in the present research were centrifuged whole to obtain albumin concentrations.

It was identified in the literature that tear sample collection by Schirmer strip led to an increase in measured albumin concentrations (chapter 3). The extent of increase was varied and may have been influenced by other factors of variation such as analysis method. In the present research, tear albumin concentrations were determined in samples collected by both microcapillary and Schirmer strip in a population of 10 subjects, to compare the two techniques further. The sensitive ELISA technique was used to determine tear albumin concentrations as it was hypothesised that higher reported Schirmer strip albumin concentrations could have been partially related to the use of less sensitive assay methods. All sampling was conducted within the same hour to negate any time related variables identified in chapter 4.

The results from the comparison of the tear collection techniques were consistent with the literature and the data exhibited large differences between microcapillary and Schirmer strip-sampled tear albumin concentrations. Microcapillary collected tear albumin concentrations in the study population were between 0.75-21.5 μg/ml but the majority of samples ranged between 5-10 μg/ml. Corresponding Schirmer strip tear albumin concentrations were 3->400μg/ml for the whole population and 90->400 μg/ml for the majority of samples. The differences between microcapillary and Schirmer strip samples are made more pronounced by looking at concentration distributions between the two techniques. Fig 5.3 shows albumin distribution in 457 samples collected from asymptomatic subjects by microcapillary during the present research. The distribution of Schirmer strip concentrations collected during the

research is shown in Fig 5.4. All samples were analysed by Immunology Consultants Laboratory ELISA assays.

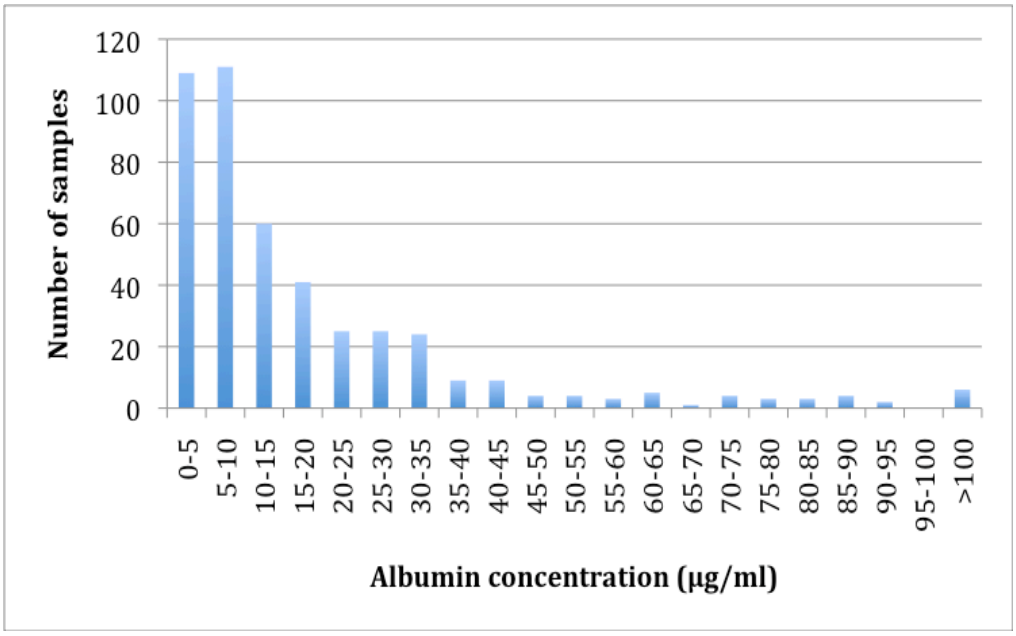


Fig 5.3. Equal interval distribution of albumin concentrations in 457 tear samples collected by microcapillary.

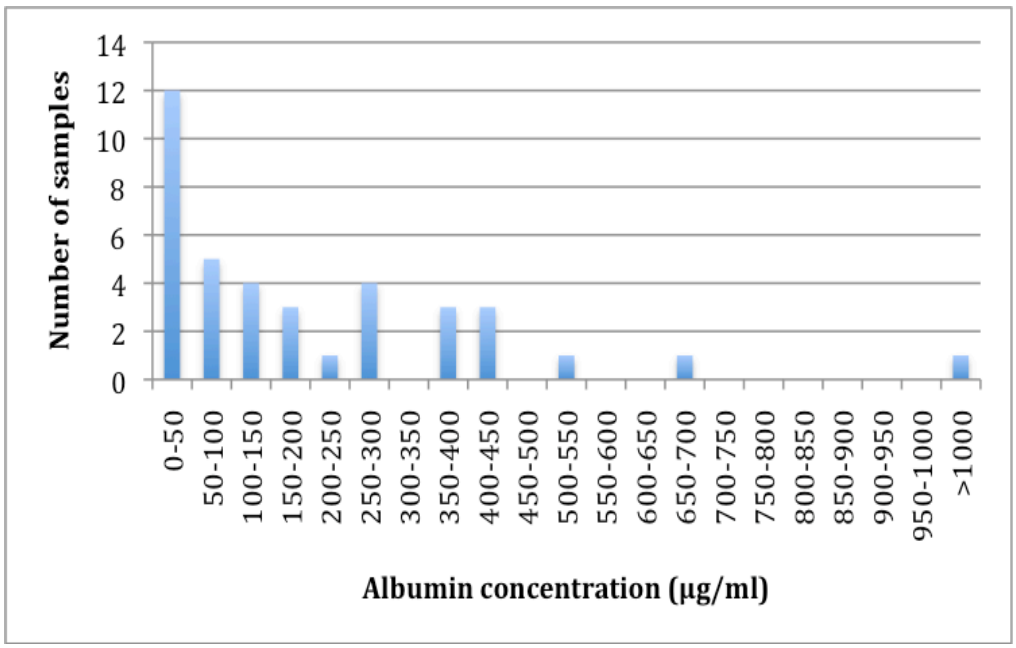


Fig 5.4 Equal interval distribution of albumin concentrations in 38 samples collected by Schirmer strip sampling.

The vast majority of assayed microcapillary albumin concentrations in the present research were below 40 µg/ml (Fig 5.3). Published microcapillary collected mean tear albumin concentrations range from 10.3-200µg/ml (Table 18). Of these, mean tear albumin levels determined by ELISA were 14.60-45.1µg/ml. This range correlates well with ELISA-derived concentrations obtained in the present research. Interestingly, the data gathered from the 457

individual sample determinations indicates that many previously reported albumin concentrations sampled by microcapillary are in fact significantly overestimated. This is potentially a result of the use of less accurate assay techniques. Schirmer strip obtained tear albumin concentrations determined by ELISA (Fig 5.4) were also lower than the literature concentrations (3-1087 μg/ml vs. 600-1830 μg/ml), although concentrations still represent a significant increase compared to the microcapillary value. Importantly, both sampling techniques also exhibit a similar concentration distribution, which is different from a typical Gaussian curve. There was no apparent direct correlation between microcapillary albumin concentrations and Schirmer strip albumin concentrations though, which suggests that the two techniques cannot be used interchangeably of one another. The results presented in Table 30 and Fig 5.4, however, show that discrimination in albumin concentration between subjects sampled by the Schirmer-strip is still possible.

The tearing response induced in subjects by each sampling technique was also investigated. This was of interest as the technique specific effects of stimulation of tear flow and of irritation was difficult to disentangle from the literature. Excessive or decreased lacrimation by subjects in the present research appeared to affect albumin concentrations, as would be expected from previous research on total protein dilution, but this was only limited to extreme cases (Table 30). Instead there was the suggestion of individual susceptibility to stimulus and inherent lacrimation enhancement factors when subjected to the two sampling techniques (Table 31). A subject experiencing reflex tearing upon microcapillary sampling is consequently not predestined to experience excess wetting and diluted concentrations when sampled by Schirmer strip. The results do however show that individual albumin concentrations could be compared, if subjects were compared with the same sampling technique (Table 30, Fig 5.4). The range of values exhibited in Fig 5.4 also show that clear distinguish between individuals with higher and lower albumin concentrations could be obtained by centrifugation extraction of worn Schirmer strips and subsequent ELISA analysis. Because differences between albumin concentrations in subjects could be identified using either sampling technique, Schirmer strip sampling and microcapillary sampling were both deemed suitable further evaluation with point-of-care analysis.

The difference in albumin concentrations between subjects sampled by Schirmer strip may be of particular interest as it is a reflection of how the individual eye deals with the mild insult of filter paper insertion. The Schirmer strip response could then potentially be extrapolated to how individuals deal with other ocular challenges such as contact lens wear. Subjects with higher or lower albumin therefore represent population subsets, which would be of interest to study.

5.7 Conclusion:

Albumin concentrations should be compared within a specific sampling technique as microcapillary and Schirmer strip concentrations cannot be related to each other with certainty. It was possible to distinguish between subjects sampled by Schirmer strip using the present extraction and assaying protocol. It is therefore of interest to investigate both microcapillary sampling and Schirmer strip sampling for point-of-care analysis.

CHAPTER 6
DEVELOPMENT AND MODIFICATION OF EXISTING POINT-OF-CARE TECHNOLOGY TO INCORPORATE TEAR ALBUMIN ANALYSIS

6.1. Aim

The aim of this study was to evaluate the viability of using commercially available point-of-care testing equipment designed for urinary albumin analysis, for tear albumin analysis. Currently there are no point-of-care systems in the contact lens clinic that directly assay albumin, or any other protein in the tear film.

6.2. Introduction

The distribution profile of microcapillary-sampled tear albumin concentrations (section 5.7), together with the albumin data sampled at the designated baseline in chapter 4, suggest that subjects have indigenously lower or higher tear albumin levels. Results in chapter 5 also showed that subjects respond differently to sampling stimulus. Collectively, the results presented in the previous chapters, highlight that larger data sets are needed to better identify and compare population subsets with different tear albumin concentrations. This chapter evaluates existing point-of-care analysis for the novel use of measuring tear albumin concentration. The reason for this is two-fold. Firstly, the aim is to identify a suitable protocol for the collection of additional albumin data and secondly the aim is to propose point-of-care testing of tear film components as a useful aid to contact lens practitioners.

The benefits of point-of-care research are discussed in section 6.3 with respect to general tear protein analysis and the determination of tear albumin. Existing methods for tear film assessment are identified in section 6.4 and placed into context with the only current tear film point-of-care analyser - the TearLab®, which measures tear osmolarity. The TearLab® has been effectively put into practice in contact lens clinics and demonstrates that there is a clinical interest in point-of-care techniques. Importantly, the key features of this system also helped to construct the criteria used for the selection of point-of-care kits for albumin analysis.

Ultimately, two urinary albumin analysers were chosen for evaluation: the HemoCue® 201 Albumin Analyser and the Afinion™ AS100 Analyser. Research presented in chapter 5 showed that microcapillary and Schirmer strip tear sampling techniques individually provided good discrimination between subjects and were suitable for further evaluation. Furthermore, it was hypothesised that Schirmer strip-sampled albumin concentrations demonstrate the individual

subject response to a mild insult to the eye. The evaluation and comparison of Schirmer strip albumin concentrations between subjects could therefore be of clinical significance as it might predict how the eye would react to other challenges, for instance a contact lens. For this reason it was decided to evaluate both microcapillary-sampled and Schirmer strip-sampled tears on the point-of-care analysers.

6.3 Benefits of point-of-care analysis

Chapter 5 introduced the notion of using point-of-care technology to facilitate the collection of additional albumin data. The use of point-of-care testing devices has long been prevalent in hospitals and general practice surgeries. More importantly, point-of-care technology has the highest annual growth rate within the in vitro diagnostic market (~10%) and use is predicted to become considerably more widespread in the near future (Warsinke, 2009). An official definition of point-of-care testing is " The provision of a test when the result will be used to make a decision and to take appropriate action, which will lead to an improved health outcome " (Price *et al*, 2004). In practice, however, point-of-care is most often used to denote portable or small handheld automated analysers for medical analysis or diagnosis. Analysers are generally characterised by simplicity of use, short analysis time and small sample volumes. An example of a prolific area of point-of-care systems is at-home disease management for conditions like diabetes, which require the careful monitoring of blood glucose levels. (Sacks *et al*, 2002).

Other biological fluids such as saliva and urine are also presently used**,** or being investigated for use, as non-invasive alternatives to blood samples (Warsinke, 2009). Although tears are only available in small volumes, they can be obtained non-invasively and can provide valuable information on the tear film composition. Tear film composition affects stability and health, and contains useful biomarkers, as discussed in section 3.6.1. With this in mind, the advantages of utilising point-of-care technology in contact lens practices could be two-fold. Firstly, it is important to realise that contact lens practitioners do not have the benefit of the same resources for clinical analysis that hospitals do. Although some external research departments are able to analyse tear fluid, the logistics of sample transport is an issue. Additionally laboratory resources are expensive and results would only be available retrospective to the appointment and the ocular observations made at that time. One potential benefit of automatic point-of-care analysers is therefore the possibility of assessing biomarkers, and more importantly evaluating the results, during the period of a contact lens appointment. In this way, a practitioner trained in the collection of tear fluid could obtain immediate valuable information regarding the state of the tear film during health and/or at the time of a problematic contact lens wear period. Secondly, from a research point of view, point-of-care testing can facilitate the collection of

additional tear film data from larger subject populations. With suitable technology, little extra effort would be required of a practitioner to sample and analyse tear proteins. Consequently, this could simplify the implementation of larger scale clinical studies. Equally, point-of-care analysis could bring more exclusive data sets to the specific investigation of the role of albumin in the tear film. The potential benefits are collated in Fig 6.1.

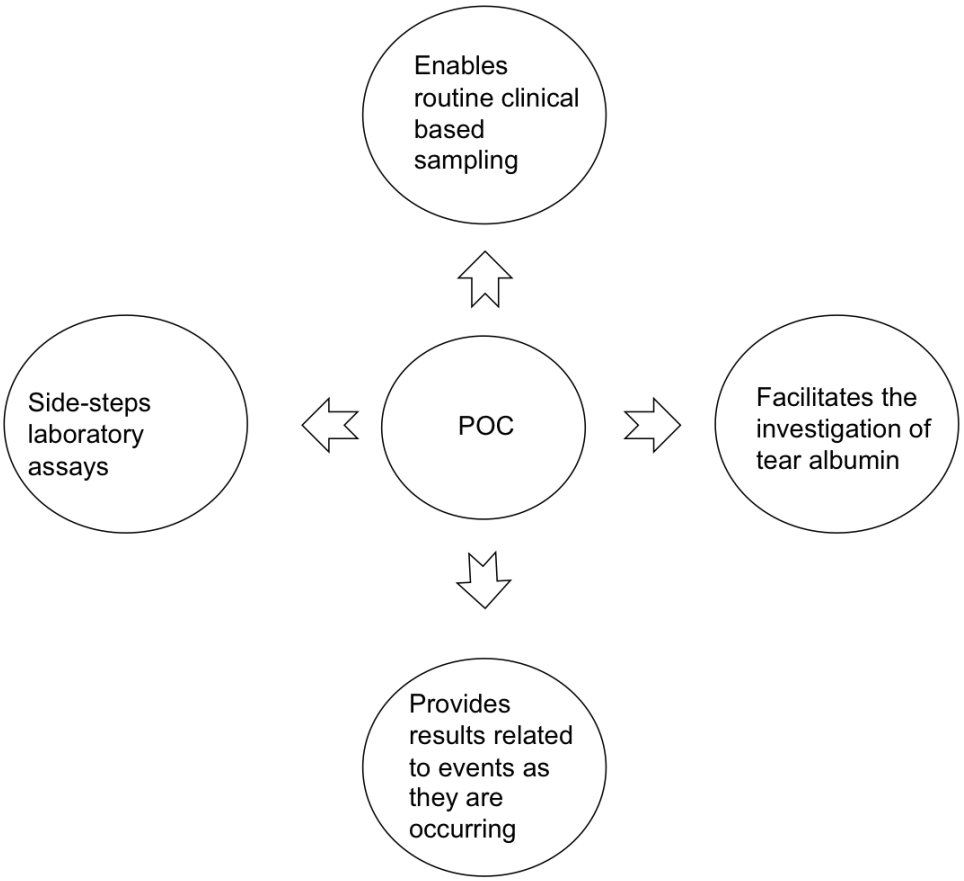


Fig. 6.1. Diagram of the potential of point-of-care analysis (POC) and how it can aid albumin investigation in tears.

There already exist point-of-care techniques for the measurement of albumin in urine. These are designed to detect microalbuminuria, which is a predictor of diabetic complications such as diabetic nephropathy and retinopathy. Microalbuminuria can be diagnosed in three different ways (Rowe et al 1990):

1. Urinary excretion levels of albumin are between 20 and 200 μg/ml in a first morning sample.

2. Urinary excretion levels of albumin are between 20 and 200 μg/ml in a 24-hour sample or on more than two or three occasions in a month.

3. Urinary excretion levels of albumin are between 20-200μg/min during an overnight timing.

The point-of-care measuring range for urinary albumin corresponds well with the range of microcapillary-sampled albumin concentration in tears reported in the literature (10.3-200 μg/ml) and determined in chapters 4 and 5 (0.75-153 μg/ml). For this reason, the automated urinary albumin analysers are of significant interest for potential use in the evaluation of tear albumin.

6.4 Existing tear film assessment techniques in the contact lens clinic

Some tests currently exist which are used routinely to evaluate the tear film during a contact lens appointment. The most common clinical techniques and the extended tests available for dry eye evaluation are detailed in Table 32 (Guillon, 2002).

Table 32. Table showing the current common clinical tests in contact lens practice and their diagnostic relevance.

Test	Target observation
Biomicroscopic observations:	
Tear meniscus	Appearance and height of meniscus
Particulate matter	Quantity of mucosal and cellular debris in the tear film
Interference	Thickness and appearance of the lipid layer
Tear film break up:	
Tear Break Up Time (TBUT)	Integrity of the tear film during prolonged eye opening with the instillation of fluorescein
Non-Invasive Tear Break Up Time (NITBUT)	Integrity of the tear film during prolonged eye opening using a spectral grid pattern projected onto the eye
Staining Agents:	
Fluorescein	Stains irregularities in the corneal epithelium
Rose Bengal	Stains cell debris and mucus fibrils
For suspected dry eye:	
Schirmer test	Tear flow with filter paper strips
Phenol red test	Tear flow with stained cotton thread
Tear dilution test	Dilution of colour over time in staining agents (Fluorescein and Rose bengal) instillated into the eye
TearLab®	Tear film osmolarity

Whilst the tests in Table 32 can be carried out during a clinical appointment, most of them cannot be classified as point-of-care tests. This is because the tests used are often subjective and rely on visual observations and grading scales where interpretation can differ between practitioners. The exception is the TearLab®, which quantifies tear film osmolarity.

