Effect of some intranasal formulations used in the management of allergic rhinitis on mucociliary function

A thesis submitted in partial fulfilment of the requirements of the University of Brighton for the degree of

Doctor of Philosophy

Marwa M. R. R. Ayoub

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Abstract

In the management of allergic rhinitis, drugs are delivered to the nasal cavity for a local effect. Mucociliary clearance (MC), the airway's primary innate defense mechanism against inhaled foreign particles, clears the drug formulations and undesirably decreases their residence time. Certain formulation variables are hence designed to optimize the intranasal (IN) residence time. It is therefore important that the effect of IN formulations on MC is understood. MC is attained via ciliary input (cilia length and density and ciliary beat frequency (CBF)) and airway surface liquid (periciliary fluid underlying mucous blanket of optimum amount, depth and viscoelastic properties). In the present study, ovine *ex-vivo* models were used to investigate the effect of a number of anti-allergic IN formulations, by GlaxoSmithKline (GSK), on different components of MC, namely mucin secretion, CBF and mucociliary transport rate (MTR). In addition, the *in-vitro* cytotoxic effect of these formulations on the human bronchial epithelial cell line, 16HBE14o-, was examined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) viability assay.

Mucin secretion and CBF were measured using sheep tracheal epithelial explants cultured on collagen coated nitrocellulose permeable supports at an air-liquid interface that maintained mucociliary differentiation. To measure mucin secretion, explants were incubated with and without test pharmaceuticals at their off-shelf IN concentration, followed by analysis of timed collections of medium for mucin content using an enzyme linked lectin-assay. CBF was measured using high speed videomicroscopy of perfused explants, which was combined with image-analysis techniques to derive CBF values at given time points before and after the addition of test pharmaceuticals. MTR was assessed by tracking the movement of carbon particles on the surface of whole sheep trachea maintained at 37 ºC in a humidified atmosphere before and after the exposure to test pharmaceuticals at their off-shelf IN concentration that were either applied by instillation or sprayed using an IN delivery system.

The preservative, benzalkonium chloride (BKC), was revealed here to significantly increase mucin secretion while significantly attenuating MTR and being significantly toxic to 16HBE14o- cells ($P \leq 0.05$). The observed effect on MTR was however clearly ameliorated by application of BKC as nasal spray rather than instillation, which justified the reported IN safety of BKC *in-vivo*. Meanwhile, GSK1004723, the novel H1/H3 antihistamine recently characterized by GSK, was revealed here to be significantly cytotoxic to 16HBE14o- cells as well as detrimental to ovine MTR and to significantly increase ovine mucin secretion ($P \leq 0.05$).

With the exception of BKC and GSK1004723, the studied IN pharmaceuticals, namely ethylenediamine tetraacetic acid (EDTA), potassium sorbate, propylene glycol, polysorbate 80, Methocel™, Avicel® and fluticasone furoate, and the IN formulation pH of 5-7.3 were shown to be well-tolerated by respiratory mucosa. This study therefore raises no safety concern on the IN use of these formulation variables *in-vivo*.

Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

Dated

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List of Abbreviations

API	Active pharmaceutical ingredient
ASL	Air surface liquid
ATP	Adenosine triphosphate
BKC	Benzalkonium chloride
CBF	Ciliary beat frequency
CV	Coefficient of variation
CsCl	Cesium chloride
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
ECG	Endothelial cell growth supplement
EDTA	Ethylene diamine tetraacetic acid
EIA	Enzyme immunoassay
ELLA	Enzyme-linked lectin assay
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FD70	Fluorescein isothiocyanate dextran (Molecular weight= 70,000)
FF	Fluticasone furoate
GSK1004723E	The naphthalene salt of the antihistamine GSK1004723
GSK1004723D	The dihydrochloride salt of the antihistamine GSK1004723
HBSS	Hanks' balanced salt solution
HNEC	Human nasal epithelial cells
HPA-biotin	Helix Pomatia Agglutinin–biotin conjugate
HPMC	Hydroxypropylmethyl cellulose

HRP-HPA	Horseradish-Peroxidase-labelled Helix Pomatia Agglutinin
HCl	Hydrochloric acid
IMS	Industrial methylated spirit
IPA	Isopropyl alcohol
KH	Krebs-Henseleit buffer
LBA	Ligand binding assay
LDH	lactate dehydrogenase
MC	Mucociliary clearance
MEM	Minimum Essential Medium Eagle
MTR	Mucoiliary transport rate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide
NA	Not available
Na-CMC	Sodium carboxymethylcellulose
NR	Neutral red
OD	Optical density
PG	Propylene glycol
PS	Potassium sorbate
PABS	School of Pharmacy and Biomolecular Sciences, University of Brighton.
PBS	0.01 M phosphate buffered saline pH 7.3 ± 0.2
PCF	Periciliary fluid
PEG300	Polyethylene glycol 300
PMT	Photomultiplier tube
RMM	Relative molecular mass
RIA	Radioimmunoassay
RO	Reverse osmosis
ROI	Region of interest

RSD	Relative standard deviation
RE	Relative error
RT	Reference treatment
Streptavidin-HRP	Streptavidin-peroxidase polymer

Chapter 1

Introduction

1.1 Intranasal Drug Delivery

Intranasal (IN) administration provides a non-invasive route of drug delivery. Therapeutic agents are delivered to the nasal cavity to act locally in case of local conditions, or to otherwise act systemically. Glucocorticosteroids, antihistamines, sodium cromoglicate and sympathomimetics represent locally acting drugs that are applied intranasally to manage conditions such as allergic rhinitis without the risk of systemic side effects [4, 5]. Macromolecules such as calcitonin (a labile peptide in the gut), IN vaccines such as Flumist® and the anti-migraine 5HT1 agonists such as sumatriptan (Imigran®) and zolmitriptan (Zomig®) are all examples of drugs administered intranasally to reach the systemic circulation [6, 7]. In addition to the convenience of administration and the low manufacturing costs, bioavailability via the nasal route offers the advantages of bypassing the hepatic metabolism (first-pass effect) for delivery of labile molecules, rapid onset of action as in the treatment of migraine or in emergencies like the treatment of opioid overdose (using naloxone) and bypassing the blood-brain barrier for delivery to the central nervous system (CNS) that is being extensively researched at present and demonstrates much potential [2, 5, 8-11].

Anatomically, the nasal cavity progresses from the nostrils through the nasal vestibules to the constriction of the nasal valve, behind which the cavity extends to the nasopharynx (Figure 1.1). The nasal septum medially divides the nasal cavity whose lateral walls behind the nasal valve contain the nasal turbinates (conchae), these foldings that result in a nasal cavity of about 160 cm^2 area while only 15 ml volume . This large and highly vascular surface area physiologically functions to rapidly heat and moisturise the inhaled air, and it additionally provides a good route for drug delivery [5, 12-14].

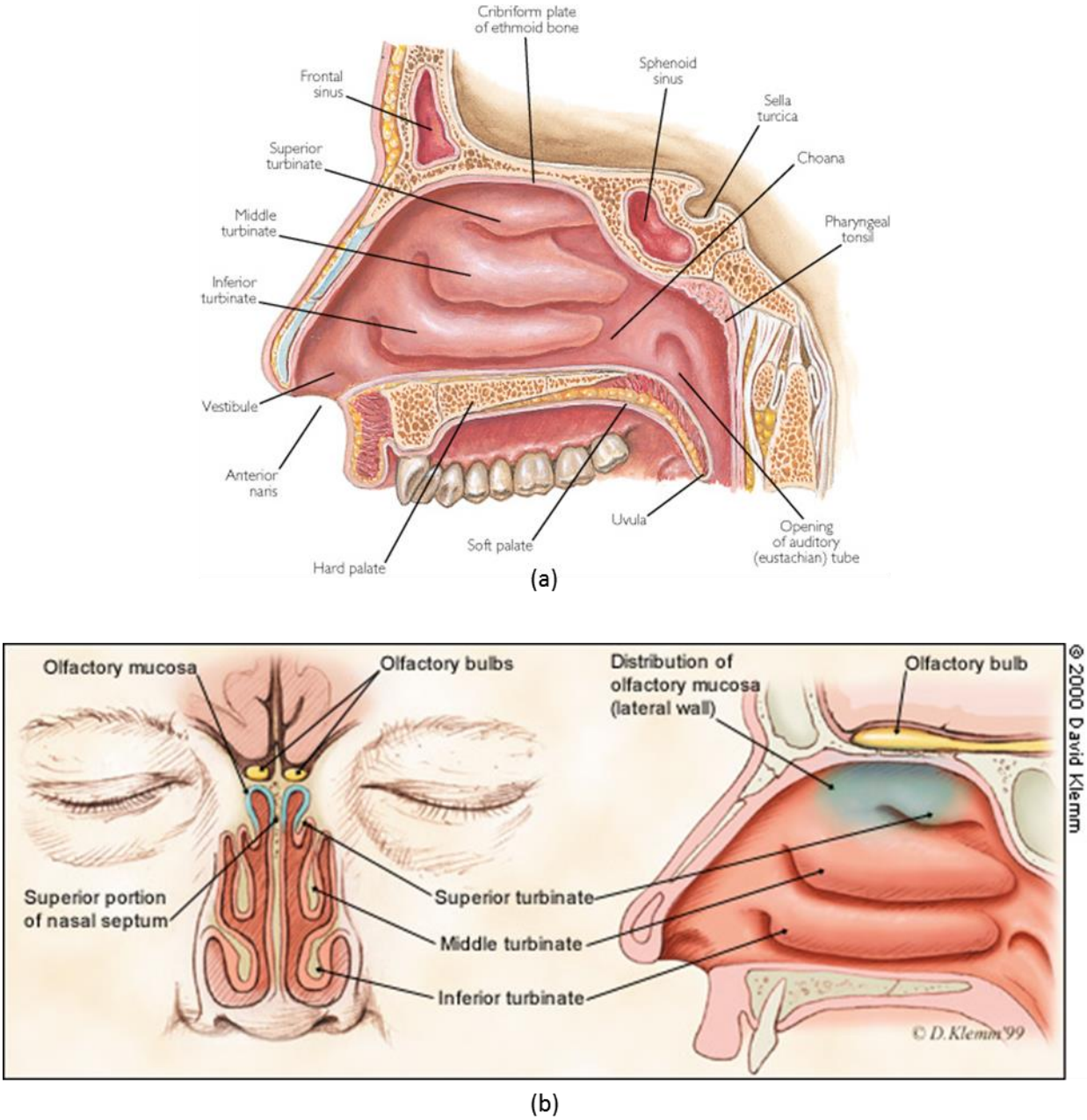


Figure 1.1 Anatomy of the Human Nasal Cavity

(Reproduced from ent4students.blogspot.com)

(a) The lateral wall of the nasal cavity. (b) A coronal (left) versus sagital (right) sections of the nose.

The lining of the nasal cavity gradually changes from being skin (keratinized, changing to non-keratinized, stratified squamous epithelium [15]) in the vestibules (these open to the face through the nostrils), where coarse hairs (vibrissae) function to filter large particles, to pseudostratified columnar epithelium (the typical respiratory epithelium lining 80-90% of the nasal cavity) posteriorly, where basal, columnar and mucus-secreting goblet cells exist (Figure 1.2). Columnar cells are covered with non-motile microvilli that contribute to the surface area of the nasal cavity and despite being non-ciliated anteriorly, many of them possess cilia thereafter. Serous and muco-serous submucosal glands as well as goblet cells are responsible for the production of nasal secretions that line the airways. [16, 17]. The mucus and cilia are major components of the primary innate defense mechanism of the airways, mucociliary clearance (MC), which is discussed below in section 1.2.

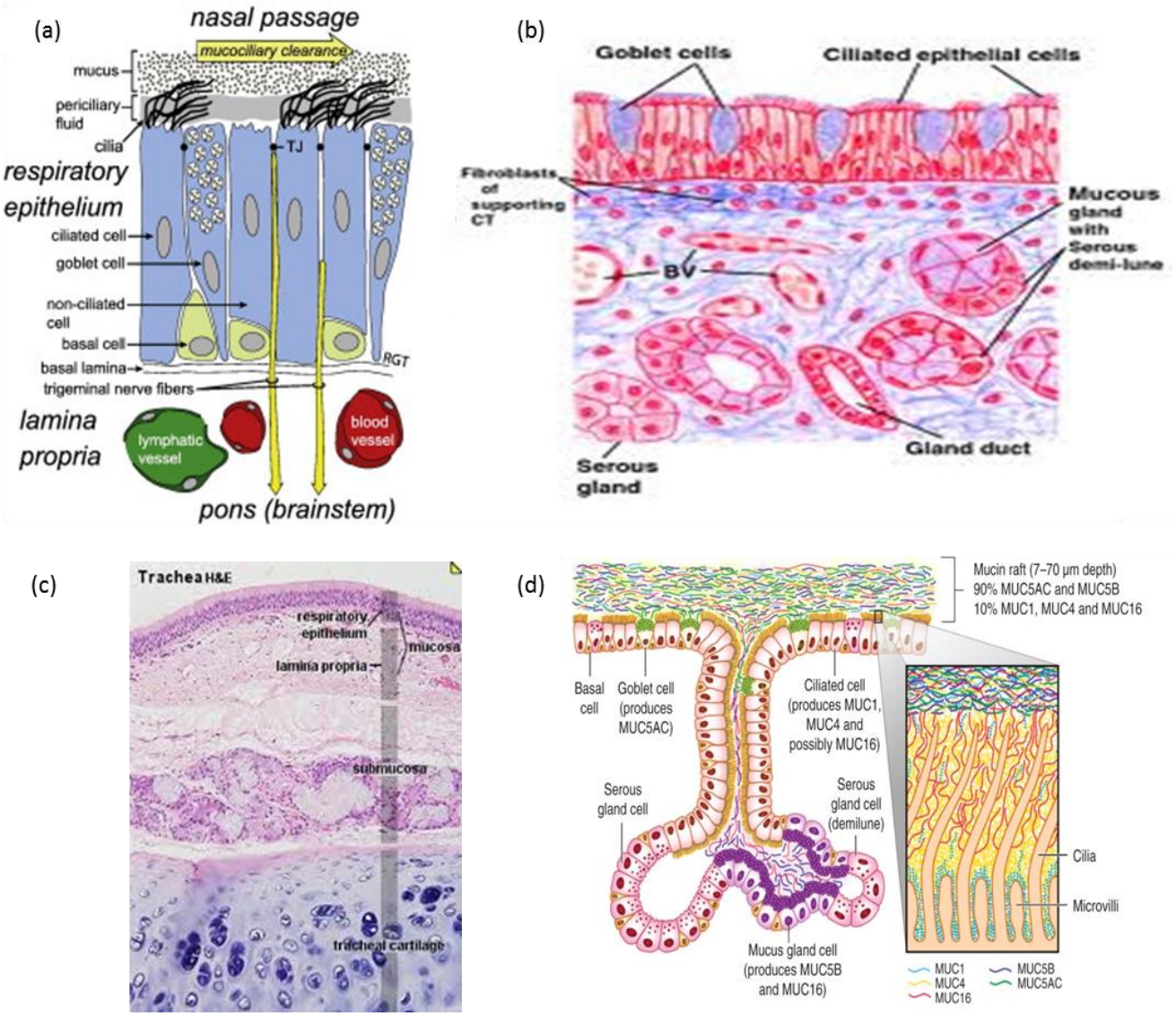


Figure 1.2 The Respiratory Epithelium

(a) The respiratory epithelium cellular components (reproduced from [2]). Four main cell types (columnar ciliated, columnar non-ciliated, basal and goblet cells) overlying the basement membrane and covered with the airway surface fluid represent the respiratory epithelium. (b) The nasal respiratory mucosa (reproduced from vetmed.vt.edu). It consists of the epithelium and the underlying lamina propria that contains blood vessels (BV), submucosal gland ducts and connective tissue (CT). (c) A haematoxylin and eosin stained section in human trachea (reproduced from lab.anhb.uwa.edu.au). It shows the typical respiratory mucosal and the submucosal airway lining that extends from the nose till before the end of the smallest airway. (d) Mucins on the respiratory epithelium (Reproduced from [3]). Variable mucin genes are expressed in different secretory cells but the mucin rafts on the surface of epithelial cells are 90 % MUC5AC (secreted by goblet cells) and MUC5B (secreted primarily by the submucosal-gland mucous cells), and 10% tethered mucins, MUC1 (located deep among the cilia and microvilli), MUC4 and MUC16.

Nasal drug delivery is therefore intended in the respiratory region of the nasal cavity with its large surface area, whereas the anterior part of the cavity (the vestibule) shows poor drug permeability particularly near the nostrils' being keratinized with sebaceous glands [5, 13]. Nevertheless, the typical respiratory epithelium is associated with the activity of MC, where the cilia beat beneath the mucus lining to propel it and any deposited material to the pharynx to be swallowed or expectorated. This results in compromised contact (residence) time for the drug at the absorption site (in case of systemic delivery) or the site of action (in case of locally acting drugs), and hence, deficient bioavailability and/or efficacy [18].

Diffusion through the mucus raft also presents another barrier against the availability of drugs from the nasal cavity especially with the range of enzymes present in the nasal secretions as well as in the epithelium (to a much lesser extent than the metabolic activity in the gastrointestinal tract though) [5]. IN formulations are therefore designed with those physiological barriers in consideration and certain formulation variables are adjusted to achieve optimum activity. For example, viscosity modifiers are used to attain a formulation consistency that would increase its residence time in the nasal cavity.

Meanwhile, the physicochemical characteristics of the drug are the main determinants of its intranasal availability. The drug needs to be in solution at a concentration that is high enough to achieve effective bioavailability from the small volume allowed in the nasal cavity. It is also preferred to be in the non-ionized form as this is better absorbed by diffusion [5, 19]. The design of the IN formulations hence takes these factors in consideration, which might require the inclusion of a solubilizer like polyethylene glycol 400 (PEG 400, approved by the FDA in IN at a maximum concentration of 20 % w/w [20]) or buffering the formulation to maintain the drug in the non-ionized form without causing mucosal irritation or encouraging microbial growth. As reviewed by Rakhi et al. [21], IN formulations in the pH range of 4.5-6.5 caused minimal mucosal irritation . It is worth noting that the physiological pH of the nasal

secretions is 5.5-6.5 [22, 23], at which the lysozymes are active against bacterial growth. Lysozymes are inactivated under alkaline conditions [24]. Meanwhile, the pH at the nasal epithelium is around 7.3 [5, 25]. England et al. found human mucociliary flow rates to be stable within the nasal physiological pH range [26].

The design of IN formulations therefore involves optimization of certain formulation variables (summarised in table 1.1 [5, 20, 27]) to achieve the required biological efficacy (locally or systemically) while causing minimal and tolerable side effects and maintaining the formulation stability. Variables such as tonicity can influence nasal retention and absorption. Some excipients can irritate and dry the nasal mucosa resulting in an unpleasant odour or after-taste, which would lead to patient tolerability and compliance issues [28].

Table 1.1 Intranasal Formulation Variables

Formulation Variables	Comments and Examples
Preservative	Most IN preparations contain a preservative to stop microbial growth. e.g benzalkonium chloride (BKC), potassium sorbate (PS). Vaccines such as Flumist® have no preservative because they are single use.
Viscosity	Cellulose derivatives such as methyl cellulose or hydroxypropylmethyl cellulose (HPMC) are the most commonly used viscosity modifiers and suspending agents. This variable is optimised to improve the nasal retention of the formulation.
Buffer (pH)	Citrate and phosphate buffers are routinely used in IN formulations.
Tonicity	Glucose and sodium chloride are common IN tonicity adjusting agents.
Solubilizer (Cosolvent)	Propylene glycol (PG), ethanol, PEG3350 and PEG400 are examples of cosolvents used in IN preparations.
Chelator	Ethylenediamine tetracetic acid (EDTA) is used to improve the formulation stability and preservation.
Wetting agent (surfactant)	Suspensions require the inclusion of a surfactant such as Polysorbate 80 and polysorbate 20. High molecular weight PEGs such as PEG3500 act as surfactants as well as cosolvents.
Antioxidant	Ascorbic acid E300, butylated hydroxytoluene and butylated hydroxyanisole are examples of antioxidants used to optimise stability.
Humectant	Glycerin is the usual excipient used to minimise mucosal irritation.

This table summarises the formulation variables generically used for IN drug delivery. This study, however, only investigated the compounds used by GSK in the IN formulations considered in this project.

In addition to the formulation variables summarised in the above table, novel strategies have emerged for improving IN drug delivery. Absorption enhancers act by increasing the permeability of the respiratory mucosa (e.g. chitosan) and might simultaneously enhance the drug solubility (e.g. surfactants such as cyclodextrins and polyethylene glycols) [5, 7, 13, 29]. Mucoadhesive nasal delivery systems present another arena for improvement of nasal formulations. They act by improving the nasal residence time, intimacy of contact with the nasal mucosa and additionally can increase the mucosal permeability [30]. Cellulose derivatives are the most commonly used mucoadhesive/viscosity enhancers. Various new dosage form technologies are currently evolving in this regard, such as nanostructured particles and *in-situ* gelling systems.

1.2 Mucociliary Clearance

The respiratory system is directly exposed to the external environment for life time and is therefore constantly at risk of exposure to infections and noxious agents. The primary innate defence mechanism that protects the airways against inhaled environmental stimuli is mucociliary clearance (MC). Airway surface liquid (ASL) and cilia, the functional elements of the mucociliary system, integrate efficiently to eliminate debris laden mucus to the gastrointestinal tract by swallowing or to the external environment by expectoration (see figure 1.3). The efficiency of this vital defence mechanism depends on the ciliary input (i.e. length, density, movement and coordination of cilia) and the amount, depth, composition and viscoelastic properties of the ASL (the mucous lining and the underlying periciliary fluid (PCF) [31-33]. Cell-membrane-tethered mucins primarily occupy the PCF layer [34], which embrace the length of cilia (~ 7 µm [35]) whose claws physiologically contact the mucous blanket to propel mucus with any accumulated debris during the effective stroke ciliary phase. A recovery stroke ciliary phase then follows, in which cilia bend to passively move backward through the less resistant PCF layer into its starting position [36]. A functional MC thus requires most of the ciliary length to be within the PCF while the ciliary tips reach the mucus layer. There is increasing evidence that airway luminal ATP, UTP and adenosine, which are continuously secreted by the respiratory epithelium in response to the tidal breathing mechanical stress, play a major role in regulating the ASL volume, ciliary beat frequency (CBF) and, hence, MC [35, 37, 38]. Notably, Liu et al. recently reported an increase in ciliary beating and mucociliary transport, via a calcium-dependant autoregulatory mechanism, upon reducing the depth of PCF following an increase in mucus load up to a physiological limit, beyond which this autoregulatory mechanism failed and MC abrogation was observed [39].

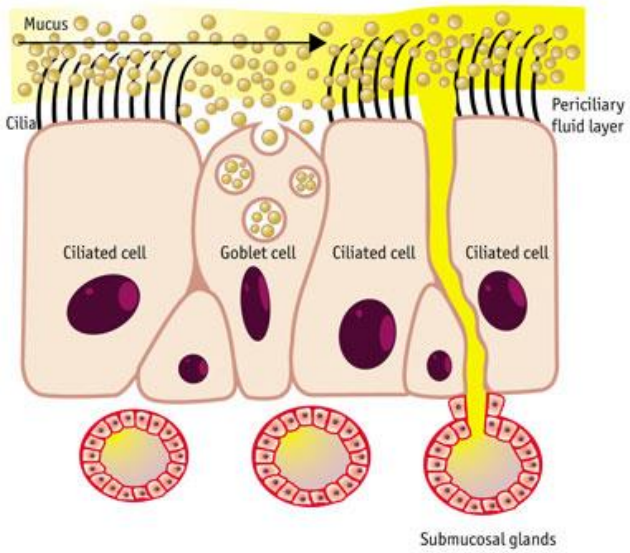


Figure 1.3 Components of the Respiratory Mucociliary Function (reproduced from [1]).

The functional components of MC are ASL (mucus blanket that traps airborne-debris overlying PCF that facilitates ciliary beating for efficient transport) and cilia.

Impairment of MC is potentially detrimental and may result in respiratory tract infections, inflammation and damage. Mucociliary dysfunction, arising from fluid imbalance and hence defective ASL, has recently been elucidated as a primary pathophysiology in cystic fibrosis (CF) [40-42]. Mucociliary dysfunction was also recognised in inflammatory hypersecretory diseases, such as asthma, bronchiectasis and chronic bronchitis , and in primary ciliary dyskinesia (PCD) owing to defective cilia [31-33, 43], which often contributes to the severe complications of these diseases. The backup defence reflexes, sneeze and cough in the nose and lower airways respectively, become prominent in disease [43-45]. Nevertheless, as reviewed by Fahy et al. and Mall et al., dehydration of ASL, such as in CF, impairs both mucociliary and cough clearance mechanisms [43, 46, 47]. In addition, cough receptors only exist in large conducting airways and hence hypersecretion in the bronchioles leads to obstruction of the air flow, which exacerbate the severity of a disease such as CF [48].

1.3 Allergic Rhinitis

Allergic rhinitis (AR) is an inflammation of the nasal passages that is triggered by an allergen such as pollen (causing AR commonly known as hay-fever), dust, certain animal dander or mould. It can be seasonal or perennial and can occur independently or with asthma (causing chronic allergic respiratory syndrome or united airway disease) [49, 50]. The condition affects about 40% of the USA adult population and 25% of the children [51, 52] causing sneezing, rhinorrhoea (runny nose), nasal congestion (blocked nose) and nasal pruritis (itching that also affects the eye, soft palate and inner ear) with the latter characterising allergic rhinitis from other types of rhinitis [53, 54]. Oral H1 antihistamines are usually effective in attenuating the pruritis, rhinorrhoea and sneezing symptoms, which usually relieve mild to moderate conditions of AR, whereas intranasal corticosteroids, which can be combined with oral or intranasal antihistamines, are the first line management for more severe conditions as they are more efficient in relieving nasal obstruction [49, 55, 56].

Mucociliary transport has been shown to be compromised in AR, which predisposes patients to other respiratory diseases [57-63]. Interestingly, Lale et al. have reviewed reports of an increase in MC in AR patients [64]. Moreover, an increase in mice CBF was recently reported in the acute phase of allergic rhinitis while histamine, the main mediator in allergic inflammations, was demonstrated not to cause any further alteration to CBF except at high non-physiological concentration, at which ciliary beat failure was observed [65]. Meanwhile, histamine, at lower concentrations, demonstrated cilio-stimulatory effects in the middle ear of guinea-pigs (by interacting with H2 and not H1 receptors) despite causing ciliary impairment at higher concentrations [66]. Interestingly, histamine was also demonstrated to stimulate mucociliary activity in rabbit maxillary sinus [67] by interacting with H1 rather than H2 receptors, an effect that was sustained in the presence of an anticholinergic agent. Meanwhile, Lee et al. have recently shown histamine to stimulate calcium ion channels, which despite

enhancing respiratory mucus secretion had no effect on ciliary beating in primary human sinonasal cultures [68]. Histamine-induced airway mucus secretion was also reported by Yanni et al., who elucidated this to operate at the H2-receptors whereas enhanced airway mucus secretion by first generation H1 antihistamines was attributed to their anti-muscarinic action [69, 70]. Notably, newer-generation H1 antihistamines were shown to directly inhibit calcium-ion channels as reviewed by Lehman et al. [4] but there is no body of evidence that they suppress mucus secretion. Kim et al. has, however, recently shown H1 antihistamines to inhibit histamine-induced MUC2 up-regulation [71] while histamine was also reported to up-regulate MUC5AC [72]. It can therefore be seen that the regulatory mechanisms of otorhinolaryngeal MC in allergic reactions are not fully understood and the state of MC in AR has yet to be established, which is quite similar to the lacked clear understanding of the mechanisms underlying compromised MC in asthma [73].

Antihistamines are first-line pharmacotherapy in mild to moderate cases of AR. Owing to their poor H1-receptor selectivity, first generation (sedating) antihistamines thicken mucous secretions and decrease ciliary beat frequency via their anticholinergic effect on muscarinic receptors [69, 74-76]. These agents were therefore observed to depress MC [77, 78]. Second and third generation non-sedating H1 antihistamines are thus the better choice in AR. However, the effect of individual drugs in this class on MC is variabile. Loratadine [79, 80], levocetirizine [81] and levocabastine [82] appeared to not alter MC. Similarly, cimetidine, a H2 antihistamine induced no significant changes in MC [77]. Azelastine, an IN third generation H1 antihistamine [83], was also shown to be non-detrimental, and sometimes beneficial, to MC [84-88]. It was also noted to enhance mucus secretion while not impacting on CBF [85], which probably indicated a within-physiological-limit increase in secretions that enhanced MC [84, 85].

Despite the agreement on the need to further investigate the long-term effects of IN steroids on the nasal mucosa, research to date demonstrated their safety on MC with a number of

studies reporting significant improvement of compromised mucociliary function in AR patients [79, 80, 89-94].

1.4 The Study Proposal

1.4.1 Aim of the Study

The aim of the present study was to examine the effect of a number of IN formulations on airway mucin secretion, ciliary beat frequency (CBF) and mucociliary transport rate (MTR) as major components of MC in order to test the hypothesis that IN formulations do not interfere with MC. In addition, the cytotoxic effect of these formulations on the human bronchial epithelial cell line, 16HBE14o- has been investigated using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) viability assay.

The significance of this study emerged from the routine design of IN formulations to slow MC in order to achieve optimum nasal residence time, and hence efficacy of intervention, while knowing that impaired MC is associated with respiratory infections [32, 44]. Importantly, therefore, the effect of such formulations on MC needed to be understood particularly that IN formulations provide management for chronic conditions such as AR, which require long-term reliance on these preparations.

1.4.2 Knowledge to Date

The effect of various formulation variables on some components of mucociliary function was reported previously. However, there was no report investigating various components in a single species, particularly a mammalian system (see table 1.2), and hence the proposal here embraced a novel approach. This had particular potential significance when one considered the known species differences in the control of mucociliary function [95-97]. Moreover, the high variability in the investigational approaches (methods and models) of MC, in which species

differences play a role, has led to difficulties in the interpretation of published literature [31, 94]. Notably, the effect of pharmaceutical formulation variables on mucus secretion has hardly been studied (see table 1.2) due to the lack of reliable methodology [33, 64], this being a novel part of the present study.

Management of allergic rhinitis usually involves corticosteroid or antihistamine APIs. This study examined the effect of fluticasone furoate (FF), the steroid API in Avamys® (GSK's most recent anti-allergic-rhinitis nasal spray), and GSK1004723, a novel H1/H3 antagonist recently characterised by GSK and currently in clinical trials, on MC. To date, no data is available in the literature on the effect of these two drugs on respiratory mucosa.

Benzalkonium chloride (BKC) is a common-practice preservative with proven efficacy in IN and ophthalmic formulations while being also used in inhalers/nebulizers preparations. BKC-induced ophthalmic and pulmonary (broncho-constricting) adverse effects are well documented [98-100]. Nevertheless, there is a lack of consensus on its effect on the nasal mucosa [92, 101-104], which fuelled a controversial debate on its IN safety [94, 105-107]. Notably, the European Medicines Agency has recently revised the label and package leaflet guidelines of BKC-containing pulmonary, ophthalmic and nasal formulations to clearly indicate the concentration of BKC and the potential adverse effects. BKC has been studied in various *in-vitro* and *in-vivo* models and the results were highly variable among the *in-vivo* as well as between the *in-vitro* and *in-vivo* data (See table 1.2). It has been argued that the respiratory *in-vivo* defence mechanisms (MC, mucous layer and surface area) were responsible for protecting respiratory mucosa *in-vivo* against the noxious effects of BKC [94, 102, 104, 107]. Nevertheless, some studies extended the *in-vitro* deleterious effects of BKC on MC to *in-vivo* models [92, 101]. Research has therefore been warranted for a better understanding of BKC IN safety.

The preservative, potassium sorbate (PS), was used by GSK in BKC-free IN formulations and its effect on MC has yet to be established. Nasal irritation caused by PS was reported in rats' nasal mucosa [108, 109], whereas it caused no significant changes in mammalian *in-vitro* CBF [89, 110, 111]. Studies looking at its effects on MTR and mucin secretion have been lacking, which was undertaken here.

The chelator, Ethylenediamine tetraacetic acid-disodium salt (EDTA), has routinely been included in IN formulations to aid stability and synergize preservation. The first report of EDTA-related respiratory diseases (asthma and/or rhinitis) was recently published [112], which highlighted the limited state of knowledge on the respiratory effects of this excipient. *In-vitro* animal studies showed EDTA to only mildly interfere with CBF [113-115] despite impairing MTR [116]. These effects were, however, not observed in human models studying MTR (*in-vivo*) [117-119] nor CBF (*in-vitro*) [119]. It can thus be seen that the effect of EDTA on the respiratory mucosa is not fully understood.

Only little is known about the effect of solubilizers and surfactants in IN formulations on MC. As shown in table 1.2, CBF was the only component of MC that was previously investigated under the effect of the solubilizer, propylene glycol (PG), and the surfactant, polysorbate 80, which showed the former to be minimally ciliotoxic while the latter induced a concentration-dependent ciliotoxicity. Similarly, there is very limited data on the effect of polyethylene glycol 300 (PEG300), the solubilizer under consideration by GSK for use as a universal solvent in pre-formulation studies at a concentration of 62 % w/w, on the respiratory mucosa. Vetter et al. [120] have recently reported PEG300 to cause only mild ciliotoxicity and cytotoxicity (using an LDH assay). Here, we aimed to look at the effect of these excipients on other components of MC (MTR and mucus secretion) as well as its cytotoxic effects using an MTT assay.

Viscosity enhancers, such as Methocel™ and Avicel®, are routine constituents of IN formulations and conventional means of extending IN residence times and thus of enhancing therapeutic efficacies. These compounds were established to slow CBF and MTR (see table 1.2 for available studies) although their effect on mucin secretion has yet to be known.

Table 1.2 Models Used to Study the Effects of IN Pharmaceuticals on Mucociliary Function

The MC Component (or Cell viability) / The Pharmaceutical	Mucin secretion	MTR	CBF	MTT
BKC	NA	• Rabbit Tracheae *in-vivo* [121], • Human *in-vivo* [92, 101-103, 117-119] • Rat nasal septum [122] • Frog palate [116]	• Rabbit Tracheae [121] • Chicken embryo tracheae [113, 114] • Chicken tracheae [115] • Human primaries (HNEC) [89, 93, 119, 123-125] • Cultured porcine tracheal mucosa [126] • Cultured rabbit tracheal epithelium [110]	• Corneoconjunctival cell lines [127-130] • Pimary Human nasal epithelial cells (HNEC) [131] • Human fibroblasts [132] • U937 cells [133]
PS	NA	• NA	• Human primaries (HNEC) [89] • Cultured rabbit tracheal epithelium [110]	• Human primaries (HNEC) [131]
EDTA	NA	• Human *in-vivo* [117, 118] • Frog palate [116]	• Chicken embryo tracheae [113, 114]	• Corneoconjunctival cell lines [130] • Chinese hamster fibroblasts V79 [134] • Murine macrophage culture [135]
FF	NA	NA	NA	NA
GSK1004723	NA	NA	NA	NA
Polysorbate 80	NA	NA	• Rat nasal mucosa[136] • Human nasal epithelial cells [137] • Chicken embryo trachea [138]	• Corneoconjunctival cell lines [127] • U937 cells [133] • Calu 3 cells [139] • Human umbilical vein endothelial cells [140, 141] • Human fibroblasts [132]
PG	NA	NA	• Human nasal primaries (HNEC) [120]	• Calu 3 cells [139]
PEG 300	NA	NA	• Human nasal primaries (HNEC) [120]	NA
Hydroxypropylmethyl cellulose (HPMC) polymer (e.g. Methocel™)	NA	• Human *in-vivo* [117, 142] • Frog palate [143, 144] • Bovine tracheal explants [145] • Toad palate [146] • Rat *in-vivo* [147]	• Chicken embryo tracheae [148]	• Calu 3 cells [139]
Sodium carboxymethylcellulose (NA-CMC) polymer (e.g. Avicel®)	NA	• Rabbit *in-vivo* [149] • Bovine tracheal explants [145] • Rat *in-vivo* [147]	• Human nasal primaries (HNEC) and rabbit *in-vivo* [150]	• Calu 3 cells [139]

NA means "Not Available" indicating that no references could be found.

1.4.3 The Study Approach

Ovine tracheal models were employed here for examining MC. Tracheae have generally been the most popular animal tissue for assessment of CBF [33] as they share the same features with the nasal and the bronchial respiratory epithelium while being more accessible [151], for instance ovine tracheae are regularly available as slaughtering by-product. Notably though, a recent study revealed inherent differences between nasal and tracheal murine MC [152]. Meanwhile, ovine mucociliary responses to pharmaceuticals were reported to be consistent with their human counterparts [153, 154] despite known differences in the respiratory pharmaco-physiology between the two species [155, 156].

Mucin secretion and CBF were measured in sheep tracheal epithelial explants (an *ex-vivo* model) cultured on collagen coated nitrocellulose permeable supports at an air-liquid interface, which maintained mucociliary differentiation. The nitrocellulose supports were collagen coated gel membranes that were first employed for culture purposes by Davis et al [157]. In the current study, they were prepared and validated for their permeability criteria as an evidence of their capability to maintain sufficient supply of nutrient to the explants. Meanwhile, mucin secretion investigations involved the quantitative analysis of timed collections of medium for their mucin content using an enzyme linked lectin-assay (ELLA) procedure [158]. Validation of the ELLA in the present study employed a respiratory mucin standard that was purified here from the sputum of a chronic obstructive pulmonary disease (COPD) patient using cesium chloride isopycnic density gradient ultracentrifugation [159].

Since MC is a mucosal physiological feature, the cytotoxic effect of the study formulations on respiratory epithelial cells such as 16HBE14o- was also studied in the present study to compile evidence on the formulations' collective effects on the respiratory mucosa.

Two FF formulations by GSK, namely a BKC-preserved formulation (Avamys®) and a BKC-free formulation (PS-preserved), were studied here. These formulations were similarly used by GSK to formulate the antihistamine API, GSK1004732. The excipients included in these formulations were PG, Avicel® (microcrystalline cellulose and Na-CMC), Methocel™ LV (Hypromellose; HPMC), BKC, PS, EDTA, polysorbate 80, citrate buffer (citric Acid anhydrous and Sodium Citrate dihydrate) and dextrose. These excipients were studied at their corresponding off-shelf IN concentrations, which were consistently equivalent to those used in the assigned GSK formulations.

Chapter 2

Effect of the Intranasal Formulation pH on Ovine Ciliary Beat Frequency (CBF)

2.1 Introduction

Cilia are tiny, though powerful, hair-like motile structures that line the respiratory mucosa to act as the motor of mucociliary clearance (MCC), beating in health at 7-16 Hz to propel the overlying mucus along with any trapped foreign particles [160-162]. These cylindrical cellular protrusions occur apically on the mucosal columnar epithelial cells with ~ 50-200 cilia/cell, each measuring ~ 7 µm length and 0.25-0.30 µm diameter, whose highly coordinated beating machinery employs the motor protein dynein that evokes the ciliary motility upon phosphorylation [160, 161].

The ciliary beat frequency (CBF) demonstrates a slow constitutive rate via basal ATPase activity while being controlled and modulated with a number of signalling mechanisms [160]. Various stimuli trigger cascades of second messengers including calcium (Ca^{2+}), cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which regulate the ciliary protein phosphorylation required to elicit ciliary motility [160, 161, 163]. A variety of stimuli have been shown to play roles in the regulation of ciliary activity including purinergic, peptidergic, cholinergic and adrenergic receptor stimulation, while mechanical, thermal, pH and chemical stimuli all elicited effects on CBF [160, 161, 163-165]. Importantly, it has been reported that a 16 % increase in CBF correlated with a 56 % increase in MCC in isolated whole trachea [160, 166].

This part of the project initially aimed to investigate the effect of the study pharmaceuticals on the ovine respiratory CBF. Nevertheless, owing to technical difficulties, the aim of this chapter settled for studying the effect of pH of the IN formulations on ovine CBF using sheep tracheal epithelial explants cultured on collagen coated nitrocellulose permeable supports at an air-liquid interface to maintain mucociliary differentiation and using high speed videomicroscopy to measure CBF. Intracellularly, alkalization was shown to enhance

CBF, which was conversely compromised by acidification [165]. Moreover, extracellular alkalization was also shown to be better tolerated by the airway ciliary activity than extracellular acidification [167-169].

2.2 Materials

2.2.1 Nitrocellulose Cell Supports

2.2.1.1 Preparation of Nitrocellulose Cell Supports

- Collodion: 4-8 % nitrocellulose solution in ≤ 45 % ethanol / ≥ 50 % diethyl ether - Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK (09986).
- Collodion flexible methylated: ~5.2 % nitrocellulose solution in 22-26 % ethanol / 60-70 % diethyl ether - Fisher Scientific UK Ltd, Loughborough, Leicestershire (C/7000/08).
- Ethanol - Fisher Scientific (E/0600DF/17).
- Diethyl ether - Fisher Scientific (D/2506/PB17).
- Ethylene Glycol - Fisher Scientific (E/0352/08).
- Petroleum Ether - Fisher Scientific (P/1760/15).
- 13 mm diameter circular coverslips, VWR International LTD, Lutterworth, Leicestershire, UK (631-0149).
- Purecol®, ultra-pure sterile bovine Type I Collagen - Nutacon BV, Leimuden, 2450 AB, The Netherlands (5409).
- Ham's F12 medium with L-glutamine - PAA: The Cell Culture Company, PAA Laboratories Ltd, Yeovil, Somerset, UK (E15-817).

2.2.1.2 The Nitrocellulose Cell Support Permeability Study

- 6-well microplates - Fisher Scientific (140675).

- Cylindrical plastic diffusion chambers (described in figure 2.1), supplied by Dr Michael Lethem.
- Fluorescein isothiocyanate dextran, relative molecular mass (RMM) 70,000, FD70 - Sigma (46945).
- 96-Well Plate, Black with clear base, EquiGlass. Non TC treated, individually wrapped - Genetix, Molecular Devices (UK) Limited, Wokingham, Berkshire, UK.
- Parker® Quink Bottled Ink Washable – available from retailers.
- 13 mm diameter circular coverslips - VWR International LTD (631-0149).
- Araldite® adhesive epoxy - Fisher Scientific (12715298)
- Multipurpose sealant, 732, clear, Dow Corning - Farnell Trade counter, Leeds, Yorkshire, UK (4010054).
- Hanks' Balanced Salt Solution (HBSS), without Ca^{2+} and Mg^{2+} and without phenol red - PAA: The Cell Culture Company (H15-09).

2.2.2 Ovine Airway Explants

2.2.2.1 Explant Culture Medium

- Dulbecco's Modified Essential Medium (DMEM) high glucose with sodium pyruvate and L-glutamine - PAA: The Cell Culture Company (E15-843).
- Minimum Essential Medium (MEM) with Earle's salts and L-glutamine - PAA: The Cell Culture Company (E15-825).
- Penicillin/Streptomycin (100 U/ml penicillin/100 µg/ml streptomycin) - PAA: The Cell Culture Company (P11-010).
- Trypsin/EDTA (0.05/0.02 % w/v in Dulbecco`s Phosphate Buffered Saline (DPBS)) - PAA: The Cell Culture Company (L11-004).

- HEPES buffer solution - PAA: The Cell Culture Company (S11-001).
- Foetal Bovine Serum "GOLD" EU approved - PAA: The Cell Culture Company (A15-151).
- Ham's F12 medium with L-glutamine - PAA: The Cell Culture Company (E15-817).
- Insulin bovine solution for cell culture (10 mg/ml ≡ 270 U/ml) - Sigma (10516-5ml).
- Hypurin® bovine neutral insulin for injection (3.42 mg/ml ≡ 100 U/ml) - Wockhardt UK Limited, Wrexham, Wrexham County Borough.
- Endothelial cell growth supplement (ECG) - Sigma (E2759).
- 3, 3', 5-Triiodo-L-thyronine sodium - Sigma (T6397).
- Apotransferrin human bioreagent - Sigma (T2036).
- Filter Steriflip Express plus 0.22 µm 50 ml - Fisher Scientific (FHD-355-110C).

2.2.2.2 Culturing Sheep Tracheal Epithelial Explants

- DL-Dithiothreitol - Sigma (D0632).
- Deoxyribonuclease I crude lypholized (Dnase) - Sigma (DN25).
- Collagenase/Dispase 0.1/0.8 - Roche Diagnostics Limited, Burgess Hill, West Sussex, UK (10269638).
- Cover slip type 2 borosilicate glass, 22 x 22 mm, 0.19-0.23 mm thick, Menzel® - Fisher Scientific (MNJ-450-020W).
- MEM liquid with Earle's salts and L-glutamine - PAA: The Cell Culture Company (E15-825).
- Dow Corning Sylgard® 170 silicone elastomer kit - Farnell international distribution centre, Leeds, Yorkshire, UK (101-693).
- 12-well plates, NunclonTM - distributed by Fisher Scientific (150628).
- 24-well plates, NunclonTM - distributed by Fisher Scientific (142475).

- Ham's F12 medium with L-glutamine - PAA: The Cell Culture Company (E15-817).
- High vacuum grease - Fisher Scientific (LG/8010/47970V).
- Cellulose nitrate filters, 5µm pore, Millipore® - Millipore (U.K.) Limited, Watford, Hertfordshire (SMWP04700).
- Industrial methylated spirit (IMS) - Fisher Scientific (11442874).
- Hypodermic needles, Microlance, 23G x 25 mm - Fisher Scientific (SZR-175-520U) and 23G x 30 mm - Fisher Scientific (SZR-175-525K).
- Nitrocellulose cell supports - prepared in-house, see section 2.2.1.1.

2.2.3 Measuring Ciliary Beat Frequency (CBF)

- Hanks' Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} without phenol red. PAA: the Cell Culture Company, H15-008.
- Anhydrous dextrose (D-Glucose), provided by GSK; GlaxoSmithKline, Stevenage, Hertfordshire, UK.

2.3 Methods

2.3.1 Nitrocellulose Cell Supports

2.3.1.1 Preparation of Nitrocellulose Cell Supports

Circular coverslips (13 mm) were washed in Petroleum Ether : Ethanol (1:1 v/v) to remove any grease, rinsed several times in ethanol, and dried. The coverslips were then placed on the platforms of an N_2 Chamber with N_2 running. The nitrocellulose solution was prepared by mixing the reagents listed in Table 2.2 in the order listed. Nitrocellulose solution (175.5 µl) was pipetted onto each coverslip prior to closing the chamber and leaving it with the N_2 flowing for 35-45 minutes When dry, each coverslip was transferred into a well of a 12-well

or 24-well plate and sterilised under a UV lamp with the lid off for 30-60 minutes The membranes were then washed in sterile PBS in a class II hood for 3 × 30 minutes ensuring the membranes were immersed to replace the ethylene glycol, which provides the gel structure of the membranes but has cell damaging effect. A pair of forceps sterilized in 70 % alcohol and shaken dry was used to gently immerse the membranes if they floated. The membranes were then coated with collagen by immersing in Purecol® 10 % v/v in sterile water for 20-30 minutes A minimum of 1 ml of Purecol® was used for each membrane if in a 12-well plate and a minimum of 0.5 ml if in a 24-well plate. The membranes were then stored in Ham's F12 medium in the incubator at 37 °C in 5 % CO_2 : 95 % air atmosphere to allow saturation of the membranes with medium and to reveal any contaminated membranes.

Table 2.1 Composition of the Nitrocellulose Membranes

Reagent	Order of Addition	Membrane-0	Membrane-I	Membrane-II
Ethylene Glycol	1	0.525 ml	0.500ml	0.375 ml
Ethanol	2	1.690 ml	1.000 ml	0.400 ml
Diethyl ether	3	1.035 ml	0.600 ml	1.250 ml
Collodion	4	0.250 ml	0.500 ml	0.470 ml

Membrane-0 had been employed successfully by members of our group in the past using the Fisher Scientific collodion (C/6980/08). However, this product had been discontinued by the time of this study. Collodion sourced from Sigma-Aldrich was used for Membranes -I and -II. The formulation of Membrane-I was developed by adjusting the alcohol: ether ratio while the formulation of Membrane-II was adapted from the method used by Davis et al. [157] who first used those membranes in 1992.

2.3.1.2 The Nitrocellulose Cell Support Permeability Study

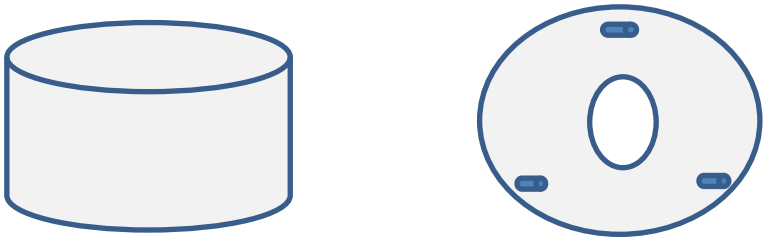
Figure 2.1 illustrates the cylindrical plastic diffusion chamber that was employed here. It had 8 mm internal height and 19 mm internal diameter, open from one end and has a central circular orifice of 4.5 mm diameter in its solid base. The base of each chamber was externally fitted with three corner-stands (made from PVC tubing, internal diameter 1 mm) to stand freely, while it acted as a donor chamber, inside a receptor chamber, which was the well of a 6-well microplate.

2.3.1.2.1 Optimisation Work

- *Adjustment of fluid level between the Donor and the Receptor Chambers*

This was achieved by adding 1 ml of water containing 50 µl of ink to the donor chamber (orifice shut with 13 mm coverslip fitted with silicone sealant) and then adding water to the receptor chamber in 1 ml aliquots (reduced to 100 µl aliquots when approaching the level of the meniscus in the donor chamber) till reaching equal levels in both chambers. The volume of fluid required in the receptor chamber was then recorded.

This was confirmed by employing another approach. 1 ml of water was added to the donor chamber and the meniscus level was marked on the outside of that chamber. Water was then added to the receptor chamber up to the level marked on the donor one.



(a) (b)

Figure 2.1 The Diffusion Chamber.

a) Lateral view. (b) The solid end of the chamber (external view) showing the orifice in the centre and the three PVC stands in the corners.

- *Validation of the Fluorescence Spectrophotometric Assay: Precision and Linearity*

The assay precision was optimised via the choice of the optimum sample size and the optimum settings for the fluorescence spectrophotometer (voltage and filter settings).

The fluorescence of different sample volumes (25 µl, 50 µl, 75 µl, 100 µl and 400 µl) was compared for precision using 0.1 µM FD70 (n = 6 for each volume). The relative standard deviation (RSD), which is the ratio of the standard deviation to the mean, was calculated for each sample volume to reflect the precision of the measurement.

The emission filter settings; Open and Auto options, and the photomultiplier tube (PMT) voltage that determined the detector sensitivity setting; 800 v, 700 v and 500 v options, were compared by measuring n = 6 of 0.1 µM FD70 at each filter-PMT setting then calculating the relative standard deviation (RSD) to reflect the precision of measurement at the settings employed.

The calibration curve of the FD70 concentration and its fluorescence reading was initially constructed using ten standard solutions in the range of 0.1 µM to 20 µM FD70 in HBSS. The concentrations had to be lowered subsequently to be in the range of 5 nM to 60 nM (Six standard solutions at 5 nM, 10 nM, 20 nM, 30 nM, 40 nM and 60 nM), which was found to be more representative of the samples' concentration range. Blank wells were filled with HBSS. Six replicate wells were assayed for each standard solution or blank. The assay precision was then reassessed at the low concentration level (5 nM standard solution). The fluorescence spectrophotometric analysis was conducted in black 96-well microplates using the fluorescence spectrophotometer, Varian Cary Eclipse, Oxford, Oxfordshire, at Ex. λ = 490 nm and Em. λ = 520 nm.

2.3.1.2.2 Measuring the Permeability of the Nitrocellulose Membranes

The permeability of the two types of nitrocellulose membrane (Membrane-I and Membrane-II) was investigated by measuring the diffusion of fluorescent dextran (FD70) across the membranes. The membranes were fitted across the orifice of a diffusion chamber (donor chamber) using silicone sealant. To fit each membrane across the chamber's orifice, a circular 13 mm coverslip was covered with the silicone sealant and pressed against the orifice of the diffusion chamber, then the coverslip was removed, excess sealant was wiped away and the nitrocellulose membrane was finally fitted to the chamber by attaching it to the orifice. HBSS (4.5 ml) was used to fill the receptor chamber (the well of a 6-well plate). The dosing solution (1 ml of 100 µM FD70 in HBSS) was added to the donor chamber that was then placed inside the receptor chamber. Samples (500 µl) were removed from the receptor chamber every 15 minutes for a period of 165 minutes Every sample taken was replaced by 500 µl HBSS. The content of FD70 in each sample was measured using fluorescence spectrophotometry as described in section 2.3.1.2.3.

2.3.1.2.3 Sample Analysis using a Fluorescence Spectrophotometric Assay

A serial dilution of standard FD70 solutions in the range of 5 nM to 60 nM (Six standard solutions at 5 nM, 10 nM, 20 nM, 30 nM, 40 nM and 60 nM) was prepared and calibration curve constructed every time a set of samples was assayed on the Varian Eclipse fluorescence spectrophotometer. Blank wells were filled with HBSS. Six replicate wells were assayed for each standard solution or blank. Following each of six permeability experiments run to study a nitrocellulose membrane type, twelve samples corresponding to twelve time points were analysed.

2.3.1.2.4 Data Analysis

The fluorescence of the samples was converted to concentration units using the regression equation of the calibration curve constructed using FD70 standard solutions that were

analysed at the same time as the samples. The concentration value for each time point was then adjusted to allow for the loss of FD70 in the 500 µl samples removed from the receptor chamber at previous time points. The mean adjusted concentrations from six independent experiments were plotted against time for each of the two membrane types assessed. The slope of the plot was subsequently used to calculate the flux across the membrane (nmoles/second). The apparent permeability coefficient (Papp, cm/s) of FD70 across each membrane type was then calculated by substituting into the equation below to demonstrate the permeability of each of the membranes [157].

$Papp = dQ/dt \,.\, 1/AC_o$

Where dQ/dt is the rate at which FD70 crossed the nitrocellulose support (nmoles/s), A is the area of the nitrocellulose support (0.225 cm^2) and C_o is the initial concentration of FD70 in the donor chamber (100 µM).

Simple linear regression (least squares method) was used to construct the linear relationship in all calibration curves. Regression equation (R^2) and p-values of the regression slope and intercept were statistically analysed using Microsoft Excel 2010, which employed a two-tailed t-test to evaluate the significance of fit of the linear model. $P \leq 0.05$ was considered statistically significant.

2.3.2 Ovine Airway Explants

2.3.2.1 Explant Culture Medium

The explant culture medium was prepared according to Davis et al [157]. It was prepared by mixing equal volumes of 3T3-conditioned medium and Ham's F12 medium. The mixture was buffered with HEPES (15 mM), spiked with 1 % antibiotics (i.e. 1 U/ml penicillin and 1 µg/ml streptomycin) plus five hormones, namely Insulin (10 µg/ml), ECG (7.5 µg/ml), Apo-

transferrin (5 µg/ml), hydrocortisone (36 ng/ml) and triiodothyronine (20 ng/ml), and finally filter sterilised.

The 3T3-conditioned medium was prepared by culturing 3T3-mouse-fibroblasts in DMEM containing 10 % FBS, 1 U/ml penicillin and 1 µg/ml streptomycin. At 50-70 % confluency, the medium was changed to DMEM containing 2 % FBS, 1 U/ml penicillin and 1 µg/ml streptomycin. This medium was left to maintain the cells for 3 days before it was harvested, filter sterilised and frozen down in 10 ml aliquots (-20 °C).

2.3.2.2 Culturing the Sheep Tracheal Epithelial Explants

The method was adapted from Davis et al [157]. Sheep tracheae were collected from a local abattoir during animal slaughter hours. Immediately after the trachea was removed from the animal, excess tissue was removed and a 7 cm length of the middle part of each trachea was transported to the laboratory in a sterile 50 ml centrifuge tube with 35 ml of sterile DMEM (or MEM) containing 2 % penicillin/streptomycin (i.e. 1 U/ml penicillin and 1 µg/ml streptomycin). In a class II hood, excess connective tissue was removed and each trachea was washed twice in fresh DMEM (or MEM). The posterior membrane was then removed and each trachea was cut between and across the cartilage rings to give a 3 cm square piece of tissue, which was opened up and fixed onto a Sylgard® elastomer solid base using sterile hypodermic needles. A sterile solution (3 ml) of collagenase (0.1 U/ml) / dispase (0.8 U/ml) in PBS (proteolytic enzymes) was then injected under the epithelium (into the lamina propria of the mucosa) alongside the cartilage rings. The tissue preparation was then covered in a freshly prepared sterile solution of MEM+ (0.1 mg/ml Dnase and 0.15 mg/ml dl-dithiothreitol in MEM or DMEM) and incubated at 37 ºC in a 5 % CO_2 : 95 % air atmosphere for 45 minute. After draining the MEM+ from the preparation, a no. 2 square coverslip was used to scrape the epithelium from the submucosal layer and float it in a dish of fresh MEM+. Using

a dissecting microscope and a sterile dissection kit in the class II hood, the sheets of epithelium were uncurled, inspected for ciliary activity, cut into ~ 5-10 mm fragments and floated out (cilia uppermost) onto a collagen-coated nitrocellulose membrane (see section 2.3.1.1) to form the explant. This was allowed to attach to the membrane by overnight incubation at 37 ºC in 5 % CO_2 : 95 % air atmosphere at an air-liquid interface, which was created by overlaying the explant-bearing membranes on to discs of cellulose nitrate filters (5 µm pore) on top of sterile stainless steel pedestals containing the explant culture medium (described above) and. The pedestals were routinely sterilised and mounted onto sterile 12-well microplates (Figure 2.2) using sterile silicone grease (a single pedestal sits in a single well) prior to preparing the explants. Ham's F12 medium was added to the wells around the pedestals to maintain the moisture level around the explants. The explants were usually checked and used experimentally after 18 hours.