6.4.1 Point-of-care testing in the contact lens clinic: The Tear Lab®

In terms of objective quantitative tests for the diagnosis or assessment of the health of the tear film, the TearLab® system is the only point-of-care test currently on the contact lens market. Recent advances in tear electrolyte analysis by fibre optic sensoring (Harvey *et al,* 2012) are however expanding the point-of-care possibilities in this area further.

6.4.1.1 TearLab® instrument specifications (TearLab® Corporation, 2012)

The TearLab® system consists of several different components: the bench-top analyser (Fig 6.2), the system pen (Fig 6.3) and the individual test card. Additional to this are control solutions and control cards.

Fig 6.2. TearLab® system reader

Fig 6.3. TearLab® system pen

The osmolarity of the tear fluid is determined by measurement of the electrical impededence, which indicates the total electrolyte concentration. A voltage is applied to the tear fluid over time and resultant impedance is then compared with a standard curve. The effect of temperature is corrected for and concentrations are given in mOsm/L. The test is performed by attaching the test card (a polycarbonate microchip) to the system pen and then using the system pen to sample 50 nanolitres of tear film from the lateral canthus of the eye. Tears are collected by passive microcapillary action and flow into microfluidic channels. These channels are equipped with gold electrodes that measure the tear fluid impedance. The system pen is then

returned to the bench top analyser docking station, where the voltage is applied and the sample is assayed.

Key features of the method are:

- Single use test cards
- Low volume of 50 nL of tear fluid required
- Short analysis time of 30 seconds
- The result is quantified automatically and shown on the display in mOsms/L.

6.4.1.2 Clinical relevance of the TearLab® system

As the tear film evaporates, the remaining tear film components are concentrated, which results in hyperosmolarity. Osmolarity measurements (total electrolyte concentration) have been mentioned as a possible international gold standard for the detection and diagnosis of dry eye (Farris, 1994). Normal tear film osmolarity values are around 302 mOsms/L. Tomlinson *et al* (2006) suggested a cut off point of 316 mOsms/L for abnormal osmolarity, derived from meta analysis of osmolarity measurements over the last 26 years. However, a 300 patient clinical trial by Foulks *et al*, (2009), suggests a lower number of 308 mOsms/L for suspected dry eye. The TearLab® measures osmolarity between 275-400 mOsms/L. A comparative analysis of osmolarity data in the literature shows that the TearLab® is generally good in distinguishing between differing severities of dry eye syndrome but has other problems (Harvey *et al,* 2012). Significant site-to-site variability was found with differences in values between studies reaching as far as 32.7 mOsm/l. Khanal and Millar (2012) also reported significant intra-assay variabilities when comparing results taken from consecutive measurements of clinical samples and TearLab® standards. According to their research, a minimum of three consecutive samples was needed to provide an osmolarity value at 95% confidence level. A further disadvantage of the TearLab® is that it is not a prospective analysis technique, able to identify at risk patients, but an additional resource used to confirm suspected dry eye. Nevertheless, its acceptance by clinicians shows that point-of-care technology for the analysis of tear fluid is a valid research area with commercial significance.

6.5. Criteria for the identification of suitable point-of-care technology

In this study, the evaluation of point-of-care testing was limited to albumin analysers that fulfilled the requirements necessary for use in contact lens practice. The TearLab® was used as a model for the identification of limitations and desirable features of the point-of-care analysers. Criteria could be divided into two categories, those specific to the needs of the practitioner such as:

- time
- cost
- space requirements
- ease of operation

and those specific to the analysis of tear fluid such as:

- volume requirements
- sensitivity
- accuracy
- suitability for microcapillary and/or Schirmer strip sampling

Based on these criteria, suitable existing point-of-care analysers for the measurement of albumin concentration in urine were identified and are compared below.

6.6 Existing point-of-care technology for the measurement of albumin

With space requirement being identified as a limiting factor, anything larger than a bench-top analyser was discarded from comparison. Available point-of-care tests for human albumin concentration in urine were compiled and compared with respect to the desired criteria as well as the laboratory gold standard – ELISA. This is shown in Table 33. In the comparison with ELISA, the cost of microplate reader, transit of samples and laboratory personnel has not been included, which would increase this cost significantly.

Table 33. Details of commercially available point-of-care kits for albumin analysis and their comparison with ELISA. Effective sensitivity indicates the sensitivity of determining albumin in a 5µl tear sample after necessary dilution factors have been applied.

Instrument	Price	Price/test	Sensitivity	Volume	Time	Effective sensitivity
HemoCue	£600-795	£1.75-2	5-150µg/ml	18 µl	1.5 min	20 µg/ml
NycoCard	£699	£5	5-200 µg/ml	50 µl	3 min	50 µg/ml
Afinion	£4,350	£7.45	5-200 µg/ml	3µl	3 min	5 µg/ml
i-Chroma	£493	£0.67	2-300µg/ml	30µl	12 min	12 µg/ml
ELISA	£260	£6.50	ng/ml	1-4µl	1.5-2 hrs	

Sensitivity was comparable across all four instruments in terms of the measuring range. Cost, sample volume and time of analysis were therefore the deciding factors in the choice of analytical technique. Sample volume is a limiting factor as the sample dilution needed to obtain enough volume alters the potential sensitivity of the instrument. This is accounted for in the last column in Table 33, which describes effective sensitivity for the assessment of a 5µl tear sample. For an example, the NycoCard analyser would need to dilute this sample by 10 to achieve a reading and therefore has an effective sensitivity of 50 µg/ml. With this in mind, the NycoCard instrument is unsuitable for tear film research. The Afinion is significantly more expensive than the competitors at £4,350 but has the lowest volume requirements and an effective sensitivity of 5 µg/ml. Additionally; the Afinion instrument is still less expensive than the TearLab instrument (£6,300 with a price per test of £10). The HemoCue and the i-Chroma are the two most cost effective instruments. Whilst the i-Chroma has a lower effective sensitivity compared to the HemoCue, the analysis time is almost 10 times as long. Ultimately, two point-of-care systems were chosen for further comparison and evaluation: the HemoCue® Albumin 201 Analyser from HemoCue Ltd and the Afinion™ AS100 Analyser from Axis-Shield Diagnostics Ltd. These kits both exhibited a shorter analysis time and lower volume requirement than the competitors.

6.6.1. The HemoCue® Albumin 201 Analyser instrument specifications (HemoCue AB, 2010)

The HemoCue® Albumin 201 Analyser (HemoCue AB) was introduced to the market in 2005 and was chosen as a primary candidate due to:

- The relatively small sample volume
- The short time frame for analysis
- The ease of operation.

The system consists of the HemoCue® Albumin 201 Analyser and individually sealed test cuvettes. Also provided are two commercially available urine albumin controls for verification and quality control:

Albutrol Low - target value: 75 mg/L (52-99 mg/L acceptable range)
Albutrol High - target value: 25 mg/L (13-33 mg/L acceptable range)

The equipment is limited to measurements between 5-150 mg/L and the analysis takes 90 seconds to perform. The HemoCue method is based on turbidity measurements. Urine is collected into a test tube, pipetted onto a piece of Para film®, and absorbed by a specially designed test cuvette containing a polyclonal rabbit-anti human albumin antibody. The antibody forms an agglutinate with any human serum albumin present in the sample and the reaction is aided by the presence of polymers in the cuvette to achieve greater sensitivity. Resultant turbidity of the cuvette is then measured spectrophotometrically at 610 nm, where the amount of scattering caused by the agglutinated particles is relative to the serum albumin concentration.

Sample volume was an initial concern with the HemoCue system, as 18 µl might prove too large for the assessment of individual microcapillary samples. Chapter 5 identified that a centrifugation protocol of worn Schirmer strips was able to give good discrimination between subjects, where tear albumin concentrations ranged between 2.63 – 1087 µg/ml (Fig 5.4). The upper measuring limit of the HemoCue is 150 µg/ml and consequently Schirmer strip samples would have to be diluted to qualify for point-of-care analysis. A 1 in 5 to a 1 in 10 sample dilution would bring the majority of Schirmer strip albumin concentrations into the measuring range of the HemoCue and, importantly, would also ensure that there was enough sample volume to meet the need for 18 µl. It was therefore decided that the HemoCue was suitable for investigation of tear albumin sampled by the Schirmer strip.

6.6.1.1 Running protocol of the HemoCue® Albumin 201 Analyser

The running protocol used for the HemoCue albumin analyser for the measurement of tear fluid is shown in Fig 6.4.

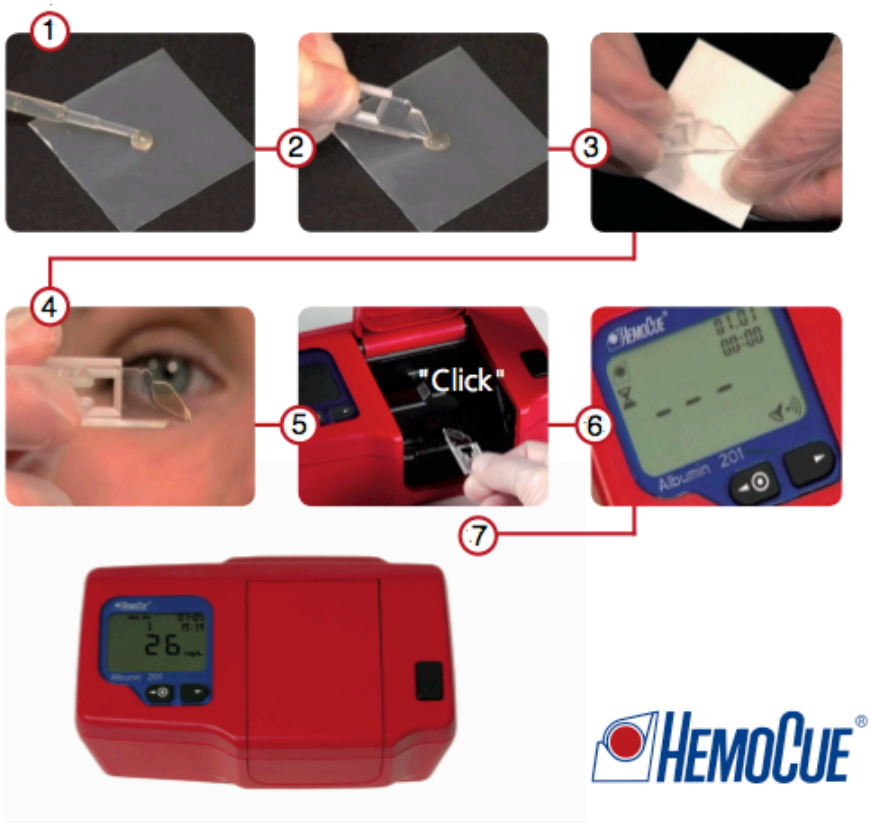


Fig 6.4. Running protocol for tear analysis on the HemoCue® Albumin 201 Analyser. (Adapted from HemoCue AB, 2010)

1. > 18 µl of the tear sample solution is expelled onto a square of Para film®.

2. The sample is absorbed into the cuvette compartment.

3. Excess fluid is wiped off the edge of the cuvettte with tissue paper.

4. The cuvette is visually assessed to be completely filled with no bubbles.

5. The cuvette is positioned into the HemoCue® Albumin 201 Analyser.

6. The sample is analysed for 90 seconds.

7. The result is displayed on the analyser screen in mg/L.

6.6.2. The Afinion™ AS100 Analyser instrument specification (Kvam *et al,* 2007)

The Afinion™ AS100 analyser (Fig.6.5) was chosen as a secondary alternative to the HemoCue® Albumin 201 Analyser with respect to the cost but is a potentially superior kit in regards to volume requirements, range and effective sensitivity. With a volume of only 3 µl this system is able to analyse single microcapillary samples neat without dilution. Other benefits include the choice of entering patient and operator ID to keep sample records easily obtainable. The range of the system is 5-200 mg/L with an analysis time of 3.5 min. The system consists of the Afinion™ AS100 analyser and individually sealed test cartridges with a detachable microcapillary (Fig 6.6).

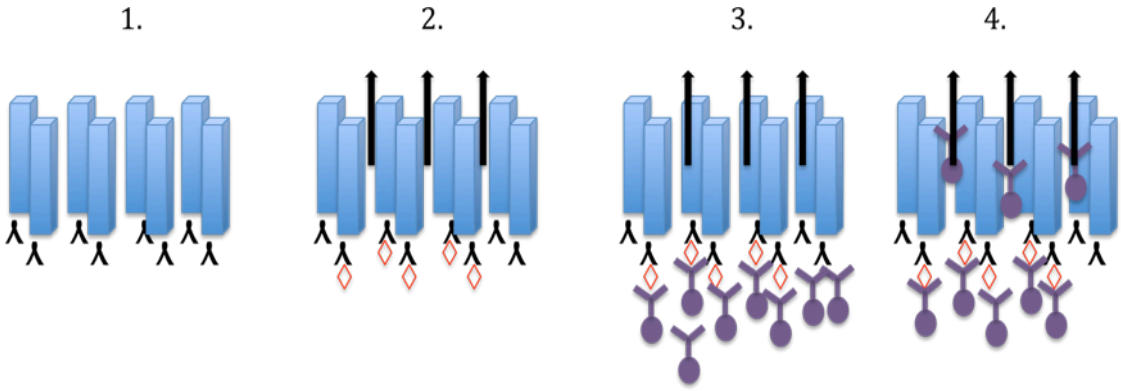
Fig 6.5. The Afinion™ AS100 Analyser. Fig.6.6 Afinion test cartridge (www.alere.co.uk)

The Afinion system also provides control solutions with the ranges below:

Control 1 target value: 12.3 µg/ml (9.9-15.8 µg/ml acceptable range)
Control 2 target value: 84.5 µg/ml (67.6-101.4 µg/ml acceptable range)

In addition to albumin analysis, the system simultaneously measures creatinine and the albumin to creatinine ratio (ACR). This ratio is used to compensate for variations in urine concentration in spot-check samples. Whereas the HemoCue determines albumin concentrations by turbidity measurements, the Afinion uses methods similar to ELISA. The sample is soaked through a membrane coated with monoclonal anti-human albumin antibodies. This membrane is then sequentially soaked with conjugate solution containing ultra-small gold particles and lastly a

washing solution (Fig 6.7). Creatinine analysis is performed simultaneously to albumin analysis by enzymatic colorimetry as described in Fig 6.8.



1. Membrane coated with mAb ( )
2. Aspirating diluted sample with antigen ( )
3. Aspirating gold-mAb conjugate ( )
4. Washing

Fig 6.7. Analysis method for albumin by the Afinion ™AS100 Analyser.

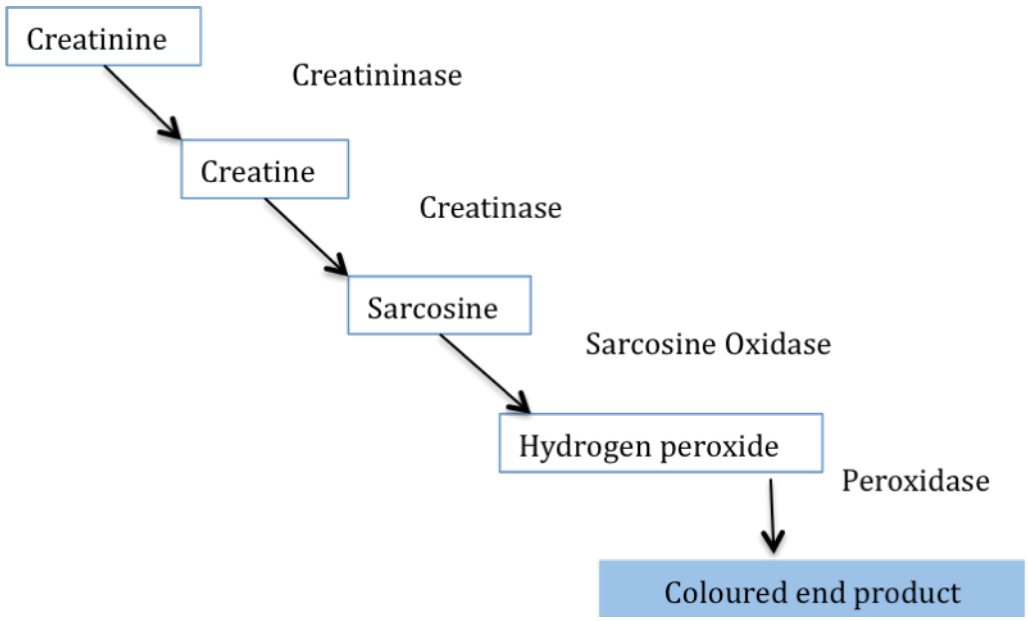


Fig 6.8. Analysis method for the detection of creatinine by the Afinion™ AS100 Analyser (adapted from Kvam *et al*, 2007). The sequential additions of enzymes to the test solution, as detailed, eventually results in a coloured end product.

6.6.2.1 Running protocol of the Afinion™ AS100 Analyser

1. 4 µl of tear sample is expelled onto a square of Parafilm®.

2. 3 µl of sample is absorbed by the detachable capillary on the Afinion™ ACR Test Cartridge.

3. The capillary is replaced into the cartridge, which is then placed in the Afinion™ AS100 analyser

4. The sample is analysed for 3.5 minutes

5. The result is given on the digital display in mg/L.

A noteworthy observation of the Afinion cartridge is the capillary design. For the protocol above, the sample has been previously taken with microcapillary and expelled onto Parafilm®. The design of the test cartridge (Fig, 5.7) would, however, make direct sampling from the eye a potential possibility. For the determination of other analytes, the Afinion analyses whole blood. The blood is collected by the detachable capillary directly from a finger prick, which demonstrates that the cartridge material has been evaluated for the risk of backwards contamination. However, as bubbles in the capillary will render it useless, direct sampling would only be truly useful for the collection of steady flow tears or reflex tears. Also confirmation of its suitability and information about the materials used in the system would be needed from the manufacturer.

6.7 Evaluation of point-of-care technology for the analysis of tear albumin

The HemoCue® Albumin 201 Analyser and the Afinion™ AS100 Analyser were compared against the ICL ELISA, which, as detailed in chapter 2, is one of the standard laboratory techniques used for tear protein analysis and has already been identified as a suitable analysis method for albumin. Disadvantages of the ELISA technique, which makes it unsuitable for use in clinics, are that it is expensive, time consuming and requires both laboratory equipment and some level of training. Benefits are that it is sensitive, with very small volume requirements and that it is highly accurate. These are desirable qualities for a point-of-care system and consequently, ELISA was used as the gold standard against which the point-of-care analysers were measured.

Prior to any tear sample research, albumin standards of known concentrations were evaluated to assess how the point-of-care kits compared with ELISA. Both deionised water and phosphate buffered saline (PBS) solutions were analysed, as PBS is closer to the tear film in terms of characteristics but water might be more practical for use in contact lens clinics. Tear samples collected by microcapillary and Schirmer strip were analysed subsequent to standards analysis. The microcapillary sample distribution profile achieved by the Afinion™ AS100 Analyser was also compared to the larger population profile determined by ELISA, detailed in section 5.7.

6.7.1 Analysis of albumin standards by point-of-care techniques: effect of phosphate buffered saline and deionised water

The aim of this experiment was to compare and contrast the two point-of-care systems with respect to the measurement of standard albumin concentrations. Consistency between sample readings was also investigated to evaluate the precision of the systems.

6.7.1.1 Methods

Albumin standards with concentrations: 200, 150, 100, 75, 50, 30, 20, 10 and 5 µg/ml were prepared by weighing out human serum albumin (Sigma Aldrich) in deionised water and PBS (Sigma Aldrich) with a target concentration of 1 mg/ml. Subsequently dilutions were made from this stock solution to reach the final concentrations. The stock solutions were analysed against an existing straight double dilution curve of albumin starting at 1mg/ml. This enabled solutions made for different experiments to be relatable to each other even if the exact concentrations differed. The prepared albumin standards were then tested on both point-of-care kits in triplicates to assess precision and comparability. Samples were measured neat according to the analyser protocols described previously.

6.7.1.2 Results

The correlation between the two techniques for deionised water is shown in Figure 6.9 and the correlation in PBS is shown in Figure 6.10. Figure 6.11 combines all the standard concentration data for both point-of-care analysers. The actual concentrations measured for both techniques are tabulated in Appendix B. Precision between triplicate measurements of the same concentration is showed by the applied standard deviation to each data point.

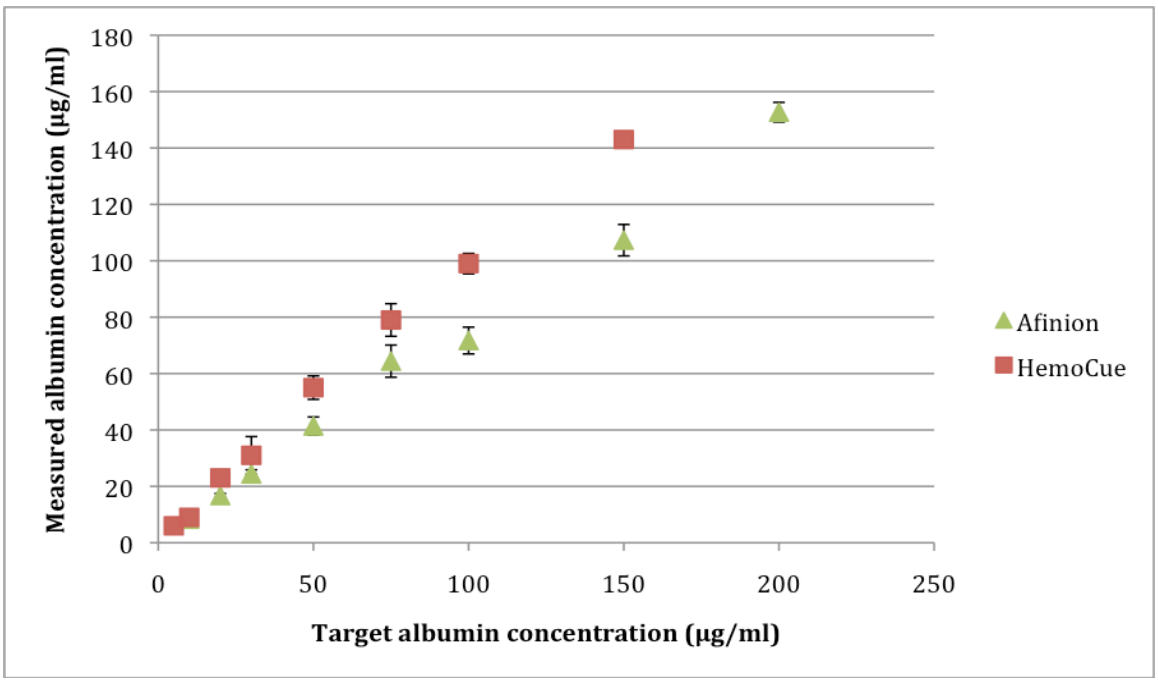


Fig 6.9. Comparison of albumin standards in deionised water measured by the Afinion™ AS100 Analyser and the HemoCue® Albumin 201 Analyser.

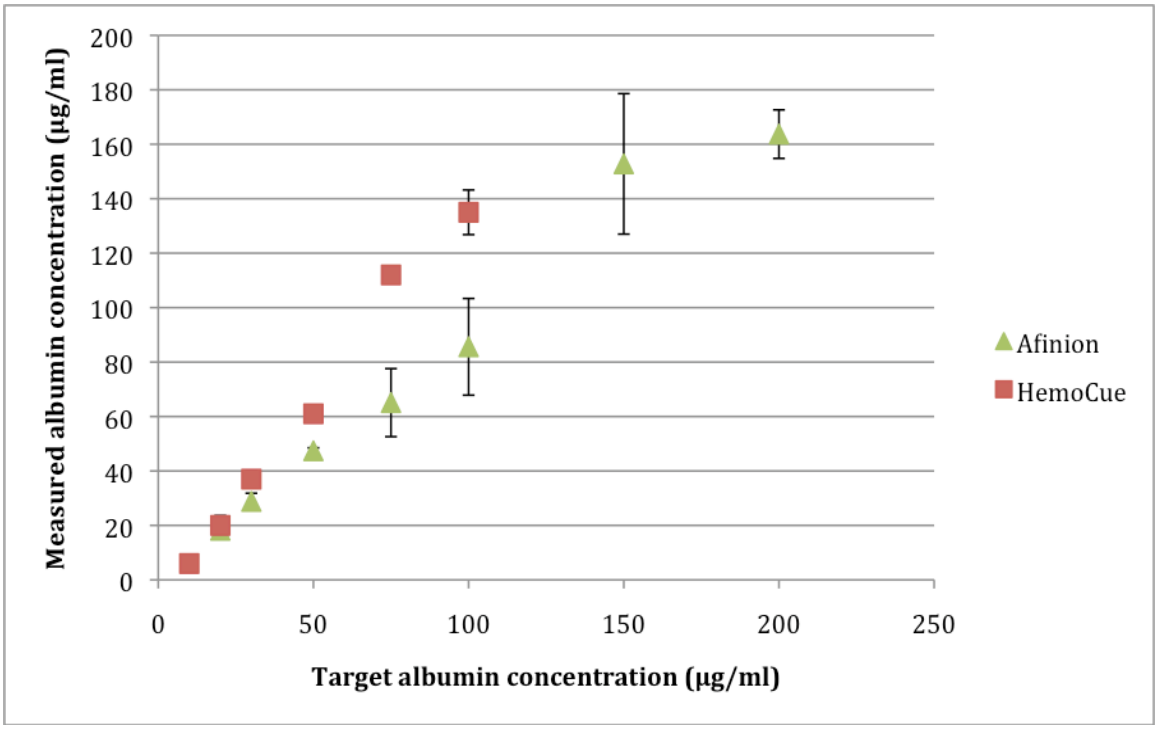


Fig 6.10. Comparison of albumin standards in PBS measured by the Afinion™ AS100 Analyser and the HemoCue® Albumin 201 Analyser.

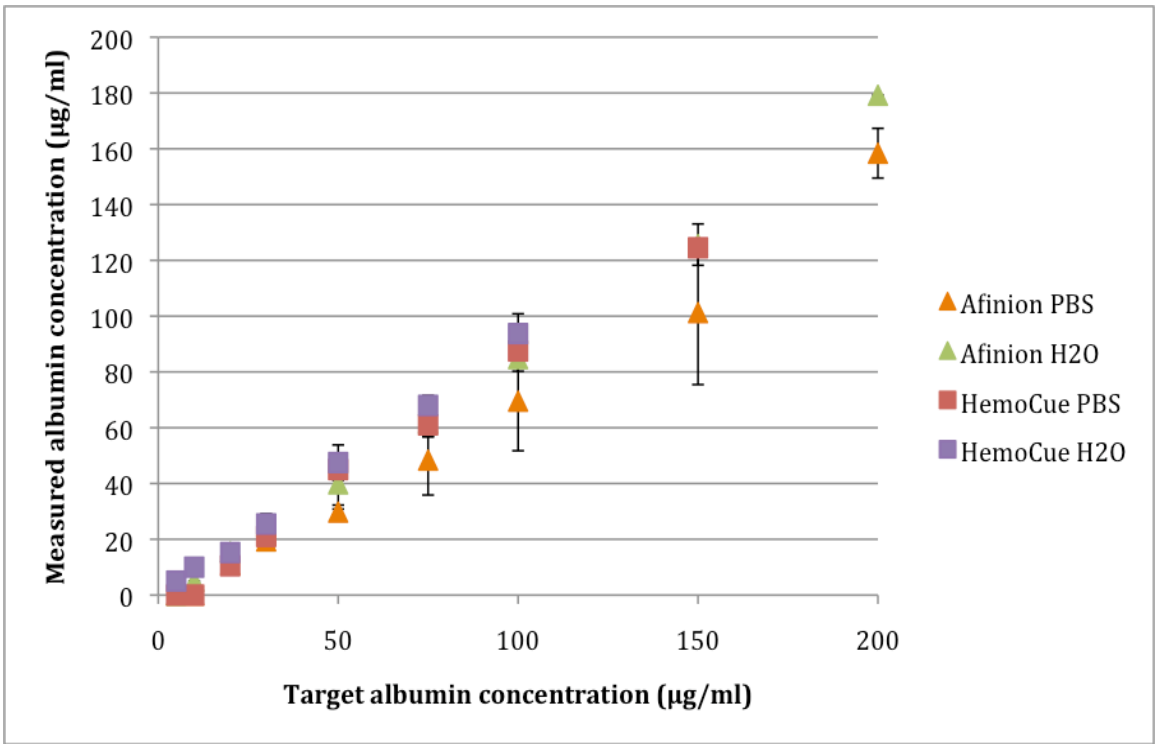


Fig 6.11. Comparison of albumin standards in both sample diluents measured by the Afinion™ AS100 Analyser and the HemoCue® Albumin 201 Analyser.

Figure 6.9 demonstrates that the two point-of-care techniques correlate well for albumin concentrations in deionised water. Both analysers also exhibited high precision between the triplicate measurements. Measurements by the Afinion analyser were consistently lower than the HemoCue, which could be a reflection of the different systems or that one system has a higher accuracy than the other. Nevertheless results obtained by both systems in this solution displayed sufficient discrimination between samples to be useful for tear sample research.

Results in PBS from the Afinion Analyser (Fig 6.10) exhibit a significant range between measurements of the same concentration. Below 50 concentrations were very precise but samples with higher values showed great disparity. The HemoCue Analyser, on the other hand, exhibited similar precision between albumin concentrations in PBS and in deionised water. A possible reason for the discrepancies between sample readings is the cartridge temperature; Afinion cartridges are very sensitive to cold temperatures. Samples were read linearly in order of dilution i.e. most concentrated samples first. Any temperature related issues would consequently have been skewed towards the higher ranges. Another issue is cartridge stability. The PBS measurements were made a short time period after the cartridge lot had reached its expiry. Degradation of kit reagents could therefore potentially have affected the higher end of the measuring range during analysis.

Collected results in Figure 6.11 show that the point-of-care techniques exhibit consistent strong positive correlation with each other and with standards in the two sample diluents. The exception is the previously mentioned poor results determined by the Afinion Analyser on albumin standards in PBS. Measured albumin concentrations were lower than the anticipated target value but, as confirmed in the subsequent section 6.7.2 this is due to human error in preparing the solutions.

6.7.2 Analysis of albumin standards by point-of-care technology and ELISA: effect of phosphate buffered saline and deionised water

The aim of this experiment was to assess the accuracy of the point-of-care systems against the established ELISA technique and also to re-assess the precision of albumin concentrations in PBS on the Afinion Analyser.

6.7.2.1 Methods

A second set of standards, with the same target concentrations as before (200, 150, 100, 75, 50, 30, 20, 10, 5 μg/ml), was prepared by diluting human serum albumin in deionised water and PBS as previously described. The standards were then analysed on both point-of-care systems and the Immunology Consultants Laboratory ELISA.

All point-of-care components had reached room temperature before the study began and concentrations were analysed in a non-linear fashion. The Afinion cartridge lot used was well within the recommended expiry date. Unfortunately, it was impossible to compare this lot with the expired lot, as there were no cartridges left from the latter. Precision for both of the point-of-care techniques has already been determined to be good in deionised water (Appendix B). Because of this only duplicate measurements were made of standards concentration in this diluent. The HemoCue Analyser also showed excellent precision in PBS and therefore duplicate measurements were considered to be sufficient for this sample diluent also. Further evaluation of the Afinion Analyser, on the other hand, necessitated continued triplicate readings of concentrations in PBS to re-assess the previously poor precision.

All point-of-care samples were analysed neat and prepared according to the analyser protocols described previously. The ELISA analysis was conducted according to the protocol described in section 2.6.5. Standard albumin solutions were diluted as follows: 200, 150 and 75 μg/ml at 1:2000, and 50, 30, 20, 10 and 5 μg/ml at 1:500.

6.7.2.2 Results

Comparisons of albumin standard analysis in deionised water and PBS on the HemoCue® Albumin 201 Analyser and ELISA are shown in figures 6.12 and 6.13 respectively. The comparisons between the Afinion™ AS100 Analyser and ELISA for deionised water and PBS are shown in figures 6.15 and 6.16.

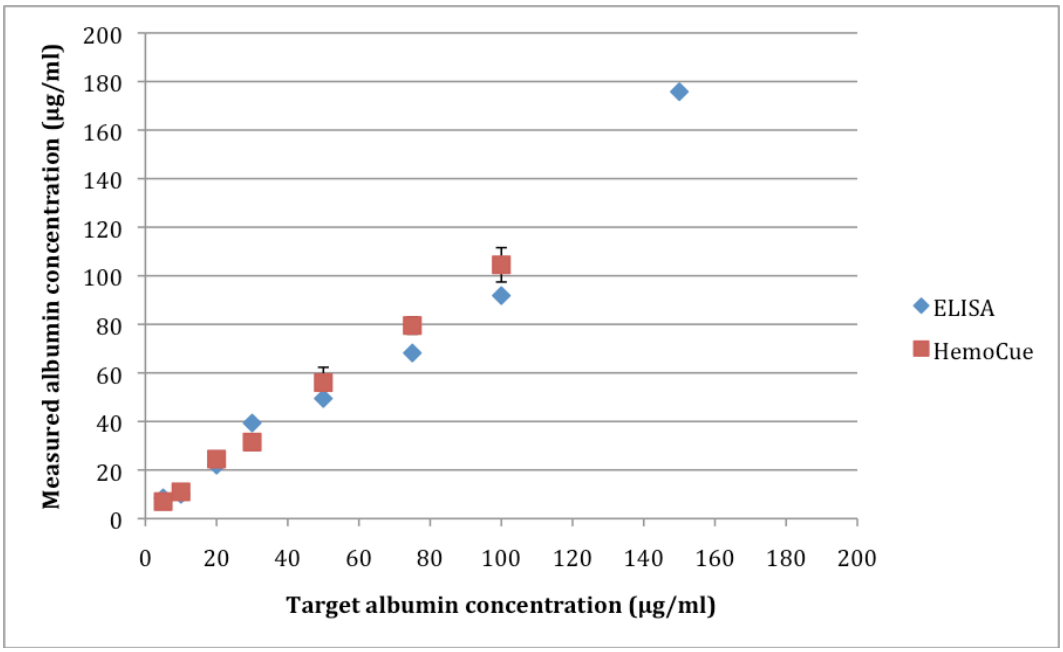


Fig 6.12 Albumin standards in deionised water measured by the HemoCue® Albumin 201 Analyser and ELISA.

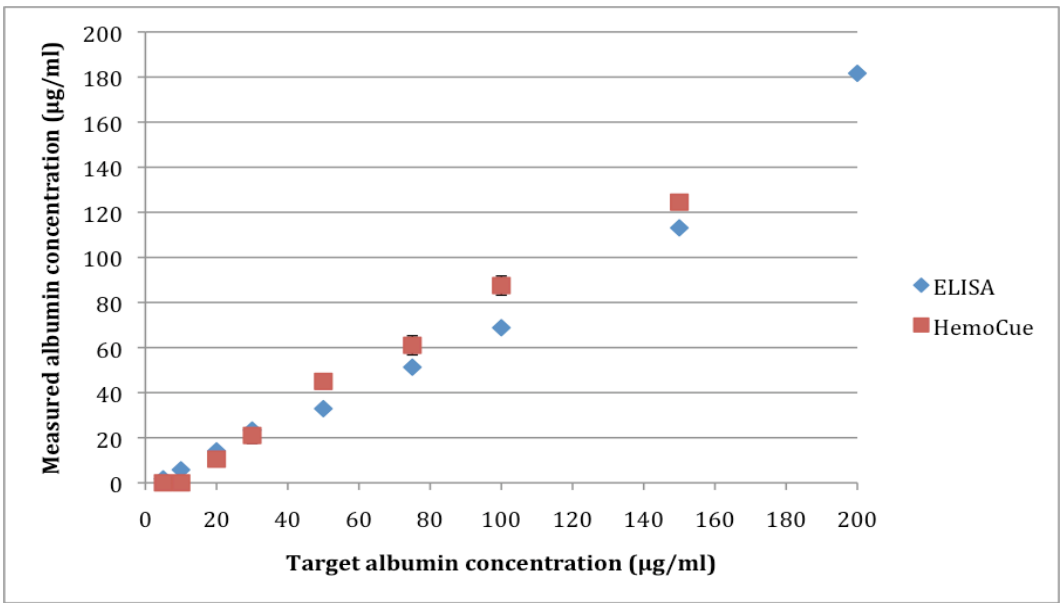


Fig 6.13 Albumin standards in PBS measured by the HemoCue® Albumin 201 analyser and ELISA.