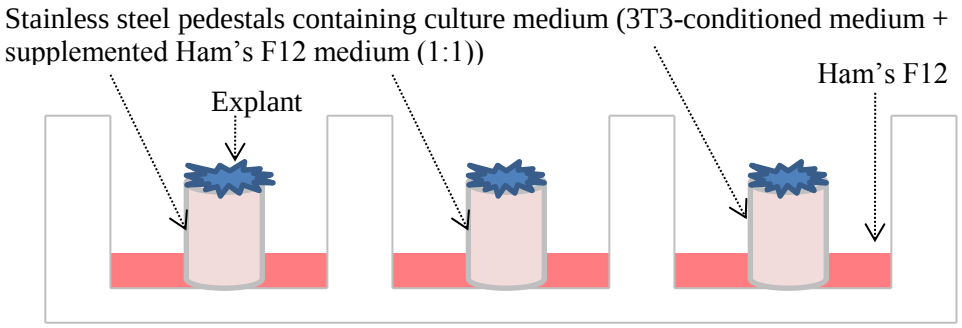


Figure 2.2 Schematic diagram of a 12-well plate (lateral view) with sheep tracheal explants cultured at an air-liquid interface.

2.3.3 Measuring Ciliary Beat Frequency (CBF)

CBF was measured using a system similar to that developed by Zhang and Sanderson in 2003 [170, 171]. The explant was observed using phase contrast microscopy at x40 magnification using a microscope mounted on an anti-vibration table. Phase contrast images were detected with a high-speed digital charged-coupled device (CCD) camera (Pulnix TM-6710) which was used with a frame grabber (Euresys Multi-Easygrab). Images were recorded and analysed using VideoSavant 3.0 software (All components were supplied by Multipix Imaging, Petersfield, Hampshire). In a typical experiment, images were recorded for 4.5 s once every two minutes at an average rate of 224 frames/s (1024 frames per recording). A region of interest (ROI), (5x5 pixels) was selected such that the variation in the grey scale data obtained from the ROI would have a high signal to noise ratio. The grey level intensity of the phase contrast image varied with the ciliary beat and by plotting the average grey-value in the ROI against time a wave form representative of CBF was obtained. Since the signal measured from the explant preparation often contained components from the beating of cilia from more than one cell, the dominant beat frequency from the signal obtained at each sampling point was calculated using a Fast Fourier Transform (FFT) analysis available in the software package Origin 6.0 (Microcal Software Inc., Northampton, MA, USA).

An experiment would routinely start by perfusing an explant with HBSS for 20-30 minutes (equilibration period) before recordings were made (once every 2 minutes) for 15 min, while continuing to perfuse the explant, to establish the baseline CBF. The solution was then exchanged to the test preparation that continuously perfused the explant for 15 minutes when recordings were made every 2 minutes to monitor the CBF changes. The explants were then re-perfused with HBSS for 15 minutes to assess the tissue recovery.

2.3.3.1 The Test Preparations

The intranasal formulations studied here (see section 4.2.4) had variable pH. In addition, 5 % w/w glucose solution was the vehicle of some of these formulations. Hence, it was decided to investigate the tolerability of ovine tracheal explants to pH changes in 5 % w/w glucose solution. CBF was therefore measured in the presence of 5 % w/w glucose solution at pH 4.78, 5.80, 6.26 (the pH of a 5 % w/w glucose solution in sterile water without any adjustments) and 7.16 was compared with CBF in presence of HBSS (pH 7.4). The Metler Toledo MP220 pH meter was used for solution pH adjustments.

2.3.3.2 Data Analysis

In each experiment, the baseline CBF values, measured during the tissue perfusion with HBSS prior to the exposure to a particular test solution, were pooled and the mean value was paired to the mean response CBF value, which was calculated using the pooled response CBF values measured during the tissue perfusion with that particular test solution. For each set of experiments investigating the effect of one particular test solution on CBF, the paired baseline and response values were compared using a Wilcoxon matched-pairs test, which statistically assessed the significance of variation. $P \leq 0.05$ was considered statistically significant. Notably, on occasions of studying two test solutions in the same experiment, i.e. using the same tissue as seen in each of the five experiments in figure 2.7 (b), the statistical analysis of each test solution was done independently of the other.

2.4 Results

2.4.1 Nitrocellulose Cell Supports

2.4.1.1 Preparation of Nitrocellulose Cell Supports

The nitrocellulose permeable supports have been used successfully at the University of Brighton for many years. However, it was found to be no longer possible to obtain nitrocellulose solution from the usual supplier (Fisher Scientific collodion product C/6980/08). The collodion obtained from other suppliers behaved differently and it was therefore necessary to develop a new formulation for the nitrocellulose supports and to test the permeability of the supports made.

A variety of membranes were prepared using slightly different formulations. Of these, two membrane types that differed slightly in their composition (Membrane-I and Membrane-II, see Table 2.1), were selected. These formulations gave membranes that were sufficiently rigid and robust to support the tracheal explants while remaining transparent (a requirement for the measurement of CBF).

2.4.1.2 The Nitrocellulose Cell Support Permeability Study

2.4.1.2.1 Validation of the Fluorescence Spectrophotometric Assay: Precision and Linearity

The acceptance criterion for the assay precision is 15 % RSD although 20 % RSD is widely acceptable near the lower limit of quantitation (LLOQ) [172-174]. The levels of precision achieved here were % RSD= 1.2 % at 0.1 µM FD70 and 8.9% near the LLOQ (5 nM FD70) using 400 µl sample volume with open emission filter and medium PMT voltage on the Varian Carry Eclipse Fluorimeter. The 25, 50, 75 and 100 µl sample volumes demonstrated out of range % RSD.

Analysis of the calibration curves indicated that a linear relationship existed between the concentration of FD70 (in the range of 5 nM to 60 nM) and fluorescence, an example is shown in Figure 2.3. A limit of quantification (LOQ) for FD70 of 0.36 nM was found. Calibration curves of FD70 in the range of 0.1 µM to 20 µM were observed to deviate from linearity.

2.4.1.2.2 Measuring the Permeability of the Nitrocellulose Membranes

The permeability of the membranes is important since they must allow the passage of nutrients from the culture medium to the underside of the explants. The permeability of the membranes was assessed by measuring the flux of FD70 across the membranes.

Figures 2.4 and 2.5 show the permeability of the two types of nitrocellulose membranes to FD70. It can be seen that there is very little difference in the rate at which FD70 crosses the two membranes. The calculated values for Papp were 1.41 x 10^{-6} cm/s for both membranes. This permeability was very close to that of Davis et al. (Papp = 1.89 x 10^{-6} cm/s) [157] and was therefore considered suitable for culturing the airway cells at an air-liquid interface. Membrane-I was chosen for the ovine tracheal explant preparation due to its superior handling qualities and the enhanced linearity of its permeability plot (see figure 2.4 and 2.5). It is worth noting that the deviation of the early time points in these plots from linearity was presumed to correspond to the initial period of equilibration between the interior of the nitrocellulose membranes and the FD70 [157].

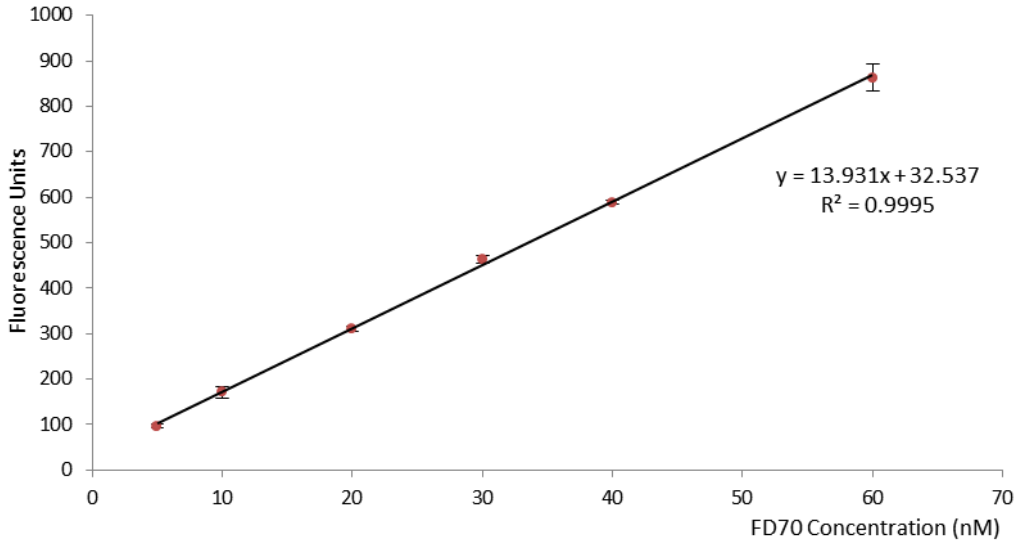


Figure 2.3 A Calibration Plot of Fluorescence against FD70 Concentration (mean ± SD, n=6)

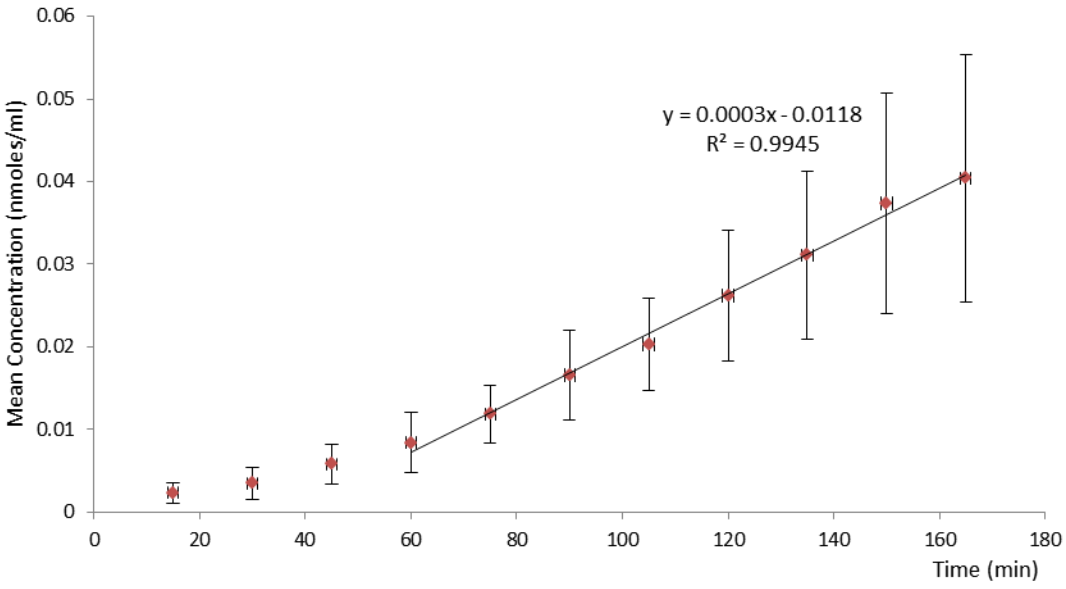


Figure 2.4 The Permeability of Membrane I to FD70 (Mean ± SD; n=6)

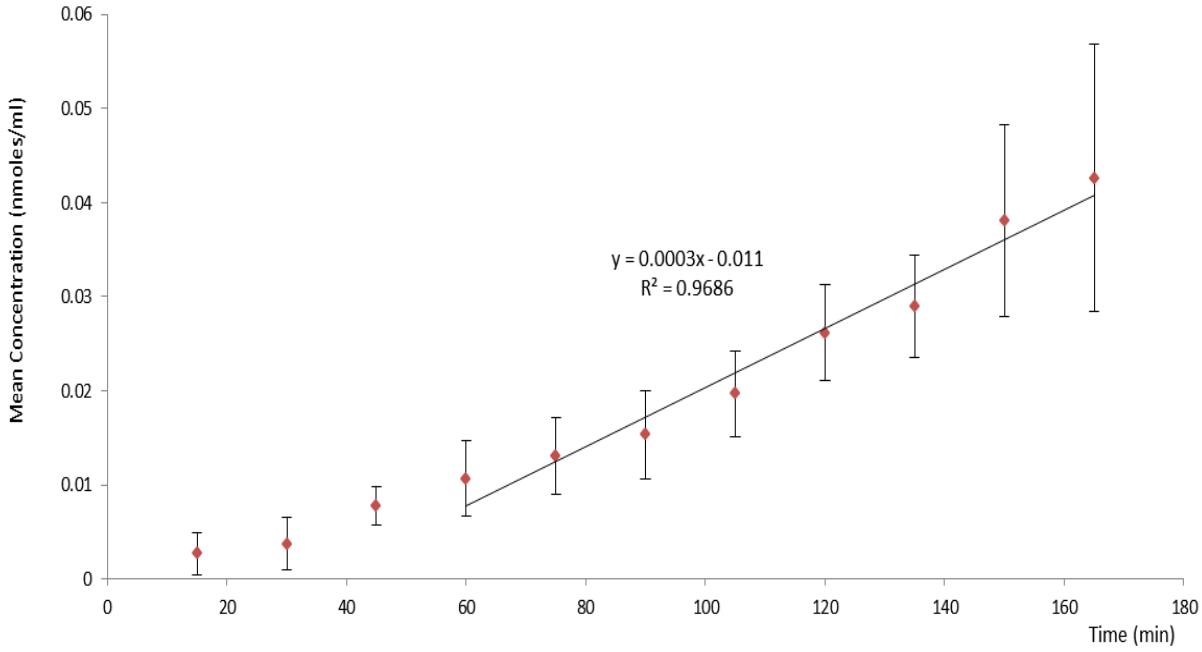


Figure 2.5 The Permeability of Membrane II to FD70 (Mean ± SD; n=6)

2.4.2 Measuring Ciliary Beat Frequency (CBF)

The CBF measurement scheme is summarised in figure 2.6 (a), whereas figure 2.6 (b) demonstrates an example of the grey scale wave form generated by the VideoSavant 3.0 software (the image analysis software).

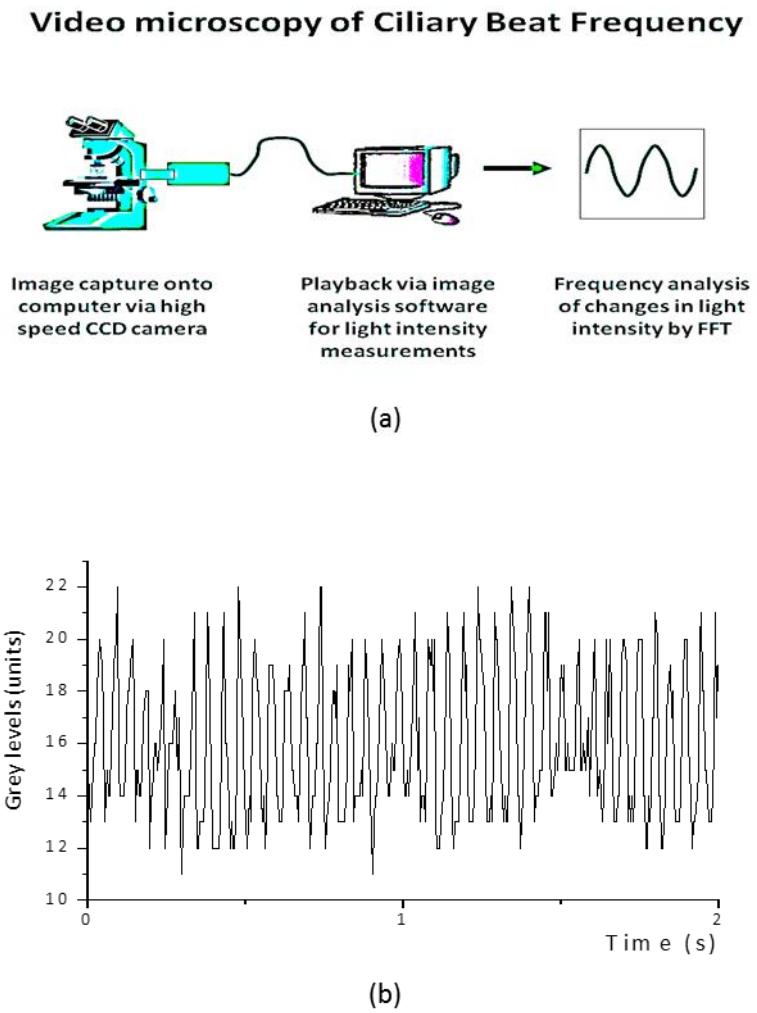


Figure 2.6 CBF Recording.

(a) CBF measurement scheme. Measuring CBF involved image capture via high-speed video microscopy followed by light intensity analysis and choice of ROI (a region with high signal to noise ratio) using VideoSavant 3.0 software, and finally frequency analysis of changes in light intensity by FFT using the Origin® analysis package. (b) An example of the grey scale signal obtained when measuring CBF using the VideoSavant 3.0 software. The waveform represents the variation in the grey intensity of the image at the ROI with respect to time and is caused by the movement of the cilia across the light source.

Many intranasal formulations employ 5.0 % w/w glucose solution as their vehicle. This solution was therefore used to study the ability of ovine CBF to tolerate changes in pH (pH 4.78, 5.8, 6.26 and 7.16), covering the pH range of off-shelf IN formulations [27] as well as the GSK formulations considered in this study (see section 4.2.4). No significant change in CBF was observed in the pH range 5.8 to 7.16. However, the preliminary results obtained at pH 4.78 indicated a potentially reversible decrease in CBF upon exposure to this pH. Figures 2.7, 2.8 and 2.9 show examples of the response of individual explant ROIs to those pH conditions (top plots), and it also summarises the overall response of a number of ROIs following exposure to such conditions (bottom plots). We thus demonstrate here that the 5 % w/w glucose solution has no effect on the ovine CBF over the pH range 5.8 - 7.16 and can therefore be used as a control solution in future work. Nevertheless, more experiments are required to establish the effect of pH 4.78 on ovine CBF.

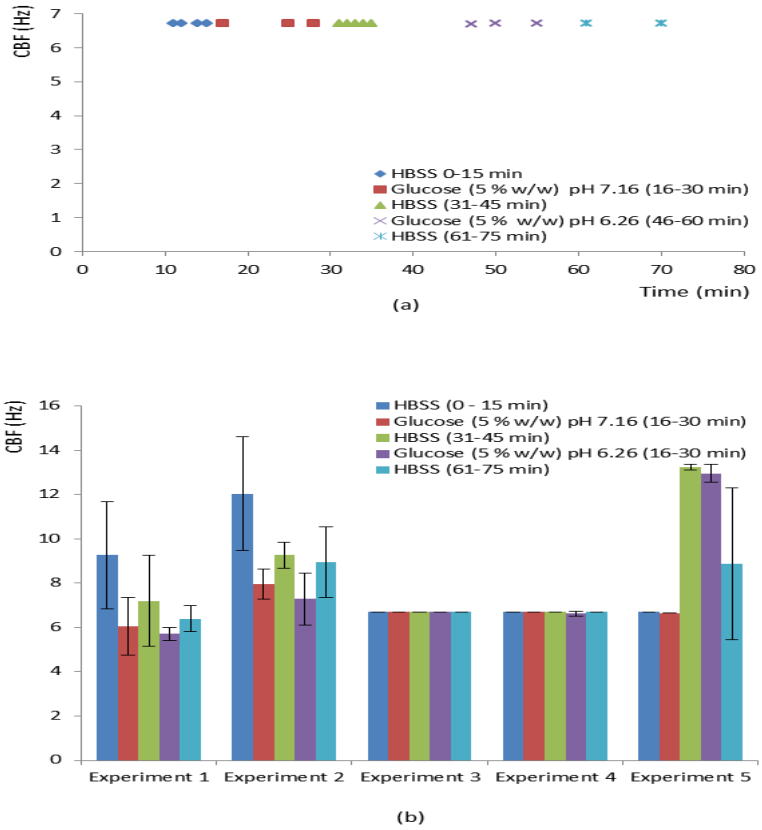


Figure 2.7 The Effect of 5 % w/w Glucose at pH 7.16 and pH 6.26 on Ovine CBF.

(a) An example of the effect of 5 % w/w glucose solution at pH 7.16 and pH 6.26 on CBF of an individual explant (Experiment 3). The first 15 minutes of the experiment gave baseline CBF recorded in the presence of HBSS. The following 15 minutes presents CBF in the presence of 5 % w/w glucose solution pH 7.16. Recordings while perfusing with HBSS were then conducted for another 15 minutes. This was followed by a further 15 minutes recording while perfusing with 5 % w/w glucose solution at pH 6.26 (the pH of 5 % w/w glucose in sterile water) and a final recovery period recording with HBSS perfusion. (b) CBF measured in five experiments (Tissue from three animals) in presence of HBSS and 5 % w/w glucose solutions (pH 7.16 and pH 6.26) each applied for 15 minutes. All values of CBF measured in each 15 minute period were pooled and the mean ± SD plotted. Statistical analysis revealed the 5 % w/w glucose test solutions to cause no significant alteration to CBF ($P > 0.05$).

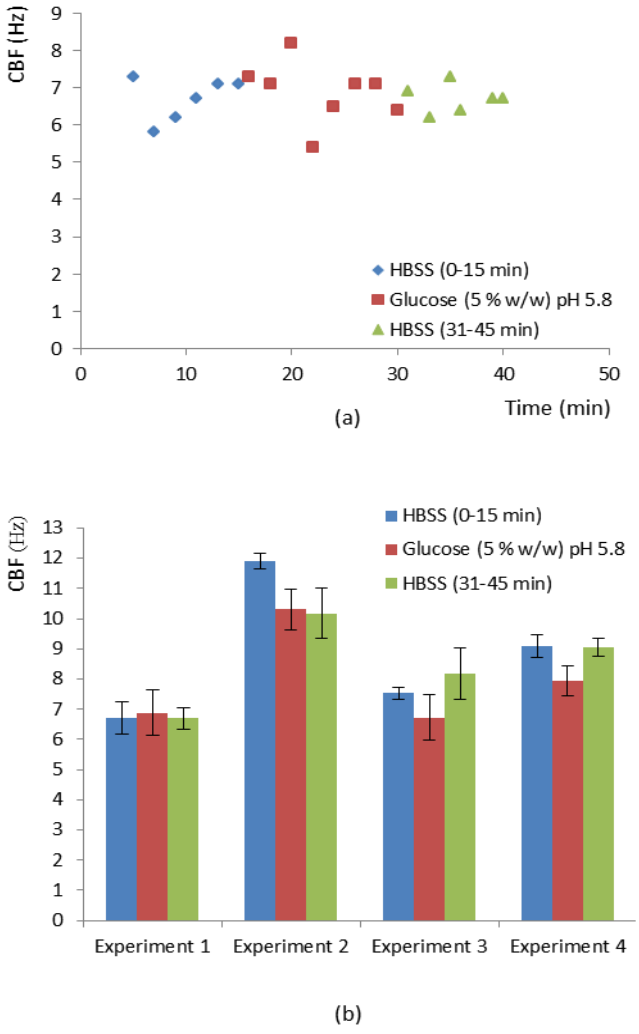
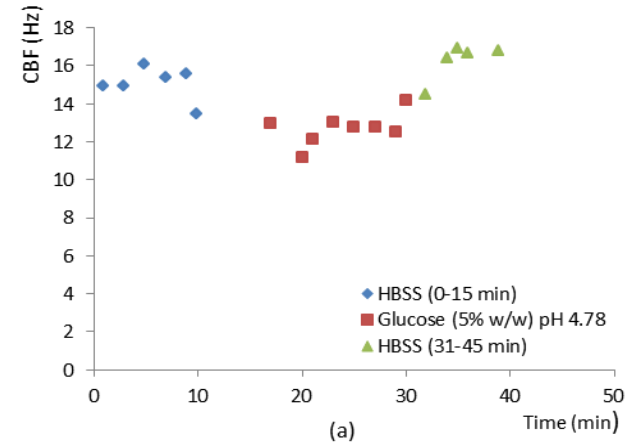


Figure 2.8 The Effect of 5 % w/w Glucose at pH 5.8 (pH of Avamys®) on Ovine CBF.

(a) An example of the effect of 5 % w/w glucose solution at pH 5.8 on CBF of an individual explant (Experiment 1). The first 15 minutes of the experiment gave baseline CBF recorded in the presence of HBSS. The following 15 minutes presents CBF in the presence of 5% w/w glucose solution pH 5.8. Recordings while perfusing with HBSS to look at tissue recovery was then conducted for another 15 minutes. (b) CBF measured in four experiments (tissues from two animals) in presence of HBSS and 5 % w/w glucose solution pH 5.8 each applied for 15 minutes. All the values of CBF measured in each 15 minute period were pooled and the mean ± SD plotted. Statistical analysis revealed the 5 % w/w glucose solution at pH 5.8 to cause no significant alteration to CBF ($P > 0.05$).



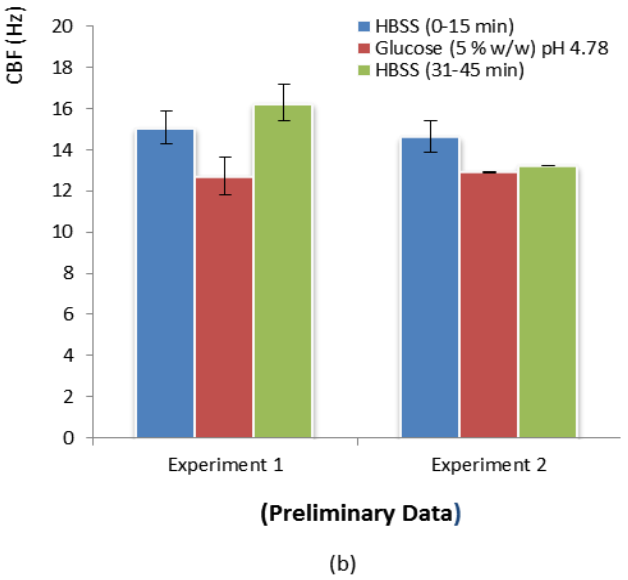


Figure 2.9 The Effect of 5 % w/w Glucose at pH 4.78 (pH of the GSK FF BKC-Free Formulation) on Ovine CBF.

(a) An example of the effect of 5 % w/w glucose solution at pH 4.78 on CBF of an individual explant (Experiment 1). The first 15 minutes of the experiment gave baseline CBF recorded in the presence of HBSS. The following 15 minutes presents CBF in the presence of 5 % w/w glucose pH 4.78. Recordings while perfusing with HBSS were then conducted for another 15 minutes. (b) CBF measured in two experiments (tissues fromone animal) in presence of HBSS and 5 % w/w glucose solution pH 4.78 each applied for 15 minutes. All the values of CBF measured in each 15 minute period were pooled and the mean ± SD plotted.

2.5 Discussion and Conclusion

This part of the project aimed to study the effect of pH of the IN formulations on ovine CBF, which required prior culturing of ovine respiratory tissue at an air-liquid interface to simulate the *in-vivo* environment necessary for physiologically functioning cells. The routine approach to establish air-liquid interface cultures usually involves culturing cells from suspension onto porous membranes (inserts or Transwells) [175-177], the procedure that is associated with loss of important physiological features (e.g. cilia and mucus seceretion) during the enzymatic digestion/dispersion of cells into suspension. These features are subsequently required to be restored (cell differentiation) prior to using such cultures in CBF or MCC studies. Owing to the lack of consensus on the optimal conditions for respiratory cell differentiation [175-180], this routine approach was not adopted here and an *ex-vivo* model was adopted instead. Ovine explants were prepared using nitrocellulose gel membranes as culture substrates, which proved economic, efficient and easy to manoeuvre (robust) during the explanting process. Moreover, they were transparent, which was necessary for measuring CBF by videomicroscopy. Nitrocellulose membranes were also recently reported valuable in continuous cell culture [181]. Nevertheless, they have compromised time-efficiency in this study as well as being quite labour-intensive. These membranes were prepared using collodion solutions, i.e. solutions of nitrocellulose in ethanol/ether mixtures. The ethanol/ether solvent content determines the physical properties of the membranes produced, such that more ether results in hard brittle membranes whereas more alcohol results in soft flexible ones [182]. Such variations in physical features were therefore likely to extend to membrane pore sizes and membrane permeability to water and nutrients [183]. Hence, the permeability of these membranes was validated here to ensure their competence in the preparation of ovine explants.

The permeability of the two nitrocellulose gel membranes described here (Papp of 1.41 x 10^{-6} cm/s) compared well with that reported by Davis et al. who used this type of support to culture canine epithelium [157]. They reported a Papp of 1.89 x 10^{-6} cm/s using bovine serum albumin (BSA), a globular protein with an RMM of 66,000 and a molecular radius of 3.62 nm. Notably, the permeability of the membranes here to FD70 (RMM = 70,000 and molecular radius = 6.4 nm) was slightly less, which could be explained by the larger molecules of FD70 compared to BSA. In agreement, Ambati et al. elucidated the molecular radius as a good permeability predictor across the eye sclera, which was more permeable to globular proteins than to linear dextrans [184].

In agreement with Clary-Meinsesz et al. who demonstrated human airway CBF to tolerate pH fluctuations in the range of 3.5 – 10.5 [168] without permanent impairment, it has been demonstrated here that the ovine tracheal CBF well tolerated pH 5.8 and 4.7 with the latter appearing to elicit a significant but reversible reduction in CBF. Nevertheless, although Clary-Meinsesz et al. as well as others showed CBF to decrease significantly in human bronchial epithelium at pH less than 6.5 [167-169], this was not evident here in ovine tracheae as the effect of pH 5.8 was not significant.

Technical issues with the high speed videomicroscopy system, used to measure CBF, precluded the progress of these experiments and interfered with fully achieving the aim of this chapter.

Chapter 3

Quantitation of Airway Mucins using Enzyme-Linked Lectin Assays (ELLAs)

3.1 Introduction

The effect of IN pharmaceuticals on airway mucin secretion was investigated (see chapter 4). However, a prerequisite for this investigation was a valid assay that could quantitatively measure the amount of mucins secreted from ovine tracheal explants in the presence of the pharmaceuticals of interest. A further prerequisite was a mucin standard to be used in the development, validation and calibration of the assay. A brief introduction to the preparation of purified mucin standards from crude sputum samples and methods to assay soluble mucins is presented below.

3.1.1 Mucins; Structure, Solubilisation and Purification

Mucins are the principal gel-forming structural components of the mucosal secretions that act as a protective diffusion barrier and lubricate all the wet-surface epithelia of the body. They are extremely large (0.25-20 MDa) heterogeneous (several mucin genes are expressed by the secretory cells) polymeric glycoproteins, the core of which is made up of tandem peptide repeats that are extensively O-glycosylated (the carbohydrate content is up to 90 % of the molecular weight) [185-188]. The sugar, N-acetylgalactosamine (GalNAc), links serine and threonine residues on the mucin central peptide backbone to various O-glycans that are often sulphated or sialylated with various sialic acids. Sialic acids are 9-carbon sugars (derivatives of neuramic acid), e.g. N-acetylneuramic acid (Neu5Ac) that is known as sialic acid, with extensive structural diversity and spatial occurrence at the non-reducing terminal of glycoconjugates [189, 190], which provide a means for the detection of mucins [187]. N-acetylglucosamine (GlcNAc), galactose, fucose and traces of mannose are other sugars occurring in mucins [191]. Interestingly, mucin glycoproteins, in addition to other proteins (including enzymes and antibodies), lipids, ions and nucleic acids, only constitute ~5 % of mucous secretions, which consist mainly of water (95%). Normal mucous secretions typically

present very little nucleic acid content, which becomes significant in sputa, the expectorated secretions in disease states, particularly purulent ones [185, 192, 193].

The structural characteristics of mucins (size, heterogeneity and gel-forming ability) result in their lack of solubility in physiological buffers, and hence the use of protein denaturants (chaotropic agents) such as guanidinium chloride 4-6 M (interestingly, guanidinium chloride appeared to stabilise, rather than unfold, native proteins at concentrations less than 0.3 M [194]), in presence of protease inhibitors such as phenyl-methyl-sulfonyl-fluoride (PMSF), is necessary to disperse mucins prior to their extraction from crude mucus or sputum samples, tissue samples or cell cultures [188, 195]. Chaotropic agents also serve in lysing any cells in the sample [196]. The conventional approach to mucin purification thereafter is a two-step cesium chloride (CsCl) isopycnic density-gradient centrifugation, in which proteins are mostly removed in the first step while the second step removes the nucleic acids [159, 195, 197]. More recent approaches have evolved (high performance gel-filtration and gel-electrophoresis/electroelution), which appear to be more time efficient than the routine CsCl procedure, however their use is yet to be widespread [195, 198-200]. The routine $CsCl_2$ procedure was used in this study to prepare a mucin standard from a crude human sputum sample.

3.1.2 Quantitative Analysis of Mucins

In the present study, the focus is on the quantitative determination of solubilised mucins rather than mucins in histological preparations, *in-situ* or *in-vivo*, which can be sought elsewhere [201-204]. Although much progress has been made in the structural analysis of various mucin gene products using various techniques such as mass spectroscopy [205-208], this is beyond the scope of this review that aims to describe methods of quantifying the overall mucin content in heterogeneous crude samples of mucous secretions.

3.1.2.1 Methods of Quantifying Mucins

Measurements of soluble mucins often involve one of three approaches, in-solution chemical assays, membrane-based (blotting) assays and ligand-binding assays (LBAs) such as immunoassays (radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs)) and enzyme-linked lectin assays (ELLAs). Based on the mucin detection principle, these approaches can be classified into chemical-stain-based, lectin-based and antibody-based. The chemical stains and lectins detect the mucin carbohydrate moieties while an antibody detects either a specific peptide sequence in the protein core of the mucins or a specific carbohydrate [187, 188, 197].

The in-solution assays are chemical-stain-based. They often rely on colour-producing reactions with the mucin sugars, such as sialic acids, which can be measured colorimetrically. Examples of these assays are the periodic acid-Schiff (PAS) and the periodate-resorcinol assays. This in-solution approach requires large sample volumes and is only seen as a semi-quantitative method due to interference of non-mucin glycoproteins with the assays. It therefore can't be used for unspecified (crude) samples, although it still provides a fast method for detection of mucin recovery during purification procedures [187, 188, 197].

The blotting approach (western blotting and slot blotting), is based on the detection of mucins retained on membrane filters, such as nitrocellulose and polyvinylidene fluoride (PVDF), using chemical stains, lectins or antibodies [187]. These techniques offer the opportunity to enhance the assay sensitivity by concentrating dilute samples (using large sample volumes) onto the membrane, to analyse a good number of samples and to filter-out interfering non-mucin components through the membrane. However, this approach can be associated with loss of mucin components that are not retained by the membrane, in addition to having a limited range of linearity, and hence it is considered to be a semi-quantitative method [187, 197].

LBAs are based on the binding of mucins, which act as antigens, to either antibodies (in ELISAs and RIAs), lectins (ELLAs) or both (in lectin-based sandwich immunoassays that employ an antibody and a lectin [199, 209]) often on a solid-phase, such as 96-well plates, beads and tubes, although they can be free in-solution. In direct LBAs, detection and quantification is achieved by labelling the lectins and antibodies (primary) with fluorophores, radioisotopes or enzymes that act on substrates to produce a measurable colour. In indirect LBAs, labelled secondary conjugates (e.g. streptavidin) or labelled secondary antibodies are employed to bind lectin-ligand conjugates (e.g. lectin-biotin) or primary antibodies respectively. LBAs are generally highly sensitive and selective quantitative methods of mucin analysis that can rapidly handle large numbers of samples, although they often require a great deal of optimisation of conditions [187].

3.1.2.2 Lectins

Lectins are carbohydrate-binding proteins that occur naturally in all living organisms and have particular sugar-specificities, through which they recognise and bind certain glycan structures [210, 211]. Despite their broad specificity and low affinity to carbohydrates, they are established probes in the analysis of glycoconjugates [211]. Mucin glycoproteins are sugar-rich glycoconjugates that can therefore be quantitatively analysed using lectins (Table 3.1), especially when measurements involve the quantification of various mucins (the global picture of mucin content) rather than a particular mucin gene product, and hence, the broad specificity is adequate [190, 211, 212].

Table 3.1 Lectins Used in Airway Mucin Determination.

Lectin (Abbreviation)	Source	Sugar specificity(s)
Dolichos biflorus agglutinin (DBA)	*Dolichos biflorus*	α-N-acetylgalactosamine (GalNAc)
Helix pomatia agglutinin (HPA)	*Helix pomatia*	α-GalNAc GalNac α (1-3)-GalNAc
Lotus tetragonolobus agglutinin (LTA)	*Lotus tetragonolobus*	α-Fucose
Peanut agglutinin (PNA)	*Arachis hypogaea*	β –Gal Gal β (1-3) GalNAc
Ricinus communis agglutinin type I (RCA I)	*Ricinus communis*	β -Gal β -GalNac
Soybean agglutinin (SBA)	*Glycine max*	α-GalNAc Galactose
Ulex europaeus agglutinin type I (UEA I)	*Ulex europaeus*	α-Fucose
Wheat germ agglutinin (WGA)	*Triticum vulgaris*	α-Neu5Ac β-GlcNAc

The listed lectins' source and specificity as well as their proven affinity to airway mucins were reported in the literature [211, 213-215]. These lectins were also reported in lectin-based assays developed and validated for the measurement of airway mucins [158, 180, 216-219]. WGA lectin demonstrates the broadest specificity as it can bind almost all glycoproteins [212, 220].

Rapid progress is being made in the area of defining the specificity of various lectins [221-225]and enhancing their affinity [226] using the glycan-array and lectin multimerization advances respectively. This could greatly enhance the role of lectins as biomedical probes enabling them to compete with antibodies.

3.1.2.3 Assay Validation

The aim of assay validation procedures is to establish a "fit-for-purpose" method [227]. The validation process often involves measuring some defined assay-performance-characteristics (validation parameters), which should comply with their widely recognised acceptance criteria (or acceptance limits) [228, 229].

Validation parameters are described by regulatory authorities, such as the US Food and Drug Administration (FDA), the American Association of Pharmaceutical Scientists (AAPS) and European Medicines Agency (EMA) [228, 230]. Moreover, international bodies, such as the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use), various conferences and workshops have also issued guidelines on the validation process [173, 174, 228]. The ICH aims at achieving a desirable harmonisation among analytical method validation standards in the USA, Europe, and Japan [231].

The validation parameters to be studied and their acceptance criteria are governed by the type and intended application of the assay. For example, the acceptance limit of the precision of a chemical method at low concentration levels is set to 20 %, compared to 25 % in case of a LBA. Moreover, the precision limit of a chemical method is 15 % if the method is intended for drug bioanalysis (the determination of drugs in biological samples) while it is only 5 % in drug pharmaceutical analysis (the quantitation of active ingredients in pharmaceutical products) [229]. In addition, in contrast to the non-linear calibration curves in LBAs, chemical

methods yield linear calibration curves, a difference that affects the required procedures and statistical analysis of the generated data [173, 232].

Quantitative assay validation parameters for biological methods (LBA such as immunoassays) are fully discussed elsewhere [172, 227, 228, 230, 233]. Validation parameters that were studied here, being relevant to the use of the ELLA in this project, are briefly described below.

1. Selectivity

Selectivity refers to the ability of the assay to differentiate the analyte(s) from components that might be present in the sample and interfere with the assay [172, 228]. It is often defined as "the ability of the bioanalytical method to measure unequivocally and differentiate the analyte(s) in the presence of components, which may be expected to be present. Typically, these might be metabolites, impurities, degradants, matrix components, etc." [173, 228].

Selectivity and specificity are often inaccurately used synonymously [172, 174, 228, 234]. Specificity is the absolute selectivity of the assay to discriminate the analyte from structurally similar compounds [172, 228]. It is not often a validation requirement for a LBA as long as the assay is fit for purpose.

2. Linearity (Calibration Curve)

Linearity is a measure of the degree of fit of a calibration curve; the graphical elucidation of the concentration-response relationship of the assay, into a straight line [172]. Since LBAs' concentration-response relationship is inherently non-linear (curvilinear or sigmoidal), this validation parameter should typically not be called linearity. Instead, it has been recently referred to as calibration curve or standard curve [227, 228, 230, 235]. In order to deduce an equation

describing this concentration-response relationship, linearization of the relationship has routinely been achieved using the log (response) versus log (concentration) data transformation. In case this linear data fitting model results in bias at the higher and/or the lower ends of the assay concentration range, the 4- and 5- parameters logistic functions are suggested to be more accurate than the conventional linearization approach [172, 227, 235].

The calibration curve is required to be established using a minimum of five to eight matrix-based standards, which are prepared in the same matrix as the intended real samples [174, 228].

3. Accuracy (Trueness) and Precision

Accuracy (systematic error or bias) is the degree of closeness between the value determined by assay and the true known value, while precision (random error or variability) is the degree of closeness between a series of measurements for replicate samples that were taken from the same homogenous sample [172, 173]. Three levels of precision are described, intra-assay (within-run or intra-batch) precision; which is often called repeatability and refers to precision under the same operating conditions and therefore it mostly assesses instrumental precision [236], inter-assay (between-run or inter-batch) precision; which can be called intermediate precision or again repeatability and refers to within-laboratory precision (different days, different analysis, different equipment, etc), and finally reproducibility, which refers to the between-laboratories variations [228, 231].

Accuracy and precision are assessed by analysis of replicate drug samples (2-5 replicates) of known concentration [172], at a minimum of three concentrations distributed over the full calibration range such that, one is near the limit of quantitation (LOQ), another near the middle of the range and a third near the upper limit of the range [173, 228].

The relative error (% RE); deviation of the mean calculated concentration (of the validation replicates at each concentration level) from the nominal true concentration expressed as percentage of the nominal concentration, is used to indicate accuracy of the obtained measurements, while precision of these measurements around their mean is expressed as relative standard deviation or coefficient of variation (% RSD or % CV) [173, 174, 227]

For LBA, the acceptance limit of accuracy and precision is ±20 % (±25 % at the lower limit of quantitation (LLOQ)) [227].

4. Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) or lower limit of quantitation (LLOQ) is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy [172, 228, 231].

5. Assay Range

The range of an analytical procedure is described as "the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity" [173, 231].

3.1.3 Aim of the chapter

Quantitative analytical procedures rely on the analysis of known dilutions of a reference standard to construct the calibration curves required for the assay development and validation and ultimately for sample analysis [227]. The early aim here was to prepare a mucin standard, by isolation of mucins from a human sputum sample, prior to the validation of an ELLA for

quantifying airway mucins in the presence of IN pharmaceuticals. This ELLA was initially aimed to be the one employed by Clancy et al. [158]. However, some of the studied intranasal excipients were found to interfere with the assay rendering it invalid for measuring mucins in samples containing these excipients. The analysis of such samples was essential in order to assess the effect of these excipients on airway mucin secretion (chapter 4). Thus, a second aim was to develop and validate a sandwich ELLA to achieve the quantitative determination of airway mucins in matrices containing these excipients.

3.2 Materials

3.2.1 Purification of Airway Mucin Standard from Human Sputum

- Guanidine Hydrochloride - Sigma (G4505).
- Ethylene diamine tetraacetic acid (EDTA), disodium salt dihydrate - Sigma (E4884).
- Disodium hydrogen orthophosphate - BDH/VWR (28026.260).
- Potassium dihydrogen phosphate - Sigma (P9791).
- Phenylmethylsulfonylfluoride (PMSF) - Sigma (P7626).
- Ultracentrifugation polyallomer "Quick-Seal" tubes (13.5ml) - Beckman Coulter, Beckman Coulter (UK) Ltd, High Wycombe, Buckinghamshire, UK (342413).
- Cesium chloride - Sigma (C4036).
- Dialysis tubing cellulose membrane flat - Sigma (D9527).
- Resorcinol SigmaUltra - Sigma (R5645).
- Copper (II) sulfate pentahydrate - Sigma (C8027).
- Hydrochloric acid concentrated, analytical grade - Fisher Scientific.
- Periodic acid - Sigma (210064).
- Bradford reagent - Sigma (B6916).
- Tertiary butanol - VWR (8.22264.1000).

3.2.2 Validation of a Direct and an Indirect Enzyme-Linked Lectin Assays (ELLAs) for the Quantitative Determination of Airway Mucins

- EIA/RIA 96-well plates, flat-bottomed high-binding certified plates, Costar - Fisher Scientific (DPS-110-080G3590).
- Phosphate buffered saline (PBS) tablets - Sigma (P4417).
- Gelatin type B from bovine skin - Sigma (G9382).
- Tween 20 (Polyoxyethylenesorbitan monolaurate) - Sigma (P1379).
- Lectin: Horseradish-Peroxidase-labelled *Helix Pomatia Agglutinin* (HRP-HPA) - Sigma (L6387).
- Lectin: Horseradish-Peroxidase-labelled *Helix Pomatia Agglutinin* (HRP-HPA) - EY Laboratories, San Mateo, CA, USA (300711-1), supplied by TCS Biosciences Ltd, Buckingham, Buckinghamshire, UK (Z8-H-3601-1).
- Lectin: *Helix Pomatia Agglutinin*–biotin conjugate (HPA-biotin) - Sigma (L6512).
- Streptavidin–peroxidase polymer, Ultrasensitive (streptavidin-HRP) - Sigma (S2438).
- *o*-Phenylenediamine dihydrochloride tablets - Sigma-Fast® - Sigma (P9187).
- Sulfuric acid - Fisher Scientific analytical grade (S/9160/PB17).
- Ham's F12 medium with L-glutamine - PAA: The Cell Culture Company (E15-817).
- Dimethyl sulfoxide (DMSO) - Sigma (D5879).
- The test formulations/excipients/APIs - provided by GSK (see section 4.2.4)

3.2.3 Validation of a Sandwich Enzyme-Linked Lectin Assay (ELLA) for the Quantitative Determination of Airway Mucins

- EIA/RIA 96-well plates, flat-bottomed high-binding certified plates, Costar - Fisher Scientific (DPS-110-080G3590).
- Phosphate buffered saline (PBS) tablets - Sigma (P4417).
- Sodium hydrogen carbonate - Fisher Scientific (S/4200/60).
- Sodium carbonate - Acros organics (207765000).
- Gelatin type B from bovine skin - Sigma (G9382).
- Tween 20 (Polyoxyethylenesorbitan monolaurate) - Sigma (P1379).
- Lectin (unlabelled): *Helix Pomatia Agglutinin* (HPA) - Sigma (L3382).
- Lectin: *Helix Pomatia Agglutinin*–biotin conjugate (HPA-biotin) - Sigma (L6512).
- Streptavidin–peroxidase polymer; Ultrasensitive (streptavidin-HRP) - Sigma (S2438).
- *o*-Phenylenediamine dihydrochloride tablets - Sigma-Fast®, Sigma (P9187).
- Sulfuric acid - Fisher Scientific analytical grade.
- Ham's F12 medium with L-glutamine - PAA: The Cell Culture Company (E15-817).
- The Test formulations/excipients/APIs - provided by GSK (see section 4.2.4).

3.3 Methods

3.3.1 Purification of Airway Mucin Standard from Human Sputum

Following the correct ethical procedure, fresh human sputum was obtained from a volunteer suffering from COPD. As a relatively small volume of sputum was required, a patient support group was approached to obtain the samples needed, which did not require the lengthy process of NHS ethical approval. Ethical approval was only needed from the School's Research Ethics Committee (the PABS REC), which was granted on December 22nd, 2009 (Ethics approval number 0905). A copy of the ethics application document, patient information sheet, consent form and the approval notification can be found in appendices 1 - 4 respectively. The Breath Easy patient support group was approached and their March 2010 meeting was attended when patient information sheets, consent forms and special containers for sputum collection were provided and discussed. It was indicated then that the sputum samples could be collected from the participant's home or from the next group meeting. In the April 2010 group meeting, the first sputum sample was collected, which was processed (solubilised in 6 M guanidinium chloride that caused lysis of any human cells) within 24 hours to comply with the Human Tissue Act requirements.

The airway mucin standard was prepared according to Carlstedt et al. [159] using CsCl isopycnic density gradient ultracentrifugation. Fresh human sputum weighing 41.9g was solubilised on the day of collection in 8 volumes (400 ml) of solubilisation buffer (10 mM phosphate buffer pH 6.5 containing 6 M guanidinium chloride, 5 mM disodium EDTA and 1 mM PMSF) by gentle stirring at 4 ºC for 24 hours. The solubilised sputum was then centrifuged at 10,000 x g at 4 ºC for 1 hour (Sorval® RC6 Plus floor centrifuge, Thermo Fisher Scientific Inc., Loughborough, Leicestershire, UK) to remove any insoluble material from solution. A volume of the solubilised sputum (113.3 ml) was diluted with 10 mM

phosphate buffer and CsCl to give a final concentration of 4 M guanidinium chloride and a density of 1.39 g/ml (the remaining volume of solubilised sputum was kept at -20 for future work). The equation below was used to calculate the amount of CsCl needed.

$\chi = v\ (1.347\rho - 0.0318M - 1.347)$

Where

χ = amount of cesium chloride in grams (g).

v = the required final volume in millilitres (ml).

ρ = the required density in g/ml.

M= the molarity of guanidinium chloride.

The solution was then loaded into twelve Beckman polyallomer "Quick-seal" tubes (13.5 ml), which were heat sealed and centrifuged (Beckman L8-55 ultracentrifuge, Beckman Coulter (UK) Ltd, High Wycombe, Buckinghamshire, UK) at 120,000 x g at 15 ºC for 72 hours. The tubes were then fractionated by piercing each tube with a needle from the top and bottom, and collecting 1 ml fractions from the hole at the bottom of each tube while controlling the flow manually by covering the hole in the top of the tube. Equivalent fractions from different tubes were combined. The density of each of the 13 fractions was measured by weighing a 100 µl aliquot of each fraction. The sialic acid, protein and deoxyribonucleic acid (DNA) contents of each fraction were measured using the periodate-resorcinol assay (section 3.3.1.1), Bradford assay (section 3.3.1.2) and the absorbance ratio 260/280 (section 3.3.1.3) respectively, as described below.

The fractions presenting a sialic acid positive peak were pooled for further purification. The pooled fractions were loaded into a 15 cm length of dialysis tubing, which had been boiled for 20 minutes, and dialysed against five changes of 10 mM phosphate buffer, pH 6.5, containing 0.2 M guanidinium chloride and 5 mM EDTA, at 4 ºC with gentle stirring for 12-

24 hours in each change. CsCl; calculated using the above equation, was then added to the dialysed fractions to a final density of 1.5 g/ml using 10 mM phosphate buffer, pH 6.5, containing 0.2 M guanidinium chloride and 5 mM EDTA as a diluent to adjust the volume. Beckman polyallomer "Quick-seal" tubes (13.5 ml) were then loaded with the solution, heat sealed and centrifuged (Beckman L8-55 ultracentrifuge) at 120,000 x g at 15 ºc for 72 hours. The tubes were then re-fractionated and fractions monitored for density, protein content, sialic acid content and DNA content as described above.

Once again, the fractions presenting a sialic acid positive peak were pooled and dialysed with gentle stirring at 4 ºC, on this occasion against four changes of previously boiled and cooled water for 12-24 hours for each change of water. This solution became the mucin standard, which was temporarily stored at 4 °C at this stage.

A sample (1 ml) of this mucin standard was dried, in the oven at 80 °C for 2 hours, and its dry weight was checked to determine its mucin concentration. Moreover, to establish the effect of freezing on the mucin integrity, an aliquot of the mucin standard was frozen down at -20 °C for 24 hours and then assayed for its mucin content using the ELLA described in section 3.3.2.1. The mucin content was then compared with that of the mucin standard that was temporarily stored at 4 ºC and with that of a previously prepared mucin standard that was obtained from a different donor and had been stored at 4 ºC in 0.02 % sodium azide solution.

After considering the data from the ELLA analysis, the mucin standard prepared above was aliquoted and frozen down for long term storage at -20 ºC.

3.3.1.1 The Periodate-Resorcinol Assay

The procedure developed by Jourdian et al [237] was adopted for the determination of the sialic acid content of each fraction following ultracentrifugation. A sample (250 µl) of each fraction (or 250 µl 10 mM phosphate buffer pH 6.5 for the blank) and 0.04 M periodic acid (50 µl)

were mixed and allowed to stand on ice for 20 minutes A volume of 625 µl resorcinol solution (54.5 ml reverse osmosis (RO) purified water, 45.5 ml of concentrated hydrochloric acid, 0.006 g of copper (II) sulfate pentahydrate and 0.6 g of resorcinol) was then added, mixed thoroughly and the mixture was allowed to stand on ice for another five minutes. The mixture was then heated to 100 ºC for 15 minutes (while contained in a microcentrifuge tube with the lid pierced for venting) using a Grant® QBT1 heat block (Grant Instruments (Cambridge) Ltd, Shepreth, Cambridgeshire, UK). Cold water was used afterwards to cool down the mixture, which was then mixed thoroughly with 95 % tertiary butanol (625 µl) to give a single phase. The mixture was then incubated at 37 ºC for 3 minutes using a water bath. Once the mixture had cooled down to room temperature, its absorbance (optical density (OD)) was measured at 630 nm using a Jenway 6300 spectrophotometer (Jenway Ltd., Dunmow, Essex, UK).

3.3.1.2 The Bradford Assay

The procedure developed by Bradford et al. [238] was adopted here for the estimation of the protein content of each fraction. Bradford reagent (950 µl) was mixed thoroughly with a volume (50 µl) of sample (or 10 mM phosphate buffer pH 6.5 for the blank) and allowed to stand for 5 minutes before reading the absorbance of the solution at 595 nm using an Eppendorf Biophotometer (Eppendorf UK Limited, Stevenage, Hertfordshire, UK).

3.3.1.3 The DNA Assay

Following ultracentrifugation, the DNA content of each fraction was estimated by measuring its absorbance at 260 nm (the nucleic acids' absorption maximum). The ratio of the fraction's absorbance at 260 nm to its absorbance at 280 nm (A260/280) was also measured to indicate the purity of the DNA in the DNA enriched fractions (1.8 to 1.9 indicates a highly purified DNA preparation [239]). A sample (120 µl) of each fraction (or of 10 mM phosphate buffer pH

6.5 for the blank) was loaded into a Uvette® cuvette and absorbance was read on an Eppendorf Biophotometer.

3.3.2 Validation of a Direct and an Indirect Enzyme-Linked Lectin Assays (ELLAs) for the Quantitative Determination of Airway Mucins

3.3.2.1 The ELLAs

The procedure developed by Clancy et al.[158] has been adapted here. Seven to eight doubling dilutions of the mucin standard, in the range of 0.39-50 ng/well, were made up in Ham's F12 medium. High binding 96-well plates were loaded with 100 µl/well of each dilution (the blank wells received Ham's F12 medium) in triplicate. The plates were then incubated either at 37 ºC for 90 minutes or alternatively overnight at 4 ºC then at 37 ºC for 45 minutes. After emptying the plates, each well was washed three times with 200 µl wash buffer (PBS containing 0.05 % w/v gelatin and 0.5 % v/v Tween 20, applied warm at 37 °C) and then loaded with 150 µl block buffer (0.1 % w/v gelatin in PBS when the Sigma HRP-HPA was used and 0.5 % w/v gelatin in PBS when either the EY HRP-HPA or the HPA-biotin was used) at 37 ºC. The plates were then incubated at 37 ºC for 1hour.

Following incubation the plates were emptied, each well was washed three times with the wash buffer as above and then loaded with 100 µl/well of HRP-HPA lectin (1.25 µg/ml in the appropriate block buffer). When the HRP-HPA lectins became no longer available, they were replaced with HPA-biotin lectin (100 µl/well of a 0.625 µg/ml solution) changing the ELLA from a direct to an indirect LBA procedure. The lectin-loaded plates were then incubated at 37 ºC for one hour. The incubation with HPA-biotin was followed by a three-time wash step as above, before loading the plates with streptavidin-HRP (100 µl/well of a 1.25 µg/ml

solution in the 0.5 % w/v gelatin in PBS block buffer) and incubating them at 37 ºC for 70 minutes.