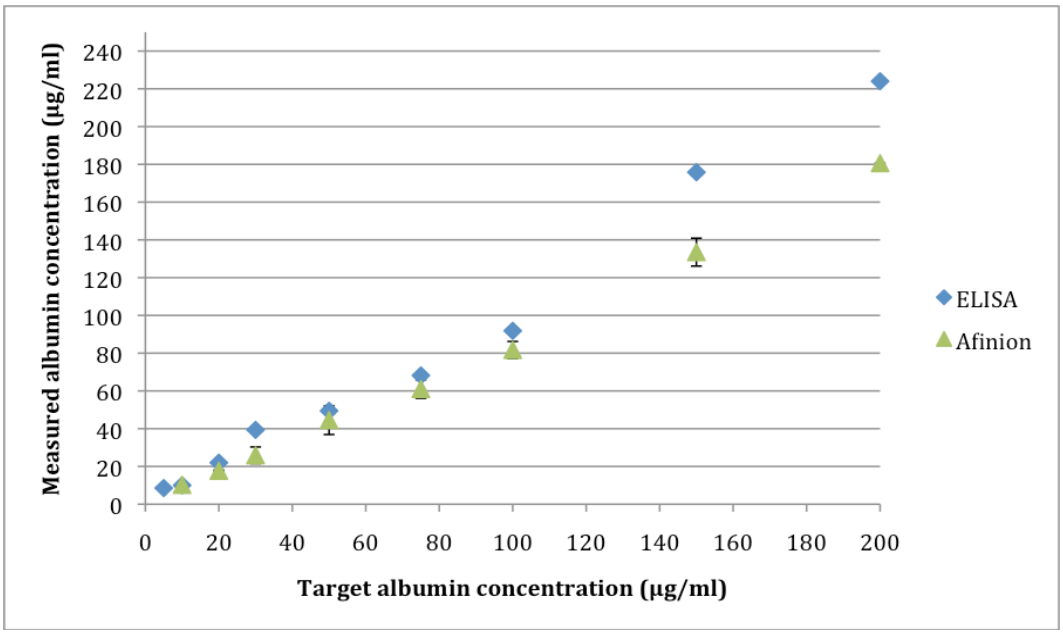


Fig 6.14 Albumin standards in deionised water measured by the Afinion™ AS100 Analyser and ELISA.

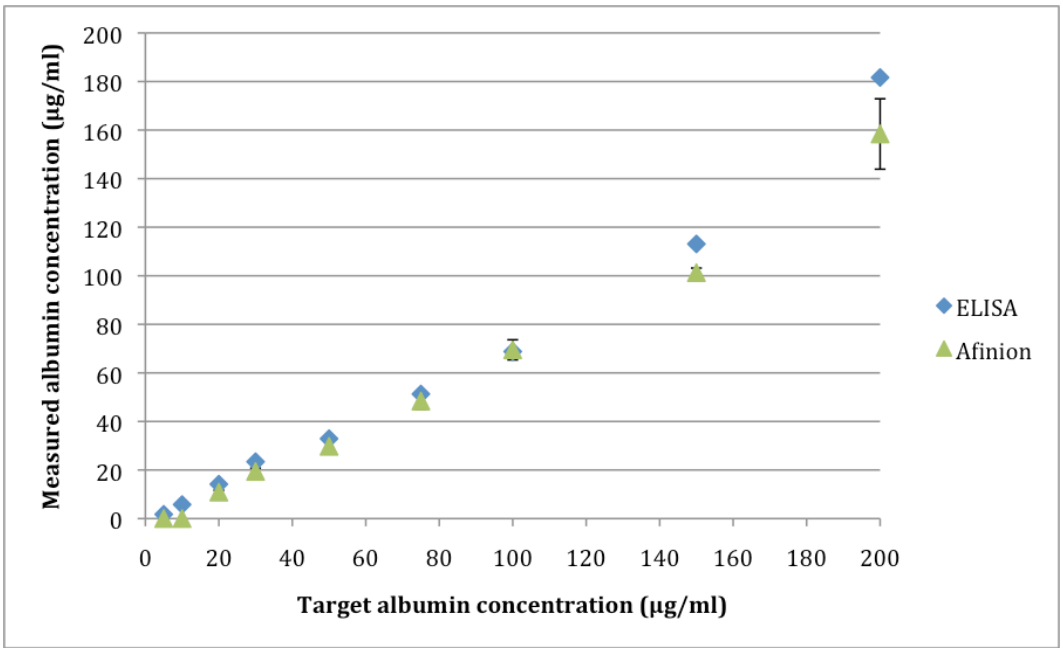


Fig 6.15 Albumin standards in PBS measured by the Afinion™ AS100 Analyser and ELISA.

The results in figures 6.12-6.15 show that the point-of-care techniques exhibit accuracy similar to the Immunology Consultants Laboratory ELISA. Concentration data provided by the HemoCue Analyser are close to perfectly aligned with ELISA concentrations. The Afinion Analyser produced slightly underestimated concentrations, especially in the higher ranges but was still consistently accurate. Precision of the point-of-care techniques, as shown by the applied standard deviation between measurements, is also overall very good. The problem previously seen with the precision of the Afinion in the analysis of PBS solutions is no longer present, which

suggests that it was related to cartridge expiry date. Actual albumin concentrations, as determined by the ELISA were less than the target concentration value. These show that discrepancies seen between target and measured concentrations in section 6.6.1.2 was due to human error during standard preparation and not a reflection of inaccuracy in the point-of-care instruments.

6.7.3 Analysis of microcapillary-sampled tears on the Afinion™ AS100 Analyser and ELISA

The aim of this experiment was to assess the Afinion™ AS100 Analyser for the determination of albumin in tear fluid sampled by microcapillary. Preliminary tear sample studies on the HemoCue® Albumin 201 Analyser showed that the 18µl volume requirement was too large to assess microcapillary samples albumin concentrations efficiently. Microcapillary tear sampling generates volumes between 1-5 µl and the dilution factor required to reach 18 µl meant that most samples ended up below the range of the assay. The point-of-care analysis of tears sampled by microcapillary was therefore only carried out on the Afinion Analyser. Resultant albumin concentrations were correlated to the established Immunology Consultants Laboratory ELISA technique.

6.7.3.1 Methods

Tear samples were collected from 8 subjects (3 male, 5 female) at various time points over three days. Sample time points were not limited to the stable daytime concentrations as it was important to produce a range of concentrations. Ultimately the aim of the experiment was to assess the Afinion Analyser for use in future tear sample research, irrespective of daytime or evening. All subjects had been well characterised in terms of their sampling response previous to the present research and represented basal, medium and reflex tear flows. Four µl were collected by microcapillary from the left eye of each subject, as detailed in section 2.3.2, and subjects were sampled at least three times during the study period. In total 29 samples were collected, which were stored at -80°C until time of analysis.

Tear samples were assayed neat on the Afinion™ AS100 Analyser according to the protocol described in section 6.3.2.1. Samples were assayed in a randomised fashion disregarding of subject or time point to avoid any bias. A total of 10 samples were measured on both the Afinion and ELISA to investigate the correlation between the two techniques. Tear samples were diluted 1:200 for the ELISA analysis and analysed according to the protocol described in section 2.7.2

6.7.3.2 Results

Creatinine levels were also recorded but were below the measuring range for all tear samples assayed. Fig 6.16 shows the correlation between microcapillary-sampled tear albumin concentrations analysed by the Afinion Analyser and by the Immunology Consultants Laboratory ELISA. Concentrations are displayed in increasing concentration.

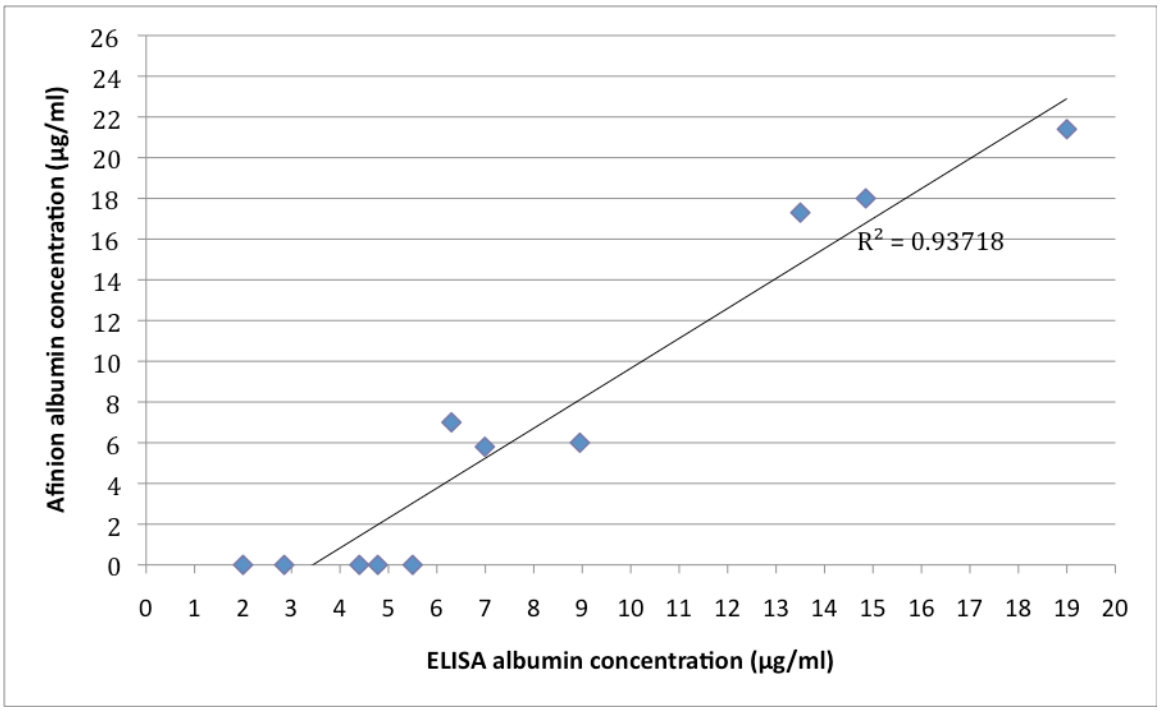


Fig 6.16. Albumin concentrations sampled by the Afinion™ AS100 Analyser and ELISA.

There is a strong positive correlation between the sample data obtained by the two techniques. Samples 1-5 reflect the inability of the Afinion analyser to quantify albumin concentrations below 5 µg/ml. For concentrations above the lower detection limit, the difference between the Afinion and the ELISA is less than 5 µg/ml. Both techniques are therefore able to provide good discrimination between samples within a very limited range of 5-25 µg/ml. All microcapillary samples analysed by the Afinion are presented in Fig 6.17. Results are displayed as equal interval data to produce a sample distribution. This distribution is similar to the microcapillary data collated in section 5.7 (Fig 6.18).

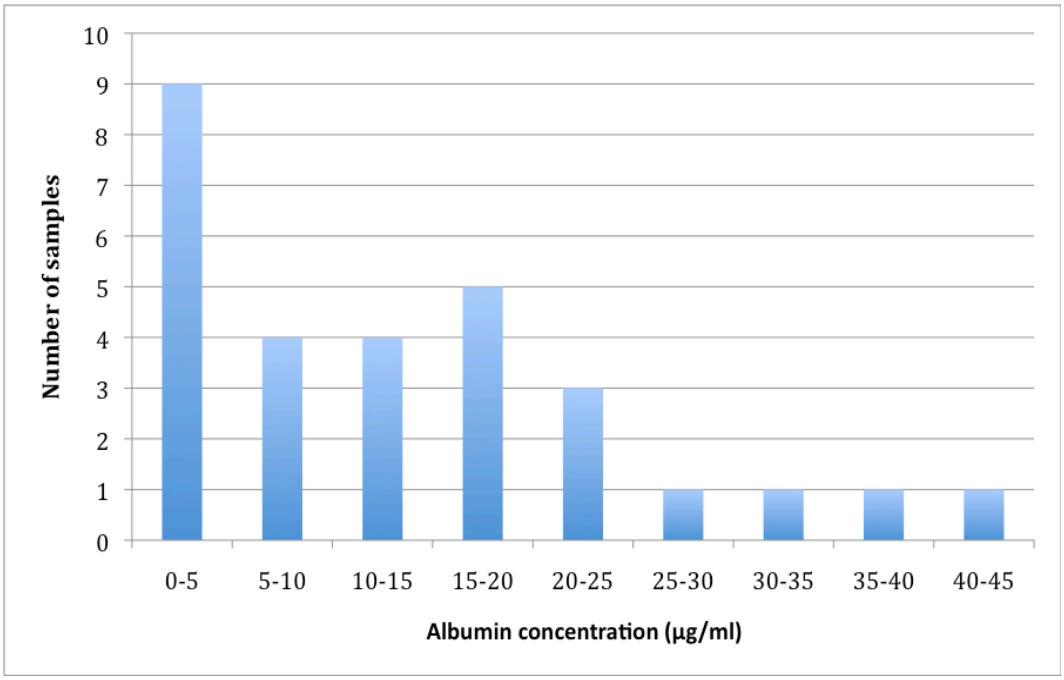


Fig 6.17 Albumin distribution in microcapillary tear samples analysed by Afinion™AS100 Analyser

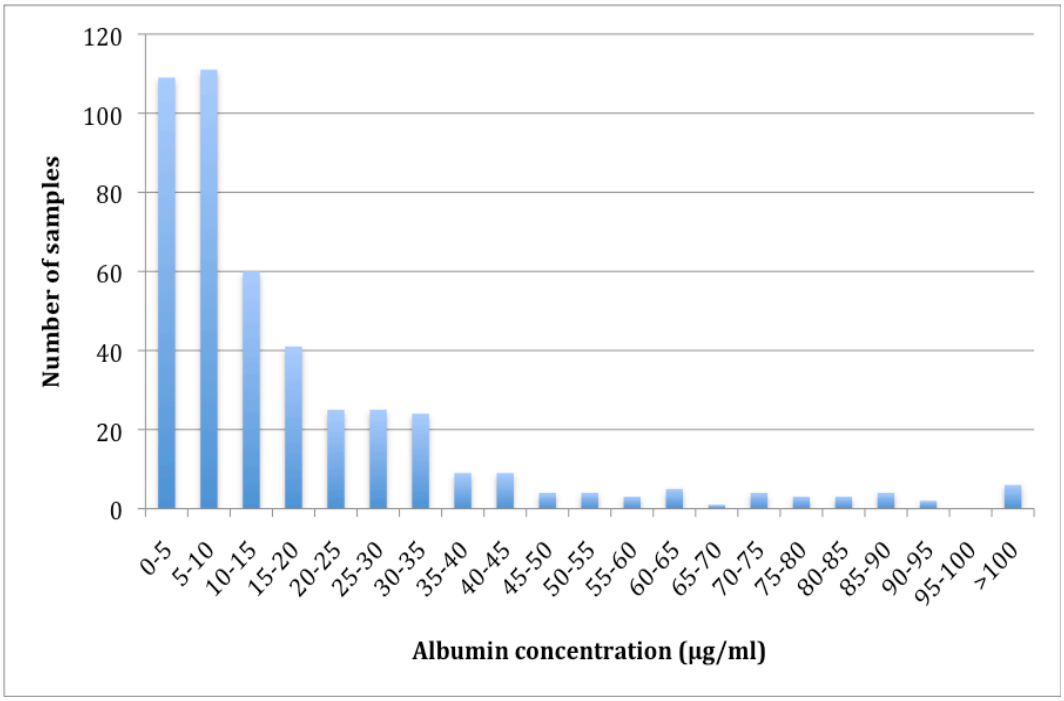


Fig 6.18 Albumin distribution in microcapillary tear samples analysed by Immunology Consultants Laboratory ELISA.

These results show that the small sample set analysed by the Afinion™ AS100 Analyser is in line with the larger population distribution.

6.7.4 Analysis of Schirmer strip-sampled tears on the Afinion™ AS100 Analyser, the HemoCue® Albumin 201 Analyser and ELISA.

The aim of this experiment was to assess the validity of analysing Schirmer strip-sampled tears on point-of-care instruments. Tear samples collected by Schirmer strips could be assayed on both the Afinion™ AS100 Analyser and the HemoCue® Albumin 201 Analyser as the high dilution factor required to bring samples into the measuring range provides enough volume for the HemoCue. The samples were also assayed by ELISA to provide a correlation between all three techniques.

6.7.4.1 Methods

Schirmer strip samples were obtained from the same clinical population that was used in chapter 5 (section 5.3.1). None of the subjects wore contact lenses and no ocular disease or disorders were observed prior to sampling. Schirmer strips represented wetting lengths between 10mm and 35mm, and were extracted whole by centrifugation according to the protocol described in section 2.4.3.

Samples for point-of-care analysis were diluted 1:9 by adding 2 µl of sample solution to 18 µl of deionised water. Water was chosen as solvent because it showed the best precision between albumin standards. ELISA sample dilutions were determined by performing the point-of-care analysis first and using that value to calculate a suitable dilution factor. The resultant sample dilutions ranged from 1:500–1:3000. All sample analysis was performed according to instrument instructions, as detailed in chapter 2. In total 18 samples were assayed.

6.7.4.2 Results

Fig 6.19 shows the comparison between the Schirmer strip samples assayed on the HemoCue Analyser and ELISA. The corresponding comparison between the Afinion Analyser and the ELISA is shown in Fig 6.20 The albumin concentrations have been displayed in order of increasing concentration.