After incubation with the HRP-HPA lectins or the streptavidin-HRP, the wells were washed five times as above. Colour development was then achieved by adding 150 µl/well of the substrate (*o*-Phenylenediamine dihydrochloride solution made up as per manufacturer's instructions) and allowing the plate to stand in the dark for 12 minutes (reaction-time if the Sigma HRP-HPA lectin was used in the previous step) or 3-4 minutes (reaction-time if the EY HRP-HPA lectin or the streptavidin-HRP was used in the previous step). The reaction was then stopped by adding 50 µl 20 % v/v sulfuric acid to each well. The absorbance of each well was then read at 492 nm on a Labsystems Multiskan Ascent microplate reader (MTX Lab Systems Inc., Vienna, Virginia, U.S.A).

3.3.2.2 Optimisation of the ELLAs

The optimisation of the ELLA initially involved the choice of an optimum lectin concentration. This was assessed by assaying seven to eight doubling dilutions of the mucin standard in triplicate or quadruplicate as described above, using various lectin concentrations. A calibration curve of absorbance against mucin concentration was then constructed for each of the lectin concentrations (1.25 µg/ml, 2.5 µg/ml, 3.75 µg/ml and 5 µg/ml for the Sigma HRP-HPA lectin, 1.25 µg/ml and 1.75 µg/ml for the EY HRP-HPA lectin, 0.625 µg/ml, 1.25 µg/ml, 2.5 µg/ml and 5 µg/ml for the HPA-biotin lectin). The lowest concentration of lectin that achieved good assay linearity and sensitivity (LOQ) was implemented in all work that followed. Similarly, different streptavidin-HRP concentrations were looked at (0.416 µg/ml, 0.625 µg/ml, 1.25 µg/ml and 2.5 µg/ml) to choose the optimum one for subsequent work.

Initial studies using the EY HRP-HPA lectin indicated that the block buffer used with the Sigma HRP-HPA lectin did not stop the non-specific binding of the lectin to the plate. A

calibration curve was constructed using different concentrations of gelatin block buffers and the one that showed the best assay sensitivity was used in all subsequent work.

The use of the EY HRP-HPA lectin also required optimisation of the reaction time as the original reaction time gave over-range absorbance readings. A calibration curve was constructed using a four minutes reaction time and another using an eleven minutes reaction time. The calibration curves were then compared.

3.3.2.3 Validation of ELLAs

The following assay validation parameters were established for the ELLAs as below.

3.3.2.3.1 Linearity (Calibration Curve)

A calibration curve was constructed using seven to eight doubling dilutions of the mucin standard as described in section 3.3.2.1. The non-linear relationship that characterises LBAs was linearized by plotting the log of the absorbance values against the log of the mucin concentrations. Simple linear regression (least squares method) was used to examine the linear relationship between the analyte concentration and response. The significance of fit of the linear model was evaluated by a two-tailed t-test [234] and $P \leq 0.05$ was considered statistically significant.

3.3.2.3.2 The Limit of Quantitation (LOQ)

The LOQ was estimated here as the concentration that produced a response ten times the standard deviation (SD) of the blank samples [228, 231].

3.3.2.3.3 The Assay Precision and Accuracy

Precision and accuracy of the ELLA were assessed by analysis of replicate mucin solutions of known concentration, at three concentrations distributed over the full calibration range [173]. A mucin standard containing 0.39 ng/well or 0.78 ng/well was analysed to represent the near

LOQ concentration, while a 20-25 ng/well mucin standard represented the middle of the range and a 50 ng/well mucin standard represented the upper limit of the range. Analysis was performed using three replicates at each concentration.

Precision of the obtained measurements (at each concentration) around their mean is expressed as relative standard deviation (RSD) [173, 174], which is the ratio of the standard deviation of measurements to its mean and is also known as "coefficient of variation (CV)".

Deviation of the mean concentration (calculated using the obtained measurements at each concentration level) from the nominal true known concentration is expressed as percentage of the nominal concentration to indicate accuracy [173, 174], which is known as the relative error (RE).

3.3.2.3.4 The Assay range

The assay was validated for mucin concentrations ranging from the LOQ concentration up to 50 ng/well.

3.3.2.3.5 Selectivity of the direct ELLA in the presence of IN Pharmaceutical Formulations and Excipients

To assess any interference with the assay, calibration curves constructed using dilutions of the mucin standard made up in Ham's F12 medium were compared to those constructed using standard dilutions made up in Ham's F12 medium that was spiked with the formulations/excipients/APIs (each independently). The excipients and FF were assessed at their working formulation concentrations (Table 3.2) while the FF IN formulations by GSK (Table 4.1) were examined at 10 % v/v and 90 % v/v dilutions in Ham's F12. The analysis was performed in triplicate and the two calibration curves to be compared were run on the same 96-well microplate, at the same time.

Table 3.2 The Pharmaceuticals Studied for their Compatibility with the ELLA.

The Excipient/ API	Concentration in Ham's F12
BKC	0.015 % w/w
EDTA	0.015 % w/w
Propylene glycol (PG)	1.5 % w/w
Potassium sorbate (PS)	0.3 % w/w (in the presence of 1.5 % w/w PG as a cosolvent)
Polysorbate 80	0.025 % w/w
Methocel^{TM} E50LV premium	1 % w/w (To achieve optimum dispersion, the polymer was initially wetted with water; one tenth of the total diluent volume, and agitated at 60-70 °C for ~ 1 hr using a magnetic stirrer before making up to the final volume in Ham's F12)
PEG300	62 % w/w
Citrate buffer	0.96 % w/w citric acid anhydrous plus 1.48 % w/w sodium citrate
Fluticasone furoate (FF)	0.0004 % w/w (~ 4 µg/ml).
DMSO	0.2 % w/w

3.3.3 Validation of a Sandwich Enzyme-Linked Lectin Assay (ELLA) for the Quantitative Determination of Airway Mucins

3.3.3.1 The Sandwich ELLA

This assay was adapted from the various lectin-based sandwich assays in the literature [209, 216-219, 240]. High binding 96-well plates were coated with unlabelled HPA lectin (0.5 µg/ml in PBS) by overnight incubation (100 µl/well) at 4 ºC followed by 45 minutes incubation at 37°C. Each well was then washed four times with 150 µl wash buffer (PBS containing 0.05 % w/v gelatin and 0.5 % v/v Tween 20, applied warm at 37 ºC), loaded with 150 µl block buffer (0.1 % w/v gelatin in PBS; at 37 ºC) and incubated at 37 ºC for 1 hour. Meanwhile, seven doubling dilutions of the mucin standard, in the range of 0.78-50 ng/well, were made up in Ham's F12 medium. After being blocked, the HPA-coated plates were washed four times as above and loaded (100 µl/well) with the mucin standard dilutions in triplicate (Ham's F12 medium in the blank wells). The plates were then incubated overnight at 4 ºC followed by 45 minutes incubation at 37 °C.

The incubation with the mucin standard dilutions was followed by washing each well four times with 150 µl wash buffer, then loading it with 100 µl HPA-biotin lectin (0.625 µg/ml in block buffer) before incubating the plate at 37 ºC for 1 hour. Each well was then washed four times with the wash buffer as above and loaded with 100 µl streptavidin-HRP (1.25 µg/ml in block buffer) before being incubated at 37 °C for 70 minutes

Finally, the wells were washed five times as above and loaded with 150 µl/well of the substrate (*o*-Phenylenediamine dihydrochloride solution made up as per manufacturer's instructions). The plate was allowed to stand in the dark for 3-4 minutes before the reaction was stopped by adding 50 µl/ well 20 % v/v sulfuric acid. The absorbance of each well was then read at 492 nm on a Labsystems Multiskan Ascent microplate reader.

3.3.3.2 Optimisation of the Sandwich ELLA

The initial coating of the high binding 96-well plate with the unlabelled-HPA lectin was optimised by comparing the assay performance using 50 mM carbonate coating buffer pH 9.6 to its performance using PBS as a coating buffer.

The use of 2 h incubation at 37 °C as an alternative to the overnight incubation at 4 ºC with its subsequent 45 minutes incubation at 37 °C was also examined.

Different block buffers (0.1 % w/v gelatin in PBS, 0.5 % w/v gelatin in PBS, 0.1 % v/v Tween 20 in PBS and 0.5 % v/v Tween 20 in PBS) were then examined to achieve the minimal background noise.

All optimisation work involved assaying seven mucin standard dilutions, in triplicate, as described above (section 3.3.3.1). A calibration curve of absorbance against mucin concentration was then constructed for each of the studied assay conditions.

3.3.3.3 Validation of the Sandwich ELLA

The same parameters described under section 3.3.2.3 were similarly studied here.

3.4 Results

3.4.1 Purification of the Mucin Standard from Human Sputum

Following the ultracentrifugation of the solubilised human sputum in 4 M guanidinium chloride, the Beckman polyallomer "Quick-seal" tubes demonstrated a yellowish fat-like top layer, a clear middle layer and a gel-like bottom layer. The fractions collected from bottom to top of the tubes (fraction one from the bottom and fraction 13 from the top) were analysed for their sialic acid, protein and DNA content as shown in Figure 3.1. It was noticed that the fat-like supernatant in fraction 13 resulted in a light-scattering effect, which falsely increased the absorbance readings of that fraction (data not shown).

The Bradford assay demonstrated that the proteins were enriched at the top of the gradient, where they start to appear in fraction 6 upwards between densities 1.29 g/ml and 1.22 g/ml. The periodate-resorcinol assay for sialic acid suggested that the mucins banded in the middle of the gradient between densities 1.37 g/ml and 1.29 g/ml (fractions 3, 4, 5 and 6).

The gelatinous bottom of the gradient was revealed to be a fairly pure layer of DNA as suggested by the high A260/A280 ratio of fractions 1-2 [239]. The DNA broadly banded up to fraction 4 (Figure 3.1) between densities of 1.40 g/ml and 1.31 g/ml.

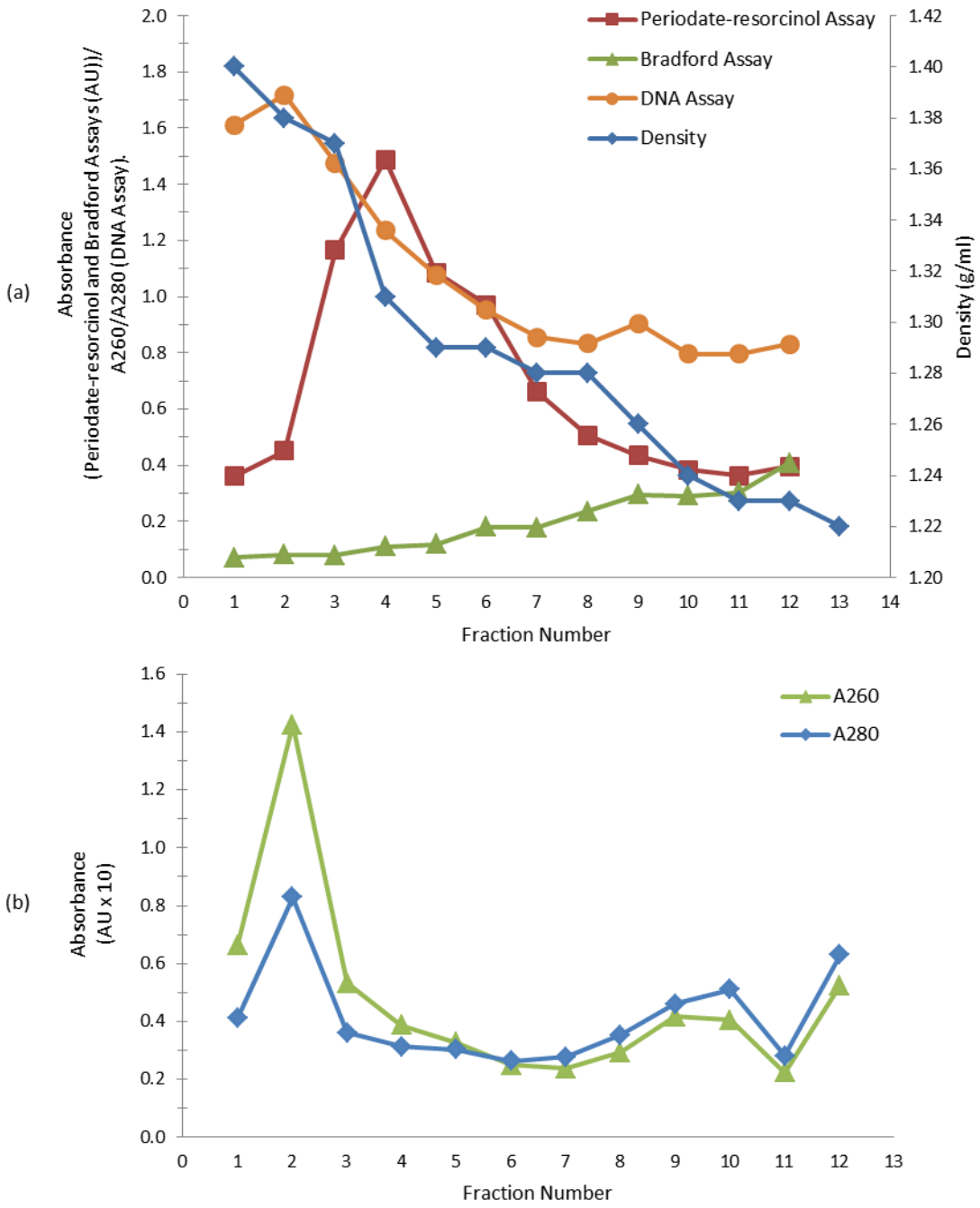


Figure 3.1 Cesium Chloride Isopycnic Density-Gradient Ultracentrifugation of Human Sputum in 4 M Guanidinium Chloride - Fractions Analysis

(a)The fractions collected from bottom to top following ultracentrifugation in 4 M guanidinium chloride were assayed for sialic acid using the periodate-resorcinol assay (absorbance measured at 630 nm), which demonstrated the mucin band. Bradford assay (absorbance measured at 595 nm) revealed the proteins band while the A260/A280 ratio indicated the DNA fractions. (b) The fractions' absorbance at 260 nm and 280 nm.

Emerging from the above data, the sialic acid positive fractions 3, 4 and 5 were pooled for dialysis and a second round of ultracentrifugation in 0.2 M guanidinium chloride (fraction 6 was excluded due to the relatively higher protein content indicated by the Bradford assay).

The second round of density gradient ultracentrifugation in 0.2 M guanidinium chloride (Figure 3.2a) achieved the separation of the remaining proteins at the top of the gradient (fractions 9 to 13 as demonstrated by the Bradford assay) at densities between 1.40 g/ml and 1.38 g/ml. The DNA was separated at the bottom of the gradient (almost entirely in fraction 1 as reflected by the dip in the absorbance of the other fractions at 260 nm and 280 nm (Figure 3.2b)) at densities between 1.48 g/ml and 1.57 g/ml. The bulk of the mucins were found in the middle of the gradient at densities between 1.40 g/ml and 1.48 g/ml (a much higher density than in the 4 M guanidinium chloride ultracentrifugation round). Fractions 5, 6, 7 and 8 thus appeared to contain the bulk of the mucins with neither proteins nor DNA contamination. They were hence pooled together, dialysed against sterile water and temporarily stored at 4 °C prior to being aliquoted (10 µl aliquots) and stored at -20 °C for long term storage.

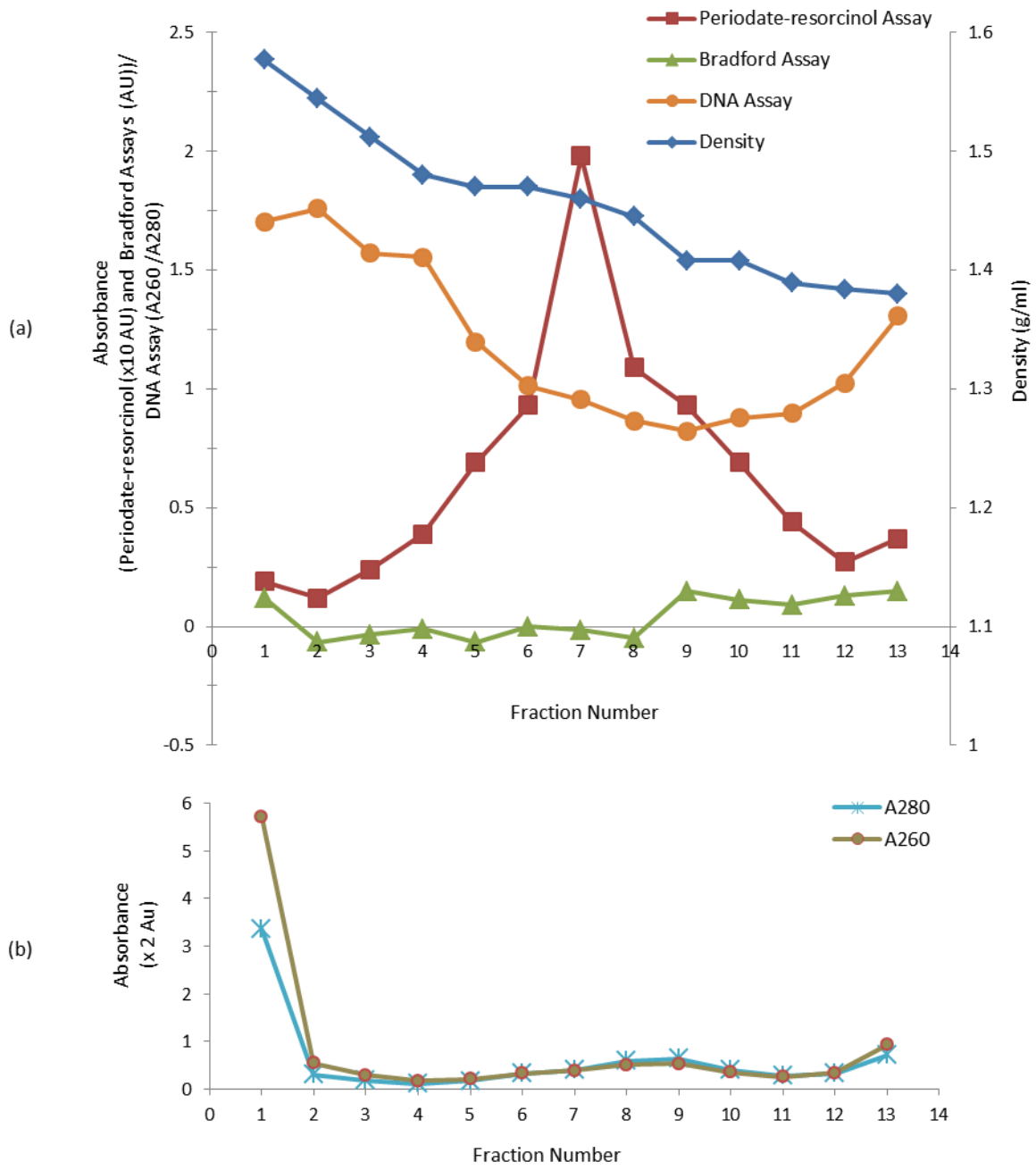


Figure 3.2 Cesium Chloride Isopycnic Density-Gradient Ultracentrifugation of Human Respiratory Mucins in 0.2 M Guanidinium Chloride - Fractions Analysis

(a) The fractions collected from bottom to top following ultracentrifugation in 0.2 M guanidinium chloride were assayed for sialic acid using the periodate-resorcinol assay (absorbance measured at 630 nm), which demonstrated the mucin band. Bradford assay (absorbance measured at 595 nm) showed the proteins band while the A260/A280 ratio indicated the mainly-DNA fractions. (b) The fractions' absorbance at 260 nm and 280 nm.

The dry weight of the purified human mucin standard revealed its concentration to be 3 mg/ml. Comparing the performance of that standard, in the assay, to the one previously prepared from a different donor and preserved in 0.02 % sodium azide at 4 °C, the behaviour was revealed to be very similar (Figure 3.3). The effect of freezing down was also looked at prior to aliquoting and storing the new mucin standard at -20 °C for long term storage. Despite observing a slight decrease in activity upon freezing (see figure 3.3), it was agreed to accept that subtle loss for the benefit of preserving the new standard for longer. The new mucin standard was therefore aliquoted (10 µl aliquots) and stored at -20 °C for use in all subsequent assay work.

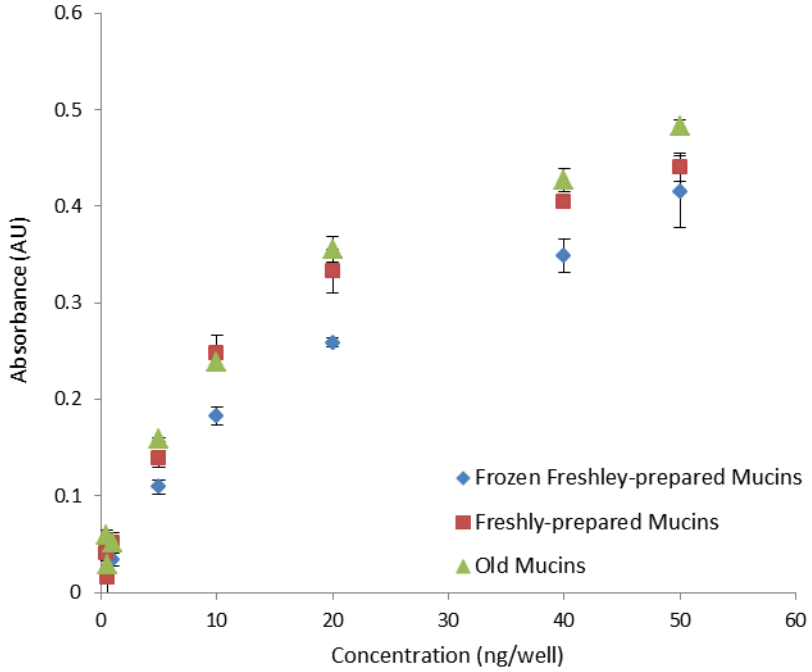


Figure 3.3 Effect of Storage Conditions on the Mucin Standard.

Three batches of purified human mucin standards (the freshly prepared batch, the frozen lot of the freshly prepared batch and the old batch from different donor) were compared by ELLA calibration curves using the Sigma HRP-HPA at the concentration of 3.75 µg/ml (mean ± SD, n = 3 - 4).

3.4.2 Validation of a Direct and an Indirect Enzyme-Linked Lectin Assays (ELLAs) for the Quantitative Determination of Airway Mucins

3.4.2.1 Optimisation of the ELLAs

An optimum working concentration of lectin in the ELLA was chosen to be 1.25 µg/ml as it was capable of demonstrating an acceptable calibration curve with no plateauing at the higher end of the concentration range and that showed linearity when plotted on a logarithmic scale (Figure 3.4).

Upon employing the EY HRP-HPA lectin instead of the Sigma one, the optimum concentration was also chosen to be 1.25 µg/ml as it still achieved the required linearity (Figure 3.5). Nevertheless, the gelatin concentration in the block buffer had to be increased to 0.5 % w/v, instead of 0.1 % w/v, to tackle the non-specific binding observed with this lectin (data not shown).

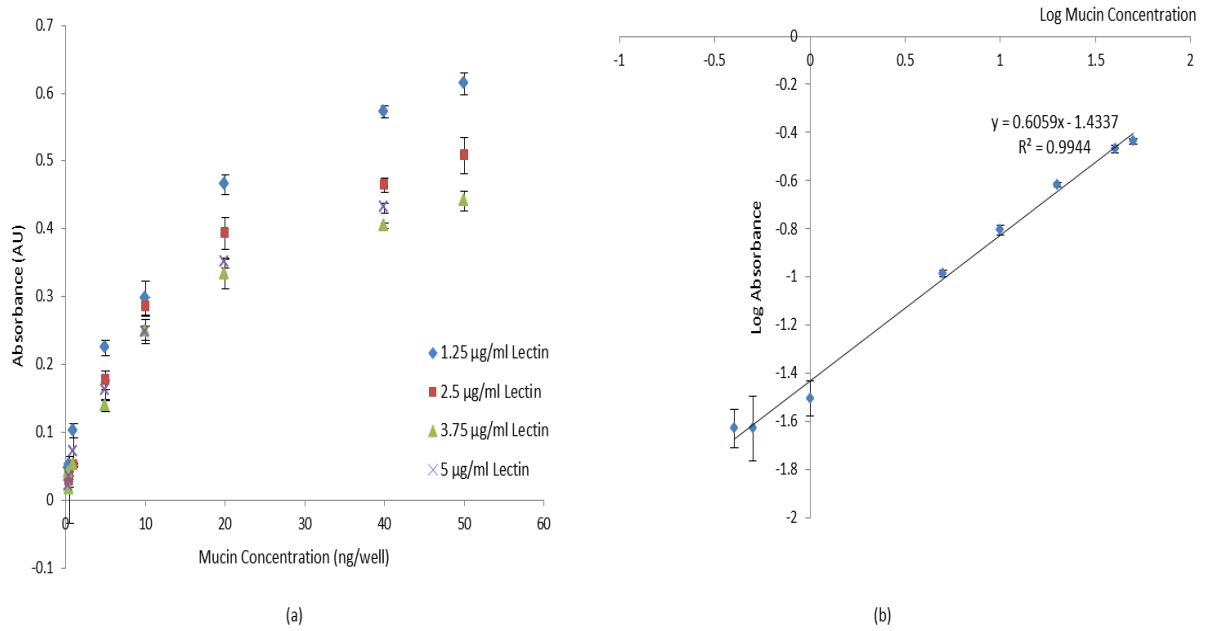


Figure 3.4 Effect of the Sigma HRP-HPA Lectin Concentration on Sensitivity of the ELLA (mean ± SD, n = 3 - 4).

(a) Four concentrations of the Sigma HRP-HPA lectin were compared using direct ELLA calibration curves. (b) A linear relationship was achieved by plotting log absorbance against log concentration (The 1.25 µg/ml lectin data is shown).

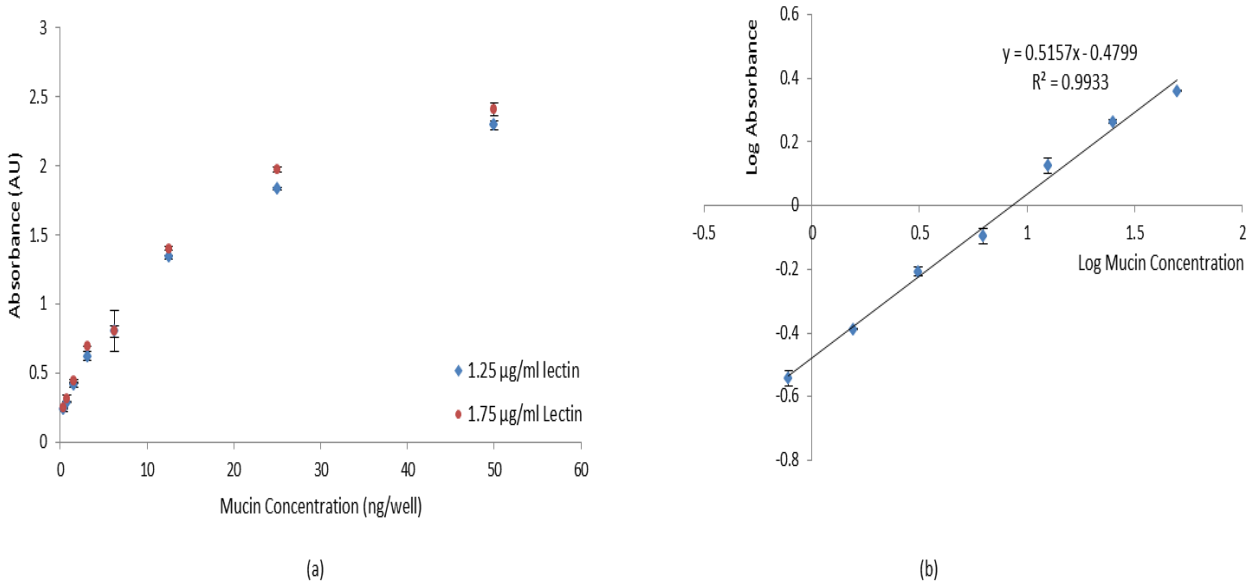


Figure 3.5 Effect of the EY HRP-HPA Lectin Concentration on Sensitivity of the ELLA (mean ± SD, n = 3).

(a) Two concentrations of the EY lectin were compared using direct ELLA calibration curves. (b) A linear relationship was achieved by plotting log absorbance against log concentration (The 1.25 µg/ml lectin data is shown).

Moreover, it was observed that the reaction time employed earlier with the Sigma HRP-HPA lectin (11 minutes) resulted in over-range absorbance readings with the EY lectin, which was demonstrated as a plateau at the higher end of the concentration range. In agreement, the plateau disappeared upon decreasing the reaction time to 4 minutes (Figure 3.6). The use of the two lectins at the same concentration indicated similar lectin activity while the different reaction time indicated higher peroxidase activity of the EY lectin. This was confirmed by the manufacturers who revealed the peroxidase activity to be 286 U/mg in case of the EY lectin and 83 U/mg in case of the Sigma lectin.

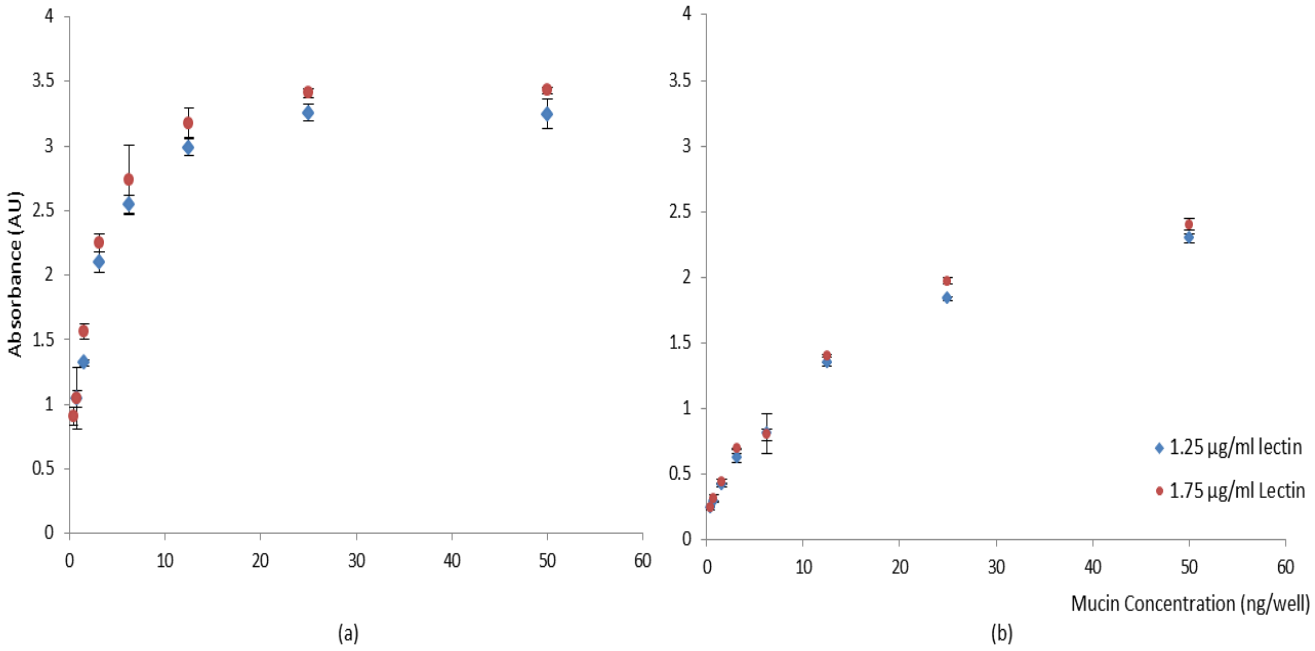


Figure 3.6 Effect of Reaction Time on Calibration Curve of the ELLA using the EY HRP-HPA Lectin (mean ± SD, n = 3).

(a) 11 minutes, (b) 4 minutes.

During the course of the study, the HRP-HPA lectins were superseded by a HPA-biotin lectin, resulting in the development of an indirect ELLA procedure. The optimum concentration of HPA-biotin lectin for this procedure was identified as 0.625 µg/ml (the lowest concentration that achieved acceptable assay sensitivity (Figure 3.7)).

Detection of the biotinylated lectin was achieved using a streptavidin-HRP conjugate, the concentration chosen to be used of which was 1.25 µg/ml (the lowest concentration that achieved acceptable assay sensitivity (Figure 3.8)) even though 2.5 µg/ml streptavidin-HRP demonstrated slightly better assay sensitivity.

The use of different block buffers (0.1 % w/v gelatin, 0.5 % w/v gelatin, 0.1 % v/v Tween 20 and 0.5 % v/v Tween 20) was also investigated with the indirect ELLA, however they were all found to behave similarly with the HPA-biotin/streptavidin-HRP detection system (Figure 3.9). A four-minute colour development reaction time was found to be optimum.

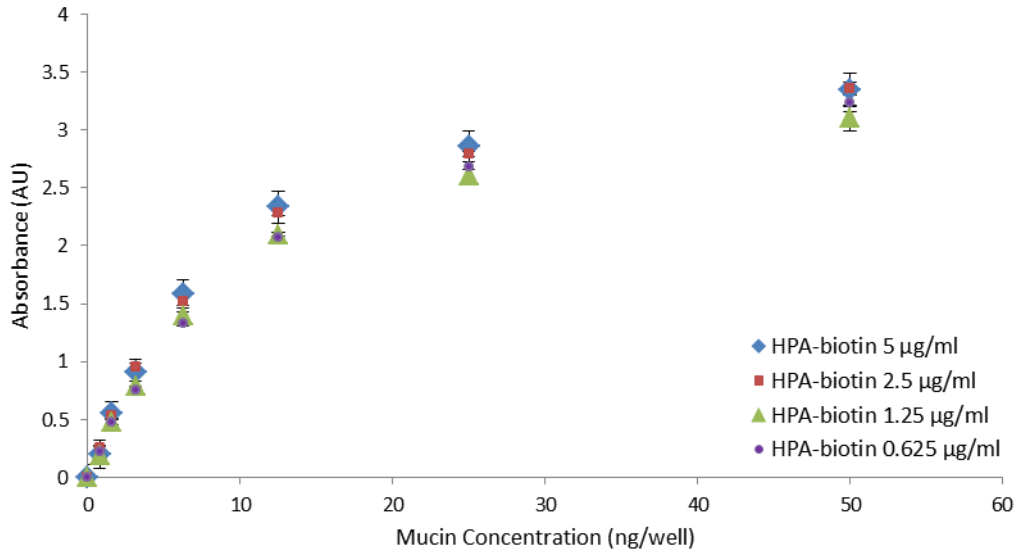


Figure 3.7 Effect of the HPA-biotin Lectin Concentration on Sensitivity of the ELLA (mean ± SD, n = 3).

Four different concentrations of the HPA-biotin were compared using indirect ELLA calibration curves with streptavidin-HRP conjugate employed at 2.5 µg/ml.

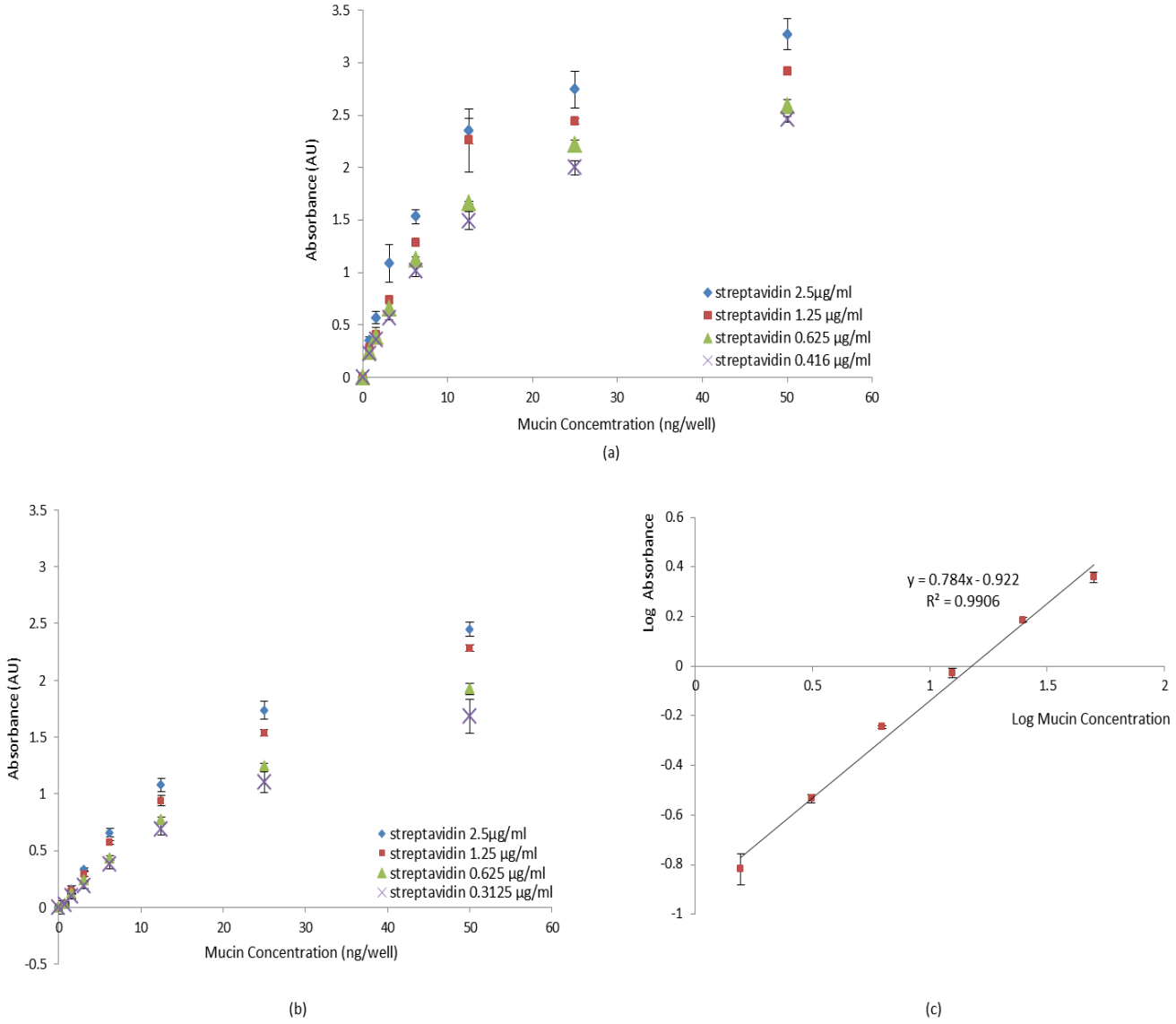


Figure 3.8 Effect of the Streptavidin-HPA Concentration on Sensitivity of the ELLA (mean ± SD, n=3).

(a) Various streptavidin-HRP concentrations, within the working dilution range recommended by the manufacturer, were compared using indirect ELLA calibration curves with 1.25 µg/ml HPA-biotin lectin. (b) The comparison was repeated using 0.625 µg/ml HPA-biotin lectin revealing the same results as in (a). (c) The streptavidin 1.25 µg/ml data in (b) was linearized.

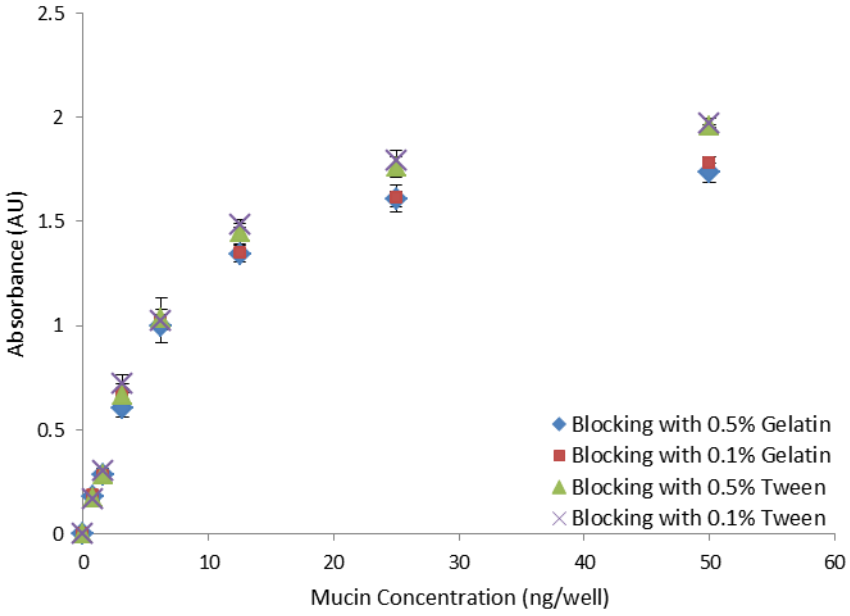


Figure 3.9 Effect of Different Block Buffers on Sensitivity of the ELLA (mean ± SD, n=3).

3.4.2.2 Validation of the ELLAs

3.4.2.2.1 Linearity (calibration Model)

As shown above, the ELLA calibration curves (Figures 3.3 to 3.9) demonstrated non-linear relationships similar to those characterising immunoassays. Those calibration curves were therefore linearized by plotting log of the absorbance against log of the concentration (Figures 3.4 and 3.5). Regression analysis revealed that more than 99 % of the reported variation in response was attributed to the corresponding variation in mucin concentration as R^2= 0.9944 using the Sigma HRP-HPA lectin (Figure 3.4), R^2= 0.9933 using the EY HRP-HPA (Figure 3.5) and R^2= 0.9906 using the biotin-streptavidin system (Figure 3.8 (c)). The P-value of the regressions is less than 1×10^{-4} using the HRP-HPA lectins and is 1×10^{-4} using the biotin-streptavidin system, which suggests a significant relationship between mucin concentration and absorbance.

3.4.2.2.2 The Limit of Quantitation (LOQ)

The LOQ was less than 0.6 ng/well when using the Sigma HRP-HPA lectin, 0.4 ng/well when using the EY HRP-HPA lectin and 0.2 ng/well when using the biotin-streptavidin detection system.

3.4.2.2.3 The Assay Accuracy and Precision

The assay % RSD was less than 3 % at the top of the mucin concentration range (50 ng/well), less than 5 % at the middle of the concentration range (20-25 ng/well) and 9.5 % or less near the LOQ (Table 3.3). Moreover, the assay has shown a % RE of less than 21.5 % near the LOQ and less than 15 % in the middle and the higher end of the concentration range.

The recommended-acceptance-range of accuracy and precision of immunoassays is ±25 % at the LOQ, and ±20 % elsewhere. The criteria demonstrated here are thus consistent with the acceptable assay criteria of immunoassays [172, 232].

Table 3.3 The Intraday Precision of the ELLAs

Mucin Concentration (ng/well)	**Absorbance minus blank (AU)**			**%RSD (%)**
	Replicate 1	*Replicate 2*	*Replicate 3*	
0.4	0.032	0.027	0.026	**9.5**
20	0.237	0.237	0.241	**2.7**
50	0.359	0.361	0.378	**2.8**
0.39	0.251	0.218	0.23	**5.85**
25	1.845	1.823	2.031	**4.9**
50	2.332	2.295	2.252	**1.4**
0.78	0.142	0.132	0.129	**4.13**
25	1.633	1.675	1.608	**1.68**
50	2.357	2.413	2.283	**2.2**

Precision of the ELLA was studied for each of the three different lectins/detection systems used in this project:

Sigma HRP-HPA EY HRP-HPA Biotin/streptavidin system

3.4.2.2.4 The Assay range

Previous work in the department on sheep tracheal explants showed mucin secretion of up to 30 ng/well. Thus, the assay range of ≤ 0.6 ng/well (LOQ) up to 50 ng/well is fit for the assay purpose.

3.4.2.2.5 Selectivity of the ELLA to Airway Mucins in the presence of Intranasal Formulations and Excipients

The assay was found incapable of detecting mucins in matrices containing many of the project's intranasal pharmaceuticals. Table 3.4 lists the formulations, drugs and excipients studied for their compatibility with ELLA and the results obtained, while figures 3.10 and 3.11 demonstrate these results by comparing calibration curves constructed in Ham's F12 to those constructed in Ham's F12 containing these pharmaceuticals.

Table 3.4 Compatibility of the ELLA with Some IN Pharmaceuticals

Formulation/ Drug/ Excipient	Compatibility with the ELLA
♦ Avamys® *. ♦ Avamys® placebo*. ♦ Fluticasone furoate (FF) BKC-free formulation*. ♦ Placebo BKC-free formulation*. ♦ Methocel (0.5 % w/w in Ham's F12) ♦ PEG 300 (62 % w/w in Ham's F12) ♦ Polysorbate 80 (0.025 % w/w in Ham's F12)	Complete assay blockage (zero absorbance)
♦ Citrate buffer (0.96 % w/w citric acid anhydrous plus 1.48 % w/w sodium citrate in Ham's F12) ♦ Polysorbate 80 † (10 mg/L = 0.001 % w/v) i.e less than its critical micelle concentration (CMC)).	Almost complete assay blockage
♦ Potassium sorbate (0.3 % w/w in Ham's F12 in the presence of 1.5 % propylene glycol (PG)) ♦ EDTA (0.015 % w/w)	Partial assay blockage
♦ BKC (0.03 % w/w in Ham's F12) ♦ PG (1.5 % w/w in Ham's F12) ♦ FF (4 µg/ml in 0.2 % w/w DMSO in Ham's F12) ♦ DMSO (0.2 % in Ham's F12)	Assay compatible (full recovery of the mucins in the calibration standards prepared in these matrices as compared to those prepared in Ham's F12 only).

* See table 4.1 for the composition of the listed formulations.

† The polysorbate 80 is a surfactant and a common excipient in all GSK's intranasal preparation studied in this project. Further to the full assay blockage observed with polysorbate 80, it was hypothesised that the mucins might be trapped in the micelles formed by this surfactant. We therefore examined its interaction with the ELLA at a concentration (10 mg/L) that is less than its critical micelle concentration (CMC = 13-15 mg/L) but there was only a little improvement in the recovery of mucins.

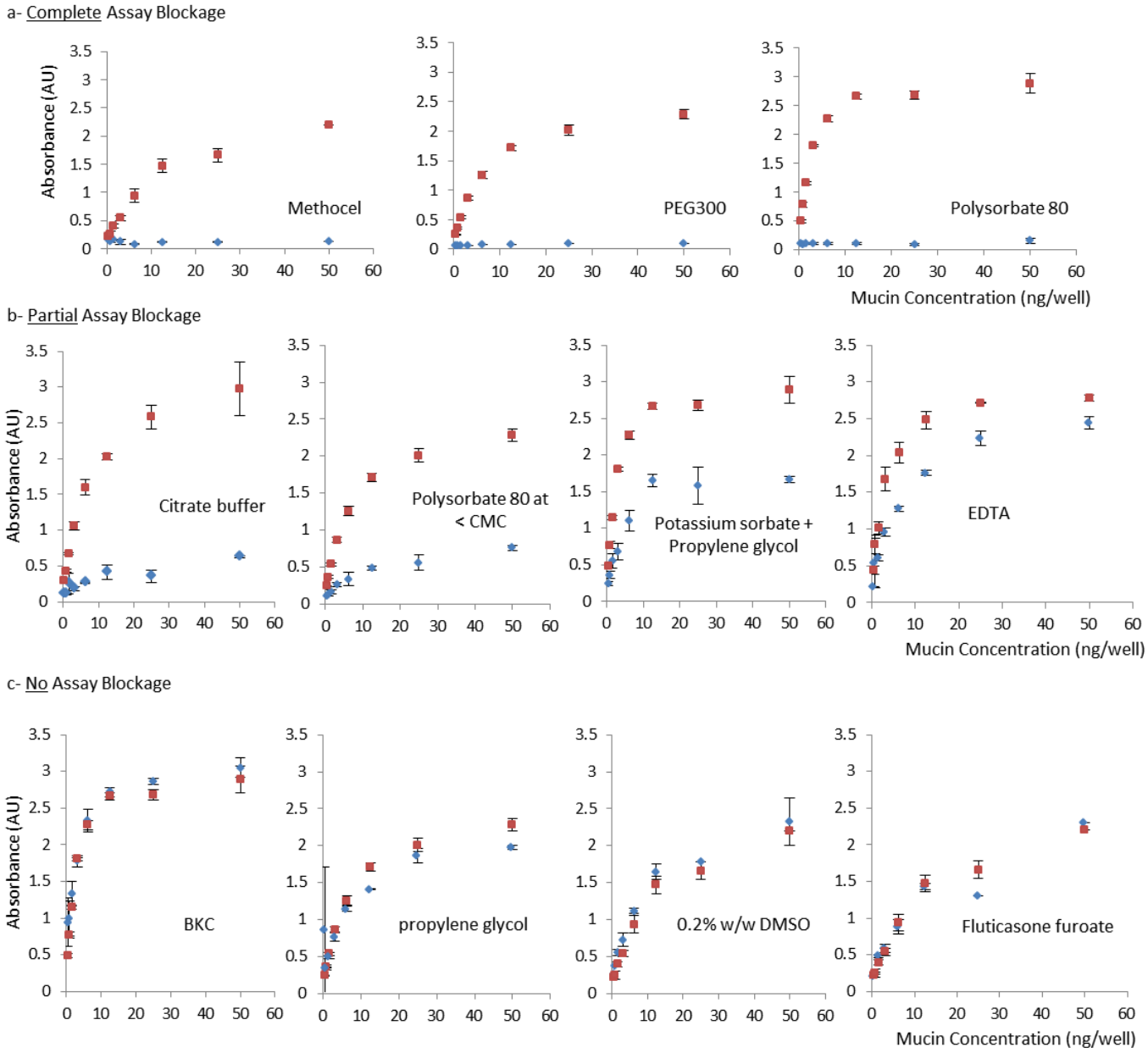


Figure 3.10 Selectivity of the ELLA for Airway Mucins in the presence of Certain IN Pharmaceuticals.

ELLA calibration plots (absorbance against mucin concentration, mean ± SD, n = 3) were constructed using mucin standard dilutions prepared in Ham's F12 containing the pharmaceutical specified on the plot (blue plots) at its working formulation concentration (Table 3.4) and compared to plots constructed using mucin standard dilutions prepared in Ham's F12 (red plots). It is worth noting that DMSO is not a pharmaceutical excipient; however it was used to solubilise FF.

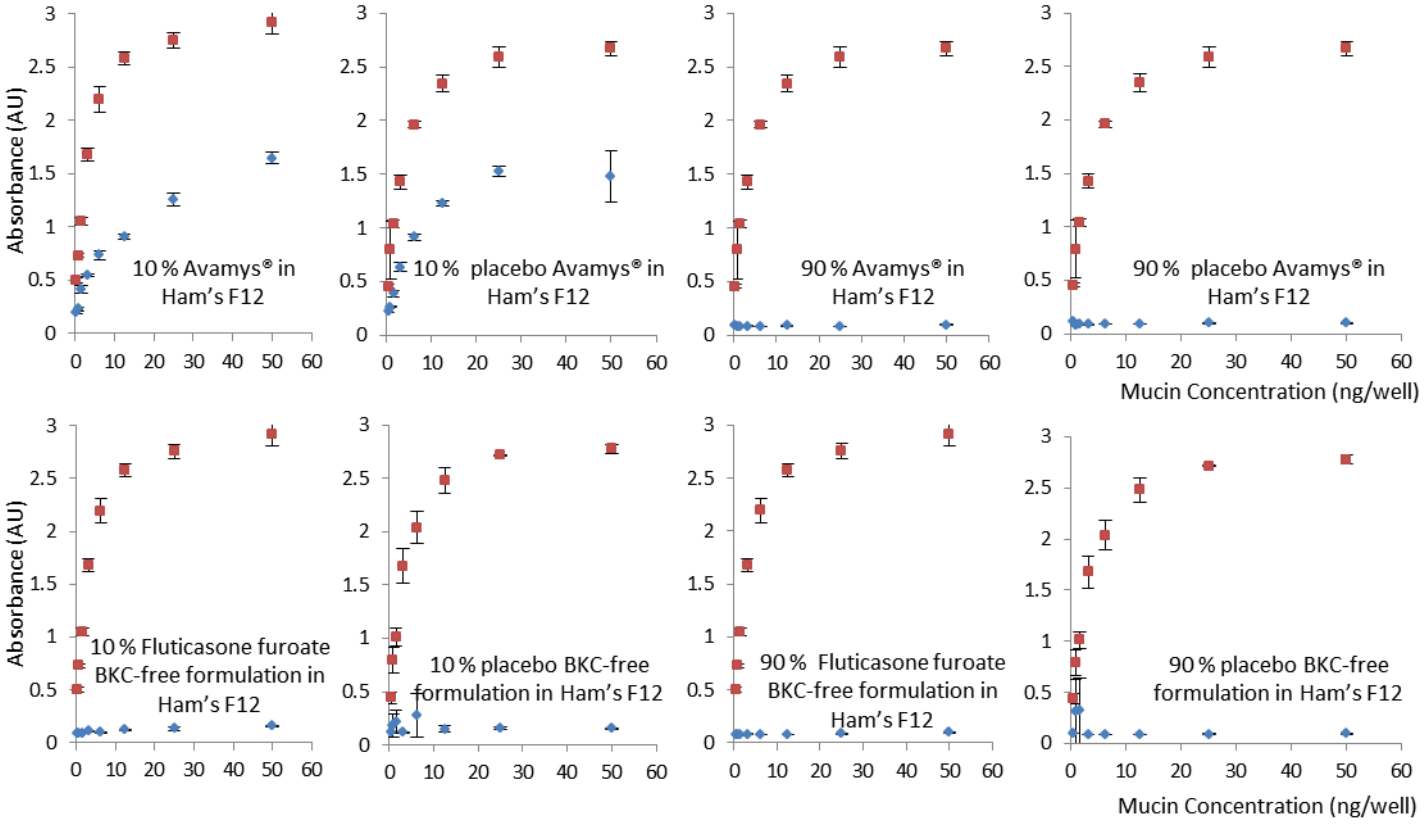


Figure 3.11 Selectivity of the ELLA for Airway Mucins in the presence of Some IN Formulations.

The blue calibration plots were generated using mucin serial dilutions made in Ham's F12 containing the formulation indicated on the plot, while the red plots were obtained using mucin serial dilutions made in Ham's F12 alone (mean ± SD, n = 3).

Figure 3.11 shows that the BKC-free formulation of FF was capable of blocking the assay even at 10 % of its concentration. In contrast, Avamys® caused only partial blockage of the assay at 10 % of its concentration while completely blocking it at 90 % concentration. This appeared to be due to the cumulative effect of more than one component interfering with the assay in the BKC-free formulation (polysorbate 80, potassium sorbate and citrate buffer) whereas only one component of the Avamys® interferes (polysorbate 80) with the assay.

It was therefore evident that airway mucins could not be assayed using the direct ELLA in the presence of those pharmaceuticals that fully block the assay. This fact presented a real obstacle to the progress of an important part of the project (The effect of these pharmaceuticals on mucin secretion), which prompted the development of a sandwich ELLA with the prospect of airway mucin quantification in the presence of these compounds. It was, however, deemed acceptable to use the standard ELLA in the presence of a compound that would only partially block the assay. This was achieved by spiking the calibration standards with the compound at a concentration identical to that in the sample.

3.4.3 Validation of a Sandwich Enzyme-Linked Lectin Assay (ELLA) for the Quantitative Determination of Airway Mucins

3.4.3.1 Optimisation of the Sandwich ELLA

The calibration curves of absorbance against mucin concentration (Figure 3.12) demonstrate that this sandwich ELLA can detect airway mucins with absorbance correlating to mucin concentration. It also shows that the initial plate-coating step of the assay with the unlabelled HPA lectin can be done in either PBS (pH 7.4) or carbonate buffer (pH 9.6) as no clear differences were observed between the two plots.

A range of concentrations (0.5 µg/ml to 4 µg/ml) of the unlabelled HPA lectin was studied in our laboratory prior to concluding that 0.5 µg/ml was the optimum concentration for coating the high-binding plates ready for the sandwich ELLA (data not shown).