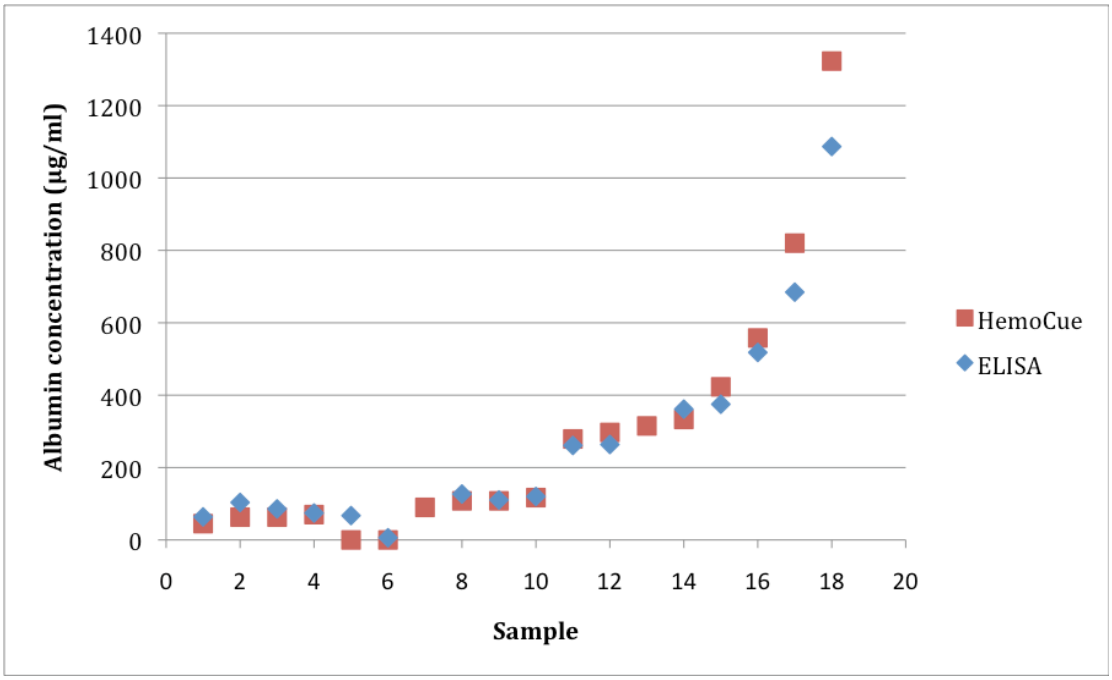


Fig 6.19. Schirmer strip samples analysed on the Hemocue® Albumin 201 Analyser and ELISA

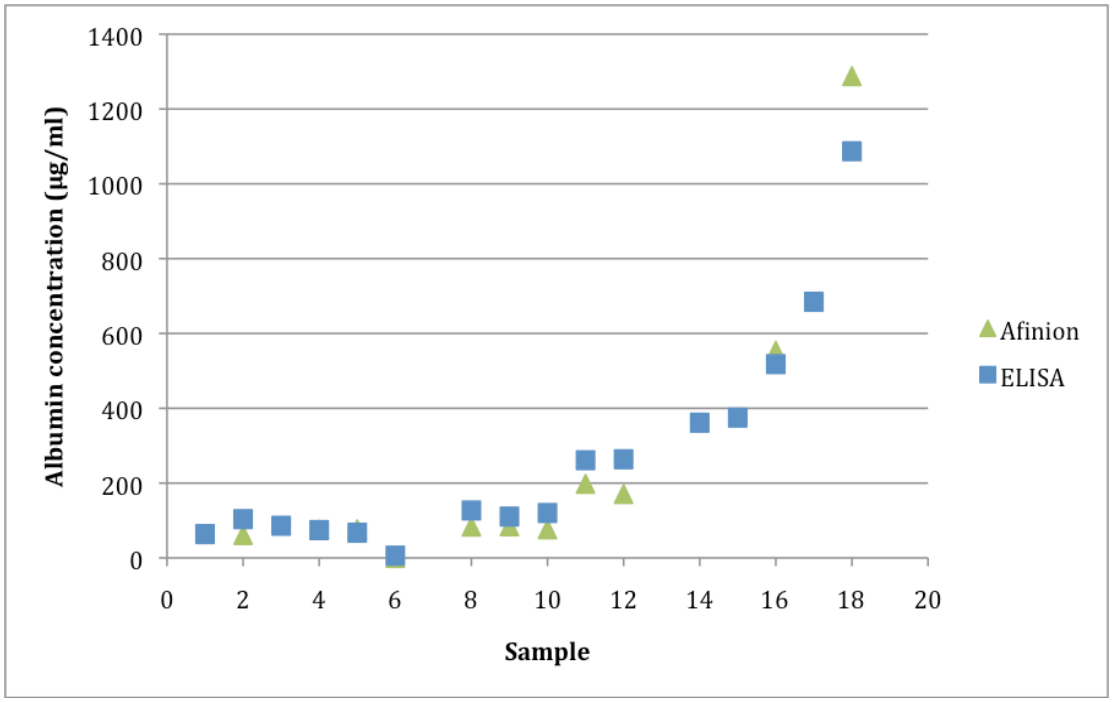


Fig 6.20. Schirmer strip samples analysed on the Afinion™ AS100 Analyser and ELISA

The results in Fig 6.19 and Fig 6.20 show excellent correlation in assayed Schirmer strip concentrations between the HemoCue and the Afinion instruments and the established ELISA technique. Concentrations >600 μg/ml showed higher disparities between point-of-care techniques and ELISA values. A possible reason for this is the higher dilution factors required by these samples for ELISA analysis, which could affect the results. Similarly, sample 5 in Fig 6.19 is merely a reflection of the HemoCue Analyser's lower range cut off value of 5 μg/ml. The actual value, with the applied 1:9 dilution factor, could be anything between 0-45 μg/ml. The corresponding Afinion results are 76.5 and the ELISA result is 67.3. Consequently, the HemoCue result could have only just fallen below the measuring range. Ultimately, all three techniques managed to provide good discrimination between albumin concentrations sampled by Schirmer strips in the present clinical population.

6.8 Discussion

Point-of-care technology in contact lens clinics could provide useful information on the tear film composition to contact lens practitioners. Additionally it could facilitate further research into the tear film and long-term studies on clinical populations. The research presented in this thesis has aimed to provide a platform for the investigation of the role of albumin in tears. Concentrations have been shown to be most stable in early daytime hours and to be affected by sampling and assaying techniques. Existing point-of-care techniques for the analysis of urinary albumin have a measuring range compatible with expected tear albumin concentrations, as identified in chapters 3,4 and 5. These were therefore suitable for investigation and potential implementation into contact lens clinics.

Two urinary albumin analysers were evaluated in this chapter: the HemoCue® Albumin 201 Analyser from HemoCue AB and the Afinion™ AS100 Analyser from Axis-Shields. Both showed high precision between triplicate measurements of albumin standards prepared in deionised water and in PBS. Initially the Afinion performed badly in PBS but a second investigation using fresh solutions and another cartridge lot produced good results similar to the ones seen for standards prepared in deionised water. Although temperature was a possible issue, the most likely source of the discrepancy in the first set of standard measurements is the quick degradation of the system components after passing the expiry date. Consequently it is recommended to only use cartridge lots within the expiry and to allow cartridges to reach room temperature well before analysis. Because the second standard measurements exhibited good precision, both deionised water and PBS were recognised as suitable solvents for the present point-of-care analysis.

Correlation of standard samples with the established ELISA technique showed good agreement between the three techniques. Afinion concentrations at the higher end of the measuring range were disparate from ELISA measured concentrations (Fig 6.14 and Fig 6.15). This is less important for clinical use, as shown from the albumin distribution in Fig 6.18, microcapillary-sampled concentrations rarely reach such high levels. Analysis of microcapillary-sampled tears on the Afinion™ AS100 Analyser supported this statement (levels were <45 µg/ml) and showed good correlation with ELISA (Fig 6.16). The error margin between the two techniques was 5≥ µg/ml and both techniques would be able to discriminate accurately between subjects in a population. The Afinion also produced a sample distribution profile similar to the microcapillary-sampled albumin profile established by ELISA analysis (Fig 6.17). Consequently the Afinion would be suitable for analysing microcapillary sample data in a contact lens clinic.

The HemoCue Analyser could not be used for the determination of tear albumin sampled by microcapillary, as the instrument requires a sample volume of 18 µl. Both the HemoCue® Albumin 201 analyser and the Afinion™ AS100 analyser were however able to analyse samples collected by Schirmer strip. Albumin samples (n=18) extracted from Schirmer strips collected from a clinical population showed excellent correlation between the point-of-care instruments and the ELISA assay (Fig 6.19 and Fig 6.20). Both point-of-care analysers were also able to show accurate discrimination between samples from the clinical subject population. The analysis time of the HemoCue is 90 seconds compared to the 3.5 minutes of the Afinion. Individual cost of analyser and test cartridges for the HemoCue are also significantly less than the Afinion (Table 21) and the cartridges have a longer expiry date. For these reasons, the HemoCue Analyser is potentially preferential to the Afinion for the analysis of Schirmer strip-sampled tear albumin in the contact lens clinic**.**

The Schirmer strip technique is already established as a routine investigation in the contact lens clinic (Table 32) and is consequently useful for use in point-of-care analysis. Chapter 5 identified that microcapillary-sampled albumin concentrations and Schirmer strip concentrations cannot be used interchangeably as the albumin levels sampled by the Schirmer strip were increased in a non-predictable manner. Discriminations between albumin concentrations in subjects sampled by the Schirmer strip were, however, still possible. The insertion of the Schirmer strip subjects the eye to a mild insult and this results in increased albumin. The magnitude of this increase appears to differ between subjects and could provide valuable information on how population subsets respond to ocular challenges. It would therefore be interesting to identify different population subsets and correlate the albumin concentrations sampled by Schirmer strips with comfort scores on the sampling experience. Another aim would be to correlate Schirmer strip

albumin concentrations in subjects with the effect of contact lens wear to see if Schirmer strip albumin could be a predictor for problems with wearing lenses.

The present research also demonstrates that, given suitable conditions, existing point-of-care technology can be utilised for determination and quantification of tear sample components. Opportunities for the cross-use of point-of-care analytical technology is likely to increase as more biomarkers are determined and techniques become more sensitive. There already exist a large variety of other analyte tests available in a point-of-care format. Examples are: hematocrit, cholesterol, triglyceride, glucose, influenza A/B, pH, hemoglobin (Nichols, 2003). A review on protein point-of-care testing by Warsinke, (2009) identified the invasive sampling techniques as one of the major hurdling blocks to current point-of-care. The sampling and analysis of tears is therefore an exciting avenue and further investigations into point-of-care technology for proteins of interest would be beneficial. An issue of using instruments that are designed for the analysis of blood and urine for tear sample analysis is the potential of contamination due to differences in fluid composition. The Afinion AS100 Analyser has been evaluated for interference with a variety of drugs, their metabolites and common urine components (Kvam *et al,* 2007). A cross-section of these substances are described in Table 34.

Table 34. A selection of substances tested for interference on the Afinion™ AS100 Analyser.

Component	Maximum concentration giving no significant interference
Ascorbic acid	3000 mg/L
Bilirubin	3.5 mg/dL
Glucose	45 mg/mL
IgG	20 mg/L
Myoglobin	20 mg/L
Ibuprofen	2 mg/mL
Salicylic acid	2 mg/mL

Whilst no evidence of interference was found during the assessment of the current point-of-care technology, the eventuality of contamination needs to be taken into consideration for any study investigating point-of-care systems for tear film research.

6.9 Conclusion

The results in this chapter show that point-of-care analysis of tear albumin is possible. Both the Afinion™ AS100 Analyser and the HemoCue® Albumin 201 Analyser showed great correlation with the already established ELISA technique. The point-of-care techniques were also able to discriminate between tear albumin concentrations within small ranges, with high precision. Tears sampled by microcapillary were best suited for analysis by the Afinion Analyser due to the small volume requirements of the technique. Schirmer strip samples on the other hand could be analysed by both point-of-care techniques but the HemoCue Analyser is preferential as it is significantly cheaper and faster than the Afinion Analyser. The next step would be to implement the HemoCue Analyser in a contact lens clinic to investigate the response of the eye to the stimulus imposed by the Schirmer strip. It would be useful to correlate this information with subsequent subject success in wearing contact lenses.

CHAPTER 7
SUMMARY AND SUGGESTIONS FOR FUTURE WORK

7.1 Introduction

Tear albumin is of interest for several reasons. Compared to other tear film proteins present in high concentrations (e.g. lactoferrin, lysozyme, lipocalin) albumin has been largely neglected in research and its role in the tear film is poorly understood. In the literature, tear albumin is largely utilised as a marker of vascular leakage, although information on mobility and transport of albumin into the ocular environment is incomplete. In plasma however, albumin is known to perform many vital functions, which include: controlling oncotic pressure, promoting binding and transport functions, and providing anti-oxidant effects (section 1.3.3). A hypothesis is that albumin might perform similar functions in tears and consequently may constitute more than a simple marker. As a result, further investigations into both albumin concentrations in the tear film and the consequences of its presence are warranted.

The purpose of the research presented in this thesis was to provide a strong foundation for further studies on albumin in the tear film. There were three main objectives:

- To collate and evaluate the literature to identify factors of variation for tear albumin concentrations.
- To investigate albumin concentrations in the tear film with regard to both diurnal and subject-to-subject variations.
- To perform a parallel investigation into the use of point-of care technology to analyse albumin concentration in tears in contact lens clinics.

The summary and conclusions of this research are described in sections 7.2-7.4.

7.2 The determination of factors affecting albumin in tears

The disparities in published tear albumin concentrations were investigated by addressing the previously neglected literature and performing a systematic investigation of collected reported human albumin concentrations in tear fluid. Factors for variation were conveniently divided into external (sampling technique and stimulation, method of analysis, time of day, open or closed eye tears) and internal (ocular disease or disorder, and subject-to-subject variation). Three major factors for variation were identified: sampling technique, ocular disorder or disease, and assay methodology.

Sampling technique was found to be the most influential factor of variation in measured mean albumin concentrations. Harsher filter paper techniques produced an increase in albumin concentration compared to microcapillary sampling. The magnitude of this increase differed between studies but an average of 50 times the microcapillary value was noted. Significantly, increased albumin concentrations were observed with harsher sampling techniques (compared to microcapillary) even with the additional factors of ocular disease and disorder. Ocular disease and disorders, as individual factors of variation, exhibited increased albumin concentrations compared to asymptomatic controls. This increase was however non-specific and high albumin concentrations, as a factor, were not directly linked to any specific disease or disorder. Assay technique has been an issue in interpreting the significance of the early tear albumin determinations (ca pre 1990). Semi-quantitative techniques such as immunodiffusion are less sensitive than modern techniques and provide higher values. More recent advances in assay techniques have lead to increased sensitivity. This has meant that, although assay techniques carry a small degree of variation, this is negligible compared to the other factors described here. The careful understanding of these factors is therefore vital in the design of further research studies into albumin in the tear film. This is particularly true when investigating subject-to-subject variation, which, as shown in chapter 3 and 4, is elusive, and of a magnitude that could easily be masked by inflicted additional albumin influx.

7.3 The investigation of diurnal variation in tear albumin concentrations

The assembled tear albumin literature displayed a consistent lack of description or comment on the time period during which tear sampling took place. Published data on when albumin concentrations were measured were insufficient to be able to draw any conclusions on this factor from the literature. Data from the overnight tear film and diurnal variations in other biological fluids did however suggest the possibility of time-related variation in albumin concentration. Consequently, albumin concentrations were sampled throughout the day to investigate this further. A population of thirteen subjects was recruited and subjects were divided into two study groups, Group 1 and Group 2. Group 1 (n=13) had tears collected by microcapillary every two hours during normal office hours for a period of 2-6 days, depending on subject availability. Group 2 (n=5) comprised a subset of subjects trained to collect their own tear samples using microcapillary. For group 2, albumin concentrations were measured every two hours: post-waking up to and including pre-retiring to bed over a number of days (9-11).

The collected data revealed previously unrecognised albumin variations, both between subjects and within some subjects during the course of the day. An apparent stable period between

10:30 and 14:30 hrs was observed, where albumin concentrations within the subjects showed little variation. Variation between subjects was however evident during these hours, which suggests subject-specific inherent differences in albumin concentration. The subset of subjects that self-sampled throughout the day (Group 2) exhibited a progressive increase in tear albumin concentration post 16:30, which culminated in a significant spike in concentration prior to retiring to bed. The inclusion of previously unstudied time points in the present work was able to demonstrate that the ocular environment in the evening is significantly different from the stable daytime environment in terms of albumin concentration. This asymmetric shift, towards higher albumin concentrations in the evening, has not been previously reported and carries two major implications.

Firstly, the identification of conditions during which albumin concentrations are relatively stable provides a baseline for comparative studies of albumin between populations. It is recommended, therefore, that sampling of albumin concentrations in future studies should therefore be limited to the time points where albumin has been shown to be most stable in order to negate any effects of diurnal variation. Secondly, the results also carry potential significance for the investigation into the role of tear film albumin, and whether it is passive or responsive in nature. Increased albumin concentration has been previously recognised in the closed eye tear film (Sack *et al,* 1992) but the present research has exhibited an earlier progressive increase during the open eye state. The consistent increase in albumin concentrations during the evening suggests a regulated rather than sporadic influx of albumin into the tear film. Sack *et al* proposed that the significant increase in IgA and albumin in the closed eye environment potentially served a protective role. This, together with the present research on tear diurnal variation of albumin, suggests a positive active association with albumin and ocular defence. Further attention should be paid to this late evening increase in albumin phenomenon to characterise this possible relationship.

Importantly, this work highlights for the first time a pattern of variation in albumin concentration throughout the day, emphasising potential pitfalls in the use of single point analyses for diagnostic purposes. The role of albumin in tears has yet to be determined; whether its presence is purely a response to stimulation or part of an adaptive mechanism remains unresolved. It is clear, however, that the concentration of albumin in tears is not stable. It is subject to change in a manner that is unlike that of any other prominent tear protein. Taken together, these factors suggest that albumin is a biomarker of considerable potential significance, which deserves further investigation and greater attention.

7.4 The evaluation of point-of-care technology for analysis of tear albumin

The identification of time periods during the day where albumin concentrations are relatively stable has provided valuable information for the design of studies involving the assessment of tear albumin concentrations between different populations. The identified time points, during which tear albumin concentrations vary insignificantly within subjects, correlate well with expected opening hours of contact lens clinics and research facilities. Contact lens clinics are ideal institutions for the recruitment of study populations for tear protein analysis, because subjects attend these on a regular basis for eye examinations. Disadvantages of sampling in commercial premises include the lack of laboratory space and trained personnel to conduct protein analysis. Time and cost of analyses are also important issues. Point-of-care technology was therefore evaluated to facilitate the collection of additional tear albumin data in contact lens clinics. The benefits of the automated point-of-care assays are that they are portable, fast, and simple to use and have a high accuracy.

Experimental investigation of point-of-care analysis was performed in two parts. Firstly the effect of tear collection technique on tear albumin concentrations was evaluated to choose a suitable sampling protocol. Secondly, point-of-care instruments were chosen and compared with the established ELISA technique for the determination of albumin in tear fluid.

Microcapillary sampling and Schirmer strip sampling were evaluated for tear collection and future use by point-of-care analysis. Tear albumin concentrations and technique-induced stimulation of tear flow was assessed in a population of 10 subjects sampled by both Schirmer strip and microcapillary. Schirmer strip samples were extracted by centrifugation of the whole strip. This protocol was suitable for clinical use as it was simple, required no buffers and results had previously been shown to produce an electrophoretic protein profile similar to microcapillary-collected samples (section 5.2.2). Furthermore, investigations of extraction protocols showed that only assaying sections of the strip only would produce an incomplete and potentially erroneous picture of the albumin concentrations present.

Subject-to-subject variation was evident, in terms of tearing response, to both the Schirmer strip and the microcapillary sampling technique in the population (n=10). However, the results showed no direct correlation between the induced stimulation of tear flow by the two techniques. Similarly, the albumin concentrations sampled with one technique could not be used to predict albumin concentrations sampled with the other. Schirmer strip sampling exhibited increased albumin concentrations compared to microcapillary sampling (3.0->400 μg/ml vs.

0.75-21.5 μg/ml) and created a far greater range of values between subjects. Both sampling techniques were however able to provide good discrepancy between albumin concentrations sampled by different subjects. The Schirmer strip is already widespread in use in contact lens clinics and would be easier to implement for albumin tear collection in clinical studies. Microcapillary sampling on the other hand requires more training but provides a non-invasive sample of the tear film. Both techniques were therefore selected for evaluation on point-of-care technology.