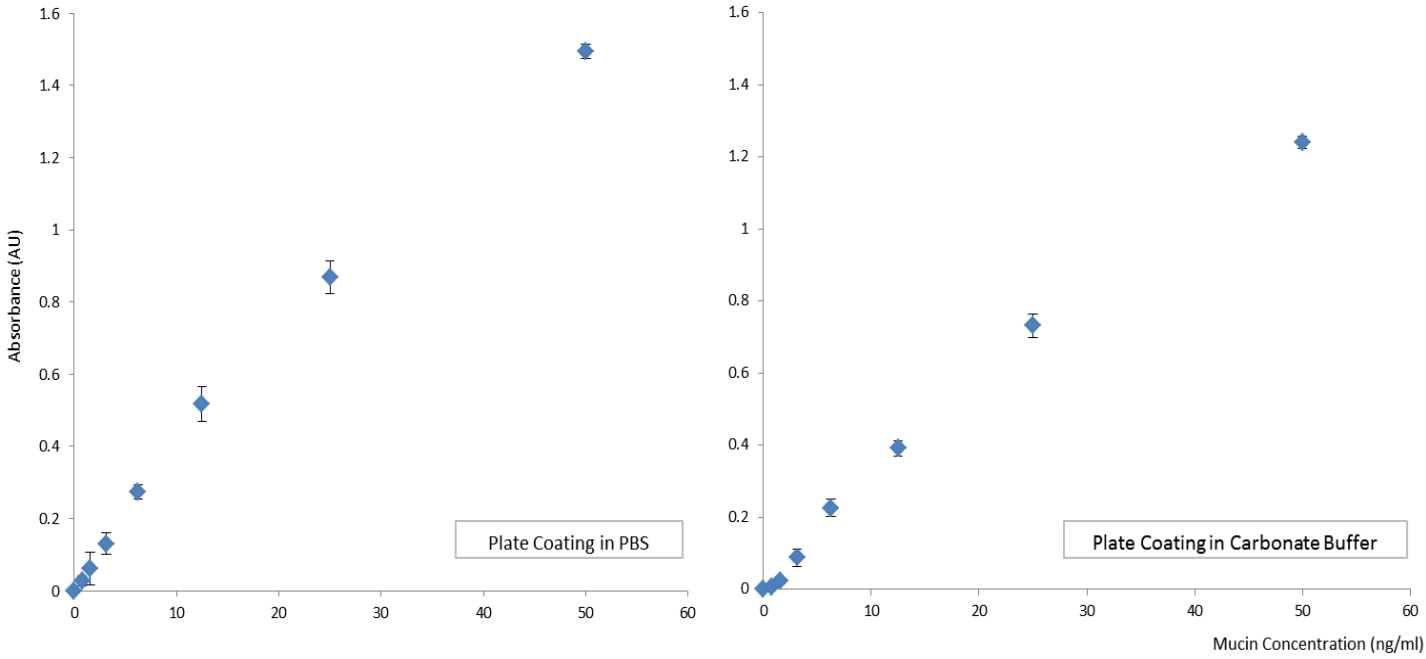


Figure 3.12 Effect of Coating Buffer on Calibration Curve of the Sandwich ELLA (mean ± SD, n=3).

The sandwich ELLA calibration was also run using various block buffers (0.1 % w/v gelatin, 0.5 % w/v gelatin, 0.1 % w/v Tween 20 and 0.5% w/v Tween 20) to minimise the background noise, which demonstrated that the 0.5 % gelatin block buffer should probably not be used in this sandwich assay as it was associated with the highest noise level (Table 3.5). All the other block buffers behaved very similarly showing an acceptable noise level although it was still higher than what was seen in the direct and indirect ELLAs described earlier.

Table 3.5 Effect of Block Buffer on Background Absorbance in the Sandwich ELLA

Block Buffer	Blank Absorbance (AU) (n = 3, mean ± SD)
0.1 % gelatin (w/v)	0.29 ± 0.01
0.5 % gelatin (w/v)	0.38 ± 0.03
0.1 % Tween 20 (w/v)	0.23 ± 0.006
0.5 % Tween 20 (w/v)	0.22 ± 0.02

Kruskal-Wallis statistical test showed the four block buffers to be significantly different ($P < 0.05$). Dunn's Multiple Comparison follow-on test showed the 0.5 % gelatin background noise to be significantly different to the 0.5 % Tween one.

The incubation periods, required for the initial coating of the high-binding plates with the unlabelled-HPA lectin and for the later binding of mucins in the sandwich ELLA, were also studied. Figure 3.13 demonstrates that an overnight incubation at 4 °C followed by 45 minutes incubation at 37 °C was almost as efficient as 2 hr incubation at 37 °C. Nevertheless, a solely overnight incubation at 4 °C substantially compromised the mucin binding at the higher end of the concentration range in all the direct, indirect and sandwich ELLAs (data not shown).

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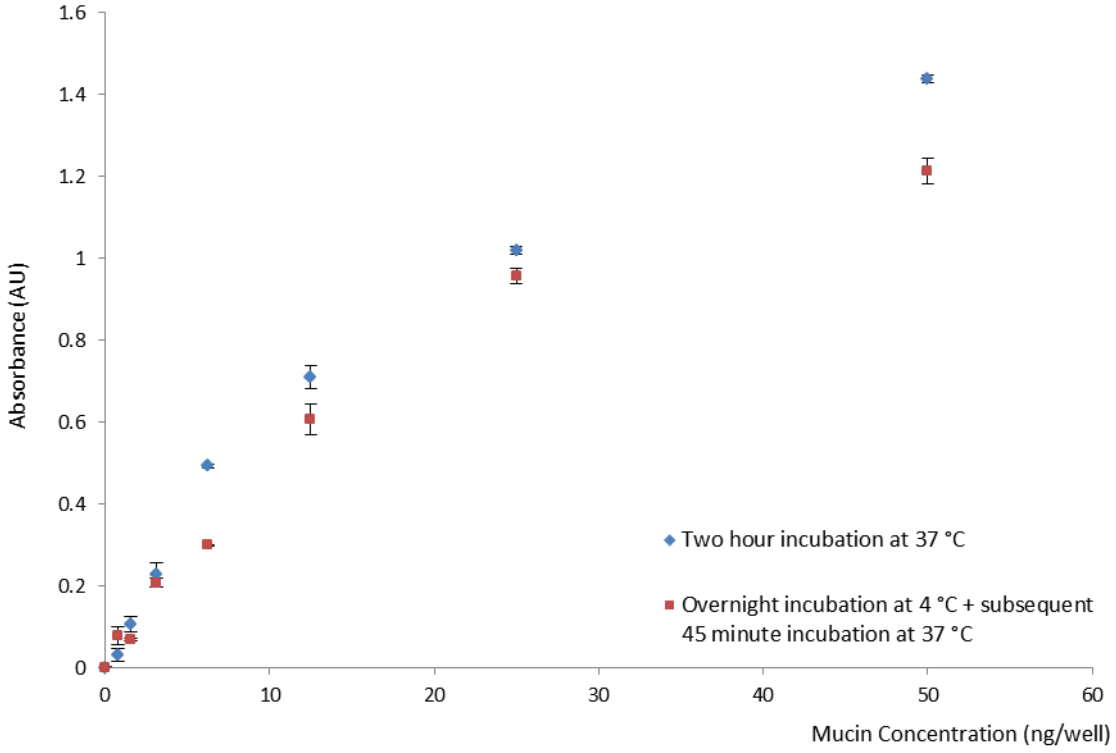


Figure 3.13 Effect of Initial Incubation Time (Plate Coating with the Unlabelled HPA Lectin) on Sensitivity of the Sandwich ELLA (mean ± SD, n=3).

3.4.3.2 Validation of the Sandwich ELLA

3.4.3.2.1 Linearity (calibration Model)

Regression analysis of a linearized Sandwich ELLA calibration curve (log absorbance against log concentration plot) demonstrated that the reported variation in absorbance was more than 99 % dependent on the corresponding variation in mucin concentration as R^2= 0.9934 (Figure 3.14). The P-value of the regression is less than 1×10^{-4}, indicating a significant relationship.

3.4.3.2.2 The Limit of Quantitation (LOQ)

The LOQ was 0.28 ng/well

3.4.3.2.3 The Assay Accuracy and Precision

The assay % RSD was less than 2 % at the higher end of the mucin concentration range (50 ng/well), less than 4 % at the middle of the concentration range (25 ng/well) and less than 5% near the LOQ (0.78 ng/well). Moreover, the assay has shown a % RE of 22 % near the LOQ and less than 11 % elsewhere in the concentration range. These criteria thus fall within the recommended-acceptance-range for precision and accuracy of immunoassays as explained above in section 3.4.2.2.3.

3.4.3.2.4 The Assay Range

The assay range of 0.28 ng/well to 50 ng/well was acceptable as the amount of mucins to be measured by the assay (secreted from ovine tracheal explants) was found over the years to fall within this range.

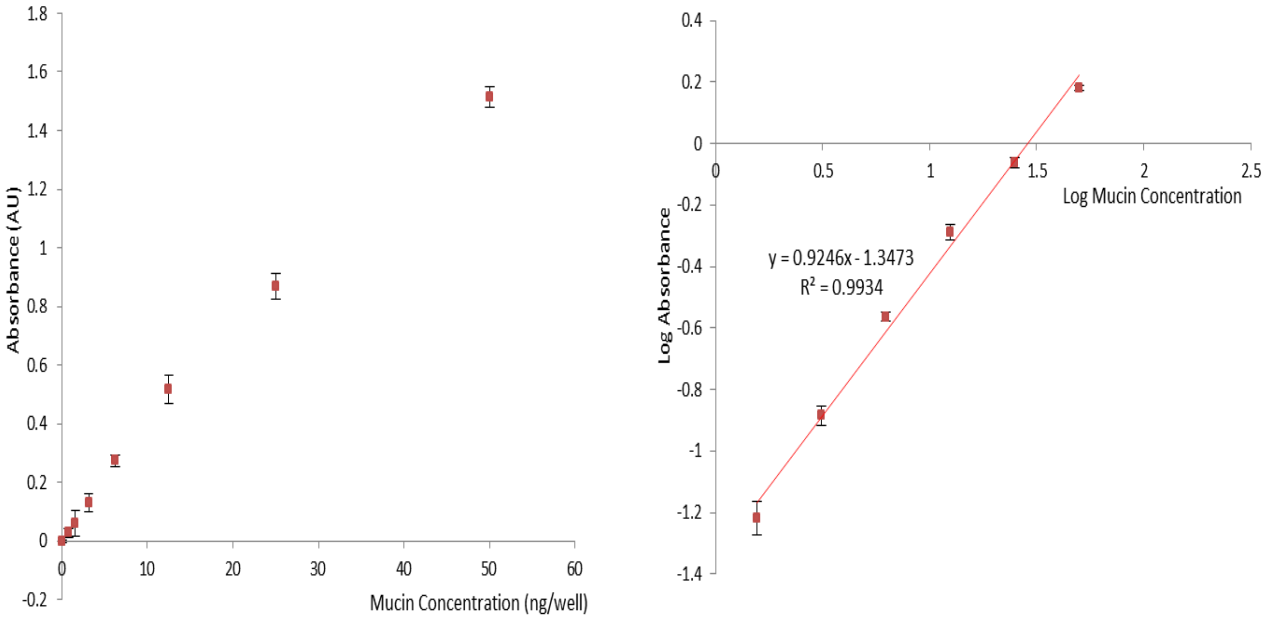


Figure 3.14 Linearity of Calibration Curve of the Sandwich ELLA (mean ± SD, n=3).

3.4.3.2.5 Selectivity of the Sandwich ELLA to Airway Mucins in the presence of IN Excipients

The assay demonstrated the ability to detect mucins in matrices containing polysorbate 80 and Methocel (Figure 3.15) that previously caused full blockade of the direct ELLA (see section 3.4.2.2.5). This indicated the sandwich ELLA was able to quantitate airway mucins in the presence of these intranasal pharmaceuticals.

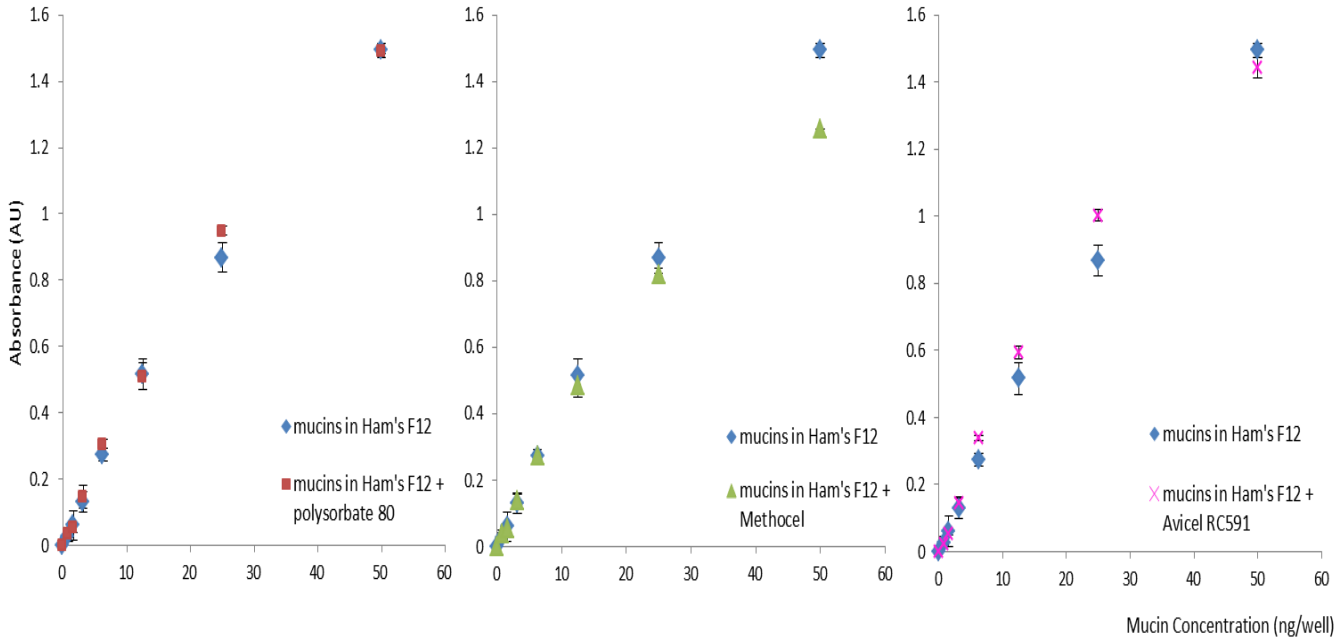


Figure 3.15 Selectivity of the Sandwich ELLA for Airway Mucins in the presence of Certain IN Excipients (mean ± SD, n=3).

Mucins were detected in the presence of the pharmaceutical specified on each plot at it working formulation concentration (Table 3.4).

3.5 Discussion and Conclusion

The object of this part of the project was to establish an ELLA that was fit for purpose. Pre-analytically, this quantitative procedure required a working reference standard. A mucin standard was prepared using the conventional procedure, CsCl isopycnic density gradient centrifugation. In agreement with previous reports, this procedure competently yielded mucins free from nucleic acids and proteins, particularly degradative enzymes whose activity was suppressed during the isolation process using proteinase inhibitors (PMSF and EDTA) in guanidinium chloride vehicle to prevent mucin degradation [159, 192, 195]. The long term storage of the purified human mucin standard was achieved here without the routine use of toxic chemicals (e.g. sodium azide) and with evidence of only subtle loss of activity following one freeze-thaw cycle. Following the development and validation of the ELLA, this human mucin standard was used for the quantitative determination of ovine mucins as a marker of ovine airway mucous secretion (See chapter 4). It can therefore be inferred that due to species differences between mucins [241], this determination was a relative rather than a definitive quantitation, in which human mucins acted as a scalar for the relative measurement of ovine mucins. This is a common approach in the quantitation of physiological macromolecules when there is difficulty in obtaining the endogenous matrix [172].

Despite the abundance of ELLAs in the public domain [209, 216-219, 240], to date, no report has employed an ELLA in the presence of pharmaceutical excipients. Major interference with the direct ELLA (using HRP-HPA lectin) was incurred when matrices containing some of the study's intranasal excipients were analysed. It was plausible that this interference would extend to the indirect ELLA (using the biotinylated lectin HPA-biotin) that superseded the direct one following the discontinuation of the peroxidase-labelled lectins HRP-HPAs. This is because the antigens (mucins) would still be expected to bind to the solid phase (96-well high-binding plates), the step that was highly compromised in the direct ELLA by some

interfering excipients to the extent of causing complete loss of signal. The selectivity profile of the indirect ELLA was therefore assumed to be similar to that of the direct one. It was not clear however, whether the interfering excipients were preferentially binding to the high-binding plates or whether they were capable of altering the mucin properties (e.g. solubilising it) and hence interfering with its plate binding ability. The latter possibility appeared more likely initially, owing to the negatively charged nature of mucins (sulphated and sialylated O-glycan structure) with an affinity to positively charged molecules (electrostatic interactions), in addition to the hydrophobic and H-bonding interactions that could attract mucins to other molecules [187, 242]. Nevertheless, as the issue of the interfering excipients was later resolved using the sandwich ELLA, in which the mucins were captured by the unlabelled-HPA-lectin for subsequent detection with the labelled-lectin, it seemed highly likely that the excipients were preferentially binding to the plate in the direct ELLA precluding the mucin retention on the plate. This theory appeared more plausible than the presumed excipient-induced mucin-solubilisation as the latter would have interfered with the capture of mucins by the unlabelled-HPA-lectin, unless the affinity between mucins and unlabelled-HPA-lectin was sufficient to reverse this solubilisation. Regardless of the mechanism, the sandwich ELLA was able to quantitatively determine airway mucins in the presence of the interfering IN pharmaceutical excipients, a prerequisite for studying the effect of these pharmaceuticals on airway mucin secretion as will be described in the next chapter.

All the ELLAs validated here were proved highly sensitive with an LOQ of as little as 0.2 ng/well and a useful range of up to 50 ng/well in all the ELLAs. The biotin-streptavidin detection system clearly enhanced the assay sensitivity owing to the high affinity between streptavidin and biotin [243], which is associated with signal amplification. It is worth noting that the concentration of the capture lectin (unlabelled HPA) in the sandwich ELLA should not be too high as this compromised the assay sensitivity. This was presumably because

fewer binding sites on the mucins remained available to bind the labelled HPA lectin when higher concentrations of the unlabelled HPA lectin were used, which therefore compromised the assay sensitivity.

The assays here also demonstrated adequate accuracy over the assay range using a linear function based on a log-log data transformation. Nevertheless, other approaches of data transformation, such as the 4- and 5- parameters logistic functions [227, 228, 235], were reported to offer enhanced accuracy.

A high level of background noise can be a problem in ELLAs. This is due to the presence of carbohydrate-contaminants in most commonly used blocking reagents. Such contaminants can bind lectins creating a false positive signal or masking original signal [211]. Bovine skin gelatin was used here in the block buffer of all the ELLAs. It demonstrated low background noise in the direct and the indirect ELLAs and hence, it was unlikely to contain any carbohydrate contaminants. This was particularly evident when the concentration of the gelatin in the block buffer had to be increased from 0.1 % w/v to 0.5 % w/v to suppress the increased background noise observed upon replacing the Sigma HRP-HPA with the EY one. However, upon employing the sandwich ELLA, a relative increase in the background noise was demonstrated, which was partially decreased by reducing the gelatin concentration to 0.1 % w/v. This observation raised doubts regarding whether the background noise was due to specific lectin binding to carbohydrate contaminants or merely due to protein-protein non-specific binding. The noise level observed with the 0.1 % w/v gelatin persisted at the same level when Tween 20 replaced the gelatin at two different concentrations in the block buffer. If the noise observed with the gelatin had emerged from any contaminant, it would have shown a variation in the noise level between the gelatin and the Tween 20, and if there had been any contaminant in the Tween 20 as well, the noise level would have increased with the higher concentration of Tween 20. It was therefore evident that the observed noise was the

minimal inherent noise of the sandwich assay due to non-specific rather than specific interactions, and that the increase observed with the 0.5 % w/v gelatin block buffer was most likely due to non-specific protein-protein interactions. The absence of specific interactions between the HPA lectin and the blocking reagents could also be further confirmed in the future by assessing the background noise in presence of the HPA lectin-inhibitory-sugar, GalNAc. This sugar will preferentially bind the lectin and preclude its specific interactions with any other glycans, leading to the disappearance of any signals due to specific lectin binding while signals due to non-specific bindings will persist [211, 213].

The sandwich ELLA procedure presented here is the first report, to our knowledge, of a sensitive and selective quantitative method of analysing airway mucins in the presence of certain pharmaceutical excipients.

Chapter 4

Effect of the Intranasal Formulations on Ovine Mucin Secretion

4.1 Introduction

4.1.1 Airway Mucins; Types, Sources and Secretion

The respiratory tract is comprised of conducting airways (nasal cavity, pharynx, larynx, trachea, bronchi, bronchioles and terminal bronchioles) and respiratory airways (respiratory bronchioles, alveolar ducts, and alveolar sacs). It can also be divided into proximal cartilaginous airways (from nasal passages to bronchioles) and distal non-cartilaginous airways (terminal bronchioles, respiratory bronchioles, alveolar ducts, and alveolar sacs)[244]. The airways are lined with mucosal epithelium that varies a great deal throughout the length of the airways. The epithelial cell types, distribution and abundance depend on the airway region in which they exist. The proximal large airways starting from the nasal passages down to the bronchi are lined with pseudostratified columnar ciliated epithelium, which is comprised of ciliated cells, secretory cells (serous and goblet cells), basal cells and undifferentiated columnar cells. Notably, the occurrence of goblet cells is infrequent in healthy airways; however they greatly increase during inflammation. Moreover, in the proximal airways, invaginations of the epithelium form submucosal glands, which are comprised of serous and mucous secretory cells. The branching of the bronchi to bronchioles in the proximal cartilaginous airways and then to terminal bronchioles in the distal non-cartilaginous airways is associated with a gradual change in the epithelium initially to become more ciliated with less secretory cells and less glands, prior to becoming simple cuboidal epithelium in the terminal bronchioles with the emergence of Clara cells; the major secretory cell type in the distal airways. Brush cells and pulmonary neuroendocrine cells are two microvilli-bearing minor cell types that also occur in the conducting airways. In the respiratory bronchioles, which possess both bronchiolar and alveolar features, the epithelium gradually shifts to the alveolar epithelium with type-I and type-II cells. [244-247]

Mucins line the body's mucosal surfaces to provide innate protection. These glycoproteins occur either as membrane-tethered (transmembrane or cell-surface) mucins or as secreted mucins. In the airways, transmembrane mucins are primarily MUC1, MUC4 and MUC16 [248, 249], although the expression of MUC11, MUC13, MUC15 and MUC20 has been observed [185]. These mucins form the glycocalyx that provides immediate protection of the apical surface of epithelial cells, whereas the airway secreted mucins (MUC2, MUC5AC, MUC5B, MUC7, MUC8 and MUC19 [185, 250]) lie on top of the glycocalyx to provide dynamic protection [195].

Transmembrane mucins typically consist of a larger wholly extracellular subunit and a smaller subunit that consists of an extracellular region, a transmembrane domain and a cytoplasmic tail [195]. MUC1 and MUC4 transmembrane mucins appear to be present in all airway surface epithelial cells [193, 251], although there is accumulating evidence that they occur predominantly at the apical surface of ciliated epithelial cells [185, 248, 249, 252], which possibly also express MUC16 [249, 253].

Secreted mucins constitute 90 % of the mucin content of sputum. With the exception of MUC7 and MUC8, they all have polymeric gel-forming properties [250]. Notably, MUC5AC and MUC5B (the former being the highest expressed [246, 250]) are the principal gel-forming mucins; they are the most prominent secreted mucins in healthy human airways and have been shown to be conserved between species [193, 248-250]. MUC5AC is the predominant secretion of goblet cells, which also normally secrete some MUC5B. MUC2 is also normally secreted by goblet cells in the bronchi [250], although it is secreted elsewhere in inflammatory conditions. MUC5B is, however, primarily expressed in the mucous cells of submucosal glands, which also produce MUC8 and MUC19 [254], while MUC7 (a non-gel-forming secreted mucus) is expressed in the serous cells of submucosal glands. [185, 248, 252]

4.1.2 Regulation of Airway Mucin Secretion

Mucins are secreted constitutively at a low basal rate in healthy airways (via exocytosis from epithelial secretory granules). Finely regulated secretory machinery maintains the balance between mucin production (intracellular synthesis and storage in secretory granules) and secretion, which prevents much intracellular accumulations of mucins. In response to rising levels of extracellular secretagogues, the mucin secretion rate is increased (stimulated mucin secretion). MUC5AC and MUC5B are both secreted constitutively in human, and hence they both contribute to the mucus barrier and clearance functions. In contrast, mouse Muc5ac was hardly detected constitutively in murine airways, although both Muc5ac and Muc5b increased in inflammation.[46, 202, 246, 255]

Mucin exocytosis is regulated independently from mucin production. In the airway surface epithelium, various secretagogues, such as purinergic agonists, cholinergic agonists, inflammatory mediators, proteases and arachidonic acid metabolites, elicit exocytosis to release mucins [46, 256], among which purinergic agonists (adenosine triphosphate (ATP) and uridine triphosphate (UTP)) have been identified as the chief mucin secretagogues of goblet cells, which have been shown to be poorly innervated [257, 258]. Constitutive ATP release occurs from ciliated cells (a paracrine stimulus) in response to mechanical shear stress, as well as from the secretory granules (an autocrine stimulus) along with uridine nucleotides [259]. ATP and UTP act directly on the G-protein coupled purinergic P2Y2 receptors on the apical membrane of secretory cells, leading to the stimulation of secondary messengers with the release of intracellular calcium to elicit mucin exocytosis. It has, however, yet to be established whether the other agonists, e.g. histamine and acetylcholine, act similarly on secretory cell receptors or alternatively act on smooth muscles to release ATP [46, 246, 259].

On the other hand, secretions of the airway submucosal glands, which are highly innervated by the sympathetic, parasympathetic and non-adrenergic non-cholinergic nervous systems,

are regulated by neurotransmitters (Cholinergic, adrenergic and non-adrenergic non-cholinergic including substance P, vasoactive intestinal peptide, neurokinin A and nitric oxide) and inflammatory agents e.g. histamine [46, 96, 257, 258], the dominant of which is the cholinergic (muscarinic) neural control [96].

4.1.3 Airway Mucin Secretion in Disease

Cystic fibrosis (CF), asthma and chronic obstructive pulmonary disease (COPD) including chronic bronchitis and emphysema are all respiratory conditions associated with airway mucus pathologies such as hypersecretion. In the proximal cartilaginous airways of a healthy human adult, submucosal glands constitute one third of the inner airway wall while goblet cells occur occasionally and become scarce in the bronchioles. In addition, goblet cells are absent in the healthy human distal non-cartilaginous airways. Nonetheless, in hypersecretory diseases, submucosal glands and goblet cells have been observed to increase in size (hypertrophy) and number (hyperplasia). Moreover, goblet cells have also been observed in the distal airways of murine airways, following inflammatory sensitisation, as a transformation of Clara cells (goblet cell metaplasia) [45, 46, 96, 254, 257]. In addition, mucous metaplasia; the up-regulation and alteration of proportionality of polymeric mucins in the secretions of various secretory cells, was also associated with respiratory diseases [46, 246, 250]. Inflammatory mediators were found to play a major role in the remodelling of the airway epithelium in pulmonary diseases [45, 254, 260].

4.1.4 Animal Models of Mucin Secretion Studies

Although magnetic resonance imaging (MRI) has recently enabled non-invasive mucin secretion studies *in-vivo* [203, 261, 262], these studies have traditionally relied on *in-vitro, in-situ* or *ex-vivo* experimental models. Intact parts of a respiratory organ (e.g. ferret trachea [263, 264] and human bronchi [265]) or explants of the surface epithelium of a respiratory organ (e.g. human

turbinates [266], canine tracheae [157] and ovine tracheae [158]) are typical models in these studies, with the former being used to study mucins emanating from both surface epithelial secretory cells and submucosal glands and the latter being used to study mucins released solely from the surface epithelial secretory cells [258]. Mucin secreting cell culture models are currently an area of extensive research that bears much potential. Both the rat tracheal epithelial (RTE) cell line SPOC1 [212, 258, 267, 268] and human bronchial epithelial (HBE) primary cells [176, 217] have successfully demonstrated mucin secretion, however the culture and differentiation procedures are labour-intensive.

4.1.5 Aim of the Chapter

To date, only little is known on the effect of anti-allergic IN formulations on airway mucin secretion (Table 1.2). Fergie et al. have shown prednisolone to reduce mucus secretion from a goblet cell line [269] while dexamethasone has also been shown to suppress MUC5AC protein expression [270, 271]. The latter effect however, has been shown to be non-significant in the presence of IL-13 [272, 273].

In this chapter, the effect of a range IN pharmaceuticals on mucin secretion was studied in order to test the hypothesis that they do not interfere with the respiratory physiology by altering the rate of mucus secretion. The investigation was conducted in sheep tracheal epithelial explants cultured on collagen-coated nitrocellulose permeable supports at an air liquid interface, which maintained mucociliary function. This model permitted the measurement of surface epithelial mucin secretion (predominantly from goblet cells) without any interference from submucosal gland secretion. The ELLAs described in chapter 3 were implemented here for the quantitative determination of mucins in the surface epithelial secretions.

4.2 Materials

4.2.1 The Explant Culture Medium

See section 2.2.2.1

4.2.2 Culture of the Sheep Tracheal Epithelial Explants

See section 2.2.2.2

4.2.3 Measuring Mucin Secretion

- Adenosine Triphosphate (ATP) disodium salt - Roche Diagnostics Limited, material number 519979.
- Ham's F12 with L-glutamine - PAA The Cell Culture Company, E15-817.
- The test excipients/APIs - provided by GSK (see section 4.2.4).

4.2.4 The Study Formulations/Excipients/API

Table 4.1 summarises the pharmaceuticals supplied by GSK to be studied in this project.

Table 4.1 The Study Pharmaceuticals.

The Formulation/ API/ Excipient	Composition
Fluticasone furoate (FF)	This is a steroid anti-inflammatory API that was supplied by GSK as powder.
GSK1004723D	This is the dihydrochloride salt form of the GSK's novel antihistamine GSK1004723, which was supplied as a 20 % w/w solution in water.
GSK1004723E	This is the naphthalene salt form of the GSK's novel antihistamine GSK1004723, which was supplied as powder.
Avicel® RC591	An FMC Biopolymer product (powder); microcrystalline cellulose and Na-CMC - GSK Comet item ID RM100232.
Avicel® CL611	An FMC Biopolymer product (powder); microcrystalline cellulose and Na-CMC - GSK Comet item ID RM100231.
Methocel™ E50LV premium (Methocel™ Premium hypromellose)	A HPMC polymer (powder) - GSK Comet item ID RM100191.
Anhydrous dextrose	D-glucose powder - GSK Comet item ID RM100061.
Benzalkonium Chloride (BKC)	A 50 % solution - Sigma–Aldrich (63249).
Potassium Sorbate (PS)	Granules - GSK Comet item ID RM100392.
Propylene Glycol (PG)	Liquid - GSK Comet item (ID not known).
Polysorbate 80 (Tween 80)	Liquid - Fluka Analytical Switzerland (59924)
Ethylenediamine tetraacetic acid - disodium salt (EDTA)	Powder - GSK Comet item ID RM142104.

Polyethylene glycol 300 (PEG300)	Liquid - Fluka Analytical Switzerland (81162)
Citric acid, anhydrous	Powder crystalline - Sigma (C1857)
Trisodium citrate, dihydrate	Powder crystalline - Sigma (S1804)
Avamys® (Veramyst® in the US)	This is a FF (0.05 % w/w) IN spray formulation (pH 5.8) that is preserved by 0.015 % w/w BKC. It also contains 1.5 % w/w Avicel RC-591, 0.015 % EDTA, 0.025 % w/w polysorbate 80 and 5 % w/w anhydrous glucose in water.
Placebo Avamys®	This is the same as Avamys® but without API (FF).
Fluticasone furoate (FF) BKC-free formulation	This is a FF (0.05 % w/w IN spray formulation (pH 4.7) that is preserved by 0.3 % w/w PS. It also contains 0.015 % w/w EDTA, 2.4 % w/w Avicel CL611, 0.096 % w/w citric acid anhydrous, 1.48 % w/w sodium citrate, 0.025 % w/w polysorbate 80, 0.3 % w/w and 1.5 % w/w PG in water.
Placebo BKC-free formulation	This is the same as the "FF BKC-free formulation" but contains no API (FF).
GSK1004723D formulation	Only guidance to the preparation of this formulation was provided. It contains 0.557 % w/w GSK1004723D, 1% w/w Methocel™ E50LV Premium, 0.015 % w/w EDTA, 0.015 % w/w BKC and 0.025 % w/w polysorbate 80 in a 5 % w/w anhydrous glucose vehicle. The pH is adjusted to ~ 5 (range 4.5 – 5.5).
GSK1004723E formulation	Only guidance to the preparation of this formulation was provided. It contains 0.734 % w/w GSK1004723E in the BKC-free placebo formulation described above.

4.3 Methods

4.3.1 The Explant Culture Medium

See section 2.3.2.1

4.3.2 Culture of the Sheep Tracheal Epithelial Explants

See section 2.3.2.2

4.3.3 Measuring Mucin Secretion

Explant discs (5-7 mm) covered with ovine tracheal epithelium were cut out of the explants using cork borer no. 2 or 3. Each disc was placed in a well of a 24-well plate containing 0.5 ml of Ham's F12 medium. The plate was then incubated at 37 ºC in 5 % CO_2 : 95 % air for two hours, during which the Ham's F12 medium was discarded and replaced with fresh warm (37 ºC) medium every 20 minutes for the first hour and every 10 minutes during the second hour. This equilibration period was followed by 4 x 10 minute periods when Ham's F12 medium (0.5 ml) was collected and replaced every 10 minutes to provide four samples containing mucins under baseline conditions for each explant disc. After the last baseline sample was collected, each explant disc was treated with 0.5 ml of warm (37 ºC) Ham's F12 medium containing the test formulation or excipient for 4 x 10 minute periods. Samples were collected and replaced at the end of each 10 minute period to provide four samples containing mucins under treatment conditions for each explant disc. The experiment was then finished by exposing each explant disc to 0.5 ml of warm (37 ºC) Ham's F12 medium containing 100 µM ATP (mucin secretagogue) for 3 x 10 minute periods, in which the medium was collected and replaced at the end of each 10 minute period to provide three positive-control samples. Baseline, treatment and positive-control samples were assayed for their mucin content using an ELLA as described in chapter 3. Samples, as well as calibration standards, were assayed in

triplicate on the same plate and a calibration curve was generated for each plate. Calibration standards were constructed in Ham's F12, which was spiked with the test compound when assaying the treatment samples containing this compound (to achieve the same matrix between the samples and the standards). The calibration curves were subsequently used for the conversion of the samples' absorbance values into mucin content values (ng/well).

4.3.4 The Test Excipients/APIs

All excipients were prepared at their working formulation concentration in Ham's F12. Table 4.2 lists all the compounds tested and explains any special procedure used during their preparation. It also indicates which ELLA procedure was used to quantify the mucin secretion response to each compound.

Table 4.2 The Compounds Studied for their Effect on Ovine Airway Mucin Secretion.

The Excipient/ API	Concentration in Ham's F12	The ELLA
BKC	0.015 % w/w	The direct ELLA
EDTA	0.015 % w/w	The direct ELLA
Propylene glycol (PG)	1.5 % w/w	The indirect ELLA
Potassium Sorbate (PS)	0.3 % w/w (in presence of 1.5 % w/w PG as a cosolvent)	The indirect ELLA
Polysorbate 80	0.025 % w/w	The sandwich ELLA
Methocel™ E50LV premium	1.0 % w/w (To achieve optimum dispersion, the polymer was initially wetted with water; one tenth of the total diluent volume, and agitated at 60-70 °C for ~ 1 hr using a magnetic stirrer before making up to the final volume in Ham's F12)	The sandwich ELLA
Avicel® RC591	1.5 % w/w (To achieve optimum dispersion, the polymer was initially homogenised in water; one fifth of the total diluent volume, before making up to the final volume in Ham's F12)	The sandwich ELLA
Fluticasone furoate (FF)	0.0004 % w/w (~ 4 µg/ml). FF was initially solubilised in DMSO (1 part in 500 parts; sparingly soluble). The DMSO containing the API was then solubilised in Ham's F12 to yield a final concentration of 0.2 % w/w DMSO.	The indirect ELLA
GSK1004723E (naphthalene salt)	0.01 % w/w The antihistamine API; GSK1004723E, was solubilised in DMSO (1 part in 20 parts), before solubilising the DMSO containing the API in Ham's F12 to yield a final concentration of 0.2 % w/w DMSO.	The indirect ELLA
GSK1004723D (dihydrochloride salt)	0.01 % w/w	The indirect ELLA
DMSO	0.2 % w/w	The indirect ELLA

4.3.5 Data Analysis

Owing to the difference in the number of goblet cells present in each explant, the baseline mucin secretion varied a great deal between explants. Thus, for the purpose of comparing different explants, the mucin content (ng/well) of samples from each explant was expressed as percentage of the mean baseline mucin secretion of that explant (% baseline). The mean baseline mucin secretion is the average mucin content (ng/well) of the pooled four mucin secretion measurements sampled following the four successive 10-minute exposures to Ham's F12 containing no test compound, which preceded the exposure of any one explant to any particular treatment. Meanwhile, the mean response mucin secretion is the average mucin content (ng/well) of the pooled four mucin secretion measurements sampled following the four successive 10-minute exposures to a particular treatment. The mean baseline mucin secretion value (in units of ng/well) was paired to the mean response mucin secretion value (in units of ng/well) of the same explant, and for each set of explants exposed to the same treatment, the paired data was compared using Wilcoxon matched-pairs signed rank test. On occasions of data proving to be parametric, a paired t-test was employed for comparison. $P \leq 0.05$ was considered statistically significant.

Only data from valid (physiologically functioning) explants was considered in this study. A valid explant was defined as any explant demonstrating the ability to secrete stimulated mucins via a clear increase in mucin secretion (at least a 1.5-fold increase above the mean secretion in the period prior to exposure) in response to ATP or the test preparation (positive control). In addition, valid explants were required to demonstrate baseline variability that did not mask the clarity of the ATP-stimulated mucin secretion response. All explants considered in this study demonstrated a % RSD of ≤ 145 % among the four baseline measurements.

4.4 Results

4.4.1 Effect of the Study Formulations on Ovine Mucin Secretion

Early experiments were conducted to look at the effect of the two fluticasone furoate (FF) IN formulations and their placebos (all prepared and supplied by GSK, see table 4.1), namely the BKC-free FF formulation, Avamys® (the BKC-preserved FF formulation), the BKC-free placebo formulation and Avamys® placebo. Each formulation was studied at two concentrations; 10 % v/v and 90 % v/v in Ham's F12 (Figures 4.1 amd 4.2). This data falsely implied a decrease in mucin secretion following exposure to these formulations because the direct ELLA failed to quantify the amount of mucins in the response samples, taken during the 4 X 10 minute exposures to the formulations. These samples contained excipients that proved to interfere with the ELLA as explained in section 3.4.2.2.5.

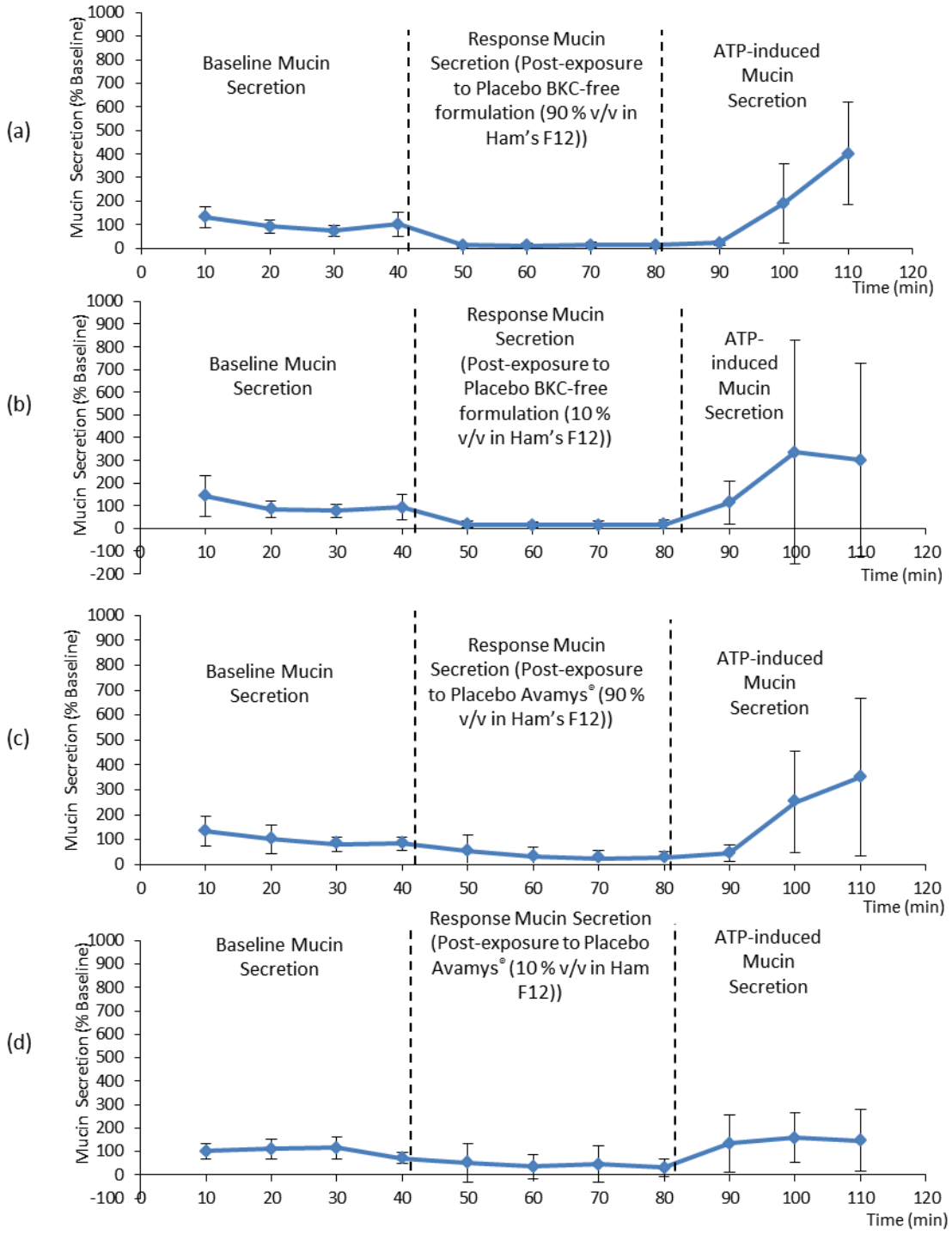


Figure 4.1 The Effect of Placebo IN formulations and ATP on Ovine Mucin Secretion.

The above plots demonstrate the increase in ovine mucin secretion in response to ATP stimulation following the exposure to two different placebo IN formulations at the concentrations of 90 % v/v and 10 % v/v (n = five explants from three animals in (a), nine explants from six animals in (b), nine explants from five animals in (c) and eight explants from five animals in (d), mean ± SD).

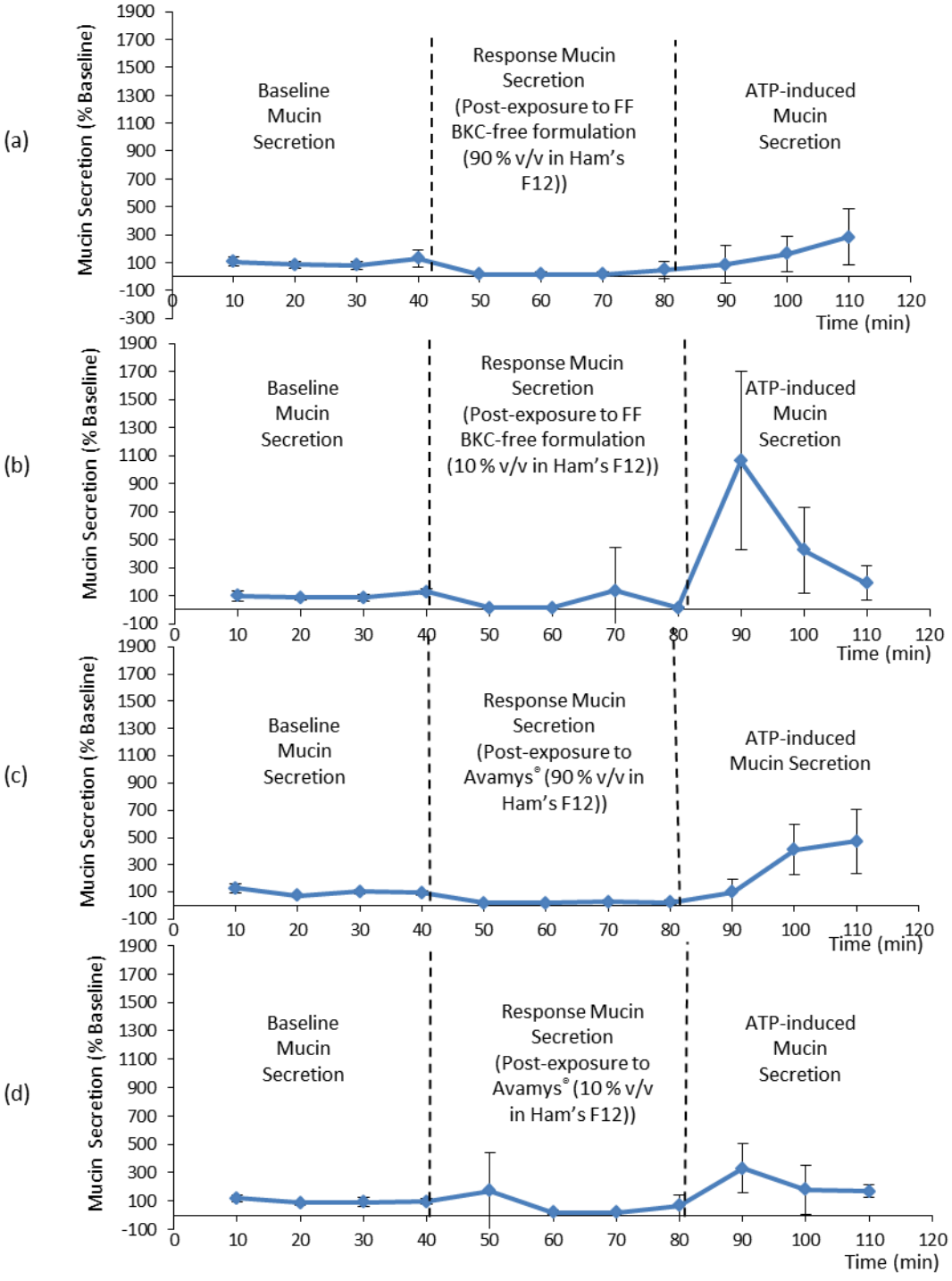


Figure 4.2 The Effect of Fluticasone Furoate (FF) IN formulations and ATP on Ovine Mucin Secretion.

The above plots demonstrate the increase in ovine mucin secretion in response to ATP stimulation following the exposure to two different FF test formulations at the concentrations of 90 % v/v and 10 % v/v (n = nine explants from four animals in (a), six explants from four animals in (b), six explants from four animals in (c), seven explants from six animals in (d), mean ± SD).

4.4.2 ATP Stimulates Ovine Mucin Secretion

The ability of ATP (100 µM) to act as a positive control that confirm the responsiveness of the tissue in each explant was demonstrated by a significant increase in mucin secretion from each explant after exposure to ATP. The effect was observed at the end of each experiment in the set of early experiments attempting to look at the effect of the intact anti-allergic intranasal formulations on mucin secretion. The tissue was then exposed to a test formulation for 4 x 10 minute periods followed by 3 x 10 minute exposures to ATP. The ATP demonstrated an increase in mucin secretion, up to 10 times its baseline level on some occasions (see figures 4.1 and 4.2).

4.4.3 Effect of Benzalkonium Chloride (BKC) on Ovine Mucin Secretion

When nine explants were exposed to BKC at its working formulation concentration (0.015 % w/w) in Ham's F12, an increase in mucin secretion was observed in all of them. Figure 4.3 (a) shows the response of three representative individual explants to BKC and demonstrates that the magnitude of the maximal response to BKC varied between explants (a maximal response between 1.6-fold and 16-fold above the mean baseline mucin secretion was observed). In addition, the exposure time required to induce that response was also variable among explants. This resulted in the observed level of variability in figure 4.3 (b), which is intrinsic to biological systems. It, however, had no impact on the significance of the reported increase in mucin secretion in response to BKC (P=0.0039) as demonstrated by comparing the mean baseline mucin secretion of each explant to the mean response mucin secretion of the same explant in the nine tested explants (Figure 4.3 (c)). Figure 4.3 (c) also confirms the evidence provided in figure 4.3 (a) on the variability of the response magnitude among explants.

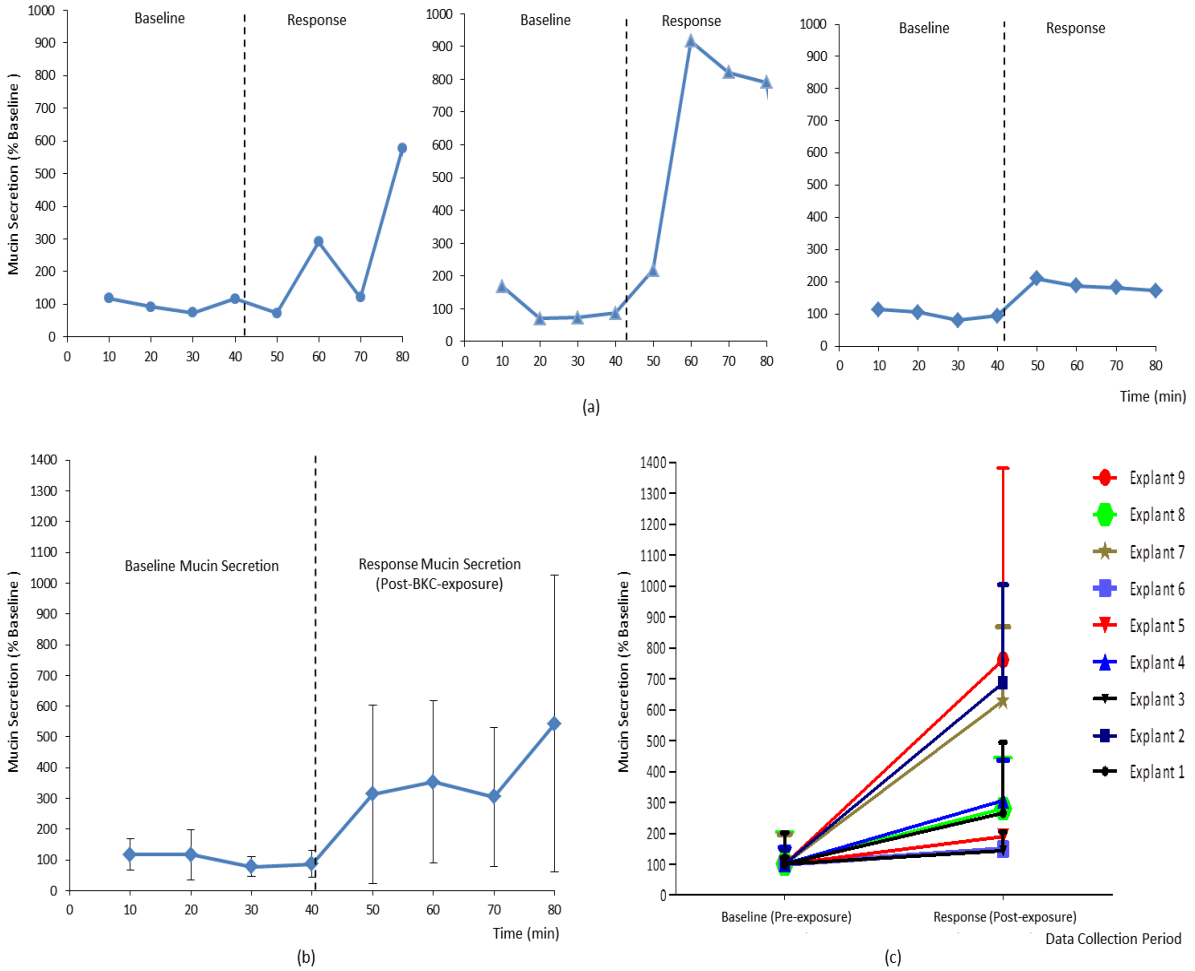


Figure 4.3 The Effect of BKC (0.015 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to BKC exposure. (b) The average mucin secretion response of 9 explants (3 animals) upon exposure to BKC (mean ± SD). (C) The four baseline mucin secretion measurements of each of the nine explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.4 Effect of Ethylenediamine Tetraacetic Acid (EDTA) on Ovine Mucin Secretion

The effect of EDTA (0.015% w/w) on mucin secretion was studied in eight explants from three animals. Two explants from two different animals demonstrated a decrease in mucin secretion below the mean baseline secretion during the exposure to EDTA (Figure 4.4 (a), left plot). However, an increase in mucin secretion, with a maximal response between a 1.6-fold and a 9-fold increase above the mean baseline secretion, was observed in six explants, which occurred at different time points following exposure to EDTA (Figure 4.4 (a), middle and right plot). The mean response of the eight explants to EDTA exposure is shown in figure 4.4 (b) while figure 4.4 (c) demonstrates that this response was not significantly different to baseline mucin secretion ($P = 0.0781$).

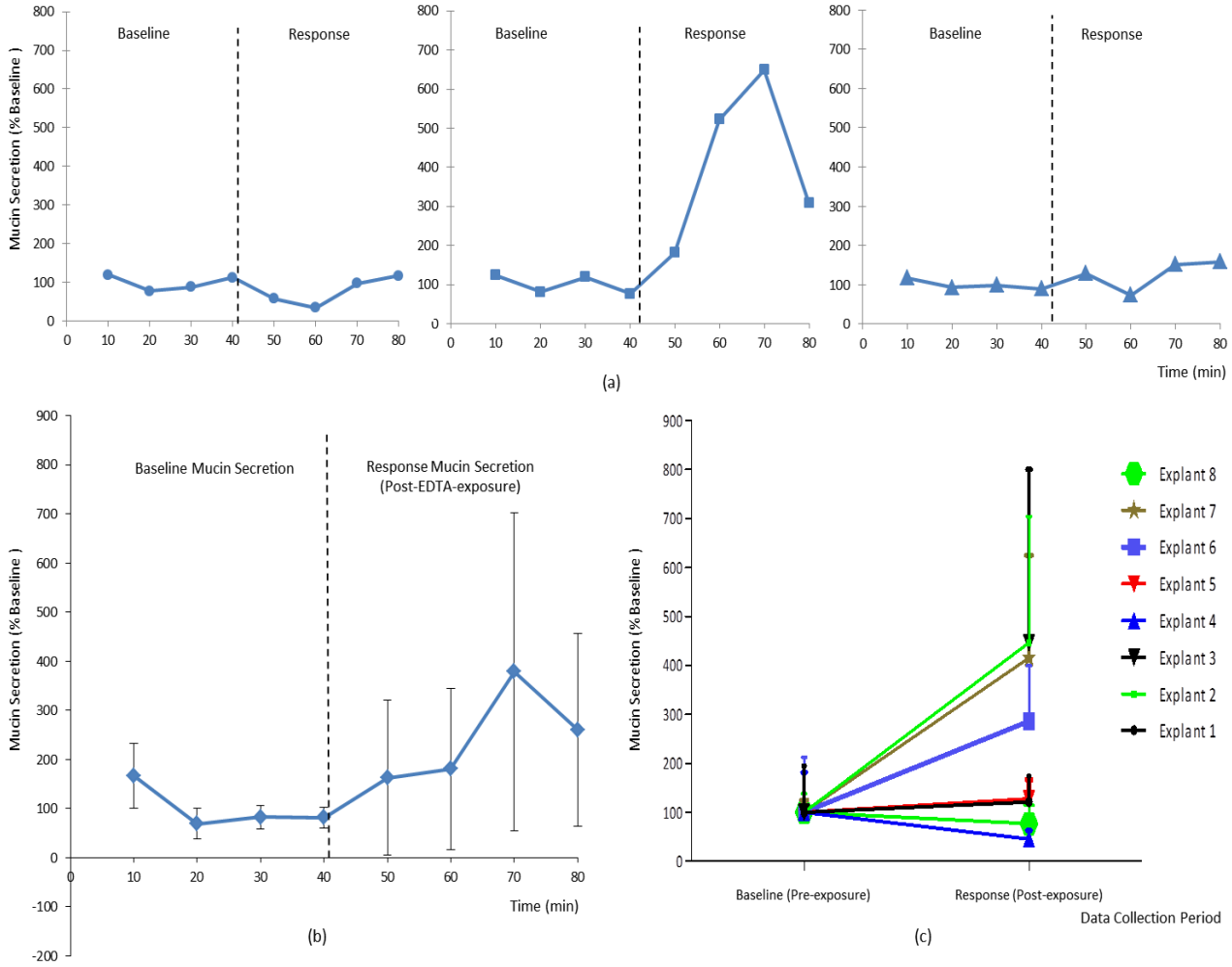
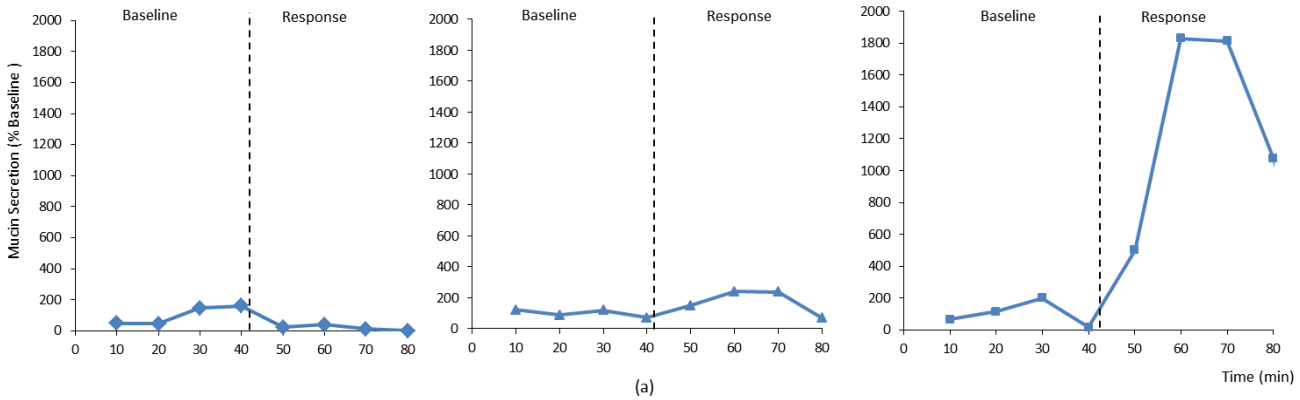


Figure 4.4 The Effect of EDTA (0.015 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to EDTA exposure. (b) The average mucin secretion response of 8 explants (3 animals) upon exposure to EDTA (mean ± SD). (c) The four baseline mucin secretion measurements of each of the eight explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.5 Effect of Propylene Glycol (PG) on Ovine Mucin Secretion

The effect of PG (1.5 % w/w) on mucin secretion was studied in seven explants from three animals (Figure 4.5). One explant demonstrated a decrease in mucin secretion below the mean baseline secretion during the exposure to PG (Figure 4.5 (a), left plot). However, an increase in mucin secretion, with a maximal response between 2-fold and 20-fold above the mean baseline secretion, was observed in six explants after 20 to 30 minutes exposure (i.e. at the 60 and the 70 minute time-points) to PG (Figure 4.5 (a), middle and right plot). This increase appeared to be transient as it declined at the 80 minute time-point in all six explants despite the continued exposure to PG. The mean response of the seven explants to PG exposure is shown in figure 4.5 (b) while figure 4.5 (c) demonstrates that this response was significantly different to baseline mucin secretion ($P = 0.0469$).