A review of available information identified two suitable point-of-care instruments for albumin determination: the HemoCue® Albumin 201 Analyser and the Afinion™ AS100 Analyser. The analytical ranges of the point-of-care systems (5–150, 5-200 μg/ml) and the analysis times (1.5 min, 3 min) make both instruments compatible for use in contact lens clinics. Evaluation of the instruments using albumin standards in deionised water and PBS showed that both techniques exhibited precision and accuracy similar to the established sandwich human serum albumin ELISA from Immunology Consultants Laboratory Inc (ICL). Results for the Afinion™ AS100 Analyser in PBS, however, implied that reagents used below room temperature and after the expiry date affected both accuracy and precision negatively.

Microcapillary-sampled tears were considered for use on both instruments but the volume requirement of the HemoCue® Albumin 201 Analyser was too large (18μl) to make analysis on this instrument viable. The Afinion™ AS100 analyser has a volume requirement of 3μl, which enabled the measurement of neat microcapillary collected tear samples without dilution. Tear albumin concentrations from 8 subjects, were determined by the Afinion™ AS100 Analyser and showed strong positive correlation with ELISA derived results.

A subsequent sample distribution of albumin concentrations analysed by the Afinion showed that results exhibited a similar range and distribution profile to samples analysed on the ELISA. Schirmer strip-sampled tears were analysed on both point-of-care techniques, as dilution of the collected samples was necessary to produce albumin concentrations within the measuring ranges of the instruments (5-200 μg/ml). Samples were diluted one in nine (2μl + 18 μl), which generated enough sample volume to enable analysis by the HemoCue Analyser. Both point-of-care instruments showed excellent correlation with ELISA-derived results for albumin concentrations determined from clinically obtained Schirmer strips (n=18). Results also exhibited good discrimination between albumin concentrations from different subjects.

Collectively these results show that point-of-care analysis of tear albumin, using the HemoCue® Albumin 201 Analyser or the Afinion™ AS100 Analyser is viable and instruments demonstrate

assayed concentrations similar to the ELISA technique. Precision between standard replicates is also excellent. In terms of practicality, the HemoCue Analyser is preferential to use, as it is has smaller dimensions, and is cheaper and faster to run. Sample volume is however a limiting factor. The Afinion Analyser is therefore suitable for the assessment of microcapillary-sampled tears whereas both instruments are able to accurately determine albumin concentrations in Schirmer strip-sampled tears.

There are several benefits of point-of-care analysis of tear albumin. The simplicity of the point-of-care analytical procedure, and the lack of sample transit between clinic and laboratory, make tear albumin determination faster and more cost effective than ELISA analysis. This facilitates the analysis of study populations that visit contact lens clinics and may make the collection of additional data sets more accessible. There is also an opportunity to correlate albumin tear concentrations with results of eye examinations, which may provide more information about albumin as a biomarker. The consistent increase of albumin concentrations in the evening tear film, identified in the research presented in this thesis, indicates that albumin might be actively recruited into the tear film as part of the ocular defence system. The measurement of tear albumin levels in subjects could therefore have potential clinical significance, although further research is needed.

7.5 Conclusion

The research presented in this thesis has provided several new insights into the parameters that dictate the measurement of albumin in tears. It has also provided new areas for investigation and facilitated the collection of additional data. In summary, the contributions are:

- The unique collation of the available tear albumin literature and the evaluation of factors affecting albumin in tears.
- The identification of a previously unrecognised asymmetric shift in albumin concentrations in the evening.
- The further evaluation of tear sampling techniques with respect to effect on tear albumin concentration.
- The proposition and evaluation of point-of-care analysis for determination of tear albumin concentrations in contact lens clinics.

7.6 Suggestions for future work

The research presented in this thesis provides a firm basis for continued albumin research and suggestions for further work are as follows:

7.6.1 A larger study of diurnal variation

Diurnal variation, with a progressive increase in albumin concentrations in the evening was evident in the sampling population. There were however differences between subjects in terms of the starting point and extent of the evening increase in albumin. The present study measuring tear albumin concentrations throughout the waking day was conducted using 5 subjects and it would be beneficial to repeat the study with a larger population. This would partially help to confirm the present results but also to investigate whether the individual subject responses are subject specific anomalies or representations of population subsets with different coping mechanisms to the ocular environment pre-retiring to bed. It is recognised in the present research that other unknown factors for albumin variation may exist. A study of this magnitude would thus also benefit from the inclusion of a questionnaire assessing the individual's ocular comfort score and lifestyle to investigate other possible influencing factors.

7.6.2 Point-of-care technology in the contact lens clinic

The results presented in this thesis show that albumin is a protein that has been neglected compared to its fellow major tear proteins in the tear film. It would be valuable to analyse albumin variations between subjects at the defined stable time points to identify eventual population subsets with differing albumin mechanisms. The research presented in chapter 6 identified existing point-of-care technology, which could be utilised to accurately determine albumin concentrations in tears. This laboratory-based investigation should be extended with a trial incorporating point-of-care into the contact lens clinic. This would enable the quick assessment of albumin levels in a larger population and also the correlation of albumin concentrations with a wide range of other factors determined during a contact lens appointment.

The aim would be to use the instruments to perform two separate investigations. Microcapillary-sampled albumin concentrations are collected non-invasively and consequently denote the baseline tear film environment. Baseline albumin concentrations could be assessed in contact lens clinics using the Afinion™ AS100 Analyser and gather more data on albumin

distributions in populations. The insertion of the Schirmer strip represents a small physical insult to the eye and produces an increase in albumin concentrations compared to microcapillary sampling. The extent of the resultant increase in albumin concentration differs between subjects and it would be interesting to characterise this response. Potentially, the albumin concentration increase by Schirmer strip sampling signifies how the eye of an individual subject reacts to insults and deviations from normal ocular conditions. This information could be of clinical significance as it might be able to identify subjects with better or worse abilities to withstand ocular challenges such as contact lens wear. The HemoCue® Albumin 201 Analyser would be suitable for the investigation of Schirmer strip-sampled albumin concentrations in contact lens clinics.

7.6.3 Albumin extraction from sampling materials

There were clear inconsistencies in lacrimation responses and albumin concentrations between subjects sampled with different tear collection techniques. What confounds the issue further and have not been dealt with fully in this thesis is the relative absorptive and extraction qualities between the different sampling materials. Schirmer strip was used as an absorptive material in the present research but there are other materials available (e.g. polyester wicks, cellulose acetate sponges,) (section 3.5.1). There is currently no extraction protocol that has been proven to have 100% efficiency on Schirmer strips or other absorptive materials. Comparative analyses of albumin extraction from sampling materials such as Schirmer strip and polyester sponges would provide further detail on material factors and lead to a more informed choice when considering sampling methodology.

Furthermore it would be of interest to evaluate contact lenses for use as tear sampling tools for the determination of tear albumin concentration. The placement of a contact lens into the eye partitions the tear film into the pre-lens tear film and the post-lens tear film. When the contact lens is removed, the liquid adhered to the front and back surface of the contact lens is consistent with a sample of the tear film. This sample has been named the tear envelope as the fluid "envelopes" the lens (Mann and Tighe, 2007). By centrifuging the lens immediately after removal from the eye, in a similar fashion to Schirmer strip extraction described in section 2.4.3, the sample can be collected. The volume can be up scaled by the addition of deionised water to the lens prior to centrifugation and in general, the process can yield sample volumes ranging between 4 and 25 µl. These volume ranges would enable the tear envelope to be analysed by one or both of the point-of-care instruments. The composition of the tear envelope sample is dependent upon the subject and the qualities of the lens material. Assaying albumin concentrations might therefore identify subject-lens combinations with higher or lower

albumin. Factors such as: loss of protein by lens deposition of albumin and extraction efficiency of the lens, would be important to evaluate prior to such a study.

7.6.4 Diurnal variation and contact lens wear

The observed progressive late day increase in tear albumin concentration identified in chapter 4 may have relevance to contact lens wear; potentially in relationship to end of day discomfort, which is a well recognised but poorly understood phenomenon. A questionnaire based study conducted by Chalmers and Begley (2006) showed that intense symptoms of dry eye discomfort in contact lens wearers significantly increased later in the day compared to two hours after waking. It would be valuable to investigate tear albumin concentrations throughout the day in contact lens wearers to see if the same effect of increase in the evening is detectable.

Similarly it may be of value to investigate extracted worn lenses. The comparison of tear albumin concentration extracted from contact lenses worn all day (including evening) to concentrations extracted from contact lenses worn during the daytime only, may be of value for the further investigation of the evening tear film. Albumin concentrations on lenses could potentially be correlated to tear film concentrations. Alternatively, deposited albumin concentrations might plateau after a few hours of wear and no correlation with day vs. evening wear is evident. Nevertheless, the differentiation between the evening tear film and the daytime tear film suggests that there is potential value in investigating the link between serum albumin leakage and end of day discomfort. Akin to the study mentioned in section 7.6.3, this would require a thorough assessment of contact lens extraction protocols.

7.6.5 Albumin ligands in the tear film

Albumin in plasma is able to bind a variety of ligands and therefore also executes ligand-mediated effects. Although this thesis has focused on albumin as a single entity, it is likely to be a simplified model and albumin should be researched further with respect to its ligand binding qualities. As detailed in the introduction, fatty acids are partly responsible for the stability of the tear film lipid layer and are extensively transported by albumin in plasma. Similarly, steroids, vitamins, and metals such as copper, magnesium, chloride and calcium have high association constants ($> 6.5 \times 10^2$) to albumin and are potentially brought across the blood-tear barrier with albumin during transport. The measurement of tear concentrations of any of these components alongside tear albumin would be useful to reveal any association between the two entities. An example would be to differentiate between delipidated albumin and the fatty acid bearing form in plasma. These results could then be used to analyse tear albumin and identify to which extent

each form is present. Statistically, albumin in plasma carries 1-2 long chain fatty acid molecules at any one time, whilst in circulation (Reed *et al,* 1975) but the actual distribution in tears is undetermined.

7.6.6 Albumin as a marker for other plasma products

Finally it is worth noting that albumin could be a marker for the recruitment of other plasma products able to pass through the blood-tear barrier. It would therefore be of interest to focus further research on the identification of plasma components that may leak alongside albumin. The tear concentration of these could then be correlated with albumin to investigate any potential relationships.

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APPENDIX A
DEVELOPMENT OF A SANDWHICH ELISA ASSAY TO MEASURE HUMAN SERUM ALBUMIN

A.1 Introduction

The research presented in this appendix details the attempt to develop an in-house sandwich ELISA for the measurement of human serum albumin. As discussed in chapter 2, commercial ELISA kits are comparatively expensive and the purpose of the laboratory constructed assay was to lower the cost of analysis of tear albumin concentrations. Chapter 2 provides an overview of the working components of the sandwich ELISA and a typical running protocol, which is not included in this appendix. The sections presented here focus on the development and evaluation of a sandwich ELISA protocol for the measurement of albumin.

A.2 Development of a sandwich ELISA for the determination of human serum albumin

A.2.1 Introduction

This section describes the development and evaluation of a sandwich ELISA targeting human serum albumin. Several criteria were determined for the production of a successful protocol. These were modelled on commercial ELISA specifications and were:

- Accurate measurements of albumin concentrations
- A blank OD similar to commercial products (0.1-0.01 OD). This was to enable good distinction between low-level albumin samples and to produce a highly sensitive assay
- Coefficient of variation < 15% between sample duplicates.
- Inter and Intra-assay coefficient of variation < 15% confirming even capture antibody coating and blocking.
- Repeatability of results

A.2.2 Initial running protocol

The Initial ELISA protocol described below was the starting point for the development of the laboratory based ELISA assay. Three antibodies were used in this first set of experiments, adopting a sandwich complex formation with a capture antibody, a detection antibody and a conjugated antibody (Fig A.1).

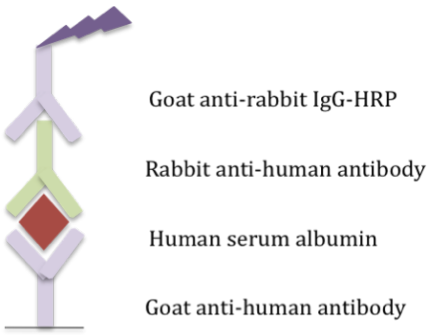


Fig A.1. Initial antibody setup

Human albumin specific conjugated antibodies are significantly more expensive that goat or rabbit conjugated antibodies and were considered only as a second alternative to the initial setup in Fig A.1.

Initial ELISA protocol (Protocol 1):

1. A Corning-half area plate was coated with goat-antihuman albumin antibody in sodium carbonate/sodium bicarbonate coating buffer at 100µl per well using a multichannel pipettor. Checkerboard titrations were made of the capture antibody to establish the most suitable working range (Fig A.2). Plates were then incubated in a moist box at 8°C for 24 hours.

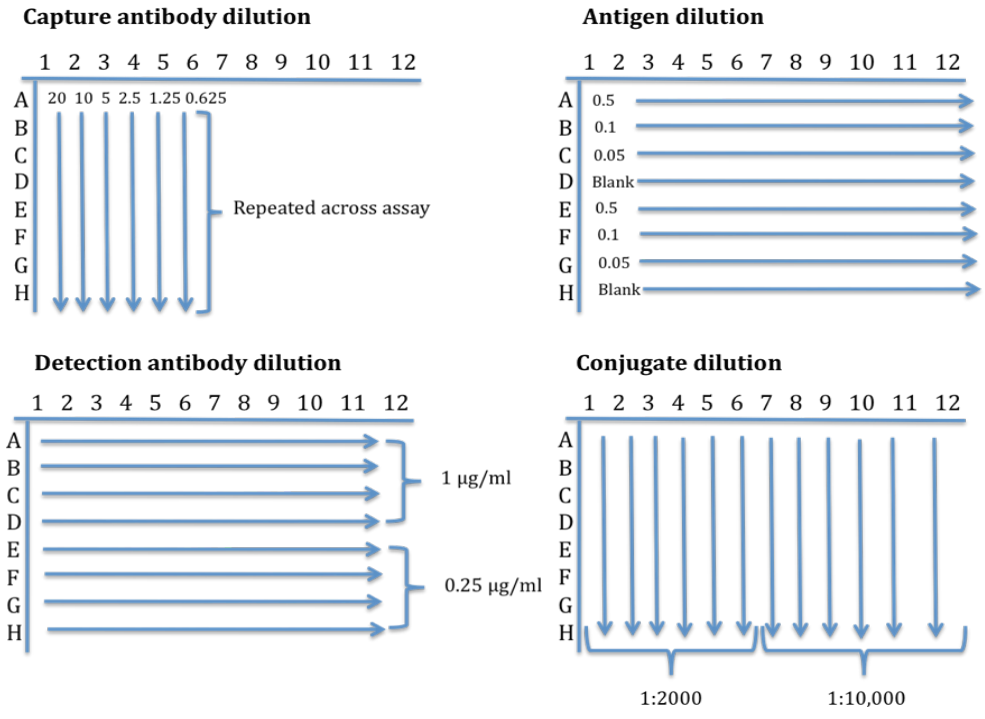


Fig A.2. Checker board titrations for assay protocol 1. Concentrations are in µg/ml.

2. The coating solution was emptied down a sink and the plate was banged dry against paper towels until no more wet spots appeared. 200µl of 3% skim milk PBS blocking solution was pipetted into each well. The plate was then incubated in a moist box at 8 °C overnight (minimum of 17 hours blocking time).

3. Blocking solution was then emptied down a sink and the plate was banged dry on paper towels until no more wet spots were visible. Human serum albumin was diluted in TBS at varying concentrations and pipetted into wells at 100µl/well. The human serum albumin represents the antigen and actual dilutions are described in Figure A.2. The plate was then incubated at room temperature for one hour.

4. After 1 hour, the plate was emptied and banged dry as mentioned previously in steps 2 and 3. 200µl of 0.1% PBS tween 20 washing solution was pipetted into each well and then plates were emptied and banged dry. This was repeated for a total of three washes, after which the plate was banged dry again before moving onto step 5.

5. Rabbit anti-human serum albumin detection antibody diluted in TBS 0.05% tween solution was pipetted into each well at 100µl/well according to the protocol in Fig A.2. The plate was then incubated at room temperature for one hour.

6. A second washing step as described in step 4 was performed to remove all unbound material.

7. Goat anti-rabbit IgG Enzyme conjugated antibody diluted in TBS 0.05% tween solution was added to each well at 100µl/well according to Figure A.2. The plate was incubated at room temperature for one hour.

8. A third washing step was performed as described in step 4.

9. 100 µl of TMB was then pipetted into each well and the plate was left to develop in the dark for ten minutes.

10. 100µl of 1M phosphoric acid was then pipetted into each well to stop the reaction. The plate was read on the spectramax M2 at a wavelength of 450 nm.

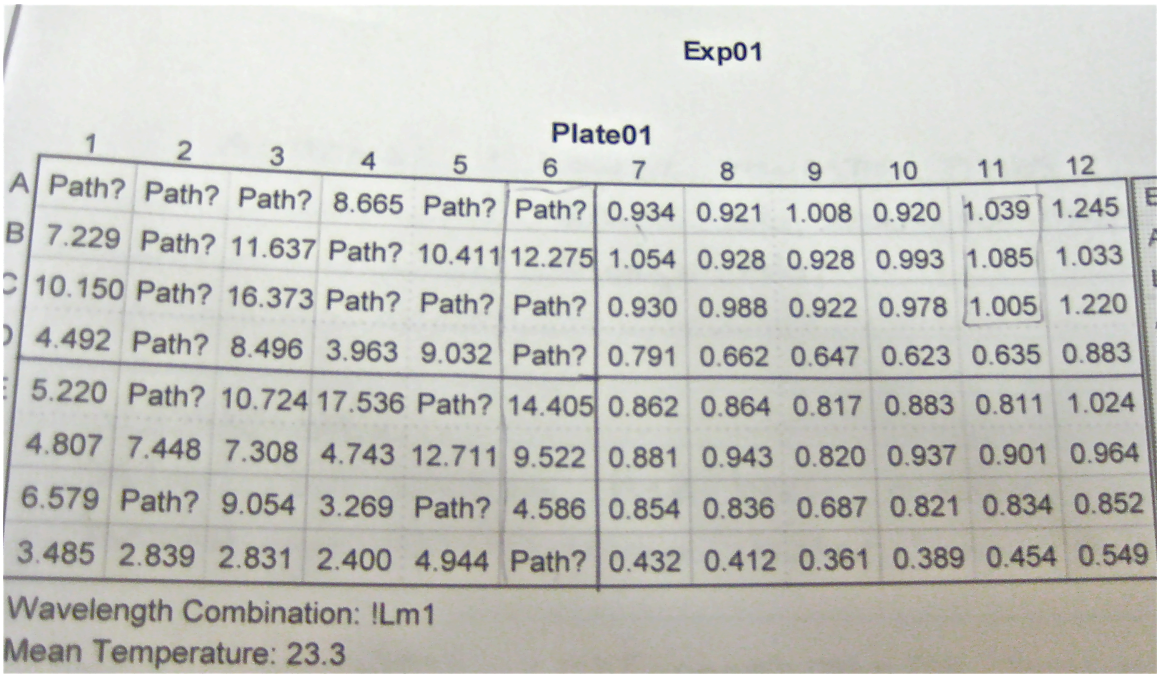
A.2.3. Modifications to initial protocol

Further modifications to the initial running protocol during development were made as detailed in the assay protocols in tables 35, 37 and 38. The first 5 protocols are described in Table 35. The protocols detail the plates used and the respective concentrations of capture antibody (Cab), antigen (Ag), detection antibody (Dab) and conjugate (Conj) for each experimental assay run. Concentrations described as numbers linked with a dash (e.g. 10-0.0098) signify a double dilution curve from the first to the last value. Incubation times, washing buffer and volumes remained the same between all protocols.