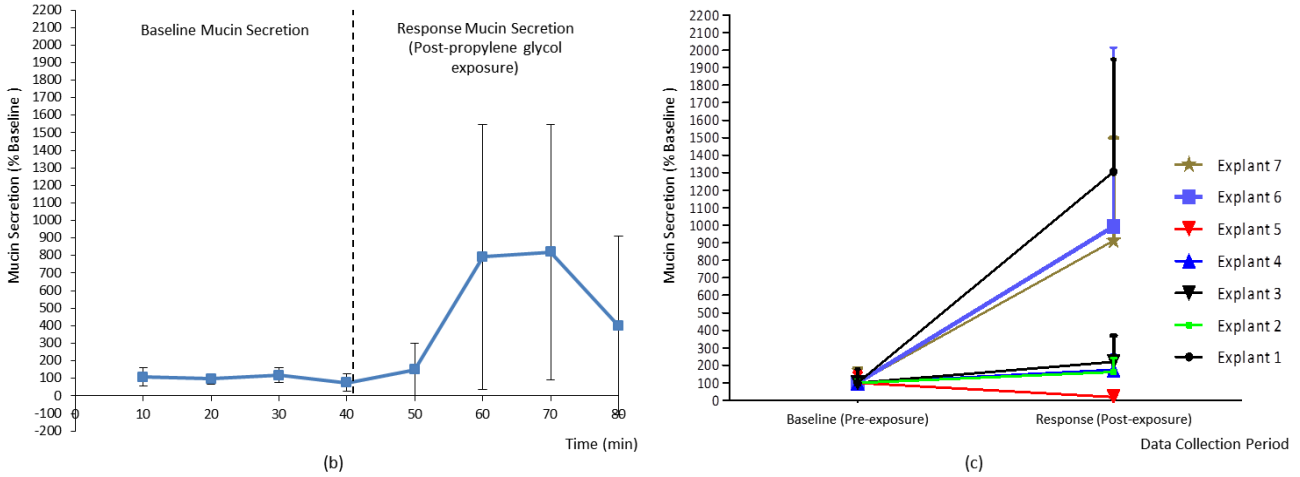


Figure 4.5 The Effect of Propylene Glycol (1.5 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to propylene glycol exposure. (b) The average mucin secretion response of 7 explants (3 animals) upon exposure to propylene glycol (mean ± SD). (c) The four baseline mucin secretion measurements of each of the seven explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.6 Effect of Potassium Sorbate (PS) on Ovine Mucin Secretion

Due to solubility issues, PS (0.3 % w/w) was tested in the presence of PG (1.5 % w/w), which was used as a cosolvent. The combined effect of the two compounds on mucin secretion was studied in twelve explants from three animals (Figure 4.6). One explant demonstrated a decrease in mucin secretion below the mean baseline secretion while another (from another animal) demonstrated no change in mucin secretion during the exposure to these compounds. However, an increase in mucin secretion, with a maximal response between a 1.8-fold and a 24-fold increase above the mean baseline secretion, was observed in ten explants, which varied in the exposure time required before observing this maximal response (Figure 4.6 (a)). The mean response of the twelve explants to this exposure is shown in figure 4.6 (b) while figure 4.6 (c) demonstrates that this response was significantly different to baseline mucin secretion ($P = 0.0068$).

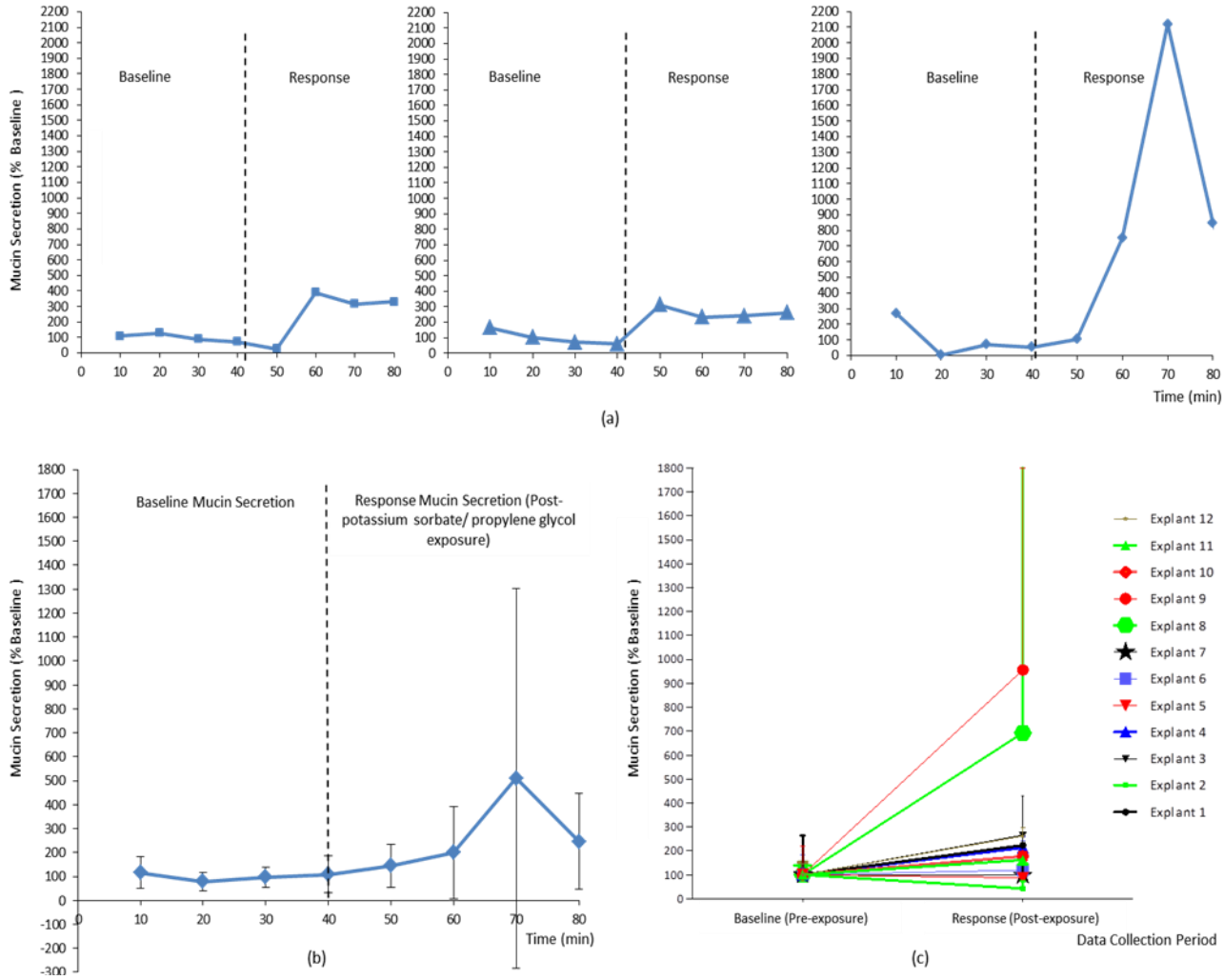


Figure 4.6 The Effect of Potassium Sorbate / Propylene Glycol (0.3 / 1.5 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to the combined exposure to potassium sorbate and propylene glycol. (b) The average mucin secretion response of 12 explants (3 animals) upon exposure to the two compounds (mean ± SD). (c) The four baseline mucin secretion measurements of each of the twelve explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.7 Effect of Polysorbate 80 on Ovine Mucin Secretion

The effect of polysorbate 80 (0.025 % w/w) on mucin secretion was studied in seven explants from three animals (Figure 4.7). An increase in mucin secretion, with a maximal response between a 1.3-fold and a 3.2-fold increase above the mean baseline secretion, was elicited in all explants. The mean response of the seven explants to polysorbate 80 exposure is shown in figure 4.7 (b) while figure 4.6 (c) demonstrates that this response was significantly different to baseline mucin secretion ($P = 0.0024$).

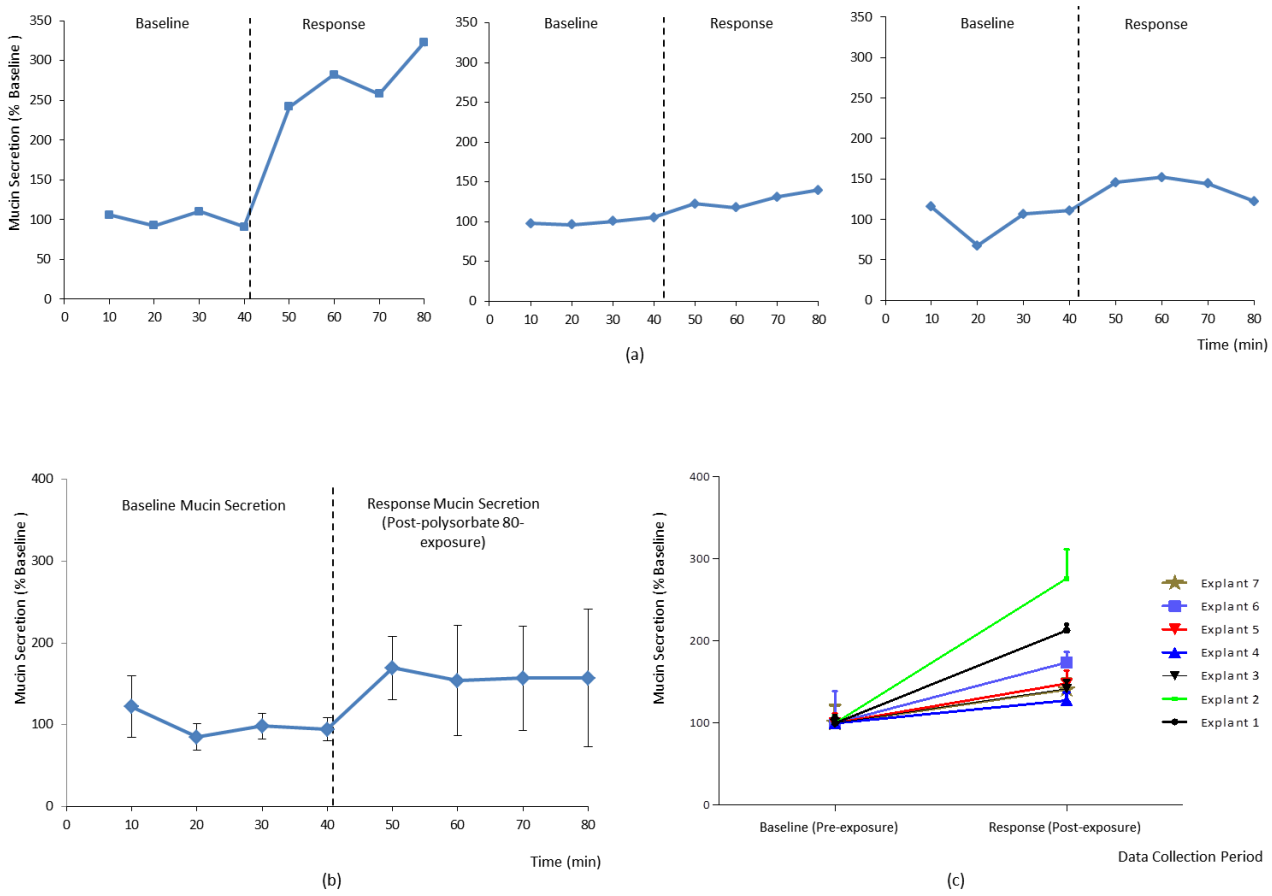


Figure 4.7 The Effect of Polysorbate 80 (0.025 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to polysorbate 80 exposure. (b) The average mucin secretion response of seven explants (3 animals) upon exposure to polysorbate 80 (mean ± SD). (c) The four baseline mucin secretion measurements of each of the seven explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.8 Effect of Methocel^{TM} on Ovine Mucin Secretion

The effect of Methocel (1 % w/w) on mucin secretion was studied in six explants from three animals. An increase in mucin secretion, with a maximal response ranging between a 1.6-fold and a 13-fold increase above the mean baseline secretion, was observed in all explants (Figure 4.8(a)). The mean response of the seven explants to Methocel exposure is shown in figure 4.8 (b) while figure 4.8 (c) demonstrates that this response was significantly different to baseline mucin secretion ($P = 0.0313$).

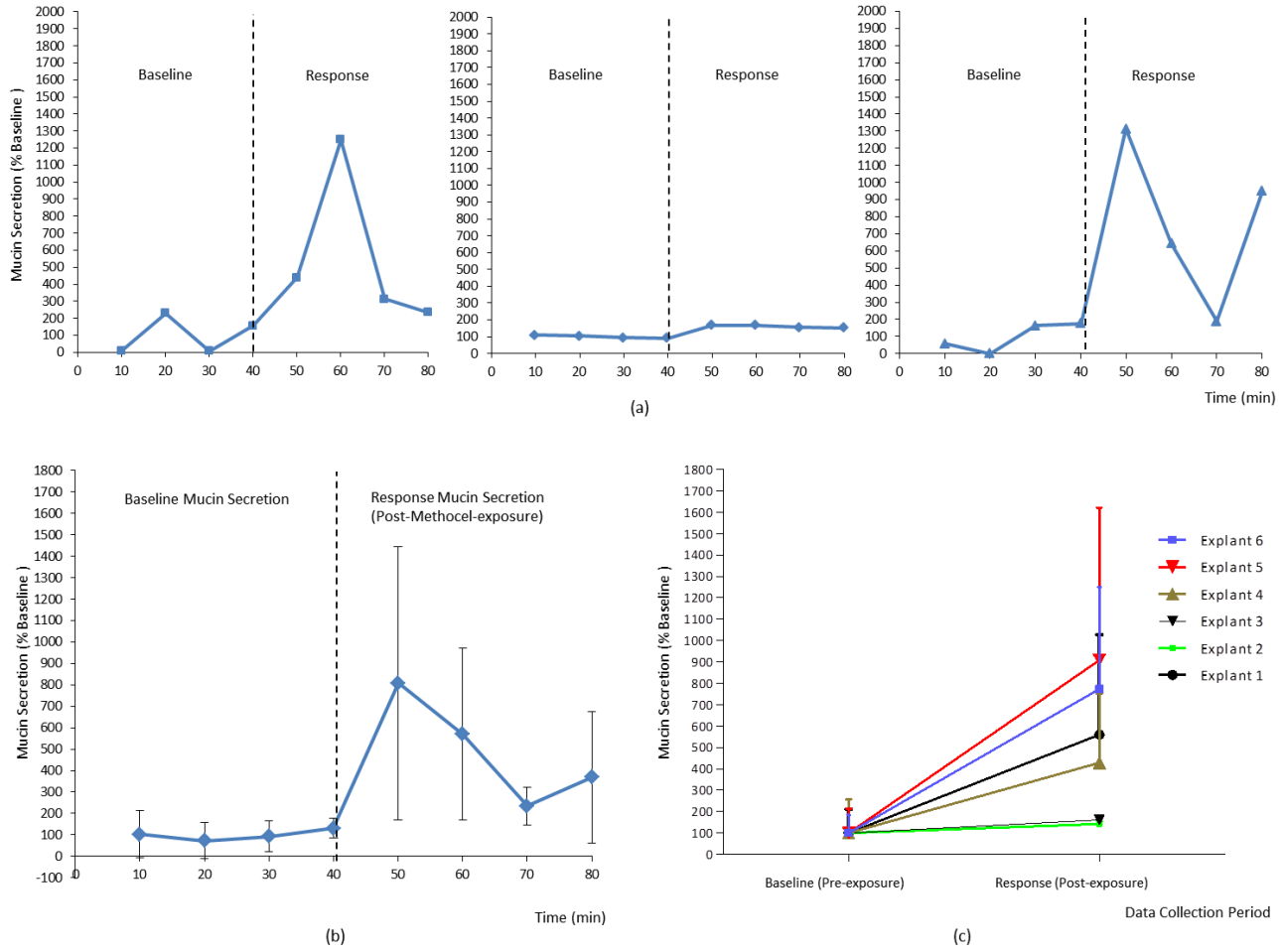


Figure 4.8 The Effect of Methocel^{TM} (1.0 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to Methocel^{TM} exposure. (b) The average mucin secretion response of six explants (3 animals) upon exposure to Methocel^{TM} (mean ± SD). (c) The four baseline mucin secretion measurements of each of the six explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.9 Effect of Avicel® RC591 on Ovine Mucin Secretion

The effect of Avicel® RC591 (1.5 % w/w) on mucin secretion was studied in eight explants from three animals (Figure 4.9). The explants' response to this exposure varied between a no-alteration in mucin secretion (observed in one explant (Figure 4.9(a), left plot)), an increase in mucin secretion (observed in four explants from two animals, with a maximal response ranging between 1.5-fold and 1.85-fold above the mean baseline secretion (Figure 4.9(a), middle plot) and a decrease (observed in three explants from two animals, with a maximal response ranging between 0.05-fold and 0.55-fold below the mean baseline secretion (Figure 4.9(a), right plot)) in mucin secretion. The mean response of the eight explants to Avicel® RC591 exposure is shown in figure 4.9 (b) while figure 4.9 (c) demonstrates that this response was not significantly different to baseline mucin secretion ($P = 0.3721$).

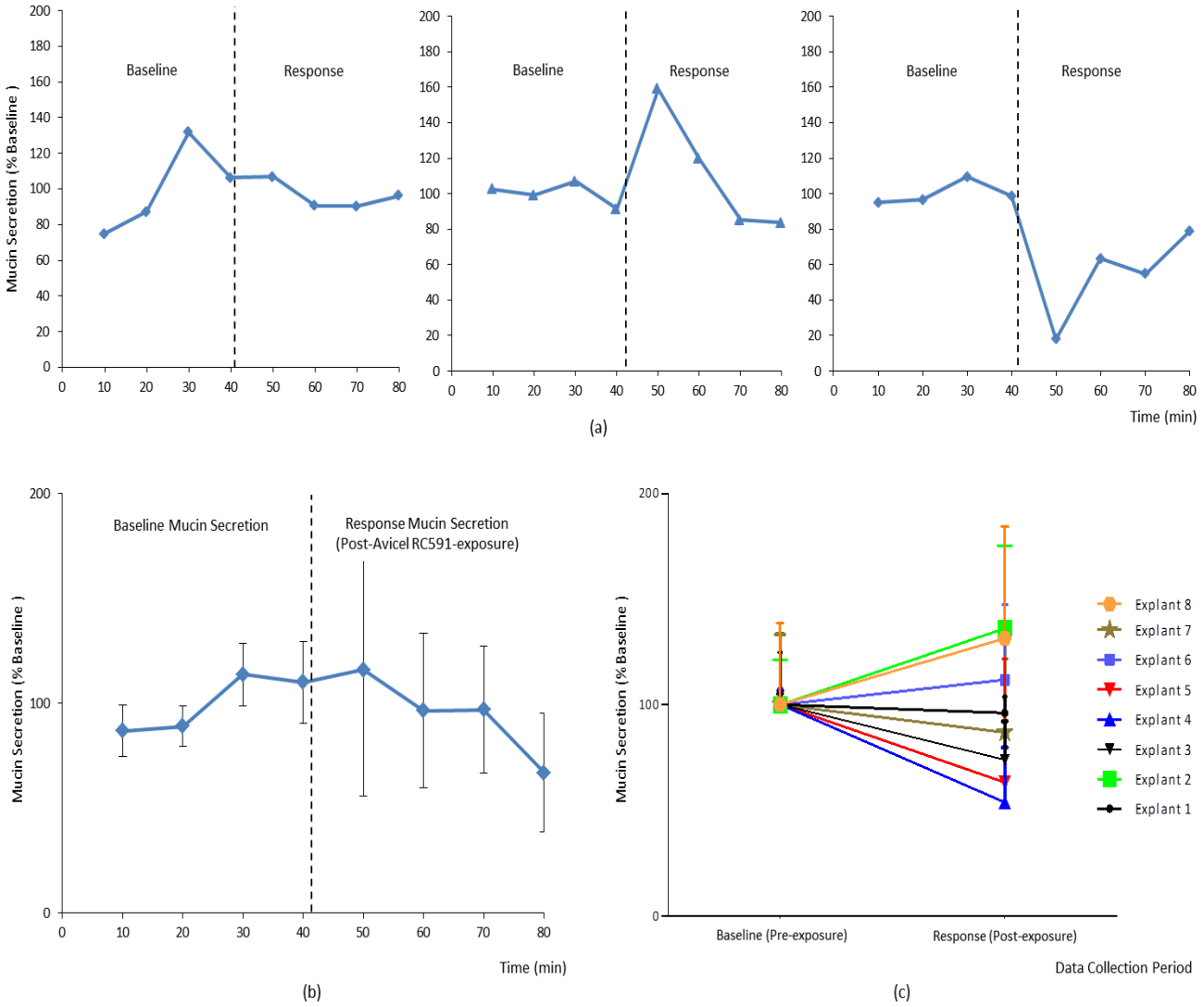


Figure 4.9 The Effect of Avicel® RC591 (1.5 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to Avicel® RC591exposure. (b) The average mucin secretion response of eight explants (3 animals) upon exposure to Avicel® RC591 (mean ± SD). (c) The four baseline mucin secretion measurements of each of the eight explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explants (mean + SD).

4.4.10 Effect of Fluticasone Furoate (FF) on Ovine Mucin Secretion

The effect of FF (0.0004 % w/w) on mucin secretion was studied in ten explants from four animals. The explants' mucin secretion response to this exposure varied between an increase (observed in seven explants from three animals, with a maximal response ranging between 1.9-fold and 3.9-fold above the mean baseline secretion (Figure 4.10(a), right and middle plot)) and a decrease (observed in three explants from the same animal with a maximal response ranging between 0.27-fold and 0.62-fold below the mean baseline secretion (Figure 4.10(a), left plot)). The mean response of the ten explants to FF exposure is shown in figure 4.10(b) while figure 4.10(c) demonstrates that this response was not significantly different to baseline mucin secretion ($P = 0.1307$).

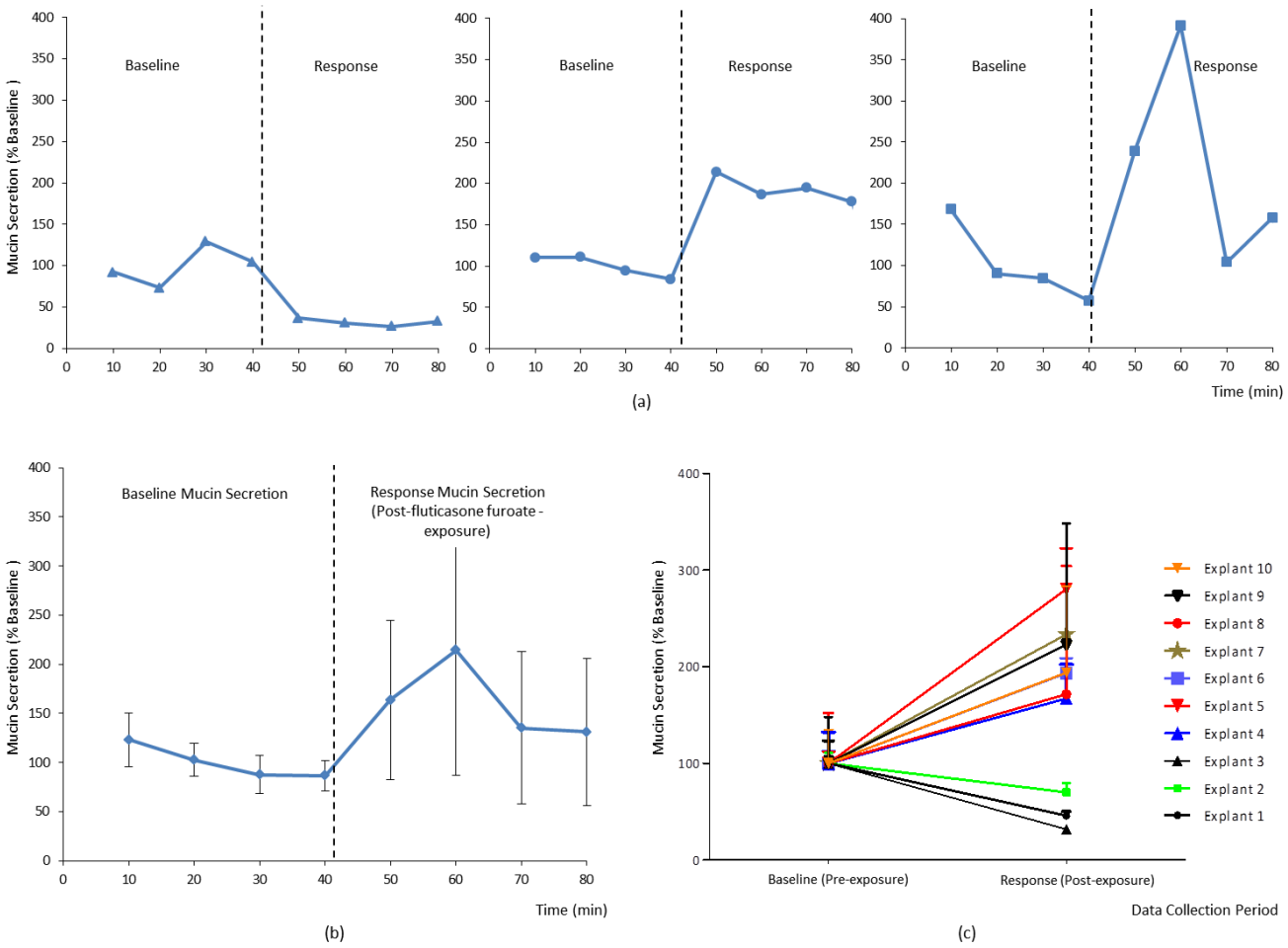


Figure 4.10 The Effect of Fluticasone Furoate (0.0004 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to fluticasone furoate exposure. (b) The average mucin secretion response of ten explants (4 animals) upon exposure to fluticasone furoate (mean ± SD). (c) The four baseline mucin secretion measurements of each of the ten explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.11 Effect of GSK1004723E on Ovine Mucin Secretion

The effect of GSK1004723E (0.01 % w/w) on mucin secretion was studied in six explants from three animals. An increase in mucin secretion, with a maximal response ranging between 2.3-fold and 16-fold above the mean baseline secretion, was observed in all explants (Figure 4.11(a)). The mean response of the six explants to this antihistamine is shown in figure 4.11 (b) while figure 4.11 (c) demonstrates that this response was significantly different to baseline mucin secretion ($P = 0.0313$).

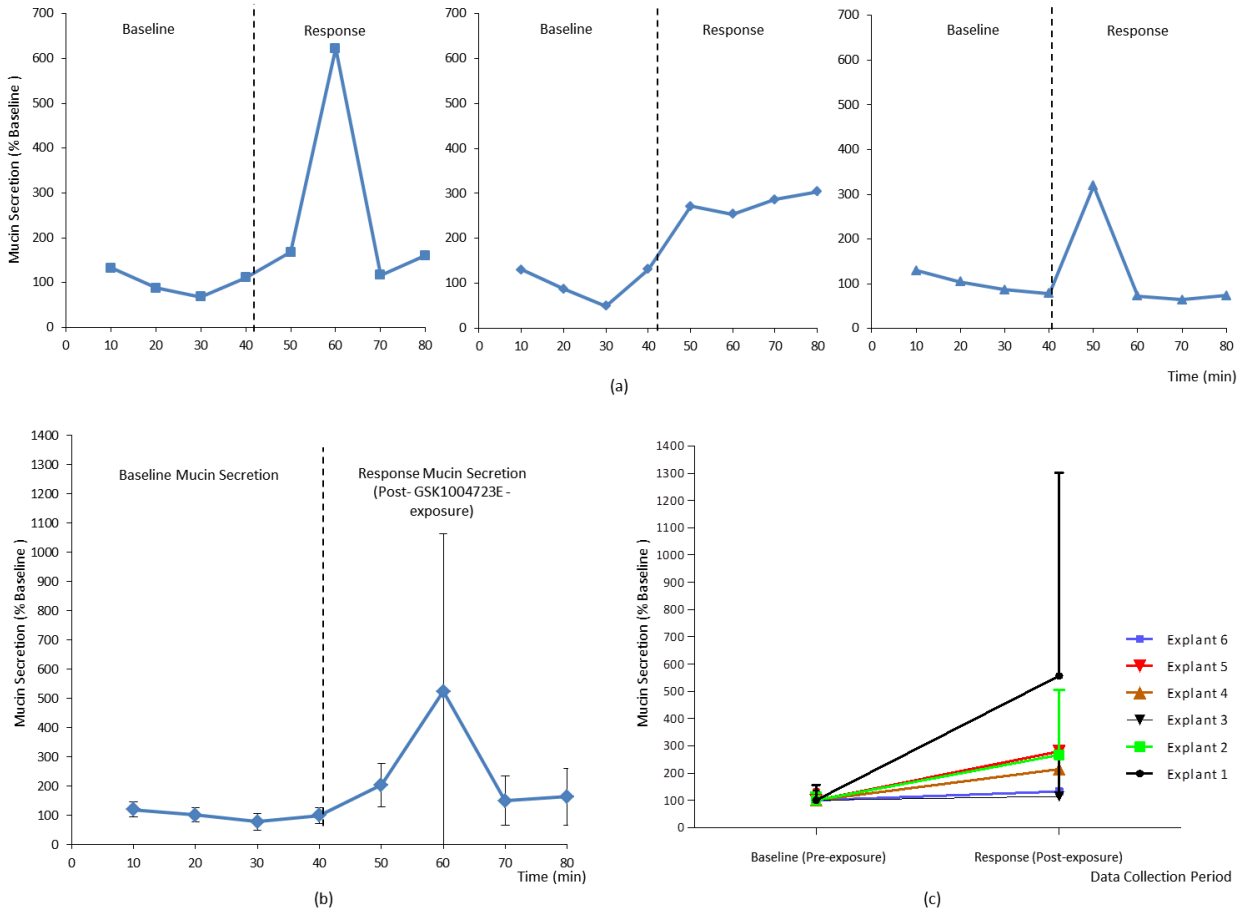


Figure 4.11 The Effect of GSK1004723E (0.01 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to GSK1004723E exposure. (b) The average mucin secretion response of six explants (3 animals) upon exposure to GSK1004723E (mean ± SD). (c) The four baseline mucin secretion measurements of each of the six explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.12 Effect of GSK1004723D on Ovine Mucin Secretion

The effect of GSK1004723D (0.01 % w/w) on mucin secretion was studied in nine explants from three animals. One explant demonstrated no change in the baseline mucin secretion following this exposure (Figure 4.12 (a), left plot) whereas three explants from two animals demonstrated subtle increases in mucin secretion (an example is shown in figure 4.12 (a), middle plot), which were mainly detectable by comparing the maximal post-exposure mucin secretion value to the last pre-exposure mucin secretion value (baseline value at 40 minute). A clear increase in mucin secretion, however, was observed in five explants (from three animals) with variable maximal response among the explants of between 2.5-fold and 3.5-fold above the mean baseline secretion (an example is shown in figure 4.12 (a), right plot). The mean response of the nine explants to this antihistamine exposure is shown in figure 4.12 (b) while figure 4.12 (c) demonstrates that this response was not significantly different to baseline mucin secretion ($P = 0.0784$).

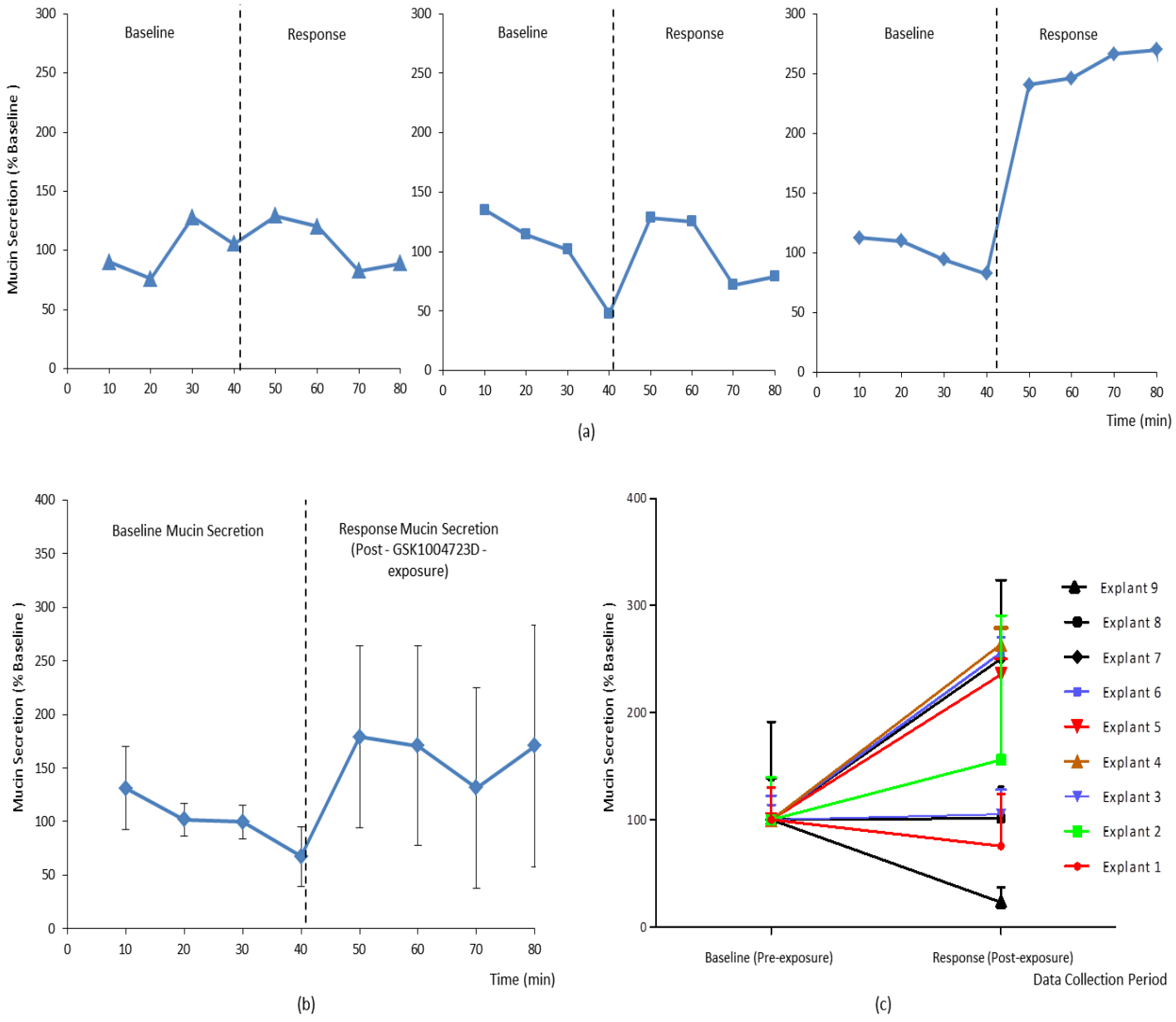


Figure 4.12 The Effect of GSK1004723D (0.01 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to GSK1004723D exposure. (b) The average mucin secretion response of nine explants (3 animals) upon exposure to GSK1004723D (mean ± SD). (c) The four baseline mucin secretion measurements of each of the nine explants tested were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant (mean + SD).

4.4.13 Effect of Dimethyl Sulfoxide (DMSO) on Ovine Mucin Secretion

Fluticasone furoate (FF) and GSK1004723E were solubilised in DMSO to give a final DMSO concentration of 0.2 % w/w. Therefore, the effect of DMSO (0.2 % w/w) on mucin secretion was studied in five explants from two animals. An increase in mucin secretion, to a maximum of a 1.46-fold increase above the mean baseline secretion, was observed in one explant (Figure 4.13 (a), left plot) whereas a decrease in mucin secretion, to a minimum of between 0.14-fold and 0.67-fold below the mean baseline secretion, was observed in four explants (Figure 4.13 (a), middle and right plots). The mean response of the five explants to DMSO exposure is shown in figure 4.13 (b) while figure 4.13 (c) demonstrates that this response was not significantly different to baseline mucin secretion ($P = 0.125$).

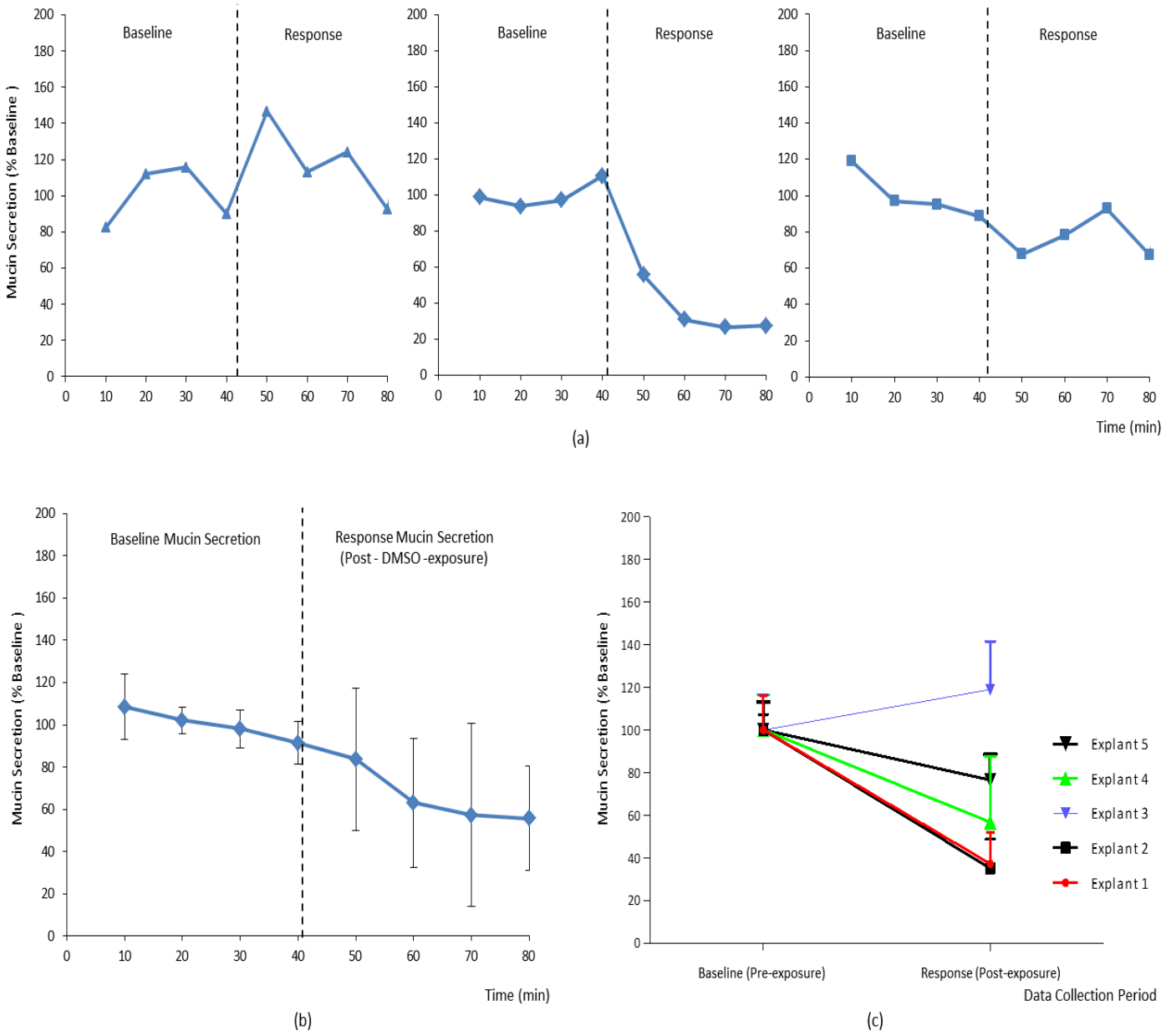


Figure 4.13 The Effect of DMSO (0.2 % w/w) on Ovine Mucin Secretion.

(a) Examples of the individual explant mucin secretion response to DMSO exposure. (b) The average mucin secretion response of five explants (2 animals) upon exposure to DMSO (mean ± SD). (c) For each of the five explants tested, the four baseline mucin secretion measurements were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements (mean + SD).

4.4.14 Summary of Findings

Table 4.3 summarises the findings in this chapter.

Table 4.3 Effect of the IN Pharmaceuticals on Ovine Mucin Secretion

The Compound	Effect on Mucin Secretion
BKC (0.015 % w/w)	Significant Increase
EDTA (0.015 % w/w)	Non-significant Increase
PG (1.5 % w/w)	Significant Increase
PS + PG (0.3 % w/w + 1.5 % w/w)	Significant Increase
Polysorbate 80 (0.025 % w/w)	Significant Increase
Methocel™ (1.0 % w/w)	Significant Increase
Avicel® RC591 (1.5 % w/w)	Non-significant Decrease
FF (0.0004 % w/w)	Non-significant Increase
GSK1004723 E (0.01 % w/w)	Significant Increase
GSK1004723 D (0.01 % w/w)	Non-Significant Increase
DMSO (0.2 % w/w)	Non-significant Decrease

4.5 Discussion and Conclusion

The effect of a number of intranasal pharmaceuticals on ovine mucin secretion was studied using ovine tracheal epithelial explants. The secretagogue ATP was used as a positive control [158, 274]. The data demonstrating the ATP-induced increase in mucin secretion appeared to be highly variable (big standard deviation error bars) at each of the time points following exposure to ATP (Figures 4.1 and 4.2). However, this can be explained by the variability in the maximal response to ATP among the explants (an increase of up to 10 times the mean baseline mucin secretion was observed). In addition, the exposure time required to induce that maximal response also varied among the explants i.e. some explants demonstrated the maximal response after 10 minutes exposure while others took longer to manifest this. These variables therefore explain the observed extent of variability (error bars) of the data, which however does not affect the significance of the observed effect.

It was noted that occasionally an animal yielded explants in which the ATP response (positive control) could not be detected in any of them regardless of how healthy the explants looked during the microscopical examination before using them the experiment. It is likely that there might be a problem with the detection rather than the secretion of mucins from such animals. In humans, HPA lectin has been reported to bind human mucins in persons with blood group A or AB and not B or O [214]. Similarly, ovine airway mucins appeared to possess some inter-subject variability, which might be linked to their blood group systems [275-280] and could have altered their binding ability to the HPA lectin used for their detection in the ELLA. Future work is warranted to investigate this theory.

Initial experiments showed that the direct ELLA was unsuitable to quantify mucin secretion in the presence of certain excipients due to interference of these excipients with the assay, which precluded examining the effect of Avamys® and the FF BKC-free IN formulations on ovine mucin secretion.

This is the first report studying the effect of the widely used preservative, BKC (0.015 % w/w), on mucin secretion. BKC elicited a significant increase in ovine mucin secretion, which was probably expected from the many in-vitro studies reporting its toxicity to various cell-types, cilia and mucociliary clearance [101, 104, 106-109, 114, 116, 123, 124, 128, 131, 281].

This study provided no evidence that EDTA (0.015 % w/w) affected respiratory mucin secretion, which was investigated here for the first time. This is consistent with the reported innocuous nature of EDTA to other components of the mucociliary system. For instance, Donnelley et al. and Epstein et al. found EDTA the least toxic, among other similarly used compounds, to rat nasal mucosa [282] and to corneoconjunctival cell lines [130] respectively. Batts et al. also did not find EDTA ciliotoxic in chicken embryo tracheae [114] nor compromising to mucociliary clearance in-vivo [118]. Moreover, Van de Donk et al. found EDTA only mildly and reversibly ciliotoxic in chicken embryo tracheae [113]. Nevertheless, a recent report associated EDTA with occupational rhinitis[112] indicating that further work is needed to assess the long term effects of EDTA.

Another novel finding here is the reported increase in mucin secretion elicited by the preservative, potassium sorbate (PS) (0.3 % w/w), in the presence of the cosolvent, propylene glycol (PG) (1.5 % w/w). PG helped solubilise sorbic acid (slightly soluble in water), the product of the conversion of PS in the test medium. PG was also observed to induce mucin secretion in the absence of PS, however with far less significance ($P = 0.0469$) than that induced by both PG and PS combined ($P = 0.0068$). It can be argued that the bigger sample size used to test the combined effect of the two compounds underpinned the observed higher significance. However, in four out of the ten explants showing an increase in mucin secretion in response to these combined compounds, the maximal increase was observed after only 10 minutes exposure, whereas PG on its own did not elicit the maximal increase after 10 minutes in any of the six explants that responded to it by an increase in mucin secretion. The maximal

response to PG was induced after 20-30 minutes exposure and was transient as explained in section 4.4.5. This suggests that PS synergised the increase in mucin secretion caused by PG leading to the appearance of a highly significant effect. Notably, Wang et al. [110] reported PS to cause an increase in rabbit tracheal CBF at the concentration level that was used here while Cho et al. [108] found PS to cause nasal epithelial lesions in rats.

The surfactant, polysorbate 80 (0.025 % w/w), was also demonstrated here, for the first time, to induce a significant, though mild (a 3.2-fold increase above the mean baseline secretion was the maximum observed response), increase in ovine mucin secretion. Similarly, Methocel™, a suspending agent comprising hydroxylpropyl methyl cellulose polymer, induced a significant increase in ovine mucin secretion.

It is, therefore, evident that excipients with surface active properties that were studied here (BKC, polysorbate 80 and Methocel™) stimulated an increase in ovine respiratory mucin secretion, which is consistent with the increase in mucin secretion induced by bile salts in different types of gastrointestinal epithelia [283]. To date, the mechanism of detergent-induced mucin secretion has not been elucidated although Klinkspoor et al. presumed it could be the epithelial cells response to protect itself against the detergent effect of surfactants [283, 284], which can cause cell lysis.

Avicel® RC591 (microcrystalline cellulose and sodium carboxymethylcellulose) demonstrated no effect on ovine mucin secretion. Notably, carboxymethylcellulose was reported to cause only mild inhibition of rabbit CBF after short-term exposure, although long-term effects are yet to be established [150].

Due to solubility issues, it was not possible to test fluticasone furoate (FF) at a concentration higher that 0.0004 % w/w (~ 4 µg/ml). FF formulation concentration is 0.05 % w/w in suspension. Nonetheless, the concentration of FF in artificial nasal fluid was 0.196 ± 0.006

µg/ml as reported by Baumann et al [285], which is much lower than the concentration tested here. It is therefore evident that studying the effects of FF at the concentration of 4 µg/ml is valid particularly in the absence of the in-vivo dilution effect of the airway secretions.

No significant alteration in ovine airway mucin secretion was detected here following the exposure to fluticasone furoate, which supports the notion that glucocorticoids like fluticasone furoate do not have a direct effect on the process of mucin production and secretion [246, 286]. Glucocorticoids are, however, effective in the treatment of hypersecretory conditions, particularly asthma, by blocking the inflammatory process that releases mucin stimulating mediators [46, 246, 260, 272, 287-289]. It is intriguing though that Mcgregor et al. reported potentiated mucin secretion in nasal lavage fluid of healthy subjects (in-vivo) following three weeks treatment with intranasal glucocorticoids [290]. This presumably contributed to the debate on the long term effects of these compounds [94], although Fokkens et al. have recently referred to the absence of deleterious effects on the respiratory mucosa following one-year exposure to fluticasone furoate [289].

Histamine, an inflammatory mediator, is known to stimulate mucin secretion [286, 291, 292]. Antihistamines are therefore expected to decrease stimulated mucin secretion in allergy models manifesting inflammation-induced mucin secretion [71]. Here, the ovine model was not challenged by any allergen, i.e. not an allergy model, and is therefore manifesting constitutive (baseline) mucin secretion, which plausibly justified the observed lack of significant alteration in ovine mucin secretion following exposure to the antihistamine, GSK1004723D (the water-soluble dihydrochloride salt). Interestingly, the antihistamine GSK1004723E (the water-insoluble naphthalene salt) elicited a significant increase in mucin secretion. To our knowledge, the definite explanation of this finding is not known, although it might be linked to naphthalene respiratory toxicity [293, 294].

Although DMSO is not a pharmaceutical excipient, it was used here at 0.2 % w/w to solubilise the two water insoluble APIs, fluticasone furoate and GSK1004723E. It was therefore necessary to assess its effect on mucin secretion in the absence of the drugs. No significant effect was detected during this exposure. Consistently, Pawsey et al. used DMSO (0.2 % w/w) as a solubiliser in their CBF analysis medium with no significant effect detected on CBF [164]. Nevertheless, it would be beneficial to expand the sample size of the explants exposed to DMSO, which could be addressed in future work.

In brief, the effect of a number of IN pharmaceuticals on airway mucin secretion has been reported here for the first time. Some compounds appeared to significantly affect ovine mucin secretion. Therefore, research was warranted to reveal which of these effects was sufficient to alter the mucociliary physiology (See chapter 5).

Chapter 5

Effect of the Intranasal Formulations on Ovine Tracheal Mucociliary Transport Rate (MTR)

5.1 Introduction

Healthy mucociliary clearance (MC) is the culmination of cilia beating effectively to transport mucus of appropriate quality (amount, depth and viscoelastic properties). Therefore, direct measurement of mucociliary clearance via measuring the mucus transport velocity, or mucociliary transport rate (MTR), is a marker of integrity of the mucociliary apparatus, which functions through healthy airway surface liquid and effective ciliary beat [34, 68]. Owing to the scarcity of *in-vitro* cell culture models with a well-characterised mucociliary system [44, 245, 295], *in-vivo*, *ex-vivo* and *in-situ* models have routinely been used for measuring MTR. For example, Sabater et al. conducted *in-vivo* mucociliary measurements in sheep to study its response to some drugs [153, 296] while De Oliveira-Braga et al. [297] and Ballard et al. [298] conducted similar studies of mucociliary measurements using *in-situ* murine bronchial and *ex-vivo* porcine tracheal models respectively. Moreover, *in-vivo* MTR measurements have been reported in rabbits [121, 149] and rats [147] in addition to humans [101, 102, 117, 118] whereas *ex-vivo* studies also used bovine tracheal explants [145], rat nasal septum [122], frog [116, 143, 144]/toad [146] palates and human inferior turbinate tissue [299].

Ex-vivo models often rely on using a tracer such as graphite or talc particles that can be visualised microscopically to measure the transport velocity on the airway epithelium [33, 297, 300]. On the other hand, human *in-vivo* measurements are often conducted using a dye and/or a saccharin particle that were placed on the inferior turbinate of the nasal cavity and the time they took to appear/ be tasted in the pharynx was used as an index to mucociliary transport velocity (saccharin transit time test [33, 44, 301]). *In-vivo* measurements were also conducted more objectively in humans using radiolabeled particles such as technetium-99, which were visualised on the airway epithelium using gamma camera to estimate the transport velocity (gamma scintigraphy[33, 44, 302]). Moreover, radio-opaque Teflon particles have also been used

in-vivo, which were visualised using an image intensifier [33]. Notably, *in-vivo* measurements have also been conducted in animals by similar techniques using fluorescently labelled microspheres [147] and gamma scintigraphy [149].

The preservative, BKC, was previously studied using both *in-vivo* and *ex-vivo* models demonstrating conflicting effects on MTR. With only a few exceptions [92, 101] , human *in-vivo* studies demonstrated no safety concern over the use of BKC in IN formulations [102, 103, 118, 303], which agreed with some animal *in-vivo* studies [121, 304] as reviewed by Marple et al. [105]. Conversely, studies using *ex-vivo* models of frog and rat demonstrated compromised mucus transport velocity in response to BKC exposure [116, 122], which was also found detrimental to respiratory mucosa *in-vitro* using human tissue [104] and *in-vivo* using rat tissue [109, 305].

Only a few investigations have studied the effect of EDTA on MTR, which reported deleterious effects only when a frog *ex-vivo* [116] rather than a human *in-vivo* [117-119] model was used.

Cellulose derivatives such as Methocel™ have conventionally been used in the IN formulation as viscosity enhancers. Some studies assessed the effects of these compounds on MTR demonstrating a consistent compromise in MTR *in-vivo* and *ex-vivo* both in human and animal models [117, 142-147], which explains their role in increasing the nasal residence time of IN formulations and hence in enhancing the IN efficacy [306].

With the exception of BKC, EDTA and cellulose derivatives, there is a gap in the literature studying the effect of the pharmaceuticals investigated here on MTR (see table 1.2). This chapter thus aimed to research these effects on ovine MTR in excised ovine tracheae (*ex-vivo* model) using carbon particles as a tracer, which was visualised under a light microscope equipped with an eyepiece graticule.

5.2 Materials

- Gassed Krebs - Henseleit buffer (KH):
    1) Sodium chloride (NaCl) analytical grade - Fisher Scientific (S/3160/65).
    2) Potassium chloride (KCl) - Sigma (P9333).
    3) Magnesium sulfate ($MgSO_4$) - Fisher Scientific (M/1050/53).
    4) Calcium chloride ($CaCl_2$) - Fisher Scientific (C/1400/53).
    5) Potassium dihydrogen phosphate (KH_2PO_4) analytical grade - Fisher Scientific (P/4800/53).
    6) Sodium hydrogen carbonate ($NaHCO_4$) - Fisher Scientific (S/4200/60).
    7) D-Glucose - Sigma (G8270).
- Carbon particles (Carbon, glassy, spherical powder, 2-12 µm) - Sigma (484164).
- Trough (reagent reservoir) - Fisher Scientific (PMP-210-050S)
- D-glucose - provided by GSK (see section 4.2.4).
- D-glucose - Sigma (G8270).
- The test formulations/excipients/APIs - provided by GSK (see section 4.2.4).
- Nasal spray bottles - provided by GSK

5.3 Methods

5.3.1 Development of an MTR Measuring Approach

5.3.1.1 Optimisation of Pre-experimental Tissue Manipulations

Sheep tracheae (~ 7 cm length from the middle of each trachea) were collected from a local abattoir, each contained in a humidified 50 ml centrifuge tube, which was then either transported at room temperature or in a vacuum flask containing water at 37 °C. Once in the lab, the trachea-containing tubes that were collected at 37 °C were maintained in a water bath

set to this temperature. During this early stage of developing an MTR measuring protocol, all tracheae were also washed with fresh KH at 37 °C before being used in any experiment, a procedure that was later excluded in the final MTR measuring approach (section 5.3.2). Tracheae were then cut longitudinally along the posterior membrane and trimmed when needed to form a semi-cylindrical segment. This was laid in a trough, which was then placed in the measuring chamber (Figure 5.1) where the trachea was maintained in a humidified environment at 37 ^{o}C using a hot water circulator (Harvard Thermocirculator, Harvard Apparatus Ltd, Fircroft Way, Edenbridge, Kent). MTR was then studied by sprinkling carbon particles on the ovine epithelium and observing their rate of transport over different areas of the epithelium using a dissecting microscope fitted with an eyepiece graticule (Bausch & Lomb Stereozoom® 4, Bausch & Lomb Inc., Rochester 2, N.Y., U.S.A). The time taken for a carbon particle to move 5 mm was recorded and later converted to transport rate (mm/min).

The effect of different collection/transportation methods of ovine tracheae, from the local abattoir to the School laboratories, on baseline MTR was studied. Five different collection environments, namely MEM at ambient temperature, KH (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.25 mM CaCl2, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose; gassed with 95 % O_2 : 5 % CO_2 for 5 minutes before use) at ambient temperature, humidified atmosphere at ambient temperature, KH at 37 ^{o}C and humidified atmosphere at 37 ^{o}C, were compared.

The effect of two x 10 minute applications of KH on the baseline MTR following an initial 20-40 minutes stabilisation time was also examined.

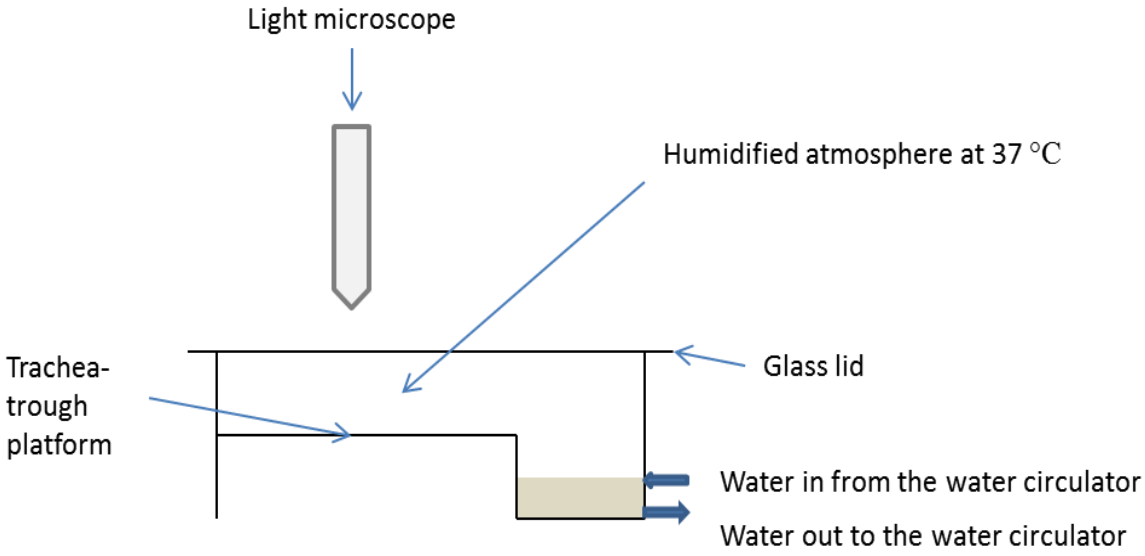


Figure 5.1 Diagram of the MTR Measuring Chamber (side view).

5.3.2 Preliminary Experiment

Preliminary experiments on the effect of Avamys® on MTR were also carried out. Following 40 minute equilibration, the ovine epithelium was covered with 5 % w/w glucose (as a control) and left for 10 minutes (exposure time). The 5 % w/w glucose solution was then removed and MTR was measured over a 10-minute period. The formulation was then applied and left for 10 minutes, after which it was removed and the MTR measured for a 10-minute period. To examine the tissue recovery, if applicable, the 5 % w/w glucose solution was applied again for 10 minutes before recording MTR measurements for a final 10 minutes.

5.3.3 Measuring MTR

Ovine tracheae, each contained in a humidified 50 ml centrifuge tube and maintained at 37 ºC after excision, were cut longitudinally along the posterior membrane, laid in a trough with epithelial side up and placed inside the measuring chamber (Figure 5.1). The epithelial lumen was then covered with 5 % w/w glucose solution (~ 3 ml) for 10 min, after which the solution was removed by gentle pipette-suction, carbon particles were sprinkled on the epithelium and MTR measurements were made from different areas of the epithelium over a 10-minute period to check tissue-viability. Each measurement was recorded as the distance moved by a carbon particle in ten seconds, which was later converted to MTR (mm/min). These measurements were then followed by another 10 minutes when the ovine epithelium was again covered with 5% w/w glucose solution, prior to this solution being removed and MTR measurements being made for a further 10 minutes (This was the baseline set of measurements made after a 30-minute equilibration time). The ovine epithelium was then exposed to the test formulation/excipient/API for 10 minutes before the test solution was removed and MTR measurements were made for another 10 minutes (This was the first set of response measurements). This exposure – measurement cycle (total of 20 min) was then

repeated once more for the test solution (second set of response measurements) before either finishing the experiment at that point or applying 5 % w/w glucose solution for 10 minutes followed by a final 10 minute period of measuring MTR. The latter was adopted to test the tissue recovery in cases when a compromise in MTR following exposure to the test formulation/excipient/ API was clearly observed.

Studying the effect of each preparation on MTR was repeated using the above protocol with the alteration of the treatment application method. Three sprays (~ 0.115 ml) of each test preparation was applied to the tissue using a nasal spray bottle instead of the three ml used above to fill the tracheal lumen.

5.3.4 The Test Formulations/Excipients/APIs

All preparations were applied to the ovine tracheae at 37 °C. They were all prepared at their working formulation concentration (see section 4.2.4) in 5 % w/w glucose (Table 5.1).

Table 5.1 Compounds Studied for their Effect on Ovine Airway MTR.

The Excipient/ API	Concentration in 5 % w/w glucose
BKC	0.015 % w/w
EDTA	0.015 % w/w
Propylene glycol (PG)	1.5 % w/w
Potassium Sorbate (PS)	0.3 % w/w (in presence of 1.5 % w/w propylene glycol as a cosolvent)
Polysorbate 80	0.025 % w/w
Methocel E50LV premium	1.0 % w/w (To achieve optimum dispersion, the polymer was initially wetted with 40-50 % of the calculated amount of water and agitated at 60-70 °C for ~ 1 hr using a magnetic stirrer before adding the glucose and making up to the final volume)
Avicel RC591	1.5 % w/w (To achieve optimum dispersion, the polymer was initially homogenised in 40-50 % of the calculated amount of water before adding the glucose and making up to the final volume)
Fluticasone furoate (FF)	0.0004 % w/w FF was initially solubilised in DMSO (1 part in 500 parts; sparingly soluble). The DMSO containing the API was then solubilised in 5 % w/w glucose at 0.2 % w/w.
GSK1004723D (dihydrochloride salt)	This drug was tested at two concentration levels: 0.01 % w/w And 0.5 % w/w
GSK1004723E (naphthalene salt)	0.01 % w/w The antihistamine API; GSK1004723E, was solubilised in DMSO (1 part in 20 parts), before solubilising the DMSO containing the API in 5 % glucose solution at 0.2 % w/w DMSO concentration.
DMSO	0.2 % w/w

5.3.5 Data Analysis

The statistical analysis was conducted using GraphPad Prism 5 software. For each trachea, the mean of the baseline set of measurements, measured during the 30 - 40 minute period of the experiment (before exposure to the treatment but after the initial 30-minute equilibration time that was started and finished by a 10-minute exposure to 5 % w/w glucose solution), was paired to the mean of the pooled sets of response measurements that were measured after exposure to the test solution (the first and second sets of response measurements were measured during the 50 - 60 minute and the 70 - 80 minute time periods of the experiment respectively). For each group of tracheae exposed to the same preparation, the paired data was compared using a paired t-test ($P \leq 0.05$).

Occasionally, the significance of the response of each individual trachea was required to be assessed (see section 5.5). Unpaired t-test was used to compare the baseline set of measurements (taken just before exposure to the treatment) to the pooled sets of response measurements (the first and second sets of response measurements that were measured after exposure to the test solution). The threshold of statistical significance was set at $P \leq 0.05$.

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5.4 Results

5.4.1 Development of an MTR Measuring Approach

5.4.1.1 Optimisation of Pre-experimental Tissue Manipulations

MTR studies required viable respiratory tissue manifesting functional mucociliary clearance. Tracheae collected at ambient temperature failed to show any transport of carbon particles on their tracheal epithelium regardless of the collection medium. In contrast, tracheae collected at 37 °C demonstrated mucociliary transport of carbon particle on their tracheal epithelium (Figure 5.2), although the tissue viability time (time from animal killing till mucociliary transport halt, which includes tissue collection/ transportation time and MTR experiment time) was variable.

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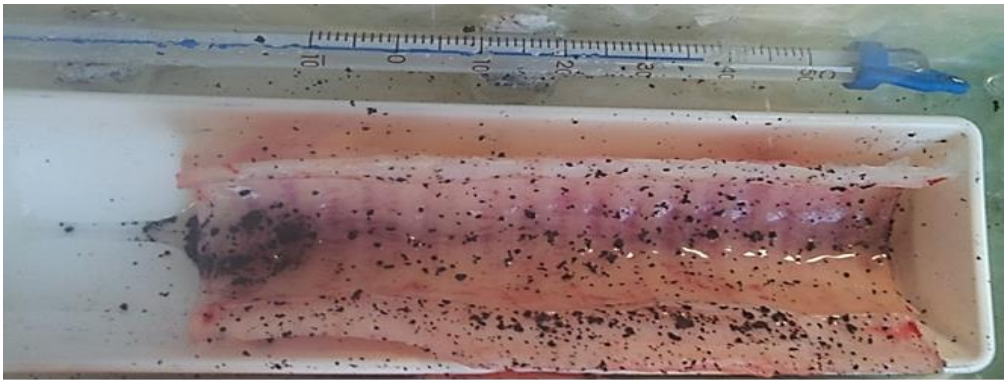
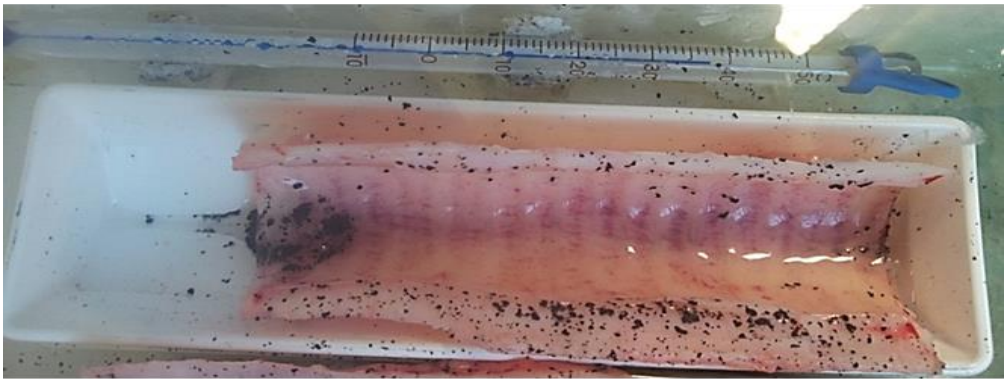


(a)



(b)

Figure 5.2 An Illustration of Mucociliary Clearance in Viable Ovine Tracheae.

(a) A sheep trachea, collected in humidified air at 37 °C, was mounted in the measuring chamber and pictured immediately after applying carbon particles. (b) The same trachea in (a) was pictured a few minutes after applying the carbon particles, demonstrating the transport of carbon particles via mucociliary clearance.

Collection in humidified air at 37 °C proved to be superior to collection in gassed KH at 37 °C (Figure 5.3) as the maximum viability time observed with the former was ~6.75 h compared to 3.5 h with the latter (data drawn from seven tracheae collected in gassed KH at 37 °C on two days and four tracheae collected in humidified air at 37 °C on two days). This was then confirmed by collecting six more tracheae in humidified air at 37 °C on another day, which showed the viability time to be between 2.75 h and 6.75 h. Collection in humidified air at 37 °C was then deemed optimum and adopted for all subsequent work in this project.

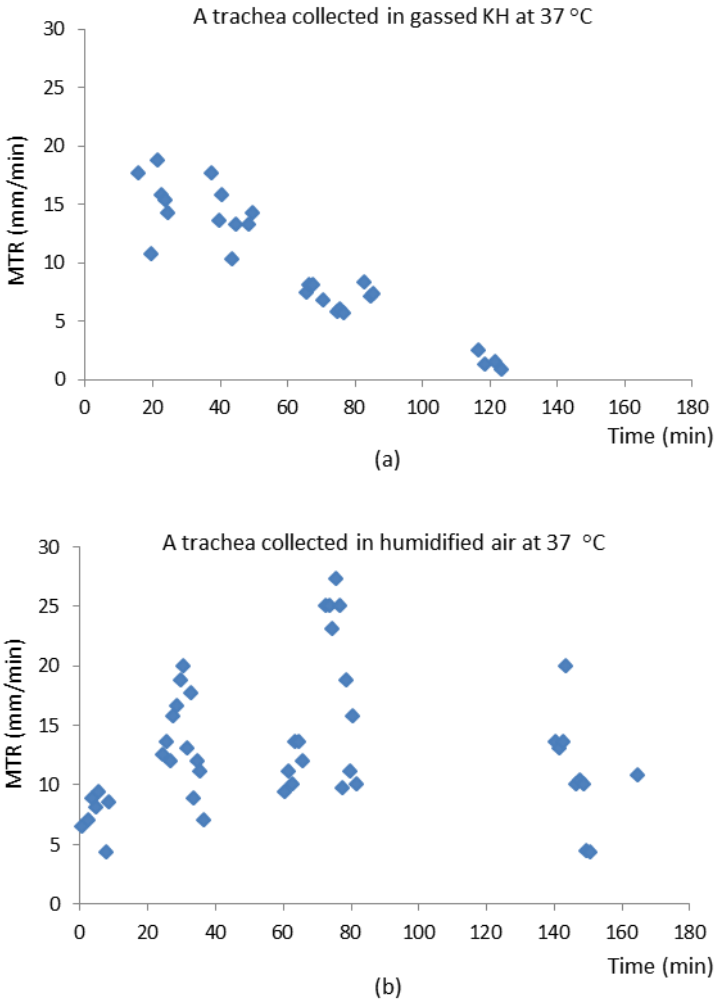


Figure 5.3 Maintaining the Viability of Ovine Tracheae Post-excision.

(a) An example of ovine MTR profile measured in a trachea collected two hours prior to examination in gassed KH at 37 °C. (b) An example of ovine MTR profile measured in a trachea collected three hours prior to examination in humidified air at 37 °C. In the laboratory, tracheae collected in KH were transferred to tubes of fresh KH at 37 °C and the tubes were maintained at 37 °C till mounting in the measuring chamber. Tracheae collected in humidified air at 37 °C were maintained at 37 °C till just before mounting in the measuring chamber when they were washed with fresh KH at 37 °C. Measurements were made from different spots on the trachea for 10 minute periods, which was repeated every ~15 minutes. No equilibration time was allowed in these tissue viability experiments. A decline in viability was observed in the top plot as demonstrated by the decreasing MTR values. On the contrary, the bottom plot showed sustained MTR during the time of examination.

Experiments studying the effect of exposure to KH for 10 minutes showed a transient increase in MTR following this exposure (Figure 5.4), which took a maximum of 30 minutes to return to baseline after removal of the KH. An equilibration time of 40 minutes after mounting in the measuring chamber was then considered appropriate for the preliminary experiments.

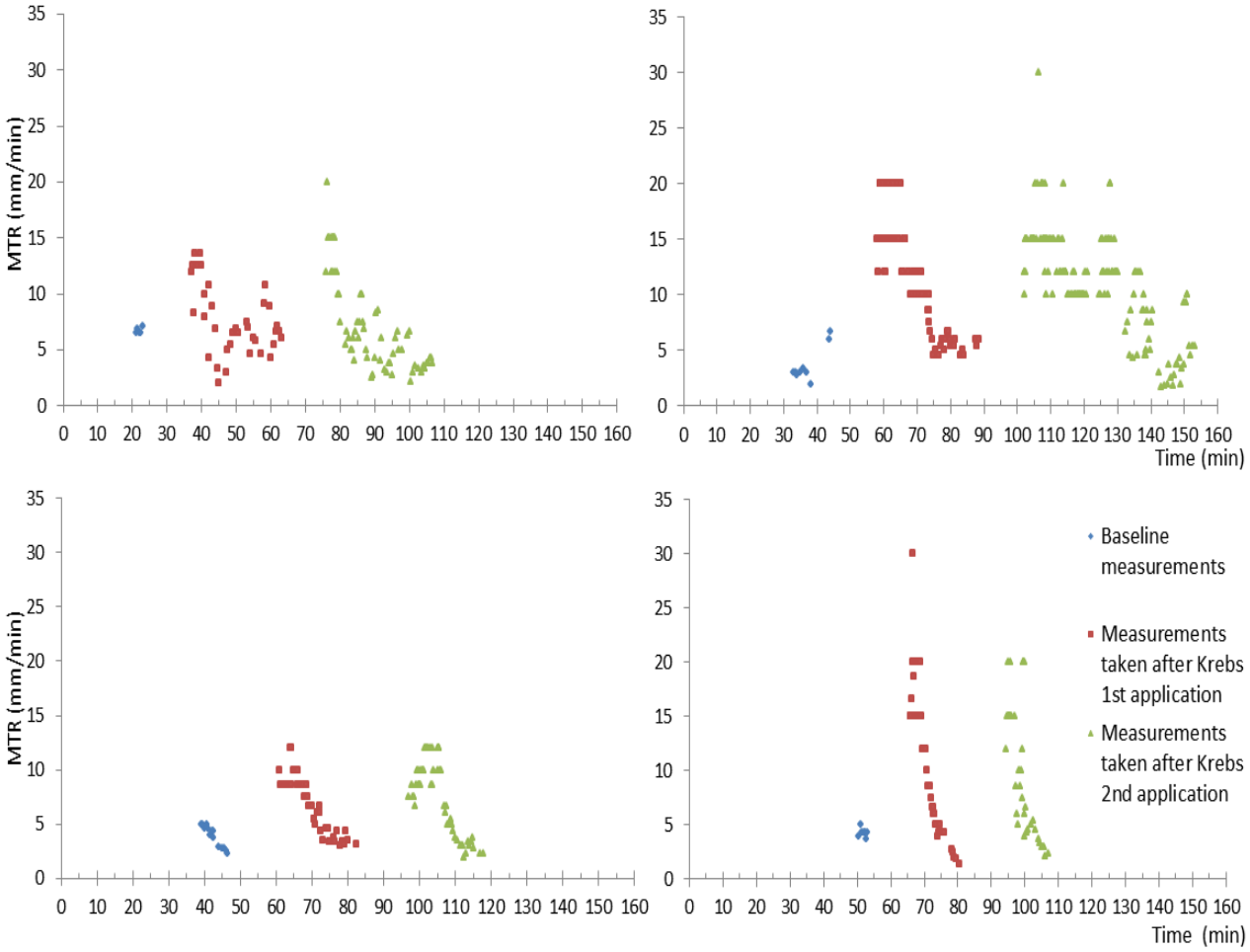


Figure 5.4 The Effect of KH on Ovine MTR.

Four examples of individual tracheal response to KH are presented here. Sheep tracheae were collected in humidified air at 37 °C (n = 9, collected over four days), rinsed with KH and mounted in the MTR measuring chamber. A 20-40 minutes stabilisation time was allowed. Baseline MTR was then measured prior to exposing the tracheae to KH (the lumen of the tracheae was filled with 2-3 ml) for 10 minutes. KH was then removed by gentle pipette-suction and MTR was measured. Upon return of the MTR to baseline, a second application of KH was made for 10 minutes, after which the KH was removed and MTR was measured until it returned to baseline values. The duration of the observed transient boosting effect of KH on the MTR varied between tracheae in the range of five to thirty minutes, after which the MTR returned to baseline values. It is worth noting that in this group of experiments the MTR was exceptionally measured from a single spot throughout the experiment.

5.4.1.2 Preliminary Data

Early experiments demonstrated a decline in MTR upon exposure to Avamys® (2-3 ml to fill the tracheal lumen) for 10 minutes (Figure 5.5). However, the significance of this effect cannot be judged due to the small sample size (n = 2).

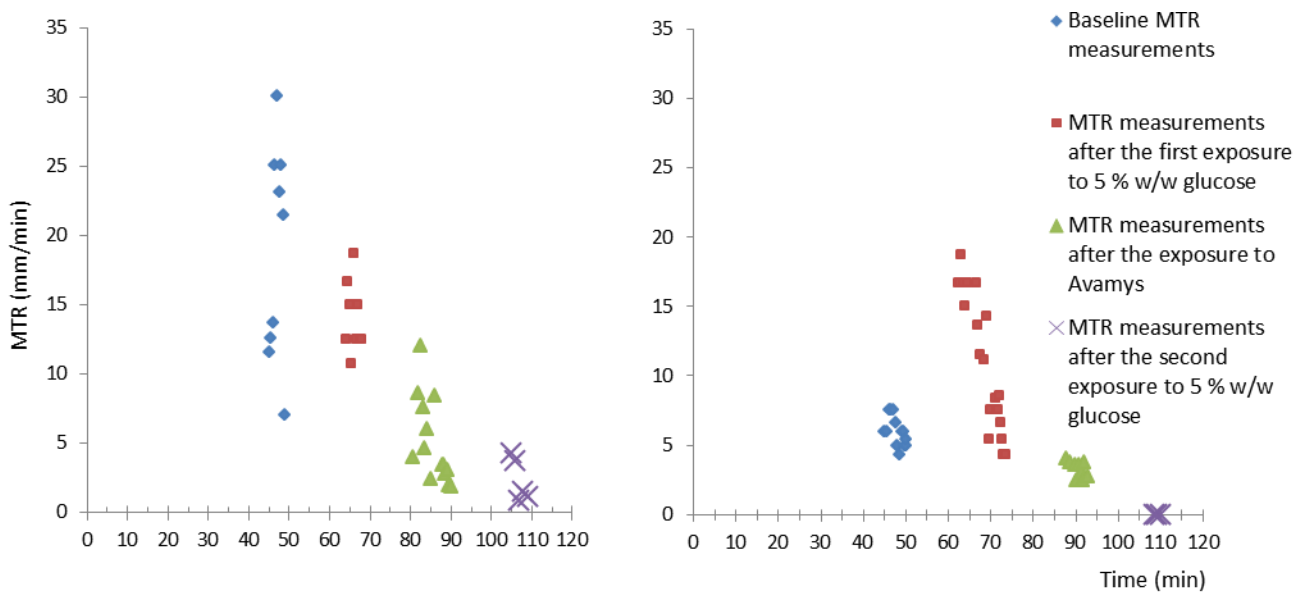


Figure 5.5 The Effect of Avamys® on Ovine MTR.

Examples of individual tracheal response to Avamys® are shown here. Sheep tracheae collected in humidified air at 37 °C (n = 2) were rinsed with KH and mounted in the MTR measuring chamber. A 40-minute equilibration time was allowed. Baseline MTR was then measured prior to exposing the tracheae to 5 % w/w glucose (a 2-3 ml volume to fill the lumen of the tracheae) for 10 minutes. The 5 % w/w glucose was then removed by gentle pipette-suction and MTR was measured. This was followed by exposing the tissue to Avamys® for 10 minutes, which was then removed by gentle pipette-suction and MTR measured. A second exposure to 5 % w/w glucose for 10 minutes was then made, followed by removal by gentle pipette-suction and measuring MTR.

5.4.2 Effect of the Excipients/APIs on MTR

5.4.2.1 Effect of Glucose (5 % w/w) on Ovine MTR

Six ovine tracheae were exposed to 5 % w/w glucose solution (~3 ml were applied to fill the tracheal lumen). No alteration in MTR was observed in three tracheae following this exposure (examples are shown in figure 5.6 (a), middle and right plots) although decreased MTR was detected in the other three tracheae after the third 10-minute exposure to this solution (an example is shown in figure 5.6 (a), left plot), which reached a partial halt in mucociliary transport (i.e. a standstill affecting only some areas on the tracheal mucosa) in one of these tracheae. It is worth noting that MTR measurements after the third and fourth 10-minute exposures were referred to here as first and second sets of response measurements respectively to simulate all experiments to-follow, in which various excipients/APIs were studied using 5 % w/w glucose vehicle, which was used as blank for measuring baseline MTR following the second 10-minute exposure period while the test compounds were introduced in the third 10-minute exposure period, after which response measurements were made.

Figure 5.6 (b) illustrates the response of the six tracheae to glucose (5 % w/w) exposure whereas figure 5.6 (c) demonstrates that this response was not significantly different to baseline MTR ($P = 0.1004$).

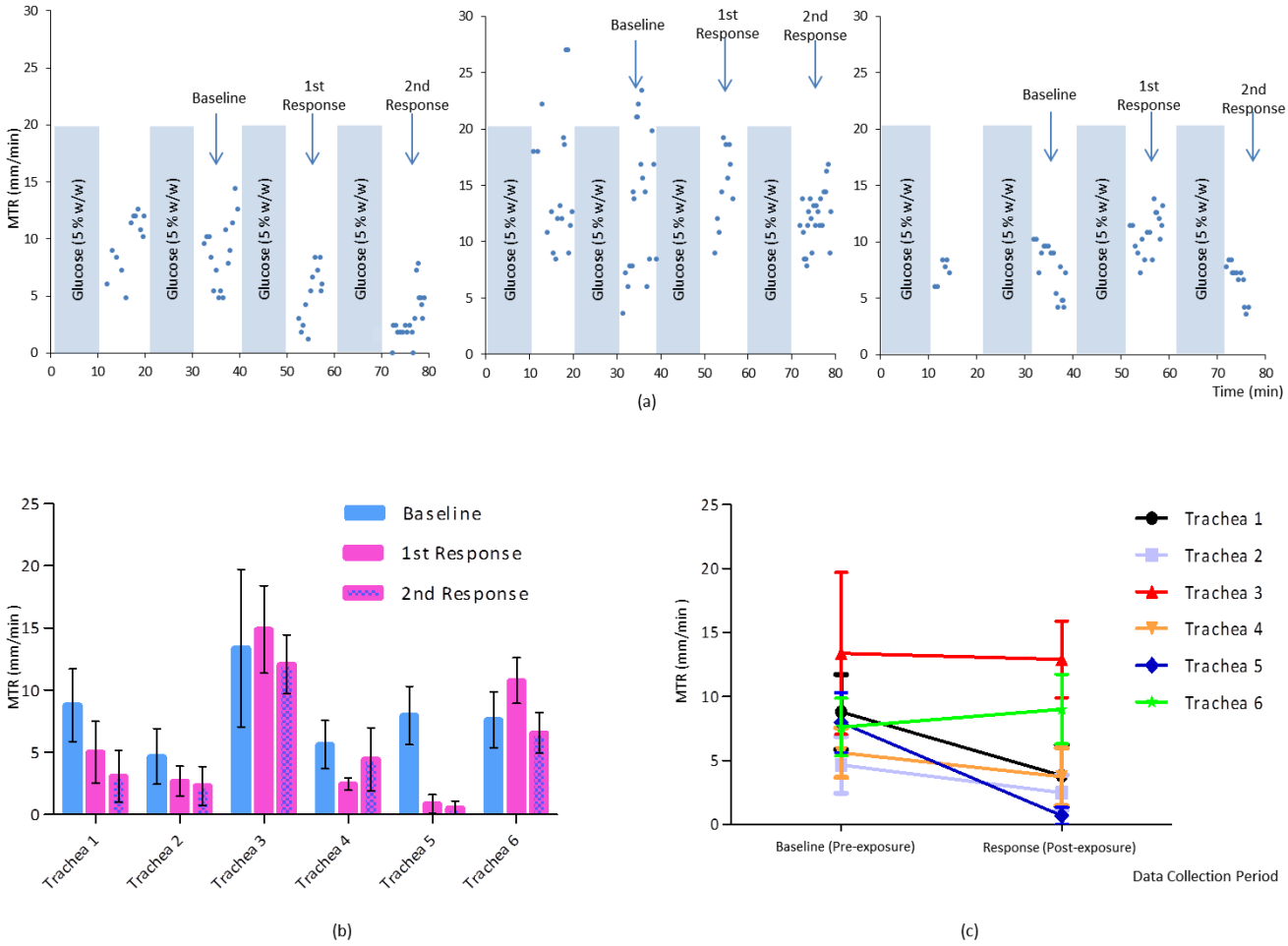


Figure 5.6 The Effect of Glucose (5 % w/w) on Ovine MTR.

(a) Examples of the individual tracheal MTR response to 5 % w/w glucose solution (~ 3 ml). The solid bars indicate the 10-minute periods of exposure to the glucose solution while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to glucose (5 % w/w) as studied in six tracheae (mean ± SD). (c) For each of the six tracheae exposed to glucose, both the first and the second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

Similarly, when six ovine tracheae were exposed to sprayed 5 % w/w glucose solution (three sprays per trachea; ~ 0.115 ml), three tracheae showed no alteration in MTR (Figure 5.7 (a), middle and right plots), whereas three tracheae showed decreased MTR following the third 10-minute exposure to this spray (Figure 5.7 (a), left plot). Figure 5.7 (b) illustrates the response of the six tracheae to this exposure whereas figure 5.7 (c) demonstrates that this response was not significantly different to baseline MTR ($P = 0.1745$)

It was therefore evident that the 5 % glucose solution was an appropriate blank for measuring baseline MTR and was hence used as a vehicle for the compounds studied here.

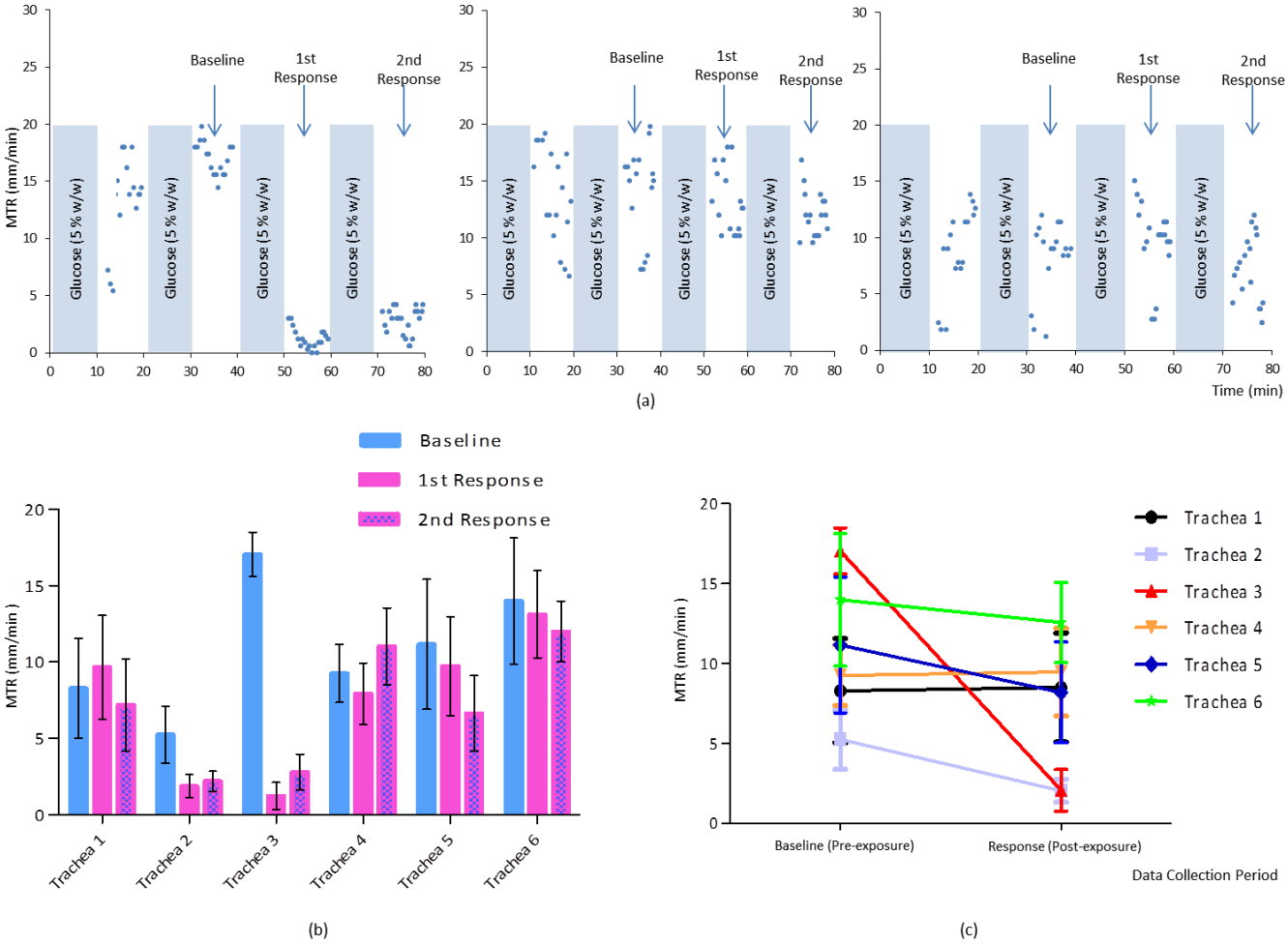


Figure 5.7 The Effect of Sprayed Glucose (5 % w/w) on Ovine MTR.

(a) Examples of the individual tracheal MTR response to 5 % w/w glucose spray (~ 0.115 ml). The solid bars indicate the 10-minute periods of exposure to the glucose spray while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to glucose (5 % w/w) as studied in six tracheae (mean ± SD). (c) For each of the six tracheae exposed to sprayed glucose, both the first and the second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.2 Effect of Benzalkonium Chloride (BKC) on Ovine MTR

When the ovine tracheal mucosa was bathed in ~3 ml of BKC solution (0.015 % w/w), mucociliary clearance halted completely in all tracheae tested. With the exception of one trachea that showed some recovery of MTR following the exposure to 5 % glucose solution (Figure 5.8 (a), left plot), the deleterious effect of BKC was irreversible (Figure 5.8 (a), right and middle plots). Figure 5.8 (b) summarises the response of the six tracheae to BKC exposure, which induced significant alteration to baseline MTR ($P = 0.0011$) as demonstrated in figure 5.8 (c).

Interestingly, when six tracheae were exposed to sprayed BKC solution (0.115 ml), three tracheae demonstrated no alteration in baseline MTR (Figure 5.9 (a), left plot) whereas one trachea showed a decrease in MTR (Figure 5.9 (a), middle plot) and two tracheae manifested a reversible halt in mucociliary transport (Figure 5.9 (a), right plot). The effect of sprayed BKC on ovine MTR is summarised in figure 5.9 (b), while figure 5.9 (c) demonstrates that the observed decrease in MTR, compared to baseline, was significant ($P = 0.0459$).

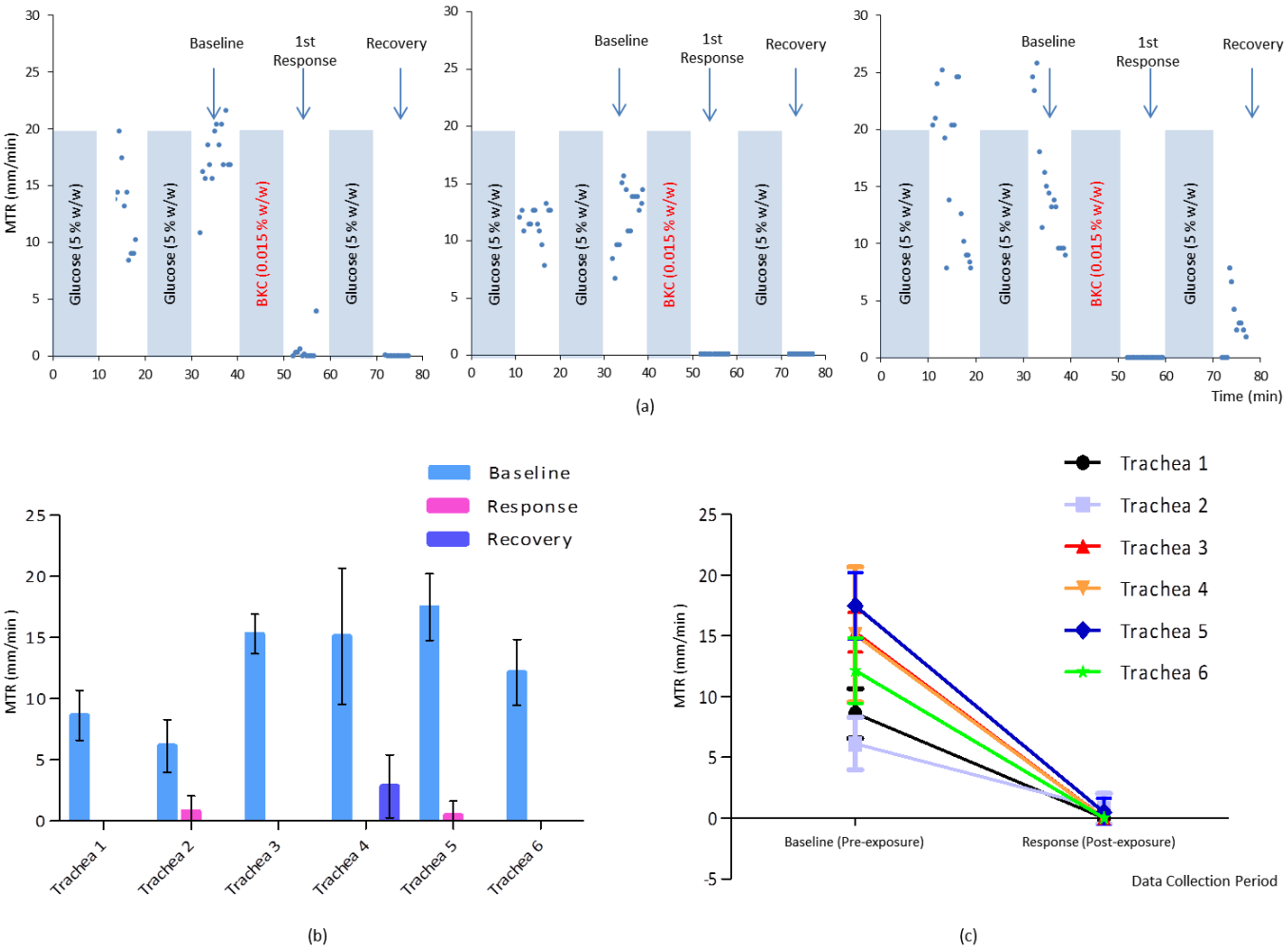


Figure 5.8 The Effect of BKC (0.015 % w/w) on Ovine MTR.

(a) Examples of the individual tracheal MTR response to 0.015 % w/w BKC solution (~ 3 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to BKC (0.015 % w/w) as studied in six tracheae (mean ± SD). MTR ceased in all tracheae except tracheae 2 and 5, where MTR decreased. Tracheae 1 and 4 to 6 were tested for recovery of mucociliary transport, however only tracheae 4 showed some recovery. (c) For each of the six tracheae exposed to BKC, the first and only set of response measurements was pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

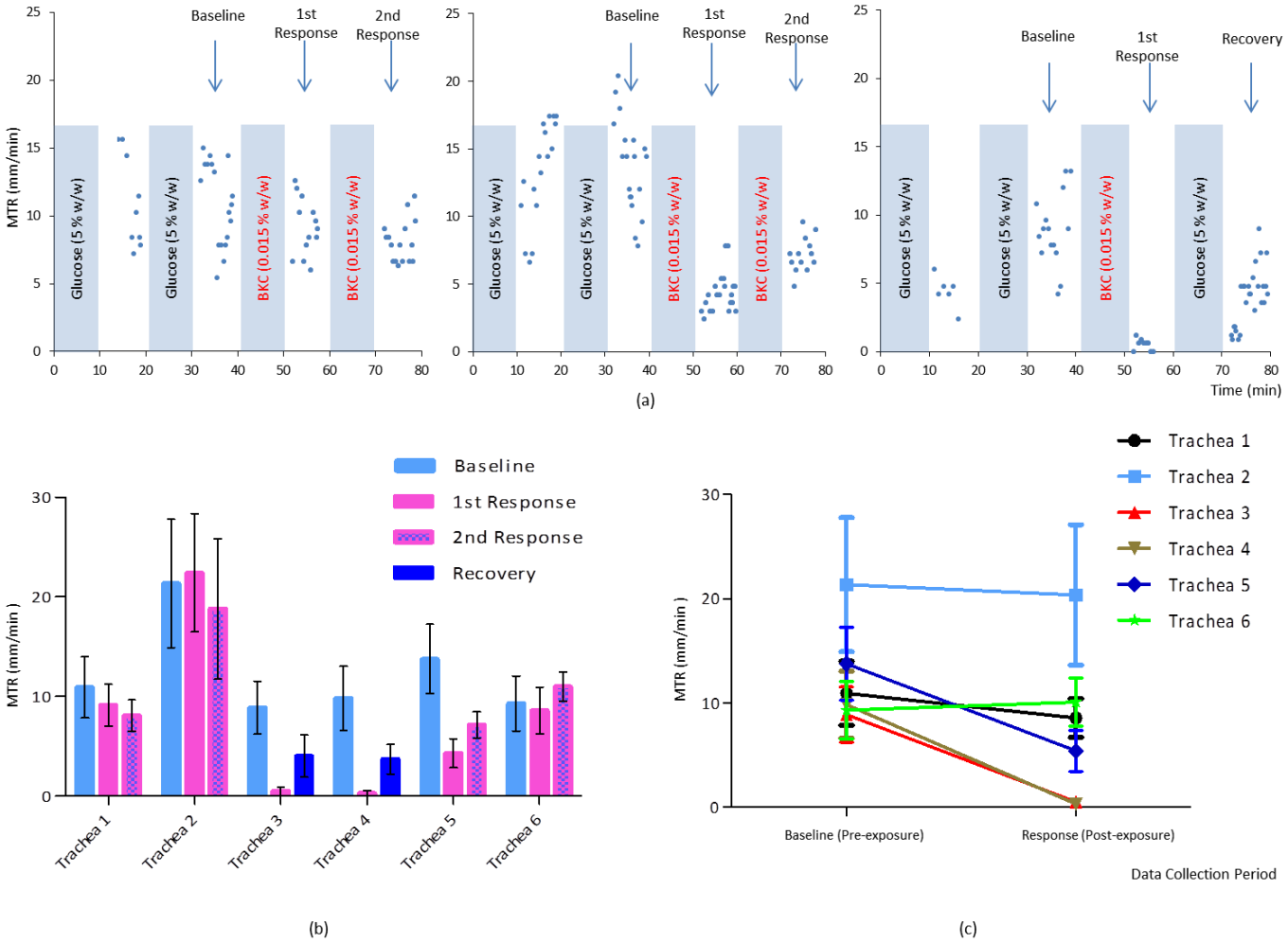


Figure 5.9 The Effect of Sprayed BKC (0.015 % w/w) on Ovine MTR.

(a) Examples of the individual tracheal MTR response to 0.015 % w/w sprayed BKC solution (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to sprayed BKC (0.015 % w/w) as studied in six tracheae (mean ± SD). Mucociliary transport almost halted in tracheae 3 and 4, however it recovered following exposure to 5 % w/w glucose solution. (c) For each of the six tracheae exposed to sprayed BKC, the first and second (if any) set of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.3 Effect of Ethylenediamine Tetraacetic Acid (EDTA) on Ovine MTR

No alteration in MTR was observed in one out of six tracheae exposed to ~ 3 ml of 0.015 % w/w EDTA (Figure 5.10 (a), left plot). However, a decreased MTR was detected in the other five tracheae (Figure 5.10 (a), middle plot), which reached a complete halt in one trachea (Figure 5.10 (a), right plot) and a partial halt (i.e. a standstill affecting only some areas on the tracheal mucosa) in two others during the second response period. This response (summarised in figure 5.10 (b)) was a significant decrease compared to baseline MTR ($P = 0.0083$) as demonstrated in figure 5.10 (c).

Interestingly, when three tracheae were exposed to sprayed 0.015 % w/w EDTA (~ 0.115 ml), their response varied between a decreased, a subtly decreased and a non-altered MTR (Figure 5.11 (a) and (b)). This response was not significantly different to baseline MTR ($P = 0.2524$) as demonstrated in figure 5.11 (c).

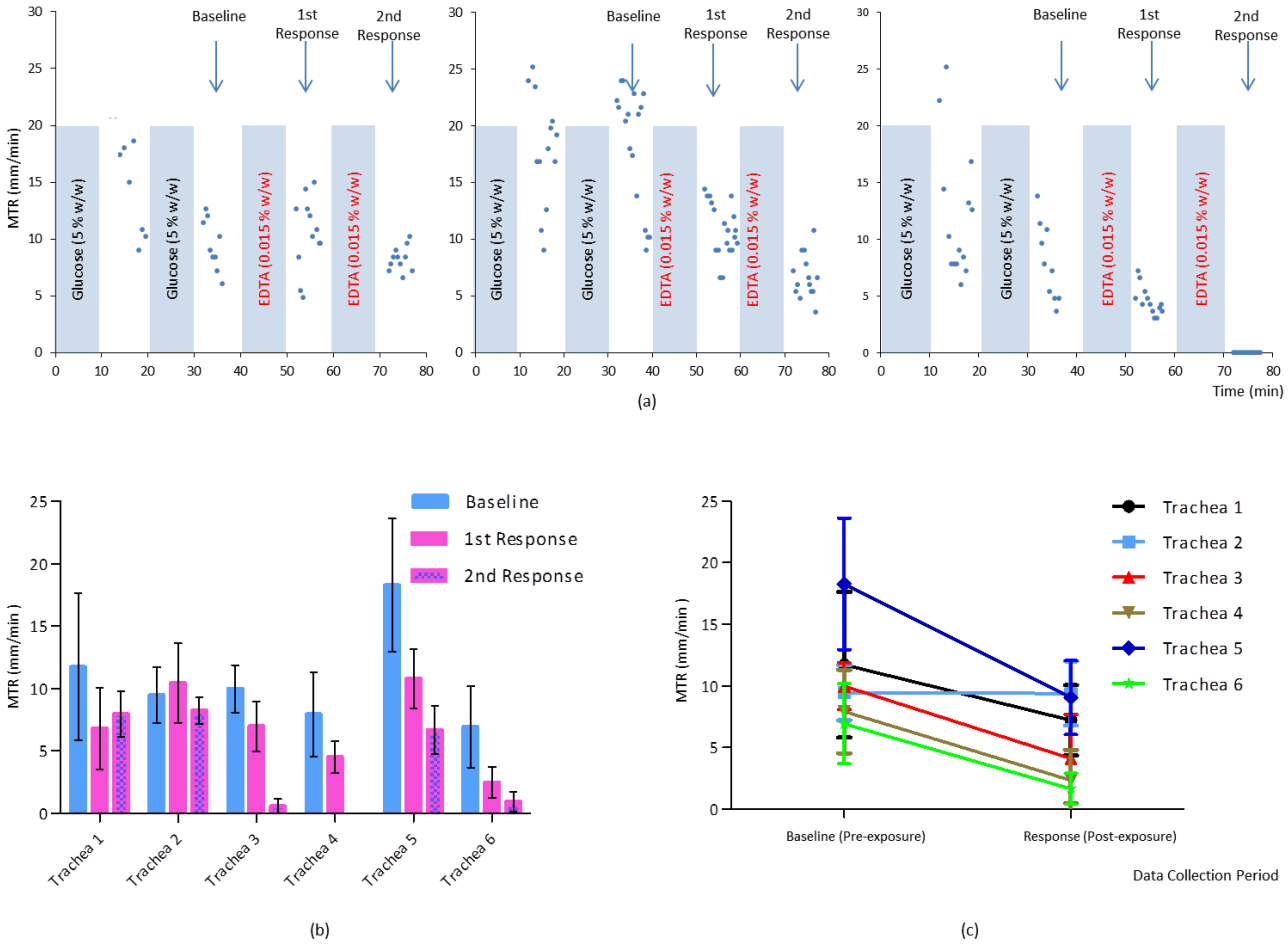


Figure 5.10 The Effect of EDTA (0.015 % w/w) on Ovine MTR.

(a) Examples of the individual tracheal MTR response to 0.015 % w/w EDTA solution (~ 3 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to EDTA (0.015 % w/w) as studied in six tracheae (mean ± SD). In tracheae 4, mucociliary transport demonstrated a halt during the second response period resulting in a hidden zero-height second response bar. (c) For each of the six tracheae exposed to EDTA, the first and second set of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

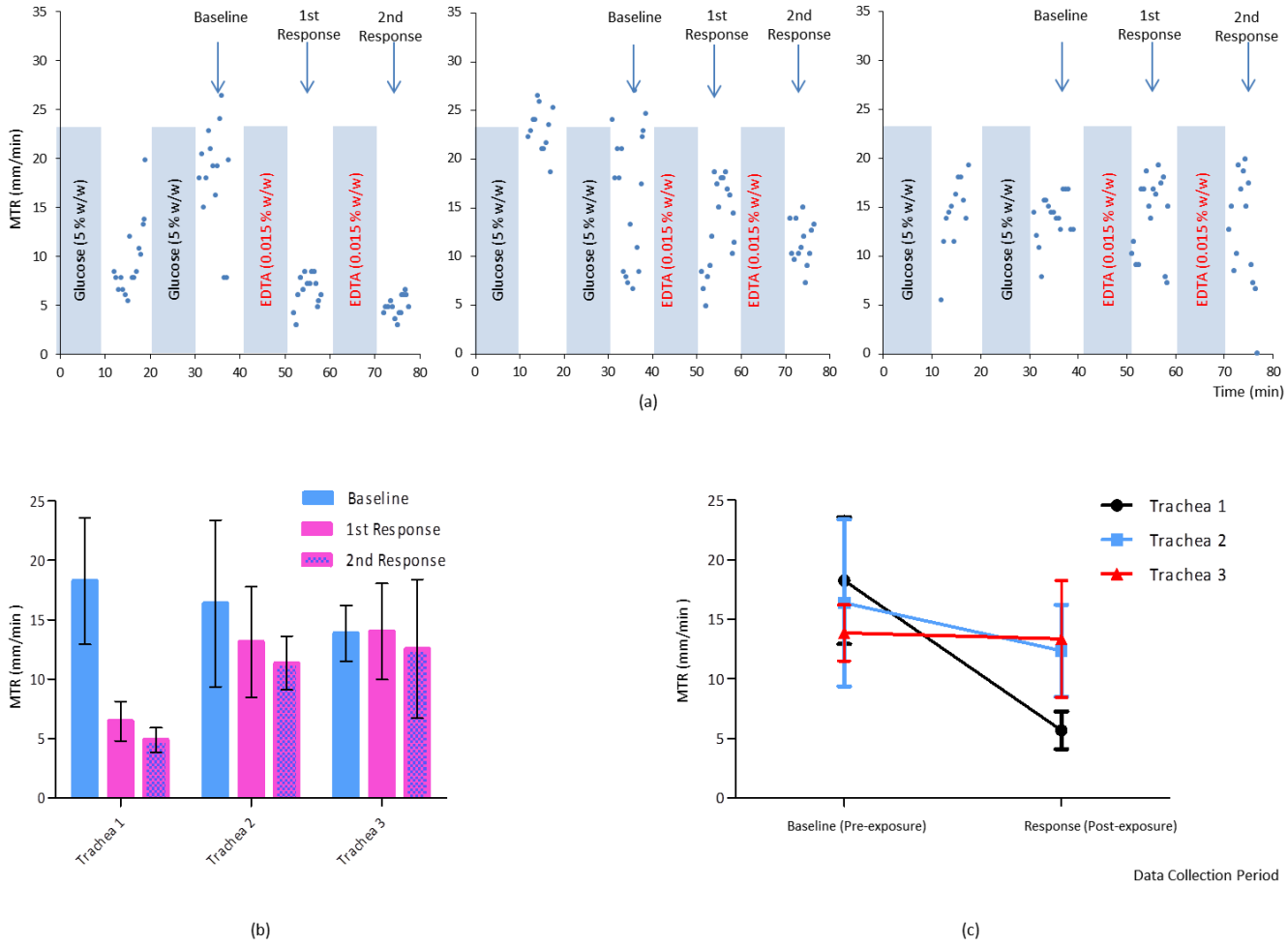


Figure 5.11 The Effect of Sprayed EDTA (0.015 % w/w) on Ovine MTR.

(a) The individual tracheal MTR response to sprayed 0.015 % w/w EDTA solution (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to EDTA (0.015 % w/w) as studied in three tracheae (mean ± SD). (c) For each of the three tracheae exposed to sprayed EDTA, the first and second set of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.4 Effect of Potassium Sorbate (PS) on Ovine MTR

The preservative, PS (0.3 % w/w), and the cosolvent, propylene glycol (PG) (1.5 % w/w), were tested concomitantly on six tracheae causing no alteration in MTR in one tracheae (see figure 5.12 (a), left plot), a subtle increase in MTR in one trachea (see figure 5.12 (a), middle plot) and a decrease in MTR in four tracheae (see figure 5.12 (a), right plot). The response that is summarised in figure 5.12 (b) was not significantly different to baseline MTR (P = 0.0666) as demonstrated in figure 5.12 (c).

Similarly, when three tracheae were sprayed with these compounds (~ 0.115 ml), only subtle changes in MTR were observed (Figure 5.13 (a) and (b)). This response was not significantly different to baseline MTR (P = 0.4480) as demonstrated in figure 5.13 (c).

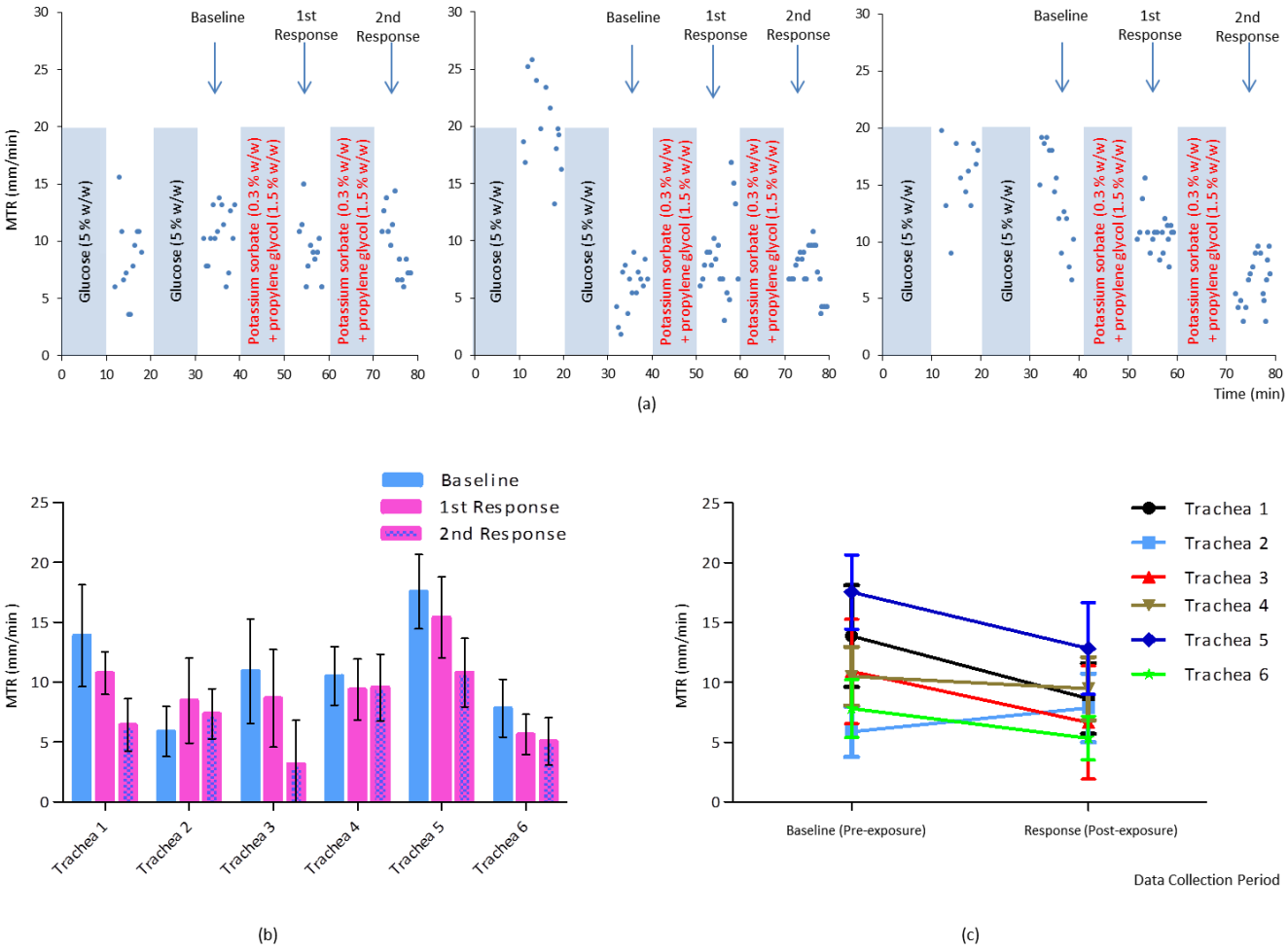


Figure 5.12 The Effect of Potassium Sorbate/ Propylene Glycol (0.3 / 1.5 % w/w) on Ovine MTR.