Table 35. Assay protocols 1-5.

Protocol no	Plate	Cab conc. (µg/ml)	Block conc.	Ag conc. (µg/ml)	Dab conc. (µg/ml)	Conj conc. (dilution factor)	Blank ok? (yes/ no)
1	Corning half area	20-0.625	3% milk	0.5, 0.1, 0.05	1, 0.25	1:2000, 1:10.000	NO
2	Sero-wel	1-0.0313	3% milk	1-0.0313	0.5, 0.25	1: 10.000	NO
3	Sero-wel	x Direct ELISA	3% milk	10-0.0098	0.25	1: 10.000	YES, high
4	Sero-wel	1-0.00098	3% milk	0.1-0.00153	0.25	1: 10.000	NO
5	Sero-wel	0.039	3%, 10%, 20%, milk 3%BSA	10-0.0098	0.25	1: 10.000	NO

The first experiment (Protocol 1) identified a suitable conjugate dilution factor, as shown in Figure A.3.



Exp01

Plate01

	1	2	3	4	5	6	7	8	9	10	11	12
A	Path?	Path?	Path?	8.665	Path?	Path?	0.934	0.921	1.008	0.920	1.039	1.245
B	7.229	Path?	11.637	Path?	10.411	12.275	1.054	0.928	0.928	0.993	1.085	1.033
C	10.150	Path?	16.373	Path?	Path?	Path?	0.930	0.988	0.922	0.978	1.005	1.220
D	4.492	Path?	8.496	3.963	9.032	Path?	0.791	0.662	0.647	0.623	0.635	0.883
E	5.220	Path?	10.724	17.536	Path?	14.405	0.862	0.864	0.817	0.883	0.811	1.024
F	4.807	7.448	7.308	4.743	12.711	9.522	0.881	0.943	0.820	0.937	0.901	0.964
G	6.579	Path?	9.054	3.269	Path?	4.586	0.854	0.836	0.687	0.821	0.834	0.852
H	3.485	2.839	2.831	2.400	4.944	Path?	0.432	0.412	0.361	0.389	0.454	0.549

Wavelength Combination: !Lm1
Mean Temperature: 23.3

Fig A.3. Recorded OD of Assay 1. The higher dilution factor of 1:10,000 produced ODs in the measuring range of the Spectramax M2 (columns 7-12). Path? refers to samples too concentrated to be analysed using the specified 450nm wavelength.

Subsequent assay protocols (2-5) (Table 35) were designed to further optimise the capture antibody and antigen concentrations. Optimal dilution for the secondary antibody (the detection antibody) was found to be 0.25 µg/ml. The initial plate selection was also rectified and the Corning plate, which had insufficient well volume, was replaced with a Sero-wel plate. A consistent issue was the lack of a true blank OD that was discernibly different from lower levels of antigen present. Assay protocol 3 substituted the sandwich ELISA for a direct ELISA (coating the antigen directly onto the solid phase, bypassing the need for a capture antibody). This protocol identified a possible cross-reaction between the capture antibody and the other assay components, as the exclusion of the capture antibody resulted in a blank OD. The achieved blank was still elevated compared to commercial blank ODs and doubling dilution of the antigen resulted in a fairly uniform absorbance value suggesting a possible antigen plateau. Higher antigen dilutions were employed in assay protocol 4 but double dilutions still exhibited uniform colour development. Assay protocol 5 attempted to improve blank OD by increasing block concentration and the inclusion of BSA as an alternative blocking agent but this did not resolve the high background. The results of assay protocols 1-5 suggested a possible cross-reaction between assay components and this factor was evaluated using double immunodiffusion and single radial immunodiffusion.

A.2.4 Principle of double immunodiffusion

Double immunodiffusion is better known as the Ouchterlony method and is based on the specific interaction between antibodies and their antigens. Antigen and antibody samples are pipetted into individual wells that have been punched out into agarose gel (Fig A.4).

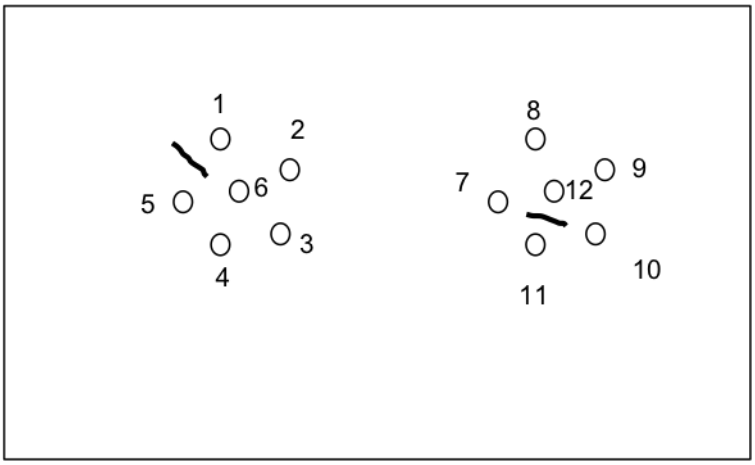


Fig A.4. An example of a typical Ouchterlony gel. This has identified a reaction between well number 1 and 5 and also between 11 and 12.

Components to be tested for interaction are positioned next to each other and allowed to diffuse through the gel. If the antibodies identify their antigen in large enough concentration, they will complex and a precipitation reaction will occur. The precipitation forms a visible line in the gel as shown in Fig A.4. Precipitation only occurs if the antigen is present in its bivalent form and not as monovalent Fab fragments as more than one antibody is required to form a visible aggregate. The Ouctherlony system is able to assess interactions between many components in a system and was used to identify potential cross-reactions between antibodies during ELISA development.

A.2.4.1 Materials

- Conical flask
- Water bath at 56°C
- Heated stirrer
- Screw top bottle (100ml)
- Centrifuge tubes (12 ml)
- Melinex sheets (7.5 x 11 cm)
- 5 mm immunodiffusion cutter
- Moist box
- Thick clear plastic rectangles (10cm x 12 cm)

- 2 plastic cartons for washing gel in
- Heavy books
- Filter paper

A.2.4.2 Reagents

- Comassie Blue stain solution
- Destain solution
- 0.9% Saline solution
- Agarose
- Polyethylene Glycol 8000 (PEG)
- Tris-Borate-EDTA
- Antibodies and analytes

All reagent components for immunodiffusion were obtained from Sigma Aldrich. The Comassie Blue stain solution was prepared by adding 1.2g of Brilliant Comassie Blue to 90ml ethanol, 90ml deionised water and 10ml glacial acetic acid. Destain was prepared by combining 150 ml ethanol, 400ml of deionised water and 50ml of glacial acetic acid.

A.2.4.3 Gel preparation

0.6g Agarose and 1.5g PEG 8000 was weighed out and added to 50 ml Tris-Borate-EDTA in a conical flask. The solution was stirred over heat until all the particulate matter was visibly dissolved. The solution was then poured into a pre-warmed 100 ml screw top bottle in a 56 °C water bath. This ensured that the agarose mixture remained liquid.

A.2.4.4 Gel pouring

Melinex® sheets were fixed to slightly larger thick, clear plastic rectangles by dabbing a bit of water on the backside of the sheet. A small triangular cut was made in the top right corner of the Melinex® sheet to differentiate it from the bottom and avoid confusion during analysis. 10 ml of the agarose solution were then poured into a 12 ml centrifuge tube kept in the water bath and then immediately poured onto a Melinex® sheet, attempting to form a uniform layer covering the sheet. It was important to place the Melinex® sheet on a level surface to ensure even flow of the gel. Gels were then left for 15 minutes to solidify.

A.2.4.5 Gel diffusion and analysis

A 5 mm immunodiffusion cutter was used to punch holes into the gel. The cut wells were removed by means of a glass pipette and discarded. Two different configurations were used: five holes in a "flower" formation with equal distance to a central well (Fig A.4.), and columns of two holes next to each other. The flower formation allows the comparison of several entities at the same time whereas the two holes on their own are used for single comparisons.

Neat samples were then pipetted into the holes, with 5µl / well, and the sheet was placed in a moistbox for 24 hours, allowing the samples to diffuse in the gel. The Gel sheet was subsequently immersed in a 0.9% saline solution overnight to stop the diffusion reaction. Filter paper was then placed onto the sheet and subsequently heavy books to flatten it into a thin film and expel excess liquid for approximately 24 hours. The flattening step was performed next to a window to achieve favourable drying conditions. Results were sometimes visible upon diffusion completion but all gels were stained to improve precipitation contrast. The sheet was removed from the plastic support for the staining procedure and completely immersed in Comassie Blue stain for 2 hours. The sheet was then transferred into a container with destain solution until all background stain was removed. Generally more than one fresh destain solution was needed to achieve this. Precipitation results were analysed visually and are described in section A.2.6.

A.2.5 Principle of single radial immunodiffusion

Single radial immunodiffusion was first described in 1965 by Mancini *et al.* and is more commonly referred to as the Mancini method. An agarose gel is prepared containing an antibody specific to the protein of analysis. Antigen added to wells made in the gel is then allowed to diffuse radially over time to bind with the antibodies present in the gel. As the antigen encounters the antibody the resultant complexes will precipitate out of solution and form visible aggregates. The expansion of the precipitation circle continues until the antigen has passed the zone of equivalence, where concentration is optimal to the antibody and the most precipitation occurs (Fig A.5). After this, the antibody is in sufficient excess to limit precipitation reactions until the process is halted completely.

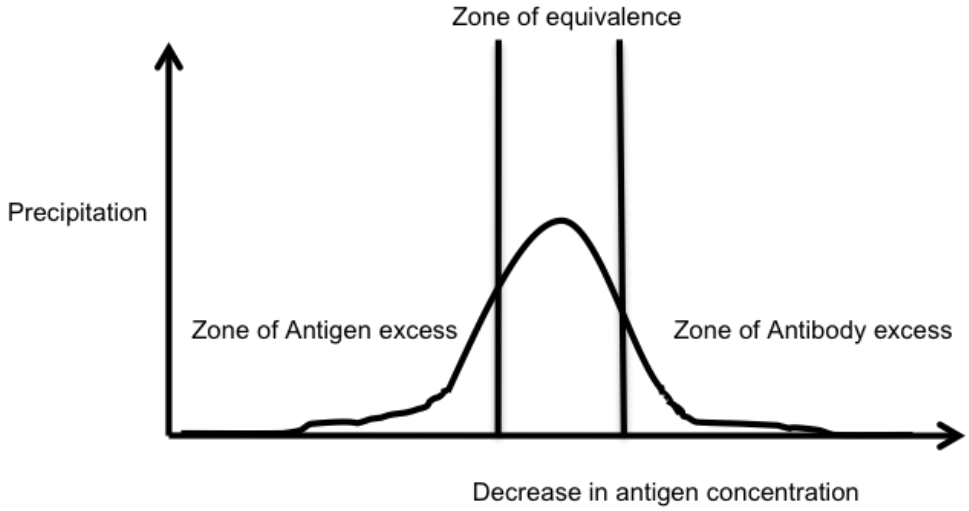


Fig A.5. Diagram of the relationship between concentrations of antigen and antibody and the resultant precipitation.

The diameters of precipitin complexes surrounding samples of antigen are directly proportionate to the antigen concentration in the sample. By introducing set concentrations of the protein, diameters provided can be used as a standard curve to calculate unknown sample concentrations from (Fig A.6). The lower limit of albumin detection using this system has been found to be an antigen concentration of 1.25 μg/ml with a standard deviation of <2% of the mean (Mancini *et al.*1965).

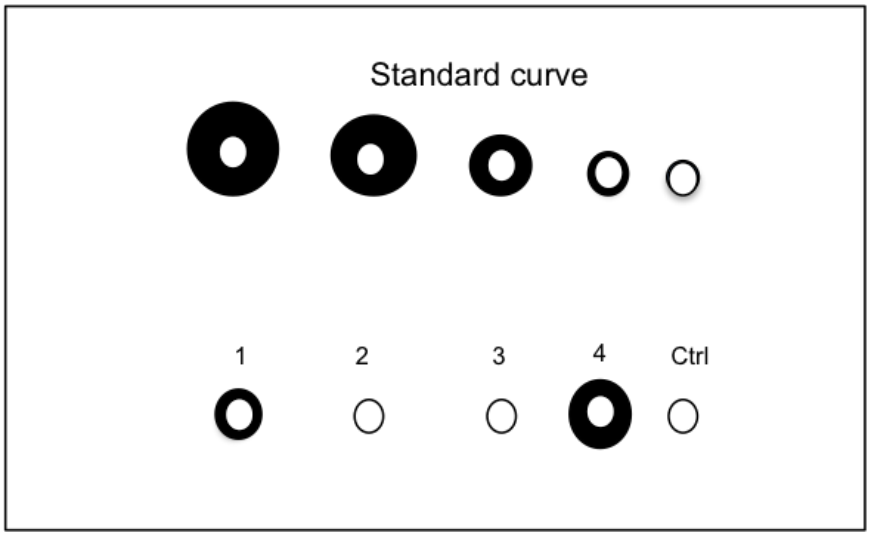


Fig A.6. Example of a Mancini immunodiffusion assay with a standard curve, control and test samples (1-4). In this example, 1 and 4 contain antigen at various concentrations and 2 and 3 are negative.

A.2.5.1 Materials and method for preparation

The materials and gel preparation of Manicini gels are identical to the Ouchterlony method with two notable differences. During the storing of the agarose gel solution in the 56°C water bath, 50µl of the desired antibody is added to the gel and gently mixed until evenly distributed. The pouring of the individual gel into the 12 ml centrifuge tubes is then done. The hole punching design also differs and was equal to the one exemplified in Fig A.6. As with the Ouchterlony gels, samples were assessed visually.

A.2.6 Immunodiffusion results for assay development

As a result of the possible cross-reaction between antibodies, Ouchterlony (Fig A.7) and Mancini gels (Fig A.8.) were run. The Ouctherlony gel was devised to detect whether there was any cross-reaction between the different antibodies used and other assay components such as coat and block buffer constituents. BSA was also included and deionised water was added as a control. The Mancini gel provided a closer assessment of the capture antibody as it had been identified as a possible source of concern in protocol 3 (Table 35).

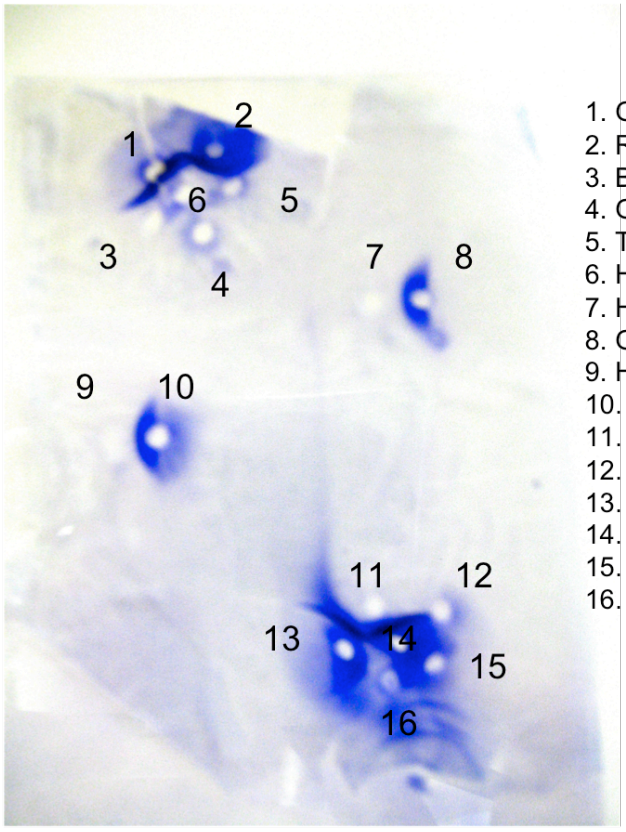
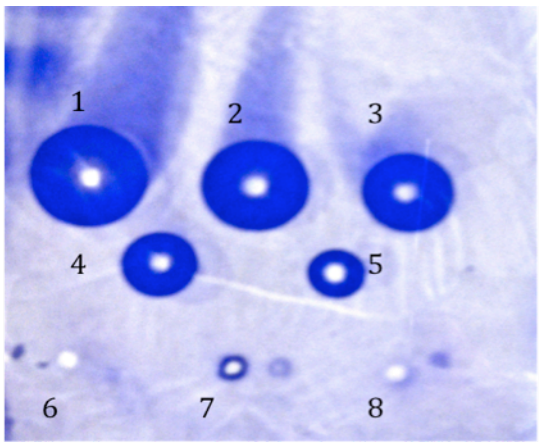


Fig A.7. Ouchterlony 1



1. HSA 1mg/ml
2. HSA 0.5 mg/ml
3. HSA 0.25 mg/ml
4. HSA 0.125 mg/ml
5. HSA 0.0625 mg/ml
6. BSA 1mg/ml
7. Rb anti-human albumin neat
8. Gt anti-rabbit IgG HRP neat

Gel: 12 ml agarose + 50 µl of Gt anti-human albumin

Fig A.8. Mancini 1.

Ouchterlony 1 confirmed that capture and detection antibodies were able to correctly identify human serum albumin. Antibodies in coating buffer also elicited a response eliminating buffer component interference as a source for concern. The reactions between goat anti-human albumin (spot 14) and the surrounding components were more difficult to discern and the possibility of cross-reactions still required further investigation. Mancini 1 assessed interactions between the capture antibody and other components. As expected, HSA in varying concentrations were compatible and BSA showed no interaction with the antibody. Spot 7, which represents the rabbit-anti human albumin antibody, exhibited some levels of complex formation although this could equally be attributed to excess of concentration and was treated as an anomaly. Consequently, questions still existed regarding the suitability of the antibody setup.