(a) Examples of the individual tracheal MTR response to a solution of these compounds (~ 3 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to potassium sorbate/ propylene glycol as studied in six tracheae (mean ± SD). (c) For each of the studied six tracheae, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

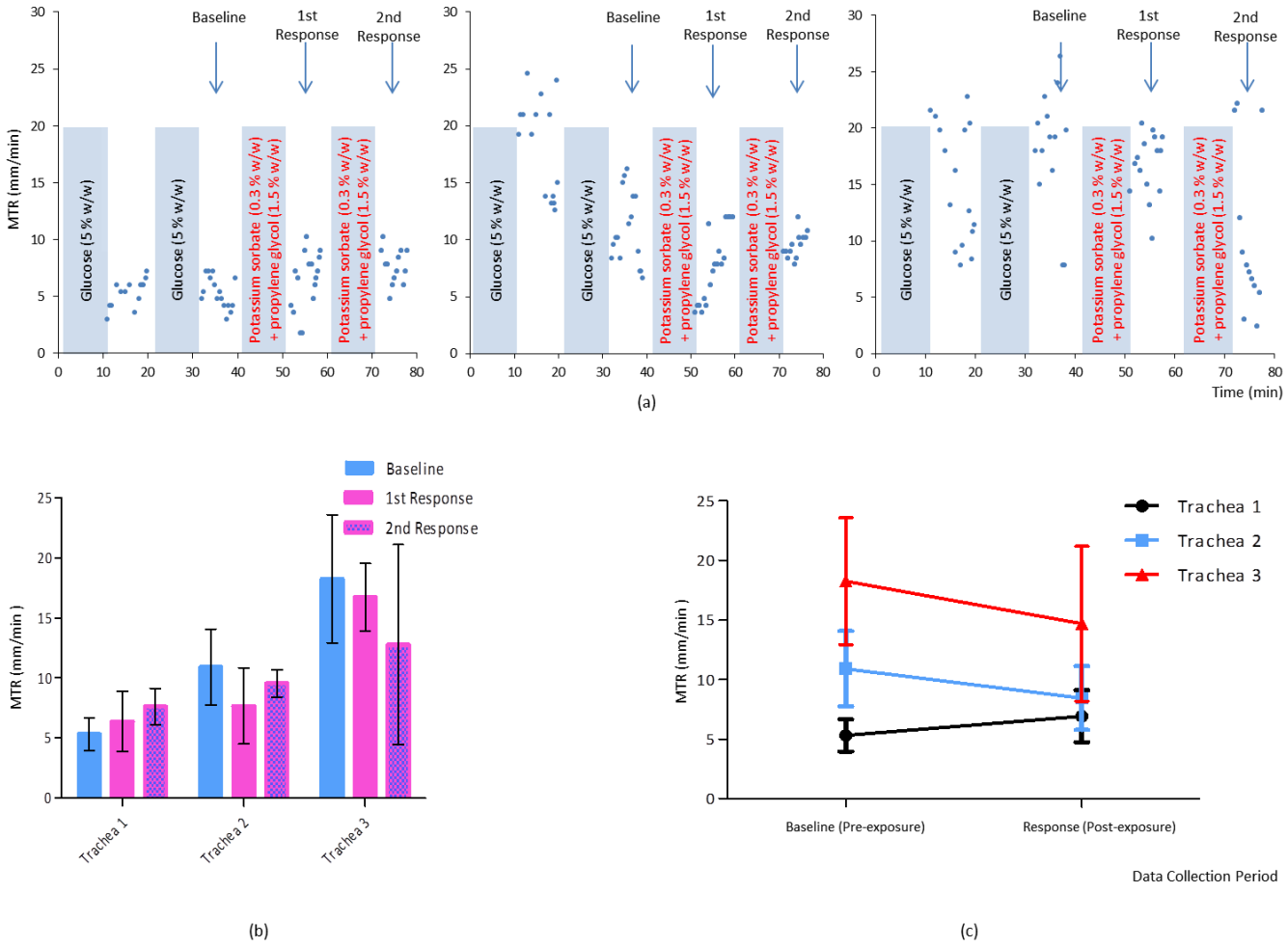


Figure 5.13 The Effect of Sprayed Potassium Sorbate/ Propylene Glycol (0.3 / 1.5 % w/w) on Ovine MTR.

(a) The individual tracheal MTR response to potassium sorbate/ propylene glycol spray (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure as studied in three tracheae (mean ± SD). (c) For each of the three tracheae exposed to sprayed potassium sorbate/ propylene glycol, the first and second set of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.5 Effect of Polysorbate 80 on Ovine MTR

Six tracheae were exposed to polysorbate 80 (0.025 % w/w). With the exception of one trachea that showed no alteration in MTR following the exposure, a decrease in MTR was detected in all the others (Figure 5.14 (a) and (b)). The observed response was significantly different to baseline MTR ($P = 0.0116$) as illustrated in figure 5.14 (c).

Interestingly, sprayed polysorbate 80 induced hardly any effect on ovine MTR when studied in three tracheae (Figure 5.15 (a) and (b)). This response was therefore not significantly different to baseline MTR ($P = 0.7603$) as illustrated in figure 5.15 (c)

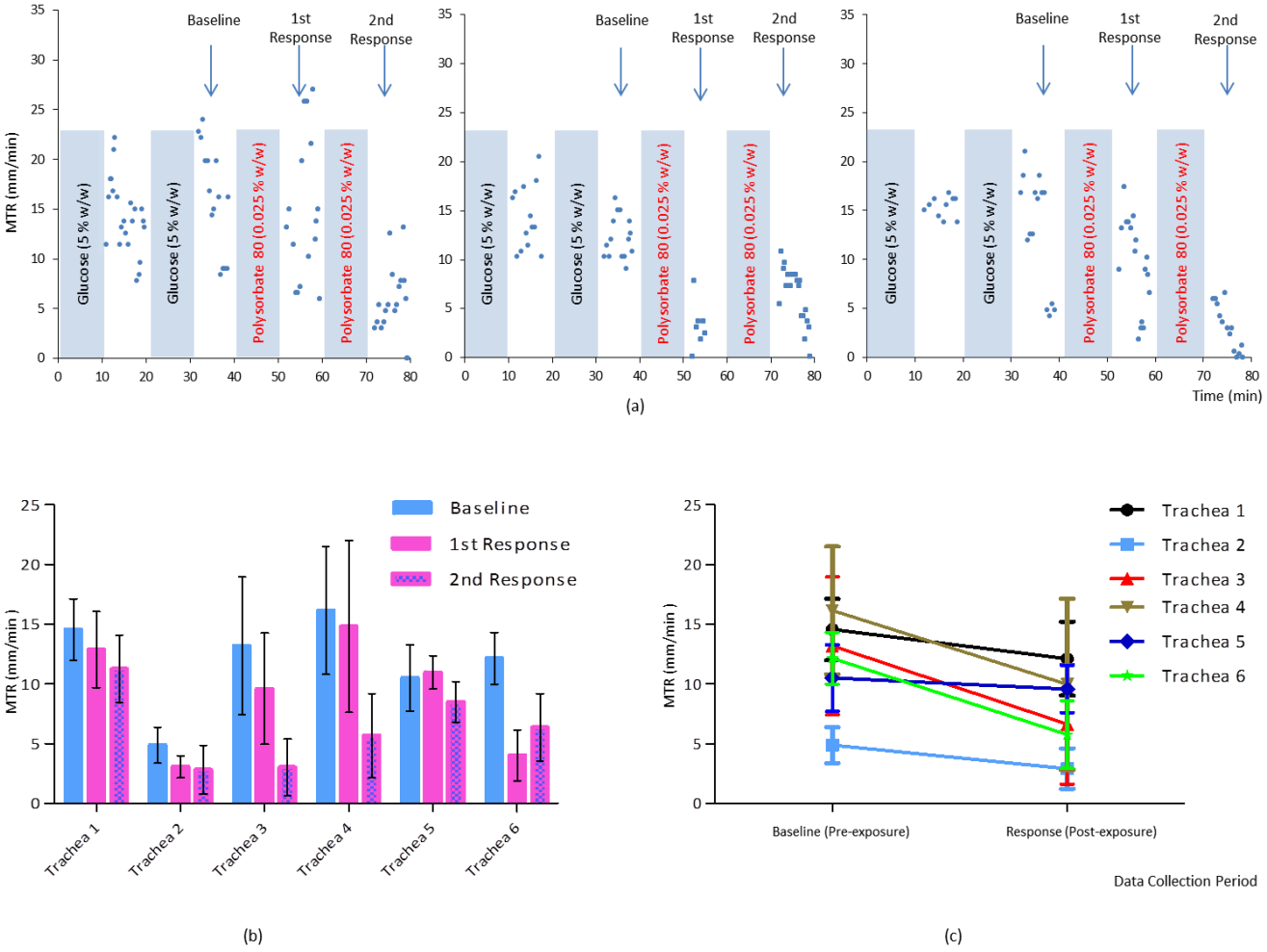


Figure 5.14 The Effect of Polysorbate 80 (0.025 % w/w) on Ovine MTR.

a) Examples of the individual tracheal MTR response to polysorbate 80 (0.025 % w/w) solution (~ 3 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to polysorbate 80 as studied in six tracheae (mean ± SD). (c) For each of the studied six tracheae, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

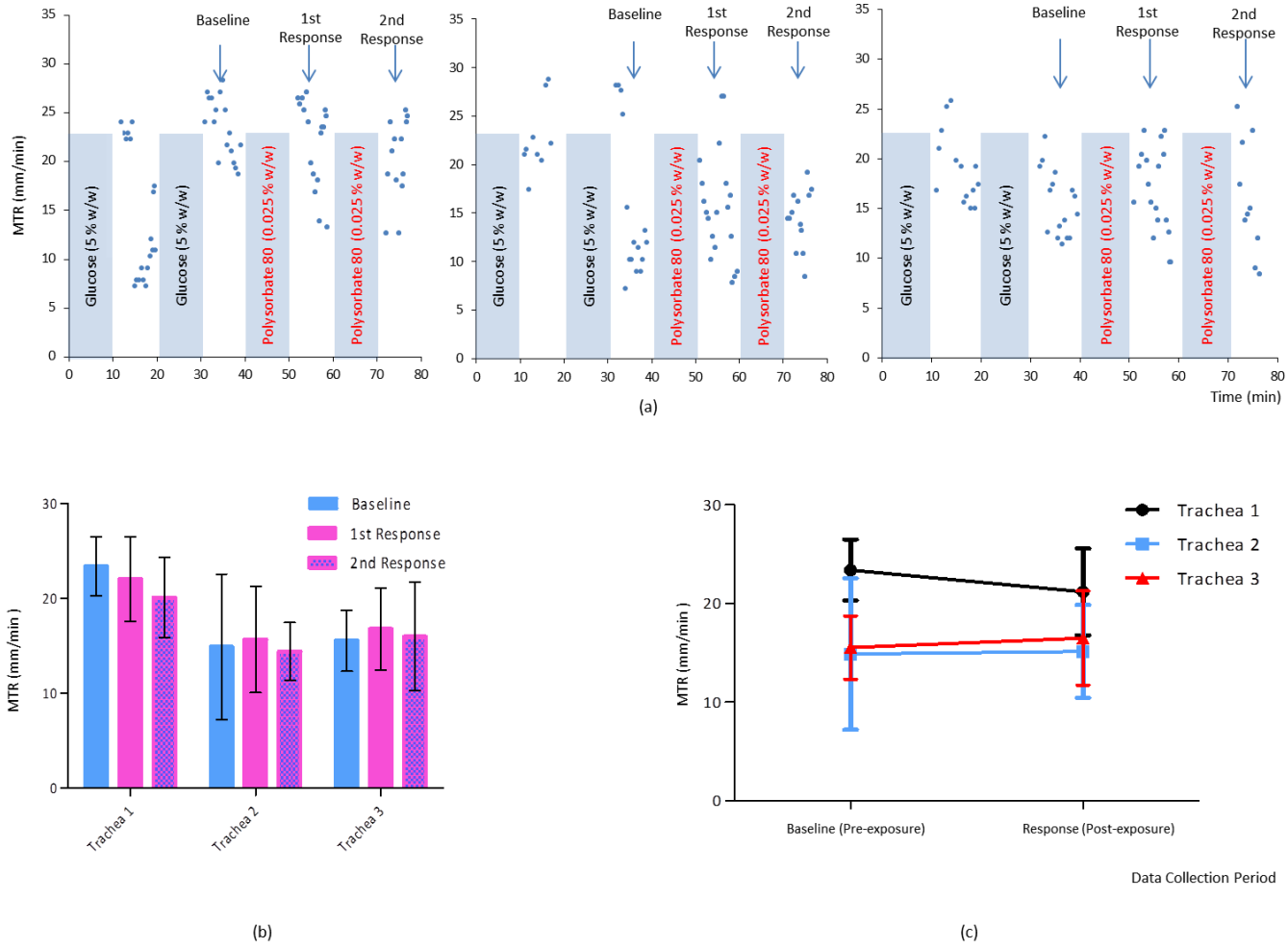


Figure 5.15 The Effect of Sprayed Polysorbate 80 (0.025 % w/w) on Ovine MTR.

(a) The individual tracheal MTR response to sprayed polysorbate 80 (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure as studied in three tracheae (mean ± SD). (c) For each of the three tracheae exposed to sprayed polysorbate 80, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.6 Effect of Methocel^{TM} on Ovine MTR

Methocel^{TM} (1.0 % w/w) elicited a decrease in MTR in each of the six tracheae exposed to a 3 ml volume of its dispersion (Figure 5.16 (a) and (b)). This response was significantly different to baseline MTR ($P = 0.0004$) as shown in figure 5.16 (c).

Similarly, sprayed Methocel^{TM} significantly decreased ovine MTR ($P = 0.0010$) as indicated by its effect on four ovine trachea (Figure 5.17). The effect appeared to be reversible as only one out of the four tracheae failed to recover from this decrease in MTR following the exposure to glucose solution (5 % w/w).

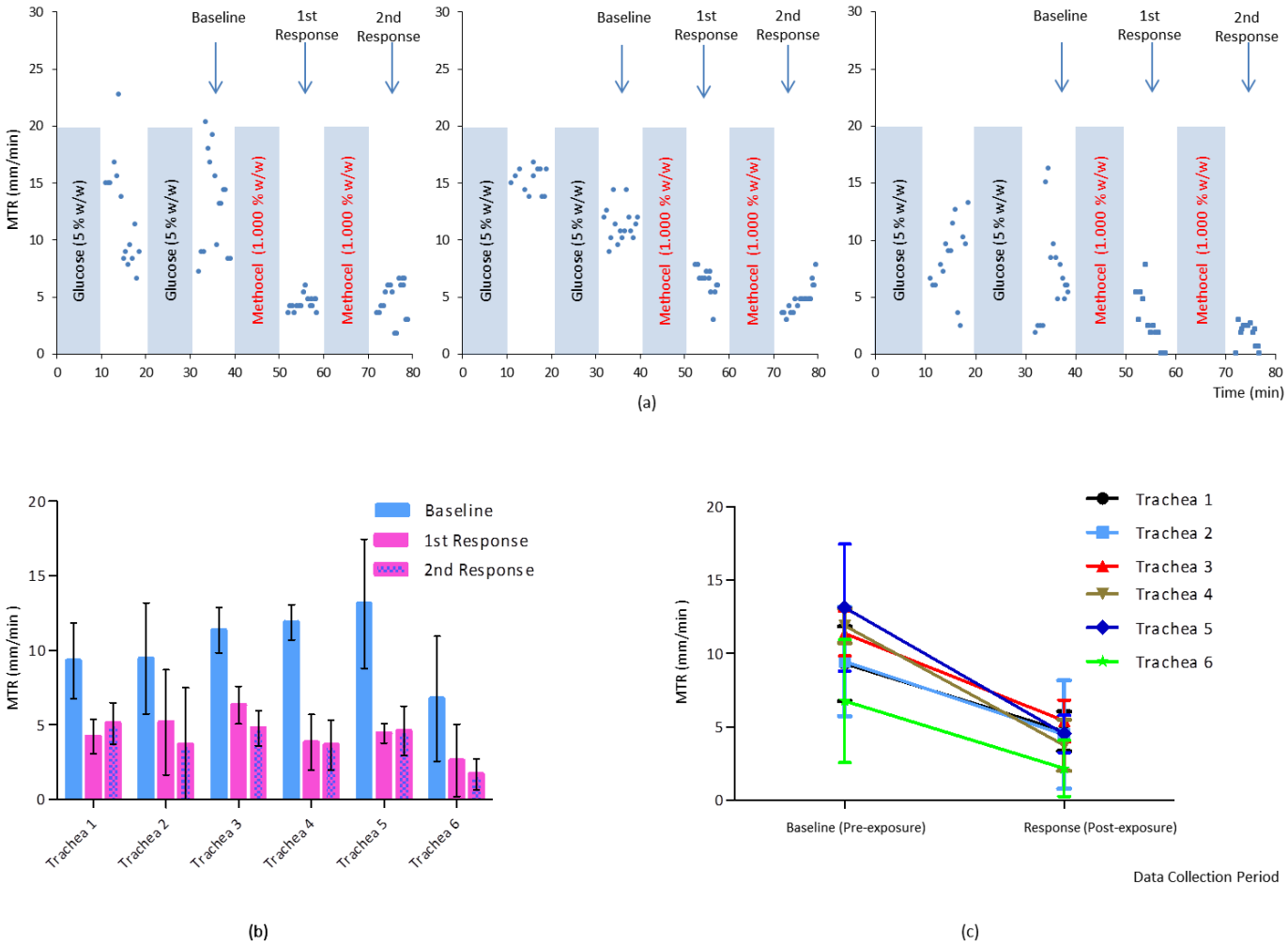


Figure 5.16 The Effect of Methocel™ (1.0 % w/w) on Ovine MTR.

a) Examples of the individual tracheal MTR response to Methocel™ (1.0 % w/w) solution (~ 3 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to Methocel™ as studied in six tracheae (mean ± SD). (c) For each of the studied six tracheae, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

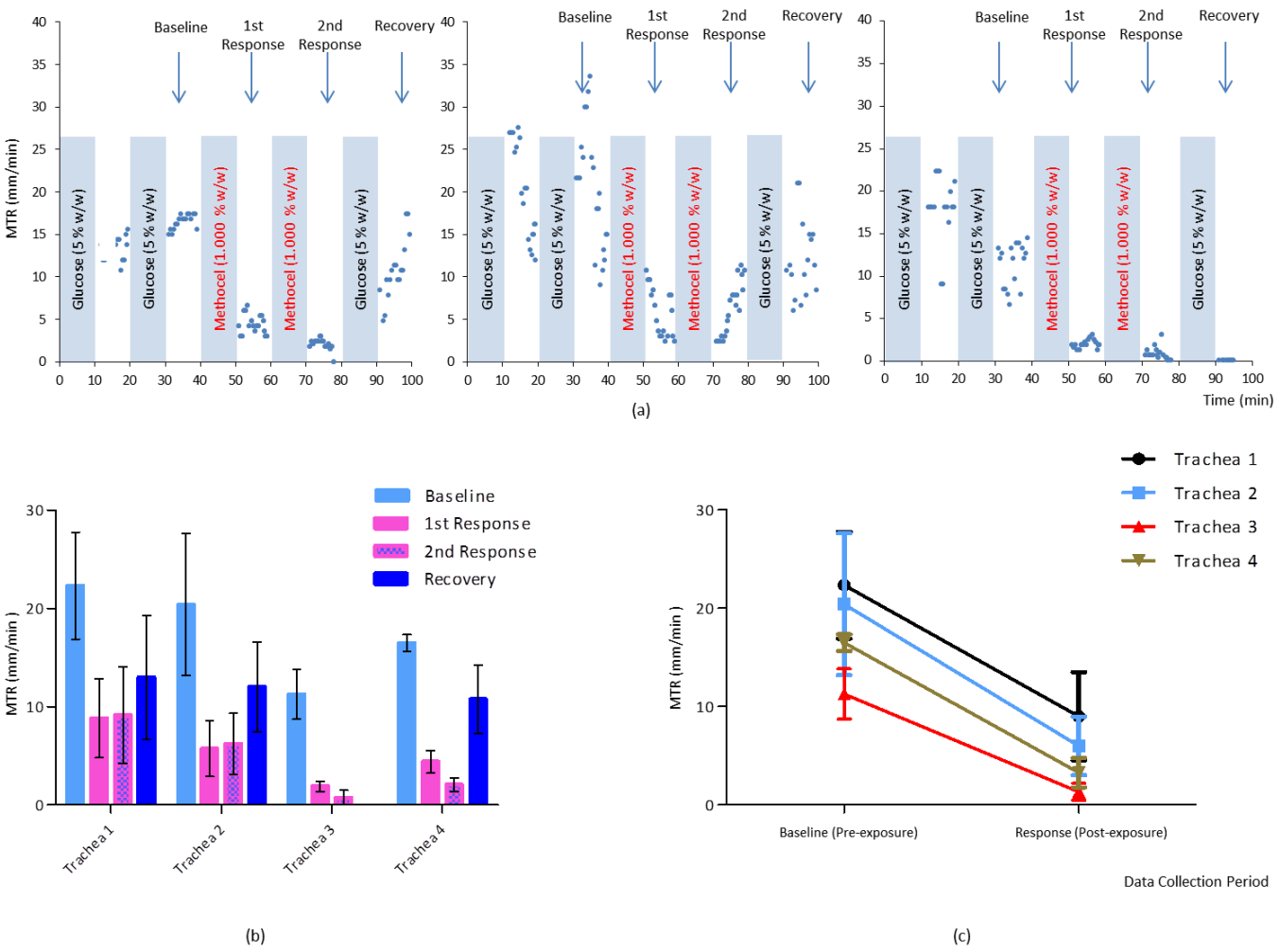


Figure 5.17 The Effect of Sprayed Methocel™ (1.0 % w/w) on Ovine MTR.

(a) The individual tracheal MTR response to Methocel™ spray (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure as studied in four tracheae (mean ± SD). Tracheae 3 demonstrated an irreversible failure of mucociliary transport. (c) For each of the four tracheae exposed to sprayed Methocel™, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.7 Effect of Avicel® on Ovine MTR

Avicel® (1.5 % w/w) spray elicited a decrease in MTR in each of the six tracheae sprayed with it (0.115 ml) (see figure 5.18 (a) and (b)). This response was significantly different to baseline MTR (P = 0.0003) as shown in figure 5.18 (c). The tracheae failed to fully recover from this decrease in MTR following 10-minute exposure to glucose solution (5 % w/w).

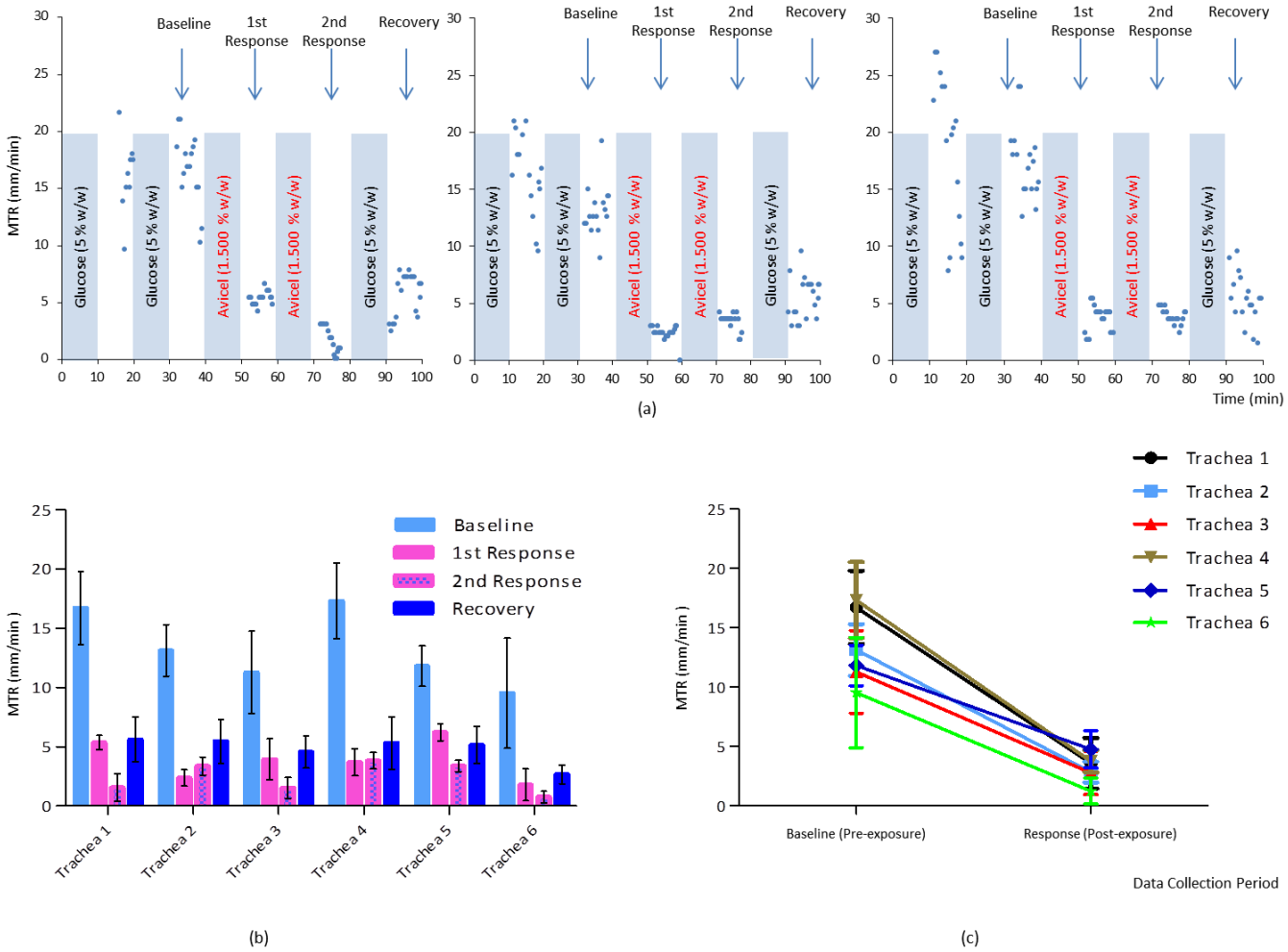


Figure 5.18 The Effect of Sprayed Avicel® (1.500 % w/w) on Ovine MTR.

(a) The individual tracheal MTR response to Avicel® spray (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure as studied in six tracheae (mean ± SD). (c) For each of the six tracheae exposed to Methocel™, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.8 Effect of Fluticasone Furoate (FF) on Ovine MTR

Three out of the six ovine tracheae exposed to FF (0.0004 % w/w, ~ 3ml that also contained 0.2 % w/w DMSO) showed decreased MTR, which reached a complete irreversible halt of mucociliary transport in one trachea (Figure 5.19 (a), left plot). Nevertheless, the other three tracheae showed subtle responses (Figure 5.19 (a), middle and right plots) to FF exposure. The response of the six tracheae (Figure 5.19 (b)) was therefore not significantly different to baseline MTR ($P = 0.1064$).

When FF (0.0004 % w/w, ~ 0.115 ml also containing 0.2 % w/w DMSO) was sprayed on the mucosa of three tracheae, a decrease in MTR was observed in each of them (Figure 5.20 (a)) although full recovery was observed in the tracheae most affected by this decrease (Figure 5.20 (b)). Interestingly, this response was significantly different to baseline MTR ($P = 0.0028$) as depicted in figure 5.20 (c).

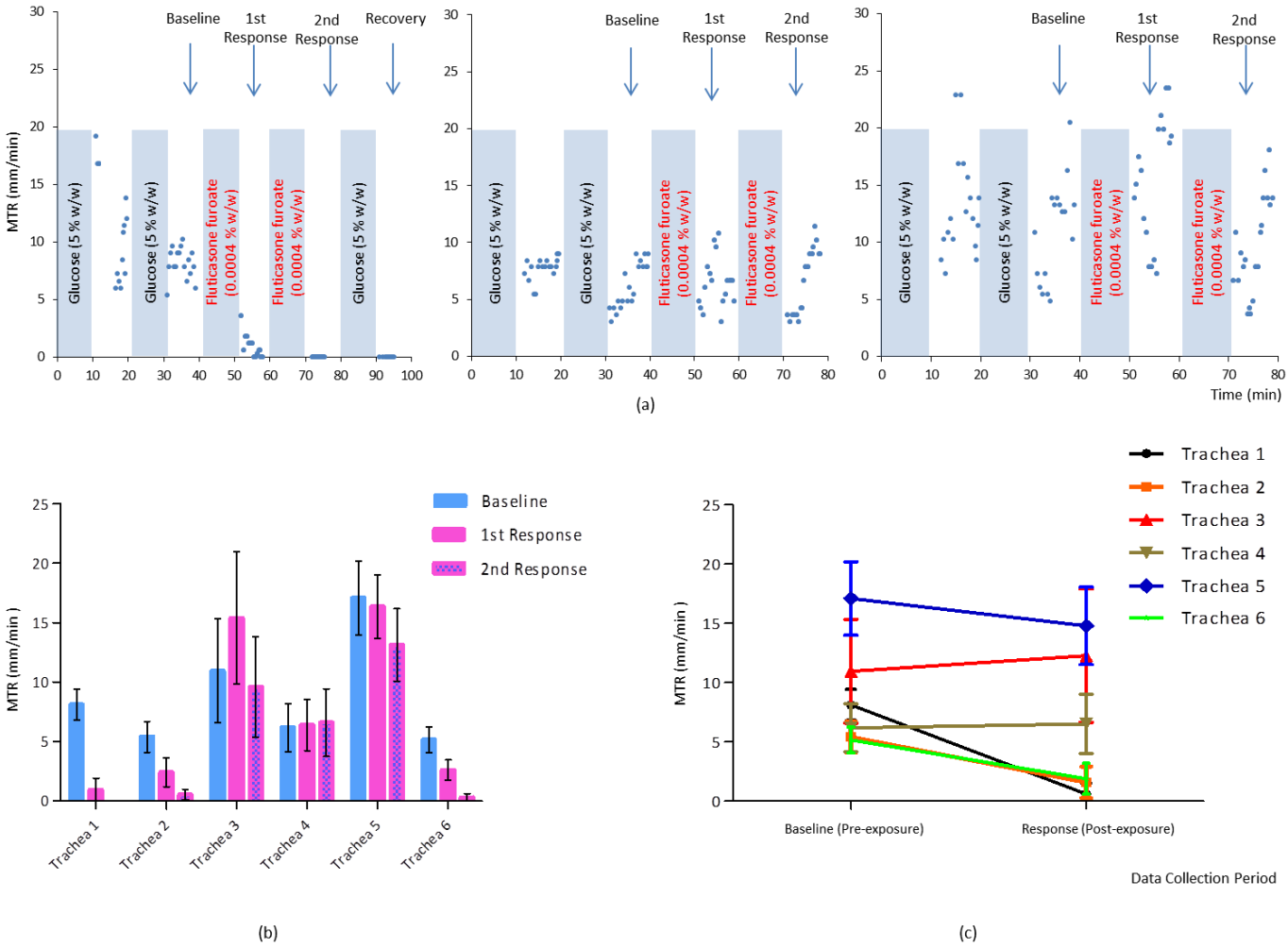


Figure 5.19 The Effect of Fluticasone Furoate (0.0004 % w/w) on Ovine MTR.

a) Examples of the individual tracheal MTR response to fluticasone furoate (FF) solution (0.0004 % w/w, ~ 3 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure as studied in six tracheae (mean ± SD). In trachea one, mucociliary transport ceased after the second application of FF. (c) For each of the studied six tracheae, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

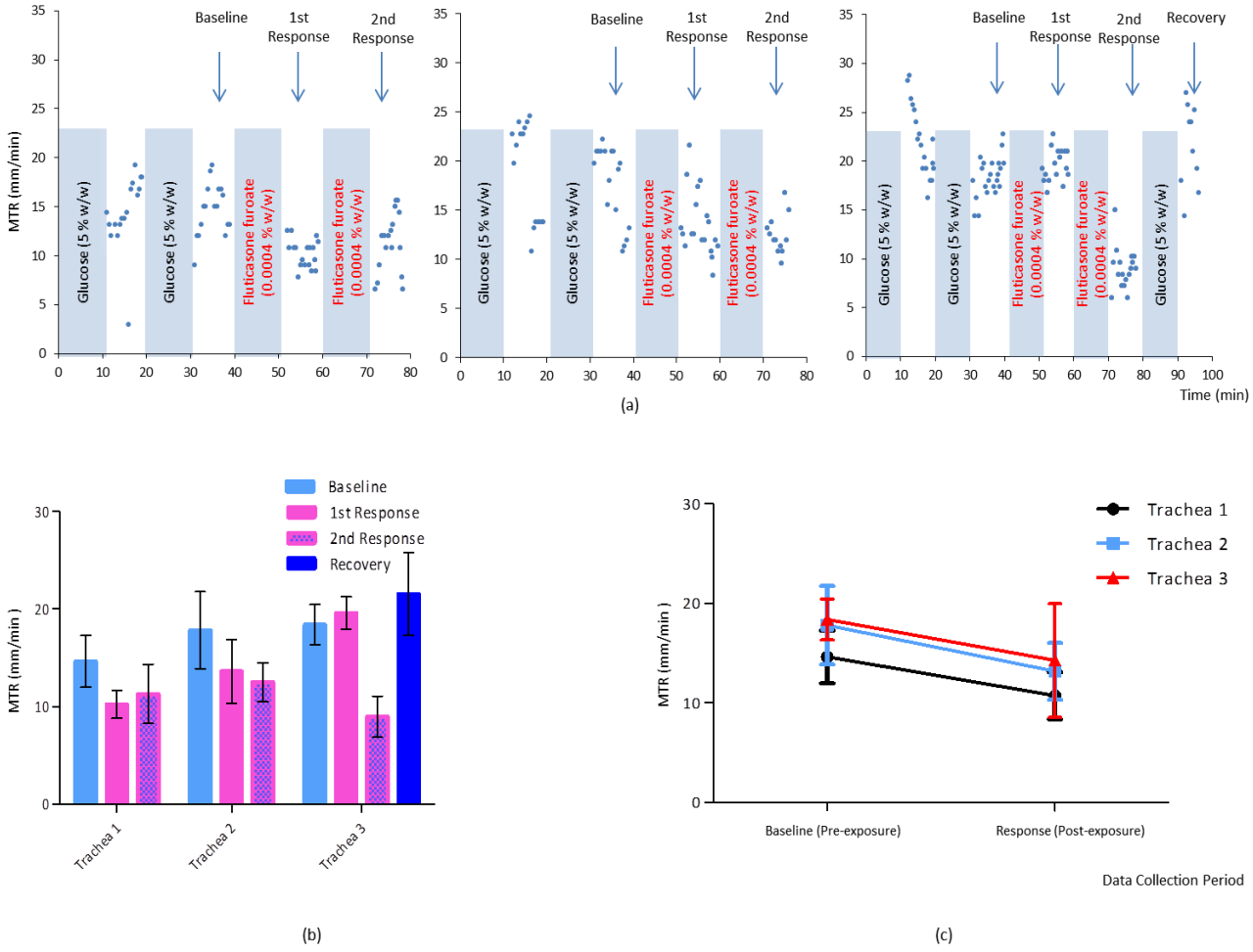


Figure 5.20 The Effect of Sprayed Fluticasone Furoate (0.0004 % w/w) on Ovine MTR.

(a) The individual tracheal MTR response to fluticasone furoate (FF) spray (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to sprayed FF as studied in three tracheae (mean ± SD). (c) For each of the three tracheae exposed to sprayed FF, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.9 Effect of Dimethyl Sulfoxide (DMSO) on Ovine MTR

Fluticasone furoate (FF) and GSK1004723E were solubilised in DMSO to give a final DMSO concentration of 0.2 % w/w. Therefore, the effect of DMSO (0.2 % w/w) on MTR was studied. A decrease in ovine MTR was observed in six out of six tracheae exposed to 0.2 % w/w DMSO (~ 3 ml) as demonstrated in figures 5.21 (a) and (b), which also showed poor recovery of MTR from this exposure. This decrease response was significantly different to baseline MTR as depicted in figure 5.21 (c) ($P = 0.0198$).

Three tracheae were then exposed to 0.2 % w/w DMSO sprays (~ 0.115 ml), which elicited a decrease in MTR in two tracheae that also showed poor MTR recovery (Figure 5.22 (a) and (b)). This decreased MTR was however not significantly different to baseline MTR ($P = 0.0913$) as shown in figure 5.22 (c).

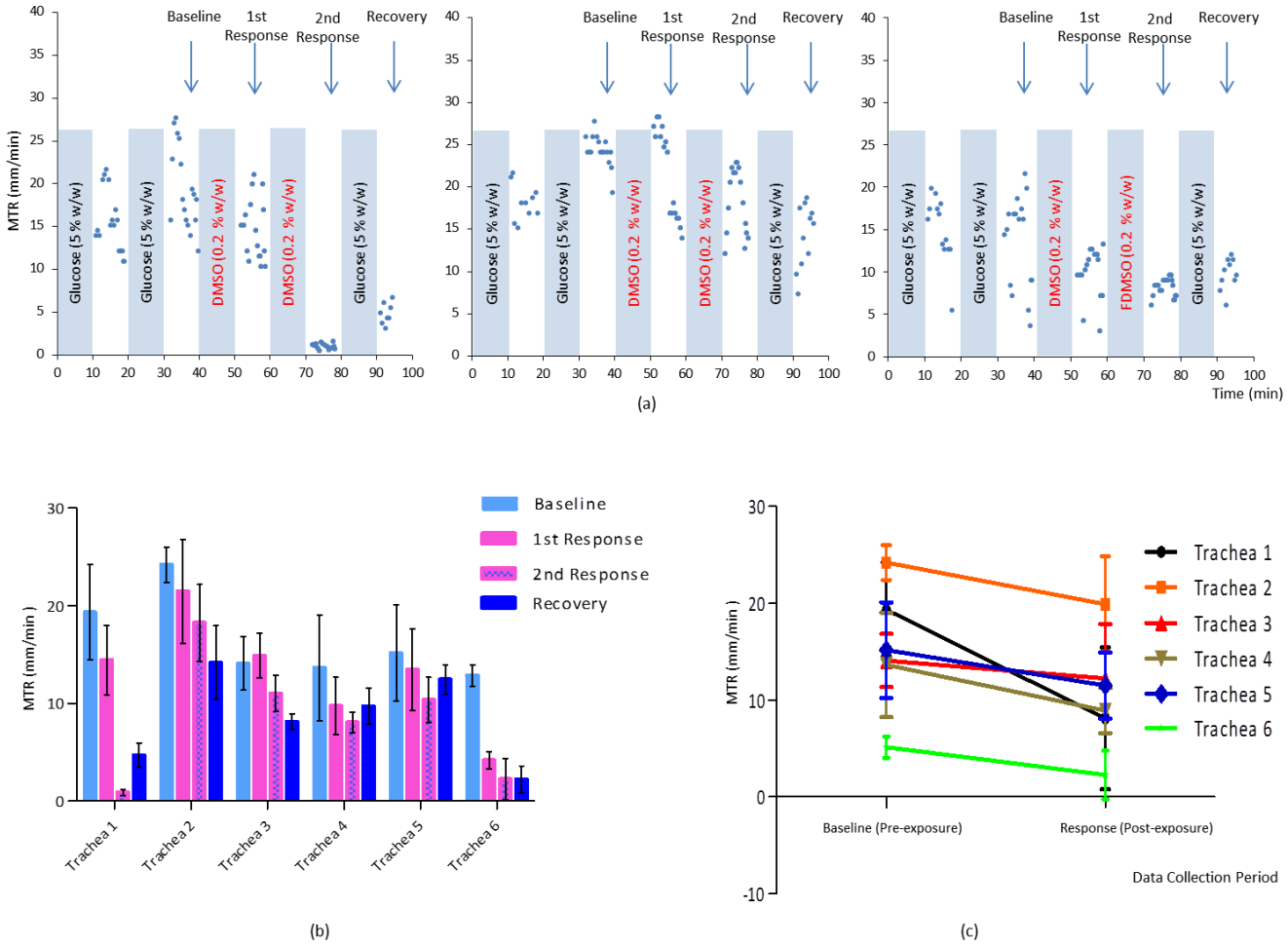


Figure 5.21 The Effect of DMSO (0.2 % w/w) on Ovine MTR.

a) Examples of the individual tracheal MTR response to DMSO solution (0.2 % w/w, ~ 3 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure as studied in six tracheae (mean ± SD). (c) For each of the studied six tracheae, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

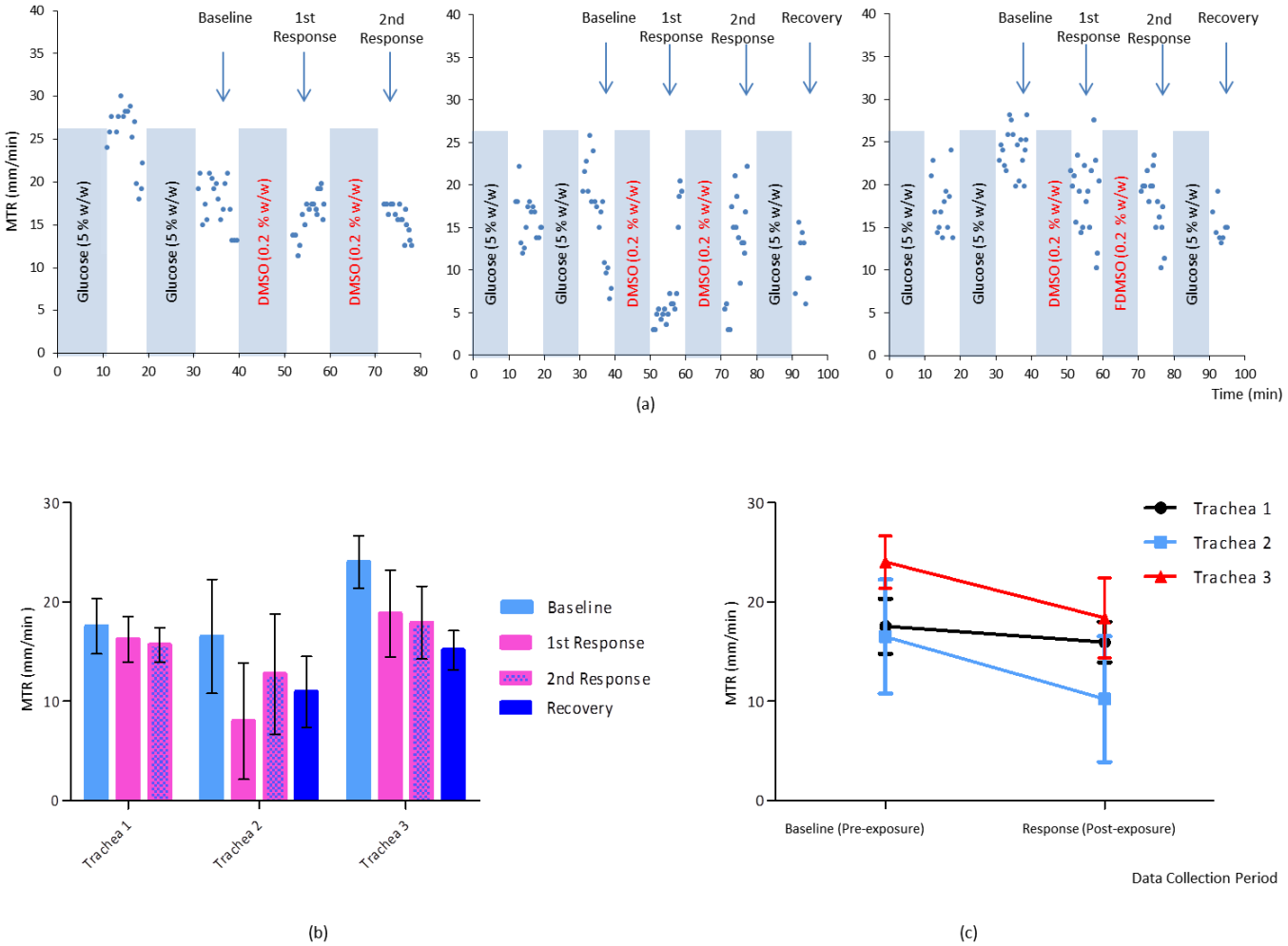


Figure 5.22 The Effect of Sprayed DMSO (0.2 % w/w) on Ovine MTR.

(a) The individual tracheal MTR response to DMSO sprays (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure summarised as studied in three tracheae (mean ± SD). (c) For each of the six tracheae exposed to DMSO, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.10 Effect of GSK1004723E on Ovine MTR

A decrease in ovine MTR was observed in five out of six tracheae sprayed with ~ 0.115 ml of 0.01 % w/w GSK1004723E, which also contained 0.2 % w/w DMSO (Figure 5.23 (a) and (b)). This decrease response was significantly different to baseline MTR (P = 0.0283) as depicted in figure 5.23 (c).

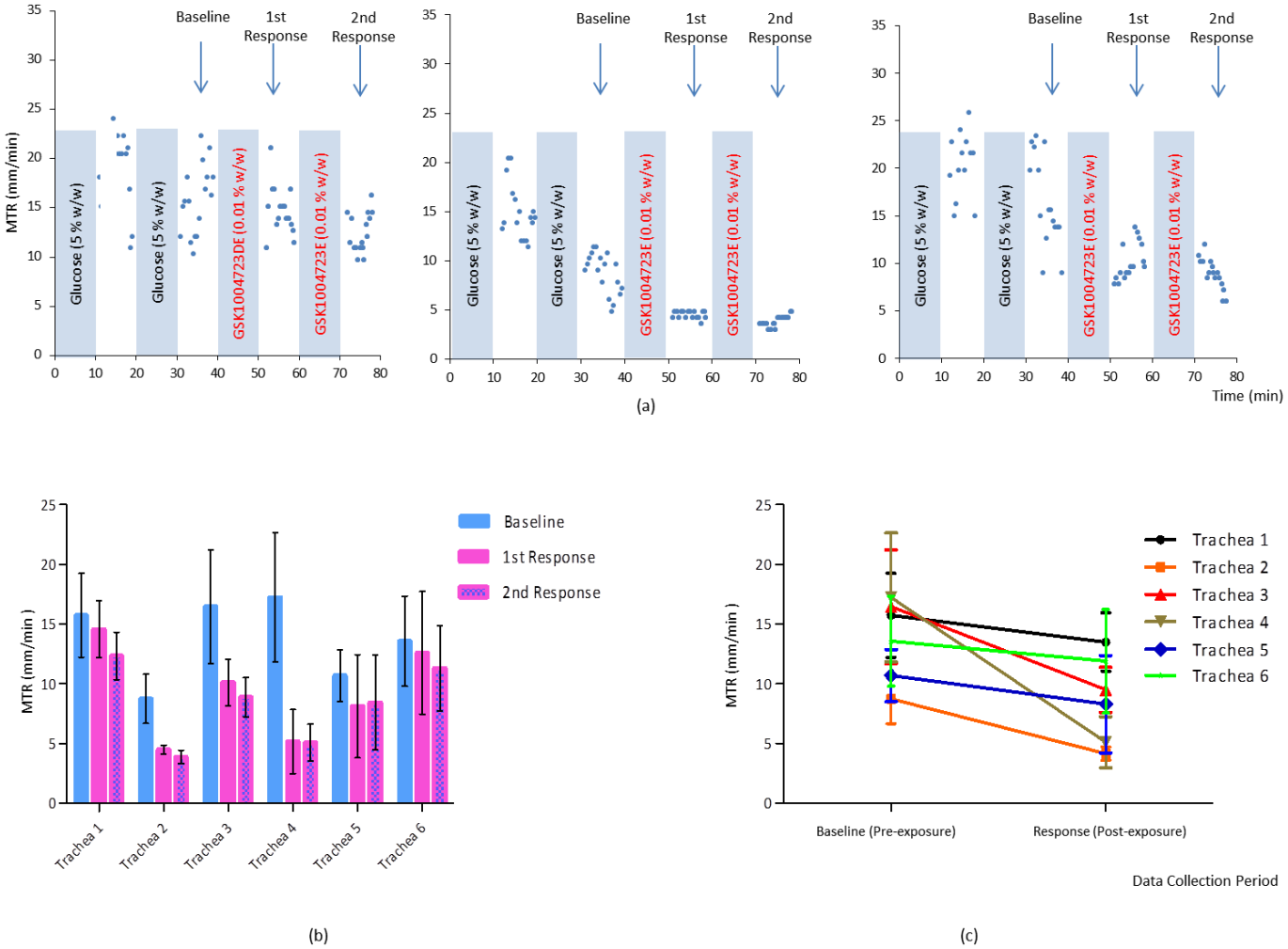


Figure 5.23 The Effect of Sprayed GSK1004723E (0.01 % w/w) on Ovine MTR.

(a) Examples of the individual tracheal MTR response to GSK1004723E spray (0.01 % w/w, ~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure as studied in six tracheae (mean ± SD). (c) For each of the studied six tracheae, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.11 Effect of GSK1004723D on Ovine MTR

Sprayed 0.5 % w/w GSK1004723D (~ 0.115 ml) elicited a standstill in ovine mucociliary transport during the first response period with no signs of recovery afterwards in three out of three tracheae (Figure 5.24 (a) and (b)). This effect was significantly different to baseline MTR ($P = 0.0021$) as illustrated in figure 5.24 (c).

On the other hand, a decrease in ovine MTR was observed in five out of six tracheae sprayed with ~ 0.115 ml of 0.01 % w/w GSK1004723D (Figure 5.25 (a) and (b)). This decrease reached an irreversible halt in one trachea during the first response period (Figure 5.25 (a), right plot). With the exception of one trachea that showed full recovery of MTR following a remarkable decrease, only partial recovery was observed in another two tracheae showing such decreases in MTR whereas one trachea failed to recover (Figure 5.25 (b)). This decrease response was significantly different to baseline MTR ($P = 0.0110$) as depicted in figure 5.25 (c).

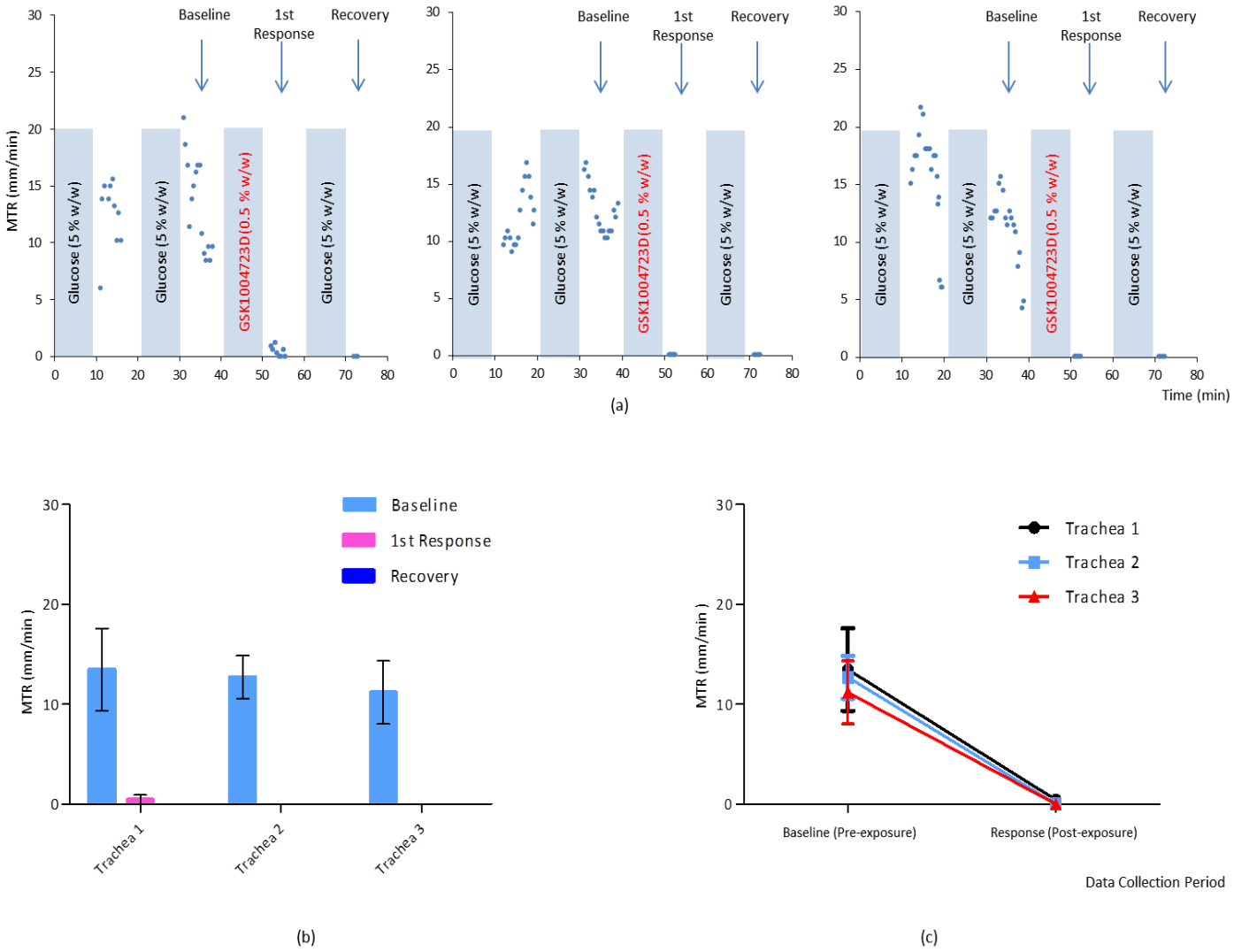


Figure 5.24 The Effect of Sprayed GSK1004723D (0.5 % w/w) on Ovine MTR.

(a) The individual tracheal MTR response to GSK1004723D sprays (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure summarised as studied in three tracheae (mean ± SD). Zero-height response and recovery bars disappeared behind the horizontal axis. (c) For each of the three tracheae exposed to GSK1004723D, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

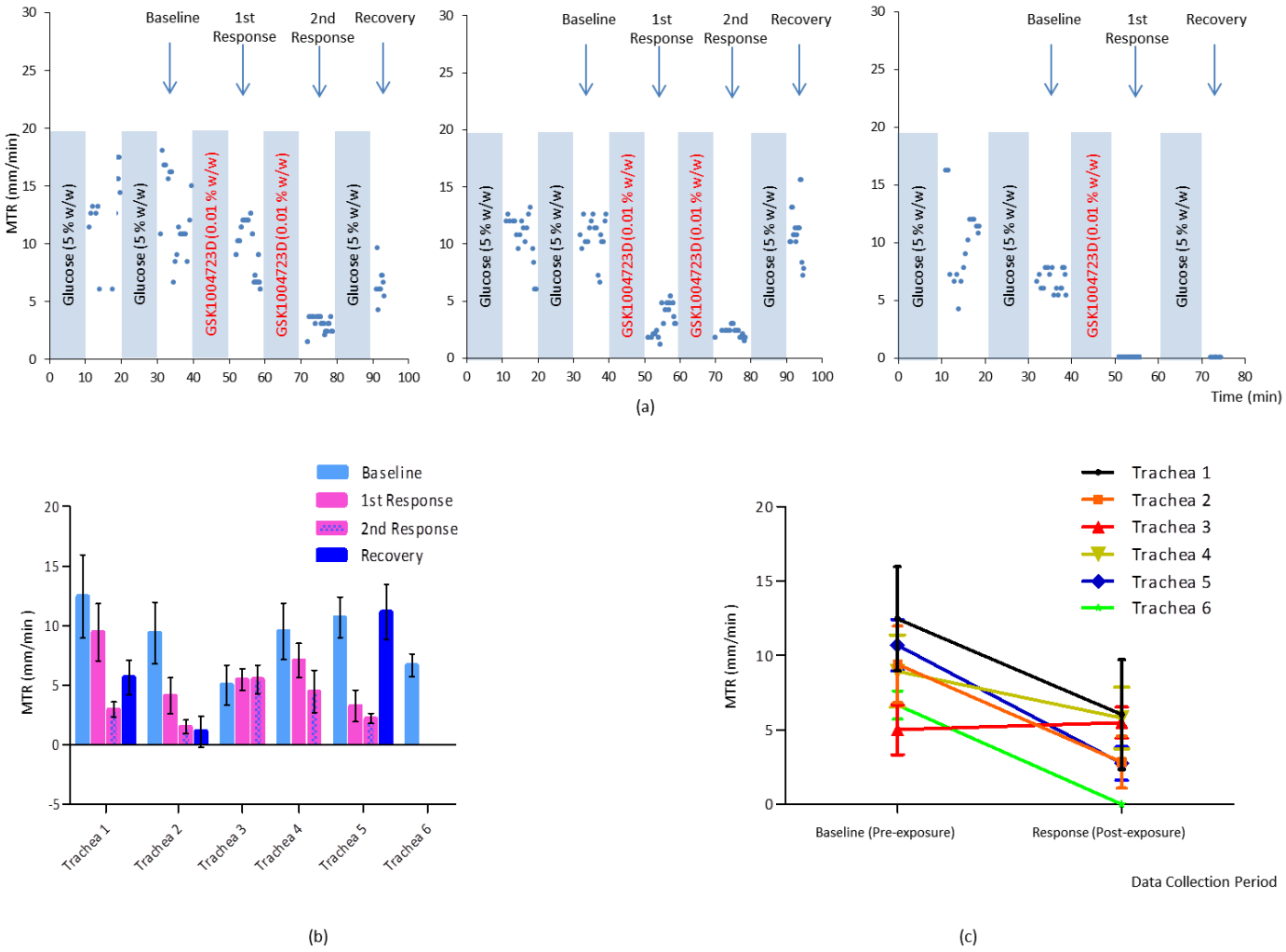


Figure 5.25 The Effect of Sprayed GSK1004723D (0.01 % w/w) on Ovine MTR.

a) Examples of the individual tracheal MTR response to GSK1004723D spray (~ 0.115 ml). The solid bars indicate 10-minute periods of exposure to solutions while the points represent MTR measurements made after each exposure period. (b) The ovine MTR response to this exposure as studied in six tracheae (mean ± SD). Zero-height first response and recovery bars disappeared behind the horizontal axis in trachea 6. (c) For each of the studied six tracheae, the first and second sets of response measurements were pooled and the mean was compared to that of the paired baseline set of measurements (mean ± SD).