A.2.7 Further assay development

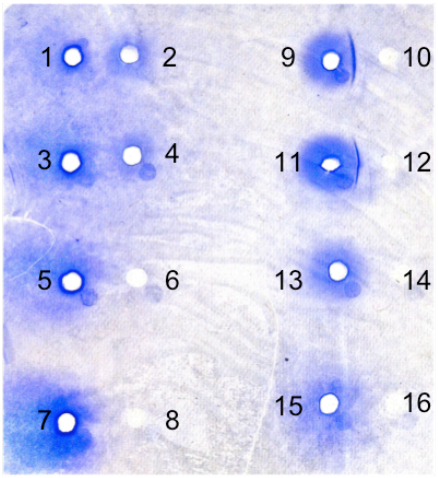
It was established that further assay development should continue with two parallel paths:

- The repetition and extension of immunodiffusion analysis to characterise antibody reactivity.
- The continued modification of the assay protocols. This involved: confirming the antigen dilutions, investigating insufficient blocking and washing as a cause for lack of blank OD and investigating the effect of restructuring the antibody complex.

A.2.7.1 Immunodiffusion analysis

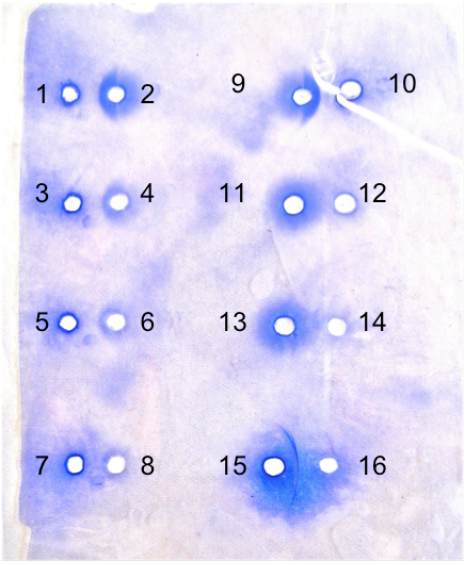
Parallel to the investigation of block buffer and washing conditions, immunodiffusion analysis was carried out to further evaluate assay component interactions and also possible interactions

with BSA (Fig A.9). Ouchterlony A investigated the cross-reactions between the antibodies in the existing setup (Fig A.1) and reactivity towards BSA. Ouchterlony B investigated cross-reactions between the capture antibody and the detection antibody in varying concentrations to eliminate reaction due to the prozone effect. Human serum albumin was included as a control.



1. Gt anti-human alb neat
2. Gt anti-rabbit IgG HRP neat
3. Gt anti-human alb neat
4. Gt anti-rabbit IgG HRP 0.5 diln.
5. Gt anti-human alb neat
6. BSA 1 mg/ml
7. Gt anti-human alb neat
8. BSA 0.5 mg/ml
9. Rb anti-human alb neat
10. BSA 1 mg/ml
11. Rb anti-human alb neat
12. BSA 0.5 mg/ml
13. Gt anti-rabbit IgG HRP neat
14. BSA 1 mg/ml
15. Gt anti-rabbit IgG HRP neat
16. BSA 0.5 mg/ml

A



1. Gt anti-human alb neat
2. Rb anti-human alb neat
3. Gt anti-human alb neat
4. Rb anti-human alb 0.5 diln.
5. Gt anti-human alb neat
6. Rb anti-human alb 0.25 diln.
7. Gt anti-human alb neat
8. Rb anti-human alb 0.125 diln.
9. Rb anti-human alb neat
10. Gt anti-human alb 0.5 diln.
11. Rb anti-human alb neat
12. Gt anti-human alb 0.25 diln.
13. Rb anti-human alb neat
14. Gt anti-human alb 0.125
15. Gt anti-human alb neat
16. HSA 1 mg/ml

B

Fig A9. Ouchtherlony gels showing possible interactions between assay components as well as BSA.

The Ouchtelony gels provided little definitive evidence about potential cross-reactions. Assay A confirmed that BSA does elicit a similar response to HSA with rabbit anti-human albumin (spots 9-12) suggesting a less specific paratope and a potential for concern. Assay B showed a definite reaction between goat anti-human albumin and HSA (spot 15-16) and suggested the possibility of a less clearly defined reaction between goat anti-human and rabbit anti-human antibodies (spots 1-2, 9-10).

A.2.7.2 Protocol modification

A commercial ELISA (Immunology Consultants Laboratory section 2.7) was run to confirm that the antigen dilutions used in the development protocols were made accurately and created a sensible curve. Results are shown in Table 36.

Table 36. Comparison between target antigen concentration and measured result.

Target antigen concentration (ng/ml)	Commercial Assay result (ng/ml)
15.7	12.1
31.3	25
62.5	59
125	120
250	>Range
500	> Range

The antigen dilutions were close to the target concentration and thus the antigenic plateau and high blank OD could not be attributed to error in dilution. A subsequent factor to evaluate was block efficiency, as poor blocking could lead to non-specific binding. A commercial blocking agent was purchased (Stabilguard®) and compared to the original 3% skim milk blocking buffer to evaluate the block efficiency. A new plate was also introduced (Costar) to evaluate the effects of block-plate combination.

In addition to block and plate variables, it was decided to re-design the sandwich antibody complex. The immunodiffusion results had not been able to conclusively establish that there were no antibody cross-reactions taking place. A new monoclonal mouse-antihuman albumin capture antibody was purchased to replace the polyclonal capture antibody and the system was re-arranged. This was an attempt to increase specificity and remove any possible previous interactions with the capture antibody and the other assay components. The available antibodies enabled two possible setups, which are described in Fig A.10. These were both evaluated in case one proved to be superior.

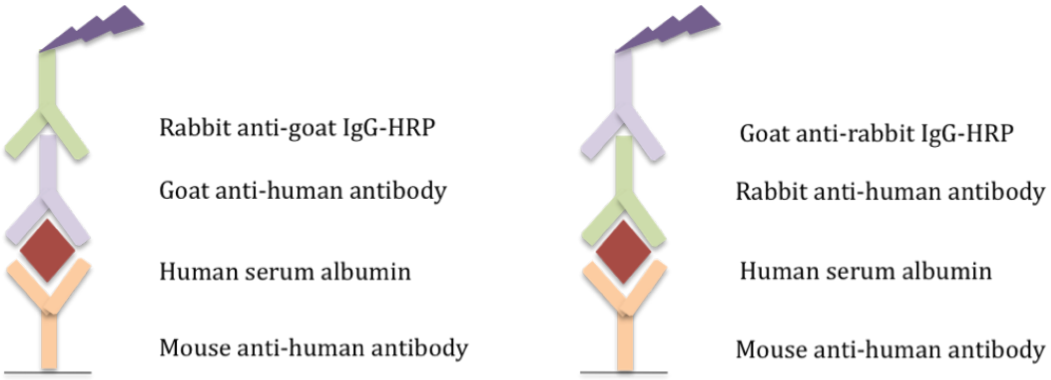


Fig A.10. New antibody setups for the human serum albumin sandwich ELISA

A.2.7.3 Outcome of protocol modification

Table 37 details the protocols used to assess the effect of block buffer and plate combination (6-9) and the protocols used to assess the new antibody complexes (8-9).

Table 37. Assay protocols 6-9.

Protocol no	Plate	Cab conc. (µg/ml)	Block conc.	Ag conc. (µg/ml)	Dab conc. (µg/ml)	Conj conc. (dilution factor)	Blank ok? (yes/ no)
6	Sero-wel	0.5-0.0157	Stabilguard	10-0.157	0.1	1:15.000	NO
7	Sero-wel	0.5-0.0157	3% milk	10-0.157	0.1	1:15.000	NO
8	Costar	10-0.157 new Cab	3% milk	0.5-0.0098	0.2 Gt dab	1: 10.000	NO
9	Costar	1 new Cab	Stabilguard	0.5-0.0039	0.2 Rb dab	1:12.000	NO

Re-assessment of OD data from the previous ELISA protocols suggested that the previously used detection antibody concentration of 0.25 was too high; colour formation repeatedly reached endpoint ODs close to 5 for the higher concentrations. Between 2.5 and 4 is a working range of colour formation closer to commercial kits (e.g. Immunology Consultants Laboratory ELISA). Detection antibody concentrations were consequently altered to 0.1 and 0.2 to reflect this. Dilution factor of the conjugate was also altered to try and reduce the endpoint colour formation. Protocols 6 and 7 compared block buffers on the Sero-wel plate and found no benefit in using Stabilguard® instead of the 3% skim milk block. Protocols 8 and 9 investigated the effect of block buffer on the Costar plate, as well as restructuring the existing antibody setup as described above. Unfortunately, this antibody regime was equally unsuccessful in producing a

blank OD. It became apparent in this study that the current protocol was not functioning as intended and that too many individual variables were assessed in each experimental setup. Ultimately it was decided to purchase an enzyme-conjugated detection antibody to limit the assay components and reduce the opportunities for cross-reaction between antibodies.

A.2.7.4 Effect of albumin specific enzyme-conjugated antibody

The detection antibody and the existing conjugate antibody were removed from the system and replaced with a polyclonal sheep anti-human albumin HRP conjugated antibody. Table 38 details the subsequent assay protocols used to characterise this new setup.

Table 38. Assay protocols 10-15.

Protocol no	Plate	Cab conc. (µg/ml)	Block conc.	Ag conc. (µg/ml)	Dab conc. (µg/ml)	Conj conc. (dilution factor)	Blank ok? (yes/ no)
10	Sero-wel	0.5-0.0157	3%	10-0.157	/	1:7500	YES, high
11	Sero-wel	0.25	6% milk	3-0.000625	/	1:8000. 1:16000	YES, high
12	Sero-wel	0.25	3% milk	0.6-0.00225 + tear samples	/	1:5000, 1:7500, 1:15000	YES, high
13	Sero-wel	0.25	3% milk	0.6-0.00225 + tear samples	/	1:7000	YES, high
14	Nunc Maxisorp	0.25	3% milk	0.6-0.00225+ 0.3	/	1:7000	YES, high
15	Sero-wel	0.25	3% milk	0.6-0.00225+ 0.3	/	1:7000	YES, high

The new antibody setup, represented by protocol 10, resulted in the first true blank OD, although this was elevated above the criteria identified in the introduction (0.2-0.3). Protocol 10 also established that the most suitable dilution for the capture antibody was 0.25 µg/ml. Further dilution analysis was performed in protocol 11 to identify antigen and conjugate concentrations. Ultimately a standard curve was constructed ranging from 600 µg/ml to 0.00225µg/ml. Subsequent assay protocols (12-15) were devised to investigate precision and the feasibility of tear sample analysis with the present format.

A.2.8 Investigating assay precision and suitability for tear albumin analysis

Three duplicates of the standard curve, along with two control samples (500 µg/ml, 12.5 µg/ml) and five tear samples were assayed under different conjugate dilutions in protocol 12 (Table 38). The majority of the tear samples produced ODs below the curve due to the elevated blank OD. Tear albumin concentrations could not be obtained for the tear fluid of those subjects. Duplicate precisions on standards were overall within the 15% CV limit (determined in the introduction) but this was not consistent across the assay, particularly with the lower level samples and controls. Assay protocol 13 adopted a similar layout to assay protocol 12, but with set conjugate dilution, to assess intra-assay precision and the importance of standard curve placement. Three standards curves were placed on different sections of the assay and the resultant ODs and curve shapes were compared. This is shown in Figure A.11.

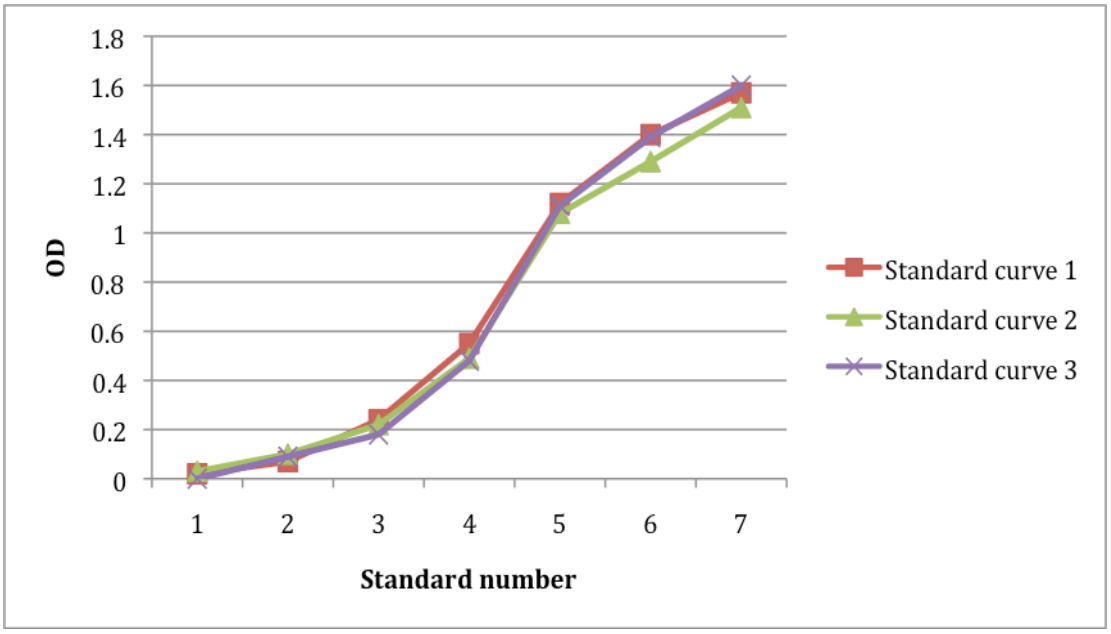


Fig A.11. Standard curve overlay on Sero-wel plate using Assay protocol 13.

Sample precision was, however, an issue with high %CVs and the blank OD values were still elevated (0.2 and 0.3). An alternative microtitre plate (Nunc Maxisorp) was assessed in an attempt to improve sensitivity and precision (protocol 14). This did not provide any improvements on the current protocol and performed considerable worse in terms of curve overlay. Assay protocol 15 was a repeat of protocol 13 with the aim to investigate intra-assay precision further. A 300 ng/ml human albumin sample was used instead of tear samples and pipetted in 42 replicates. A %CV of 17 was obtained between replicates but this was accomplished by the removal of two anomalies and high % CV on standard replicates.

A.3 Discussion of ELISA assay development

Assay protocol 15 (Table 38) represents the current protocol and its limitations as discussed above. These limitations lead to the laboratory-developed assay having no part in albumin analysis for the research presented in this thesis. It was decided that the current blocking protocol along with washing or coating protocols were potentially sub-standard. Pipetting error was considered as an alternative cause for the precision difficulties with the laboratory assay although this was discarded, as no transferred problems were observed when using the commercial alternative. Disregarding the precision issues, the running time of the laboratory assay was 48 hours compared to 2.5 for the ICL ELISA (section 2.7.3). This makes the latter significantly more time efficient as well as more sensitive and more precise. Whilst more experiments could have been undertaken to further improve the laboratory-constructed assay, the ultimate aim was to limit cost. The time spent on assay development was extensive so offsetting costs against time constraints made the choice of a commercial assay more appealing.

A.4 Conclusion

Even though the laboratory-developed assay did show promise and ability to differentiate between some levels of higher tear samples, the consistent lack of an acceptable blank OD and the precision desired lead to the conclusion it would to be too time-consuming to rely on the laboratory assay for the duration of the project. Nevertheless it provided the sought after valuable insight into the components and their interactions in the ELISA method.

APPENDIX B

ADDITIONAL DATA FOR CHAPTER 6

Table 39. Albumin concentrations in deionised water measured by the HemoCue ®Albumin 201 Analyser.

	1	2	3	Mean	SD	% CV
200	>R	>R	>R			
150	147	139	145	143	4.16	2.90
100	101	99	97	99	2	2.02
75	83	76	78	79	3.61	4.56
50	52	62	52	55	5.77	10.43
30	33	27	35	31	4.16	13.14
20	31	20	19	23	6.66	28.54
10	8	12	8	9	2.31	24.74
5	5	5	10	6	2.89	43.30
				Mean:	**3.95**	**16.21**

Table 40. Albumin concentrations in deionised water measured by the Afinion™ AS100 Analyser.

	1	2	3	Mean	SD	% CV
200	154.4	155.0	148.7	152.7	3.48	2.28
150	108.9	101.1	111.9	107.3	5.57	5.20
100	70.6	76.9	67.6	71.7	4.75	6.62
75	57.9	68.5	66.9	64.4	5.71	8.87
50	40.9	44.4	38.0	41.1	3.21	5.53
30	24.6	25.7	22.8	24.4	1.46	6.00
20	16.8	17.4	15.9	16.7	0.75	4.52
10	8.2	8.9	7.9	8.33	0.51	6.160
5	<R	<R	<R			
				Mean:	**3.18**	**5.65**

Table 41. Albumin concentrations in phosphate buffered saline measured by the HemoCue® Albumin 201 Analyser.

	1	2	3	Mean	SD	% CV
200	>R	>R	>R	/	/	/
150	>R	>R	>R	/	/	/
100	142	126	137	135	8.19	6.06
75	111	113	112	112	1	0.89
50	64	60	59	61	2.64	4.33
30	36	41	35	37	3.21	8.61
20	21	23	16	20	3.61	18.02
10	<R	<R	6	6	/	/
5	<R	<R	<R	/	/	/
				Mean:	**3.73**	**7.58**

Table 42. Albumin concentrations in phosphate buffered saline measured by the Afinion™ AS100 Analyser.

	1	2	3	Mean	SD	% CV
200	157.4	>R	170.0	163.7	8.91	5.44
150	132.6	144.0	181.9	152.8	25.81	16.89
100	66.0	90.5	100.5	85.6	17.75	20.72
75	51.6	75.4	68.5	65.1	12.25	18.79
50	48.5	46.3	47.4	47.4	1.10	2.32
30	28.2	26.1	32.1	28.8	3.04	10.57
20	18.3	18.5	18.0	18.2	0.25	1.38
10	<R	<R	<R	/	/	/
5	<R	<R	<R	/	/	/
				Mean:	**9.87**	**10.87**

PUBLICATIONS

List of publications from this work

1. Runstrom, G.K., Mann, A., Tighe, B., 2013. The Fall and Rise of Tear Albumin Levels: A Multifactorial Phenomenon. The Ocular Surface. July.

2. Runstrom, G.K., Mann, A., Tighe, B., 2013.Point-of-care Analysis of Tear Albumin Concentrations. In Preparation.