5.4.2.12 Summary of Findings

Table 5.2 Effect of the IN Pharmaceuticals on Ovine MTR

The Compound	Effect on Ovine MTR	
	When instilled (3 ml) n = 6	When sprayed (~ 0.115 ml)) n = 3 - 6
Glucose (5.0 % w/w)	Non-significant	Non-significant
BKC (0.015 % w/w)	Significant decrease	Significant decrease
EDTA (0.015 % w/w)	Significant decrease	Non-significant*
Potassium sorbate/ propylene glycol (0.3 % w/w/ 1.5 % w/w)	Non-significant	Non-significant
Polysorbate 80 (0.025 % w/w)	Significant decrease	Non-significant
Methocel™ (1.0 % w/w)	Significant decrease	Significant decrease
Avicel (1.5 % w/w)	-	Significant decrease
Fluticasone furoate (0.0004 % w/w) (containing 0.2 % w/w DMSO)	Non-significant	Significant decrease*
DMSO (0.2 % w/w)	Significant decrease	Non-significant*
GSK1004723 E (0.01 % w/w) (containing 0.2 % w/w DMSO)	-	Significant decrease
GSK1004723 D (0.5 % w/w)	-	Significant decrease
GSK1004723 D (0.01 % w/w)	-	Significant decrease

* Probably non-conclusive - see section 5.5.

5.5 Discussion and Conclusions

Mucociliary clearance has often been directly assessed through measuring mucus transport velocity in various *in-vivo* and *ex-vivo* models. Excised sheep tracheae were employed here to investigate the effect of the study pharmaceuticals on MTR. This model demonstrated tremendous sensitivity to variations in the physiological temperature, which critically threatened the tissue viability. This effect of temperature on the maintenance of mucociliary function in sheep tracheae is consistent with Kilgour et al.'s observation of total mucociliary failure in sheep tracheae with the decrease in temperature from 37 °C to either 34 °C or 30 °C [307]. It was also observed here that exposing the luminal surface of ovine tracheae to fluids (gassed KH at 37 °C) compromised the tissue viability time compared to maintaining them in humidified air at 37 °C. The latter then became the set protocol for collection and transportation of ovine tracheae in this project.

Ovine MTR was noticed to vary from one spot to another on the same trachea, which was demonstrated by the variability of measurements obtained in each 10 minute measuring period (see figure 5.3). Moreover, when some tracheae were cut along the mid-line of the ventral side into two similar halves with the prospect of using one half as a control (to confirm the viability of the trachea during the course of the experiment), the mucociliary transport was often observed to cease in one half before the other, which invalidated the use of one half as a control. Meanwhile, the particle size of the tracer (carbon particles) was observed not to affect MTR (no data was shown), which was consistently observed in a recent report [308].

Since the tracheae were washed with gassed KH at 37 °C to remove blood and debris before mounting in the measuring chamber, the effect of gassed KH on MTR was studied. This was necessary because the design of the MTR experiments should allow sufficient equilibration time for tissue recovery from any observed effect. Based on the results of this investigation,

40-minute equilibration time was allowed in the preliminary experiments that studied the effect of Avamys® on MTR. Nevertheless, experiments became too lengthy (110 minutes with only one set of response measurements made in the 80-90 minute period of the experiment) that it raised concerns of some tracheae naturally losing their viability during the course of the experiment, which would have interfered with drawing robust conclusions on the effect of exposures. Notably, ovine tracheal viability time could be as short as 2.75 h (i.e. 165 minutes including the transport time) as determined here by the early viability experiments. This issue thus prompted a radical alteration in the design of our MTR experiments. The new design used the blank solution to clean the tissue of blood and debris saving the tissue any unnecessary exposures and therefore saving time. Moreover, the tissue cleaning step (10 minutes) was integrated into a 30-minute equilibration time ahead of each experiment, which saved even more time. This equilibration time then allowed the tissue 10-minute exposure to humidified air, which relieved the strain posed on the epithelium by covering with fluids as opposed to its physiological habitat, allowed making preliminary measurements to establish tissue viability and finally simulated the actual experiment that alternated exposing the tissue to fluids and humidified air in exposure-measurement cycle of 20 minutes. The experiment time was thus cut down to a maximum of 100 minutes with two sets of response measurements, the first of which was during the 50-60 minute time period.

Despite not being conclusive due to insufficient sample size, the compromised ovine MTR observed here following the exposure to Avamys® during preliminary experiments provided evidence that studying the effect of the individual excipients/APIs on MTR was warranted.

The preservative, BKC, has often been reported to compromise MTR in *ex-vivo* models [116, 122], and this was supported in the current study where a significant decrease in *ex-vivo* ovine MTR was observed. Nevertheless, the current study also demonstrated, for the first time, some basis for the *in-vivo*/*ex-vivo* discrepancy of reports on the IN safety of BKC. Despite

irreversibly halting mucociliary transport when 3 ml of solution was applied to the ovine mucosa, BKC appeared to not affect MTR in 50 % of cases when sprayed onto the ovine mucosa using a nasal delivery system whereas it induced a reversible effect in the remaining 50 %. This indicated that dilution over a large surface area in the presence of a protective mucus lining is an important factor in neutralising the deleterious effects of BKC on the respiratory epithelium as was previously speculated [33, 107]. This finding correlates well with the lack of established evidence against BKC safety *in-vivo* in human subjects [102, 103, 105, 118]. This study therefore provides a better understanding of the *in-vivo*/*ex-vivo* conflict of data regarding the nasal safety of BKC.

In agreement with a previous study that used an *ex-vivo* model [116], the chelator, EDTA, elicited a decrease in MTR when applied in bulk (3 ml) to the ovine mucosa. Interestingly, this effect became insignificant when the compound was sprayed to the ovine mucosa, which is consistent with reports that employed *in-vivo* models [117-119]. Nevertheless, this observation might be seen as non-conclusive due to the small sample size supporting it (n = 3).

It can be argued that the sample size used in studying the effects of some sprayed compounds would benefit from an increase to six rather than three tracheae. This was restricted by the time-constraint of this project plus that it was often not necessary. For instance, the effect of potassium sorbate/propylene glycol preparation when applied in bulk was non-significant (n = 6) and continued to be non-significant when sprayed on the tracheal mucosa (n = 3), which presented a logical observation that did not necessitate a higher sample number. Moreover, a bigger sample size was also not necessary for a compound such as sprayed Methocel™, which in addition to inducing a significant effect on ovine MTR, as assessed by pairing data from four tracheae (using t-test for paired data), it also elicited a significant alteration to baseline MTR measurements in each of the individual four tracheae (using t-test for unmatched as explained in section 5.3.4). Similarly, sprayed polysorbate 80 demonstrated

non-significant alterations to the individual baseline MTR measurements in each of the three tracheae tested in addition to the non-significance of the response as assessed by pairing the data from these three tracheae, which provided enough evidence on the robustness of this effect despite the small sample size. It is worth noting that the blank (sprayed glucose) induced a non-significant alteration to ovine MTR as deduced by pairing data from six tracheae although only three out of these six individually showed non-significant alteration to their baseline. Notably, this is the first report to-date on the effect of potassium sorbate and polysorbate 80 on MTR, although the finding that cellulose derivatives (Methocel™ and Avicel® here) elicited a significant compromise to MTR, both when applied in bulk and as nasal spray, is consistent with all previous studies both *in-vivo* and in *ex-vivo* models [117, 142-147]. In contrast to Methocel™ (comprised of HPMC) that induced fully reversible effect, Avicel® (comprised of Na-CMC and microcrystalline cellulose) induced partially reversible effects. However, this was not demonstrated in the *in-vivo* reports, which demonstrated only mild to moderate effects for carboxymethylcellulose on MTR [147, 149, 150]. Meanwhile, this correlated well with the higher viscosity observed here for Avicel® than Methocel™ at their current formulation concentrations, which could have resulted in higher potency at decreasing MTR [144, 145] that presumably required longer time to demonstrate full recovery. Notably, a reversible decrease in MTR is favoured in IN formulations to prolong the nasal residence time and therefore enhance therapeutic efficacy.

Nonetheless, the above rationale justifying the testing of a small sample size on certain occasions did not apply to sprayed EDTA and sprayed DMSO (n = 3 in each of them), which would therefore benefit from a larger sample size in future work to strengthen the evidence on their non- significant effects on ovine MTR. Moreover, a larger sample size might indeed be necessary in the case of the sprayed glucocorticoid, fluticasone furoate (FF), whose significant effect on ovine MTR (n = 3) presented an unreasonable discrepancy to its non-

significant effect when applied as 3 ml solution containing 0.2 % DMSO (n = 6). Interestingly, 0.2 % DMSO solution significantly decreased MTR when applied (3 ml) to the ovine mucosa, which indicated that the presence of FF might have masked/ protected against this effect. This situation rendered the effect of sprayed FF observed here as inconclusive. Nevertheless, in light of the full recovery observed here from any deleterious effects caused by the sprayed compound and the lack of significant effects when the compound was applied in bulk, it is speculated that a larger sample size would similarly establish the lack of significant effects for the sprayed compound. Notably, other glucocorticoids were shown to improve MTR in airway allergic diseases [80, 92] although they demonstrated non-significant changes to healthy airways [103, 297, 309]. In high doses, steroids appeared to compromise mucociliary transport in healthy airways [297].

The antihistamine, GSK1004723, was initially studied here as sprayed preparations due to the knowledge of its cell toxic effect (chapter 6) at the time of this part of work, which aimed to investigate whether the presence of mucus blanket would protect the mucosa against any potentially deleterious effect. Interestingly, the sprayed preparations were sufficiently potent to compromise ovine MTR using either of the two provided salt forms (the D-form and the E-form) and at both suggested concentrations (0.5 % w/w and 0.01 % w/w). Noticeably, the IN antihistamine, azelastine, was shown to enhance MTR in allergic rhinitis patients whereas it had no effect on MTR in healthy volunteers [84].

In brief, this study showed most of the investigated IN excipients to be innocuous to mucociliary transport although it highlighted how critical is the extent of exposure in terms of the amount (dosage) applied to the respiratory mucosa. It is worth noting that long-term exposure to small amounts was beyond the scope of this study but is of tremendous interest for future work particularly since these pharmaceuticals can be used chronically for long periods of time.

Chapter 6

Effect of the Intranasal Formulations on Viability of Immortalised Human Bronchial Epithelial Cells; 16HBE14o-

6.1 Introduction

In-vitro cell culture models are often used in toxicity screening studies of pharmaceuticals [139, 245], which provide a valuable tool to rank excipients in terms of their toxicity to particular living systems and to compare novel compounds to well-known ones. The use of human cells from the appropriate living systems in these studies is considered the ideal resolution to the interspecies variability issues [245] as well as the increasing limitations on animal research [310]. It is worth noting that i*n-vitro* toxicology testing also proved valuable in elucidating mechanisms of toxicity although it does not reveal much information on adversity and reversibility of toxicity [245, 311].

Acute cell damaging effects might disrupt the integrity of cell membranes, which can be detected by measuring the presence of intracellular components, such as lactate dehydrogenase (LDH), in the extracellular fluid, e.g. the LDH assay. These damaging effects also cause cell malfunction, which would be manifested as compromised cellular uptake of the neutral red (NR) dye in the NR assays or compromised cellular reduction of the soluble yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) into the insoluble purple formazan in the MTT assays [139]. Interestingly, it has been reported that the MTT assay was the most sensitive among the above mentioned cytotoxicity assays to the effects of some pharmaceuticals on the human respiratory epithelial cell-line, Calu-3 [139]. This is a well-recognised cell culture model of the human respiratory system and is derived from a 25-year-old male Caucasian bronchial-adenocarcinoma [139]. These cells grow well in submerged and air-liquid-interface cultures to form mucus secreting monolayers [245, 310].

A cell-line that also proved valuable as a model of the respiratory epithelium and a useful tool in studying airway pathophysiology [312] as well as respiratory drug delivery and inhalation toxicity[245, 310, 313] was 16HBE14o-, which evolved from transformed human bronchial

epithelial cells from a 1-year-old male heart-lung-transplant patient [314]. These cells also grow well in either submerged or air-liquid-interface cultures although they form multi-layers and they do not secrete mucus [313]. To date, 16HBE14o- and Calu-3 cells are the two continuous cell culture models that best represent the respiratory epithelium [310, 315, 316].

Cell damaging effects were previously studied for BKC on various cell models [125, 127-130, 132, 317] although human respiratory cell models were rarely employed [131]. Similarly, a few reports studied the cytotoxic effects of polysorbate 80 on various cell models [127, 132, 133, 140, 141, 318, 319], however only one study employed human respiratory cells [139]. Moreover, despite some data on the cytotoxicity of EDTA [130, 134, 135, 320, 321], citrate buffer [322-324] and cellulose [325] to various cell models, there is a gap in the literature on their effects on human airway cells. Meanwhile, knowledge is quite limited on the cytotoxic effect of potassium sorbate [131], Methocel™ [139], propylene glycol [120, 139], PEG300 [120] and pH fluctuations [139, 317, 326] on human respiratory cells. Knowledge is also lacking on the cytotoxic effects of Avicel® and the APIs, fluticasone furoate and GSK1004723.

This chapter therefore aimed to research the potential cell damaging effects of the study IN pharmaceuticals by examining their effect on the viability of human bronchial epithelium cell line; 16HBE14o-, using an MTT assay.

6.2 Materials

- Culture flasks (175 cm^2) Nunclon^{TM} - PAA: The Cell Culture Company (PAA71175).
- Culture flasks (25 cm^2 and 75 cm^2) Nunclon^{TM} - Fisher Scientific (36196 and 78905 respectively).
- 96-well plates Nunclon^{TM} - Fisher Scientific (167008).
- Minimum Essential Medium (MEM) with Earle's salts and L-glutamine - PAA: The Cell Culture Company (E15-825).
- Trypsin/EDTA (0.05/0.02 % w/v in Dulbecco`s Phosphate Buffered Saline (DPBS)) - PAA: The Cell Culture Company (L11-004).
- Phosphate buffer saline (PBS) tablets - Oxoid Ltd, Wade Road, Basingstoke, Hampshire, United Kingdom (BR0014G).
- Foetal Bovine Serum "GOLD" EU approved - PAA: The Cell Culture Company (A15-151).
- Trypan blue - Acros Organics (189350250) distributed by Fisher Scientific (10164110).
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) - Sigma (M2128).
- Dimethyl sulfoxide - Sigma (D5879).
- Isopropanol, (Propan-2-ol, 2-Propanol, Iso-propyl alcohol) molecular biology grade - Fisher Scientific BioReagents **(**11895715).
- Hydrochloric acid 1M - Fisher Scientific (J/4320/15).
- Sodium hydroxide - Sigma (S8045).
- Sodium chloride analytical grade - Fisher Scientific (S/3160/65).
- Potassium chloride - Sigma (P9333).
- Sodium phosphate monobasic, anhydrous - Sigma (S0751).

- Disodium hydrogen orthophosphate, anhydrous - BDH Laboratory Supplies (301584L).
- Citric acid, anhydrous - Sigma (C1857).
- Trisodium citrate, dihydrate - Sigma (S1804).
- The test formulations/excipients/APIs - provided by GSK (see section 4.2.4)

6.3 Methods

6.3.1 Culture of the 16HBE14o- Cells and Optimisation of the MTT Assay

16HBE14o- cells were cultured at 37 °C in a 5 % CO_2 : 95 % air atmosphere using Nunclon® surface treated culture flasks and MEM containing 10 % FBS as culture medium. At 70-90 % confluency, the cells were washed twice with PBS and trypsinized with trypsin/EDTA (0.05/0.02 % w/v in Dulbecco`s Phosphate Buffered Saline (DPBS)). Live cell count was then estimated using 5 mg/ml trypan blue solution (1:4 mixture with an aliquot of the cell suspension) and a Neubauer haemocytometer. The cells were subsequently resuspended in the volume of culture medium calculated to give the required cell concentration ($4x10^6$ cells/ml in the early optimisation experiments and $2.5x10^5$ cells/ml in the later ones). The cells were then seeded into a 96-well tissue culture plate (100 µl/well). In the early experiments, the tissue culture plates (96-well) were seeded with a serial dilution of cell suspension (100 µl/well) over the range of $4x10^6$ to $6.25x10^4$ cells/ml (8 wells/plate for each seeding density), whereas the seeding density in the later experiments was 100 µl/well of a $2.5x10^5$ cells/ml suspension.

The plates were then incubated overnight at 37 °C in a 5 % CO_2 : 95 % air atmosphere. Following incubation, each well was spiked with 10 µl MTT solution (5 mg/ml in culture medium); this procedure was later optimised such that the medium was carefully aspirated from the plates, the cells were then washed with PBS (100 µl/well) and 100 µl MTT solution (0.5 mg/ml in culture medium) was added to each well. The 96-well plates were then incubated for 1h, which was later optimised to two hours, at 37 °C in 5 % CO_2 : 95 % air atmosphere.

Following incubation, the MTT solution was aspirated from the wells and 100 µl of either DMSO or isopropanol (IPA) acidified with hydrochloric acid (HCl) were added (the two solvents were compared). The plates were finally left to stand for 10 minutes before reading the absorbance at 540 nm using the microplate reader Labsystems Multiskan Ascent (595 nm was also used in the early experiments). The plates were re-read after 20 minutes, 30 minutes and 45 minutes to confirm the absorbance readings remained the same (indicating maximal dissolution of formazan crystals in the organic solvent). The mean absorbance value (after subtracting the blank mean absorbance) was then plotted against the seeding density to construct calibration curves that enabled the best seeding density (as well as the optimum protocol design (incubation time, solvent choice and absorbance wavelength)) to be chosen for the cytotoxicity experiments.

The most appropriate composition of the blank for the cytotoxicity studies was chosen by comparing the absorbance of various elements that might contribute to the background, namely (1) empty wells, (2) wells that only received the solvent (IPA or DMSO) in the last step of the experiment, (3) wells that went through the whole technique but had no cells in them and received no MTT solution (culture medium instead), (4) wells that went through the whole technique including receiving MTT solution but had no cells in them and finally (5) wells that had cells and went through the whole technique except that they received no MTT solution (culture medium instead).

The possibility of emptying the plates after each step of the protocol by inverting them and tapping them dry on laboratory towels rather than aspirating the liquids from the plates was also investigated (calibration curves constructed using 96-well plates handled by both methods were compared). In addition, preparing the MTT solution in sterile PBS rather than culture medium was also examined.

In order to study the cytotoxic effect of excipients with chelating properties, a centrifugation procedure had to be introduced to retain the cells detached by these excipients (see section 6.3.2.1 below). To validate this procedure, tissue culture plates (96-well) were seeded with 100 µl/well cell suspension and then centrifuged at either 500 g for 5 min, 500 g for 10 min or 580 g for 10 min. These options were then compared by counting the cells in the supernatant and choosing the one achieving zero cell count for subsequent experiments.

6.3.2 Cytotoxicity Studies

6.3.2.1 The MTT Experimental Protocol

16HBE14o- cells (passages 91-99) were cultured, harvested and counted as described above in section 6.3.1. The cells were re-suspended in culture medium to give a concentration of $2.5x10^5$ cells/ml. Cell culture plates (96-well) were seeded with 100 µl/well cell suspension to give a seeding density of $2.5x10^4$ cells/well. The plates were then incubated at 37 °C in 5 % CO_2 : 95 % air atmosphere for 24 hours. Following incubation, the culture medium was gently aspirated from each well, which was then washed with 100 µl/well PBS. The test formulation/excipient (treatment) was applied to each well (100 µl/well, n = 8 wells x 2 columns). The plates were then incubated for 30 minutes (exposure time). Following incubation, the treatment solutions were aspirated from the wells and 100 µl/well of MTT solution (0.5 mg/ml in culture medium) were added to one set of wells (n = 8) out of the two sets exposed to the treatment. The other eight wells received 100 µl/well culture medium containing no MTT (this column yielded the blank readings). The 96-well plates were then incubated for two hours. Following incubation, the MTT solution/culture medium was aspirated from the wells and 100 µl/well of DMSO was added. The plates were finally left to stand for 10 minutes before reading the absorbance at 540 nm on the microplate reader Labsystems Multiskan Ascent.

The cytotoxicity experiments involving excipients with chelating properties (EDTA and citrate ion) followed the above protocol with the addition of a centrifugation procedure (580 g x 10 min) prior to each aspiration step that occured after exposing the cells to these excipients. In addition, studying the cytotoxic effect of citrate solutions also required the introduction of a wash step (100 µl/well PBS) immediately after aspirating these test solutions from the wells to get rid of the acidic conditions that might interfere with the subsequent MTT exposure [327]. In order to achieve the consistency (in exposure times and manipulations) required for a valid comparison between the citrate solutions on one hand and the phosphate solutions, the unbuffered saline solutions and the EDTA on the other hand (in terms of any observed cytotoxic effect), it was necessary to implement these modifications (the centrifugation and the wash procedures) in experiments studying their effects.

6.3.2.2 The Test Formulations/Excipients/APIs

Sterile water was used in all preparations. Table 6.1 summarises the formulations and excipients examined for their effects on the viability of 16HBE14o- cells and the corresponding vehicles used for their preparation. It also shows the reference treatment (RT) used for each group of formulations/excipients. This was the treatment applied to a set of 16HBE14o-seeded-wells (negative-control-wells), simultaneously with the set of wells exposed to the test formulation/excipient (the experimental wells) in the same culture plate, to demonstrate normal production of formazan such that their absorbance acted as the 100 % cell viability reference for the experimental wells (as described in section 6.3.2.3).

Table 6.1 Preparation of the Formulations/Excipients Studied for their Effect on the Viability of 16HBE14o- Cells.

The Test Formulation/Excipient	The Vehicle	The RT
• Avamys® • Avamys®- placebo • BKC - 0.015 % w/w • EDTA - 0.015 % w/w • Polysorbate 80 - 0.025 % w/w • Avicel® RC591 - 1.5 % w/w • Methocel™ – 1.0 % w/w • Avicel® CL611 - 2.4 % w/w • GSK1004723D solution† - 0.01 % w/w • GSK1004723D solution† - 0.5 % w/w • DMSOΔ – 0.2 % w/w	5 % w/w glucose	5 % w/w glucose
• GSK1004723E solution†† - 0.01 % w/w • Fluticasone furoate solution* - 0.0004 % w/w	5 % w/w glucose containing 0.2 % w/w DMSO	5 % w/w glucose
• GSK1004723D suspension‡ - 0.557% w/w salt (equivalent to 0.5 % w/w base) • GSK1004723E suspension‡‡ - 0.73% w/w salt (equivalent to 0.5 % w/w base)	5 % w/w glucose containing 1.0 % w/w Methocel™ and 0.025 % w/w polysorbate 80. 2.4 % w/w Avicel® CL611, 0.025 % w/w polysorbate80 and water.	culture medium
• Potassium sorbate ** - 0.3 % w/w • Propylene glycol - 1.5 % w/w • PEG300 - 62 % w/w • PEG300 - 30 % w/w • PEG300 - 15 % w/w • PEG300 - 10 % w/w • PEG300 - 5 % w/w • Glucose - 5 % w/w • Phosphate buffered saline (PBS) pH 7.3 - 0.01 M	culture medium	culture medium
• Saline citrate (0.05 M) buffer*** pH 3 to 6 • Saline sodium citrate (0.05 M)*** pH 7.3 • Saline sodium citrate (0.03 M)*** pH 7.3 • Saline Phosphate buffer (0.1 M)*** pH 6 and 8 • Normal saline• pH 3 to 8	Saline	0.01 M Phosphate buffered saline pH 7.3

† The antihistamine, GSK1004723, was provided as two different salts, the water-insoluble naphthalene-salt (GSK1004723E) and the water-soluble dihydrochloride salt (GSK1004723D). Each of them was studied in solution and in suspension. GSK1004723D solutions were prepared at 0.01 % w/w and at 0.5 % w/w in 5 % w/w glucose solution. The pH was adjusted using 1M NaOH.

$^{\Delta}$ DMSO is not a pharmaceutical excipient. Nevertheless, it was studied here as it was incorporated into some preparations as a solubiliser.

†† GSK1004723E was solubilised in DMSO (1 part in 20 parts) and then diluted with 5 % w/w glucose to give a 0.2 % w/w final DMSO concentration. Thus the final concentration of GSK1004723E in the test solution was 0.01 % w/w.

*Fluticasone Furoate (FF) was solubilised in DMSO (1 part in 500 parts; sparingly soluble) and then diluted with 5 % w/w glucose to give a final DMSO concentration of 0.2 % w/w. Thus the final concentration of FF in the test solution was 0.0004%w/w.

‡ GSK1004723D suspension was prepared using a modification of its formulation guidelines provided by GSK (0.557 % w/w GSK1004723D, 0.015 % w/w EDTA, 1.0 % w/w Methocel^{TM} E50LV Premium, 0.15 % w/w BKC, 0.025 % w/w polysorbate80, 5 % w/w glucose and water to 100 % w/w). In this modification, EDTA and BKC were excluded due to toxic effects observed in the earlier MTT experiments. The formulation procedure involved hydrating the Methocel in water at 50-60°C using a magnetic stirrer-heater for one hour followed by cooling to room temperature. The glucose was then slowly added and allowed to dissolve. Meanwhile, the API was dissolved in a solution of the polysorbate80 in the minimum amount of water. The API/polysorbate 80 solution was then transferred quantitatively to the Methocel/glucose mixture and the weight made to 100 % w/w with water. The pH was adjusted using 1M NaOH.

‡‡ GSK1004723E suspension was also prepared according to a modification of its formulation guidelines provided by GSK (0.73 % w/w GSK1004723E, 0.015 % w/w EDTA, 2.4 % w/w Avicel CL611, 0.96 % w/w citric acid anhydrous, 1.48 % w/w sodium citrate, 0.025 % w/w polysorbate 80, 0.3 % w/w potassium sorbate, 1.5 % w/w propylene glycol and water to 100 % w/w). The modification here involved the exclusion of EDTA, citric acid anhydrous and sodium citrate (due to toxic effects observed in the earlier MTT experiments) as well as the exclusion of potassium sorbate and propylene glycol due to the very high osmolality of the suspension (~ 608 mOsmol/kg). The formulation procedure involved hydrating the Avicel CL611 in water using Silverson laboratory mixer (serial number 19822) for one hour. Meanwhile, slurry of the API was made by adding a solution of the polysorbate80 in the minimum amount of water to the weighed API while mixing vigorously. That slurry was then transferred quantitatively to the Avicel CL611 dispersion and the weight made to 100 % w/w with water.

**Potassium sorbate, demonstrated solubility issues at its working formulation concentration. Thus the effect of potassium sorbate on cell viability was studied in the presence of propylene glycol (solubilizing agent). The effect of propylene glycol on cell viability was then

compared to the combined effect of propylene glycol and potassium sorbate to deduce the effect of potassium sorbate on cell viability.

*** The citrate buffer solutions, 0.05 M at pH 3, 4, 5 and 6, were prepared by mixing calculated volumes of 0.05 M citric acid and 0.05 M tri-sodium citrate stock solutions. Meanwhile, the phosphate buffer solutions, 0.1 M at pH 6 and 8, were prepared using 0.1 M di-sodium hydrogen phosphate and 0.1 M sodium di-hydrogen phosphate stock solutions. The saline sodium citrate (SSC) pH 7.3 solutions were prepared at the concentrations of 0.05 M and 0.03 M tri-sodium citrate containing 0.1 M and 0.08 M sodium chloride respectively. The pH of all these buffered solutions were checked and finely adjusted using 1M hydrochloric acid/1M sodium hydroxide while their osmolality was adjusted to ~ 310 mOsmol/Kg by adding firstly potassium chloride (to a maximum of 4 mM) then sodium chloride.

• pH of the non-buffered normal saline solutions was adjusted using 1M hydrochloric acid/1M sodium hydroxide while the osmolality of these solutions was adjusted using sodium chloride only.

The vehicle of the preparation was generally used as its RT. For instance, some excipients were studied in culture medium, which was also used as their RT. Compounds commercially available in glucose 5 % w/w matrix were studied in this matrix, which was hence used as their RT. GSK1004723 suspensions, however, had vehicle matrices that differed among themselves and hence culture medium was used as RT to enable them to be compared to each other. Compounds were all tested at their working formulation concentrations.

The osmolality (mOsmol/kg) and pH of all solutions were measured prior to their application to the cells using the Gonotec Osmomat®030 cryoscopic osmometer and the Metler Toledo MP220 pH meter respectively.

6.3.2.3 Data analysis

The absorbance values of the eight wells exposed to a particular treatment followed by MTT were corrected for background absorbance by subtracting the mean absorbance of the corresponding blank eight wells (exposed to the same treatment but not MTT) from each of them. Each corrected value was then expressed as a percentage relative to the corrected mean absorbance of the eight wells exposed to the RT (negative control) instead of the test compound (see section 6.3.2.2). The corrected mean absorbance of the negative control thus represented 100 % cell viability and the individual corrected absorbance values of the test and reference treatments were converted to % cell viability relative to it.

The significance of alterations in cell viability following different exposures was analysed using KrusKal-Wallis test (one-way ANOVA was not valid due to considerably different standard deviations of various data sets). The data was then exposed to Dunn's test to estimate the significance of variation in cell viability between each treatment and its RT ($P \leq 0.05$). Bar charts were constructed using Microsoft Office Excel 2007 and 2010 whereas the statistical analysis was carried out using the GraphPad Prism 5 statistical package.

6.4 Results

6.4.1 Optimisation of the MTT Assay Cell Seeding Density and Experimental Design

In the early MTT assay calibration experiments, when cell exposure to MTT was carried out by directly adding 10 µl MTT solution (5 mg/ml MTT in culture medium) to the supernatant of each well incubated overnight with 16HBE14o- cells, calibration curves demonstrated linearity even at very high cell seeding densities that were clearly above confluence (Figure 6.1). In agreement, non-adherent cells that reduced MTT to formazan crystals were observed to settle loosely at the bottom of the wells. It was thus decided to aspirate the culture medium and wash the culture wells with sterile PBS prior to adding the MTT solution (0.5 mg/ml) in all subsequent work to get rid of any non-adherent cell (see section 6.5 for further discussion).

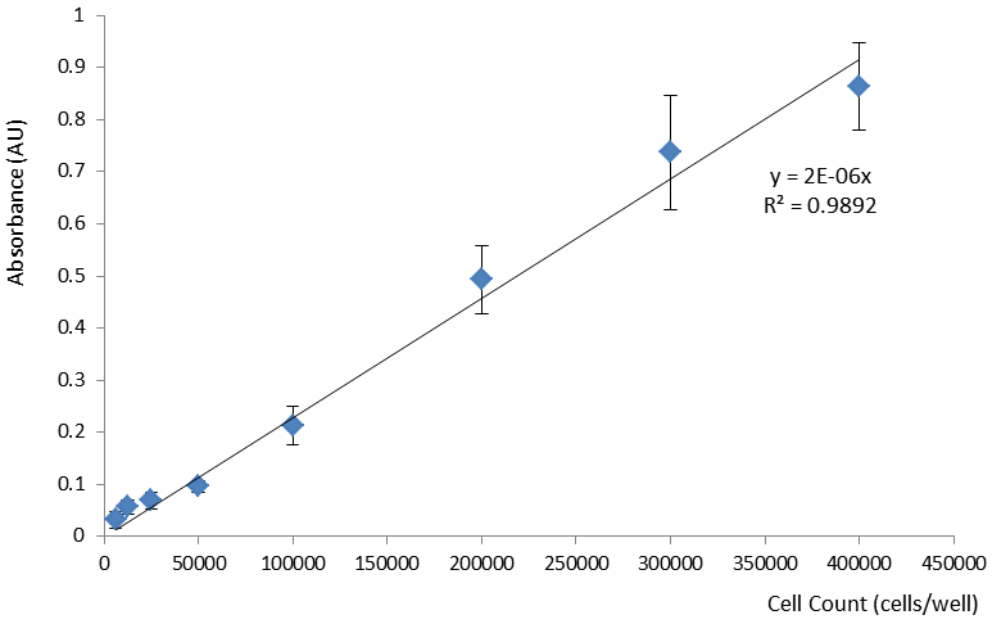


Figure 6.1 Early MTT Assay Calibration Curve.

MTT solution (5 mg/ml, 10 µl) was added to the culture medium of cells in the culture wells without change of medium or wash step. (mean ± SD, n = 8)

An improvement in assay sensitivity was observed when measuring absorbance at 540 nm as compared to 595 nm (Figures 6.2 (a)), which was more prominent when DMSO was used as the formazan solvent rather than acidified IPA (Figures 6.2 (b)). It is worth noting that the absorption spectrum is dependent on the solvent. It also became evident (Figures 6.2 (b)) that employing DMSO as the formazan solvent was generally associated with enhanced assay sensitivity as compared to acidified IPA. These observations on the MTT wavelength and solvent were supported by the data in figure 6.3, which also depicted the effect of plate manipulation techniques (i.e. the plate emptying approach) on the assay. Although the plate inversion technique appeared better than the liquid aspiration technique in emptying the wells that were assayed using acidified IPA, this situation was not observed in the DMSO treated wells, in which plate inversion only demonstrated less variability. It was therefore inferred there was no clear advantage for one technique over the other.

Emerging from the above, there was enough evidence at this stage of the study to adopt 540 nm as the MTT assay analysis wavelength, DMSO as the assay solvent and liquid aspiration as the plate emptying technique. The latter was further discussed in section 6.5.

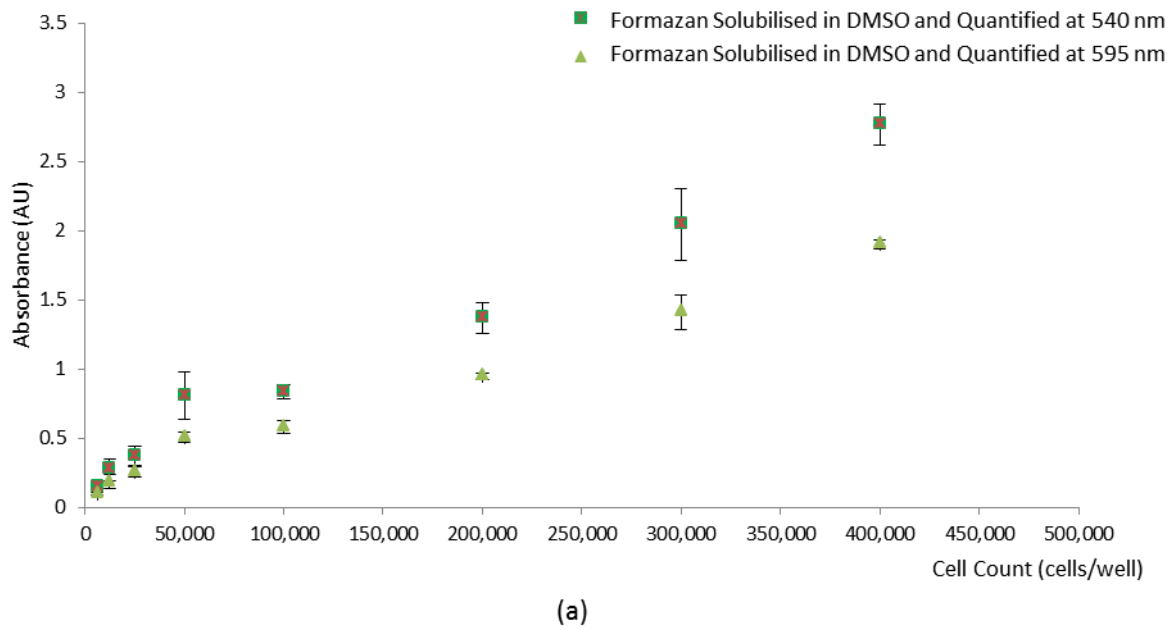


Figure 6.2 Optimisation of the MTT Assay; Analysis Wavelength and Solvent.

After two hours incubation, the MTT solution was aspirated from the cells. One plate was exposed to DMSO and the other to acidified IPA. A 45-minute solubilisation time was allowed before measuring the absorbance at 540 nm and at 595 nm. (mean ± SD, n = 8). (a) The effect of analysis wavelength on the absorbance of formazan solubilised in DMSO. (b) The effect of formazan solvent on the absorbance of formazan as measured at 595 nm and 540 nm.

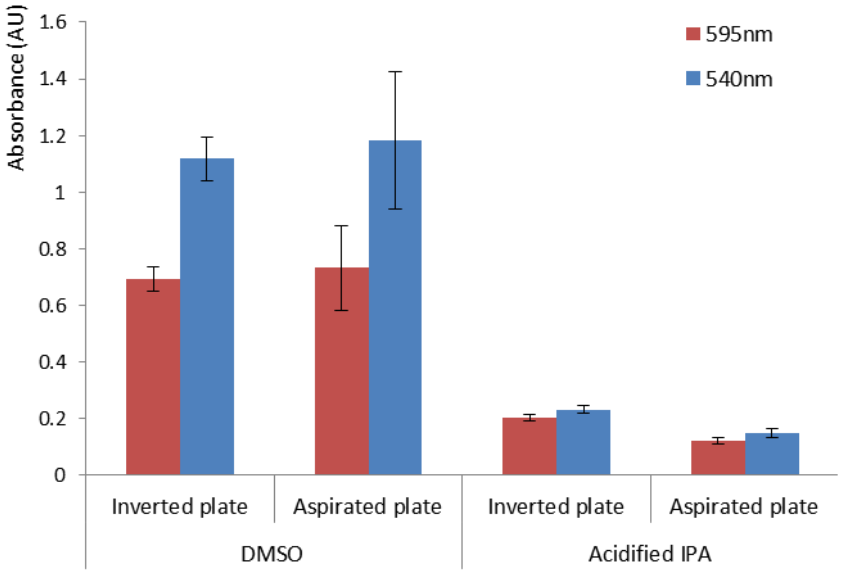


Figure 6.3 Optimisation of the MTT Assay, Aspiration Vs. Inversion

16HBE14o- cells were cultured at $2.5x10^4$ cells/well in each of two 96-well plates. Following incubation overnight at 37 °C in 5 % CO_2 : 95 % air, the wells on one plate were aspirated whereas the other plate was inverted and blot dried. Both plates were then washed with sterile PBS, treated with 100 µl/well MTT solution in culture medium (0.5 mg/ml) and incubated for two hours at 37 °C in 5 % CO_2 : 95 % air. Each plate was then emptied using the same emptying technique adopted earlier. A volume of solvent (100 µl/well either DMSO or acidified IPA) was then added to each well and the plates were left to stand for 10 minutes prior to reading their absorbance at 540 nm and at 595 nm. It is worth noting that an extra wash step using the culture medium was implemented here before adding the MTT solution. This was intended to simulate the manipulations in the actual cytotoxicity experiment where the cells are exposed to the test agent (or culture medium in the control wells) prior to adding the MTT solution. (mean ± SD, n = 8)

The incubation time with MTT was also investigated. This is often in the range of 1-4 hours. Acceptable assay sensitivity was attained after a two-hour incubation (see figure 6.4), which was demonstrated by sufficiently high absorbance values (high enough to achieve good assay sensitivity but not close to the over-range optical densities) in the linear range of the assay ($\leq 2.5x10^4$ cells/well). A two-hour incubation was then adopted for further work.

Figure 6.4 also indicated the upper limit of the linear range of the assay to fall between $2.5x10^4$ cells/well and $5.0x10^4$ cells/well (Figure 6.5 (a)). Calibration curves constructed using cell seeding densities that fell below this limit of the linear range demonstrated assay linearity (Figure 6.5 (b)). It thus became evident that an appropriate 16HBE14o- seeding density to use in this MTT assay was $2.5x10^4$ cells/well.

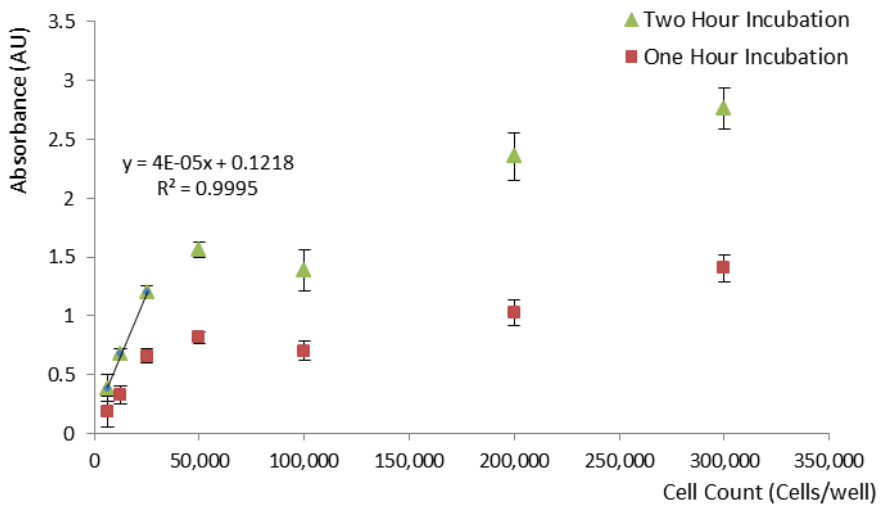


Figure 6.4 Optimisation of the MTT Assay; Incubation Time with MTT.

(Mean ± SD, n = 8)

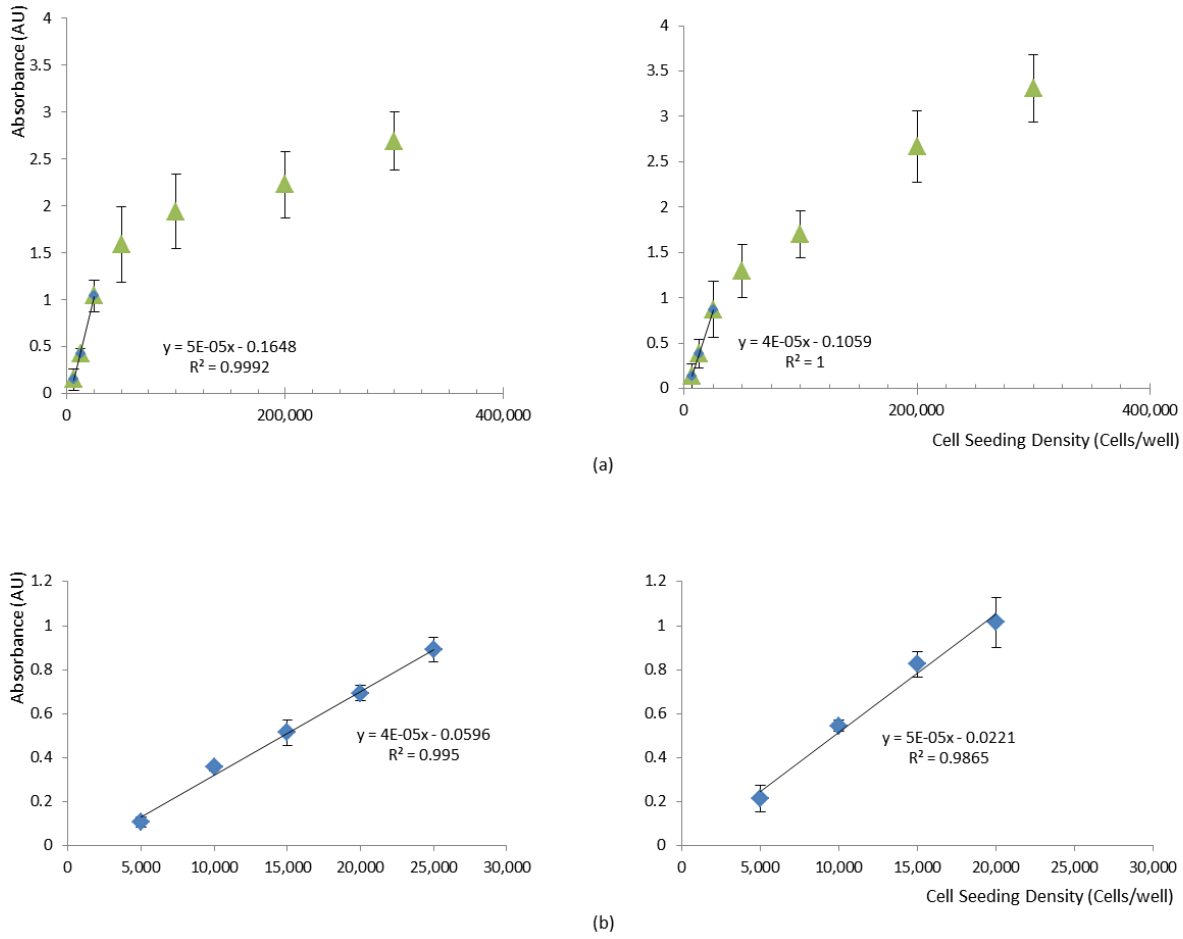


Figure 6.5 Optimisation of the MTT Assay; Cell Seeding Density.

The presented calibration curves were each constructed independently on separate days using different cell passages of the 16HBE14o- cells to provide evidence for the linear range being ≤ 25,000 cells/well. (mean ± SD, n = 8)

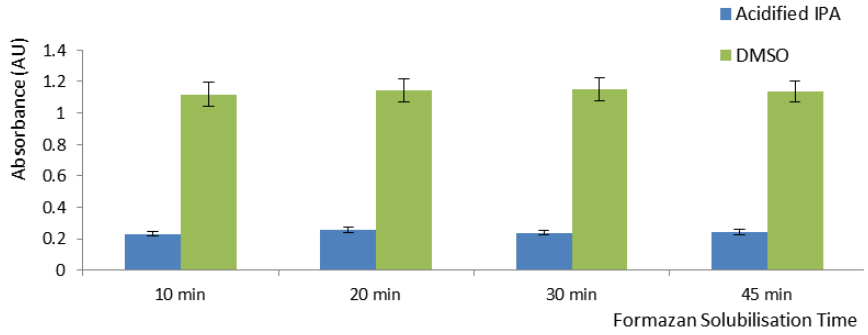
(a) Calibration curves covering a wide range of seeding densities. (b) Calibration curves within the linear range of seeding densities.

The time required for full solubilisation of the formazan crystals; the purple end product of the MTT enzymatic conversion and the assay analyte that was measured colourimetrically, was also checked. Figure 6.6 (a) showed hardly any difference between allowing only 10 minutes for formazan solubilisation and allowing any longer period. Therefore, a solubilisation time of 10 minutes was used in all further experiments.

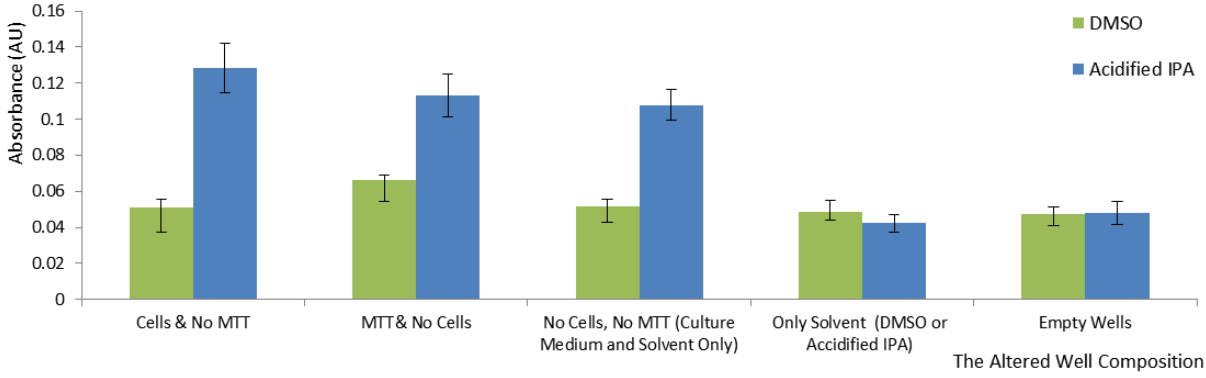
Potential sources of background noise in the MTT assay were assessed to enable optimum design of the assay's blank wells. Figure 6.6 (b) indicated that the main source of background absorbance was the well itself. The solvents, DMSO and acidified IPA, showed barely any difference in absorbance in the absence of cells, any residual culture medium or MTT solution in the well. The presence of any of these elements was associated with a considerable increase in background absorbance when acidified IPA was used as the formazan solvent although no change was detected when DMSO was used instead. This therefore confirmed that DMSO was the solvent of choice in all our subsequent MTT work.

Based on the result above, we designed our blank wells such that they were similarly seeded as the negative control and the experimental wells. They were then exposed to all assay procedures similar to the experimental wells (this included exposure to the test agent) except that they were treated with culture medium without MTT when the experimental wells were treated with MTT solution in culture medium. This design is discussed further in section 6.5.

For the purpose of studying the effect of chelating agents on the 16HBE14o- cell viability, validation of an efficient centrifugation step that achieved zero cell count in the supernatant proved the 5-minute programme to be defective whereas the 10-minute programmes (either at 500g or at 580g) were valid.



(a)



(a)

Figure 6.6 Optimisation of the MTT Assay; Solubilisation Time of the Formazan Crystals and Sources of Background Noise.

(a) Solubilisation time of the formazan crystals was studied by repeatedly measuring their absorbance in the wells after the indicated periods of time. (b) Sources of background absorbance was studied by measuring the absorbance of wells that were either exposed to all assay procedures with the exception of certain element(s), i.e. cells, MTT solution or both, or not exposed to the assay altogether (empty wells or wells containing solvent only). (mean ± SD, n = 8)

6.4.2 Cytotoxicity Studies

6.4.2.1 Effect of 5 % w/w Glucose and PBS on Cell Viability

A decrease in viability was often observed after exposure of the cells to glucose solution (5 % w/w). Nevertheless, this decrease was not significant ($P > 0.05$) compared to cell viability in wells exposed to cell culture medium (Figure 6.7). The 5 % glucose solution was therefore valid to use as reference treatment (RT).

Interestingly, a significant difference ($p < 0.05$) was observed between the viability of 16HBE14o- cells exposed to PBS (pH = 7.3) and the viability of these exposed to cell culture medium (Figure 6.8). It can however be inferred from the figure that the viability following exposure of the cells to PBS was still sufficiently high to validate the use of PBS as RT for comparable solutions such as buffers.

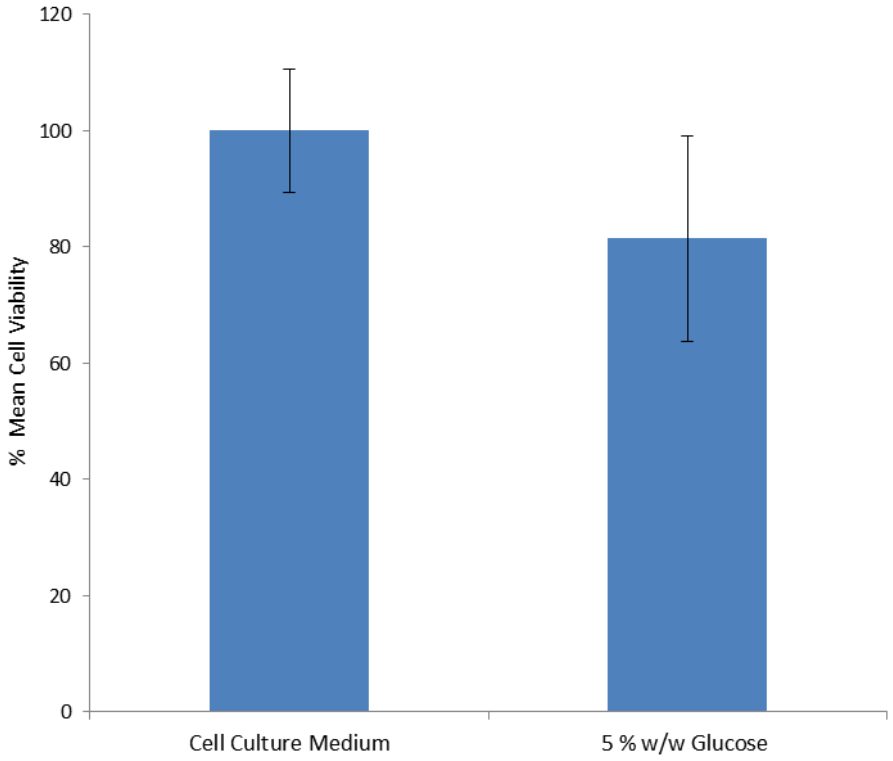


Figure 6.7 The Effect of 5 % w/w Glucose on the Viability of 16HBE14o- Cells.

(mean ±SD, n = 32 pooled from three cell passages)

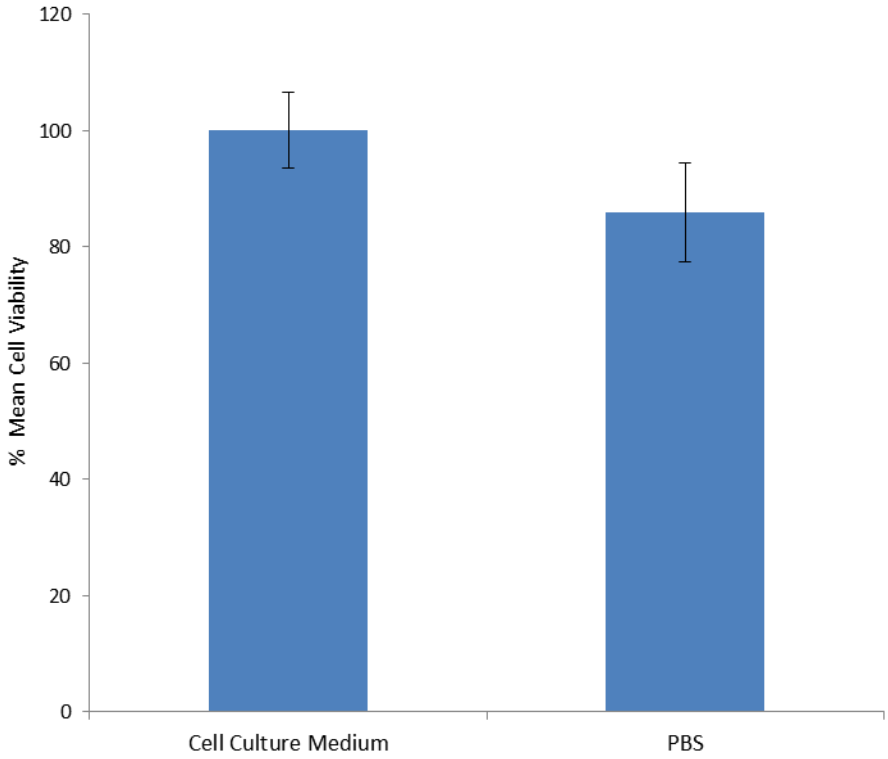


Figure 6.8 The Effect of PBS on the Viability of 16HBE14o- Cells.

(mean ±SD, n = 23 to 24 pooled from three cell passages)

6.4.2.2 Effect of the IN Pharmaceuticals Prepared in 5 % w/w Glucose on Cell Viability

As depicted in figure 6.9, most of the studied IN pharmaceuticals did not induce significant alterations to cell viability ($P > 0.05$). Nevertheless, BKC, the BKC-based formulations; Avamys® and placebo Avamys®, and the antihistamine APIs; GSK1004723D and GSK1004723E, elicited significant decreases in the 16HBE14o- cell viability ($P < 0.05$).

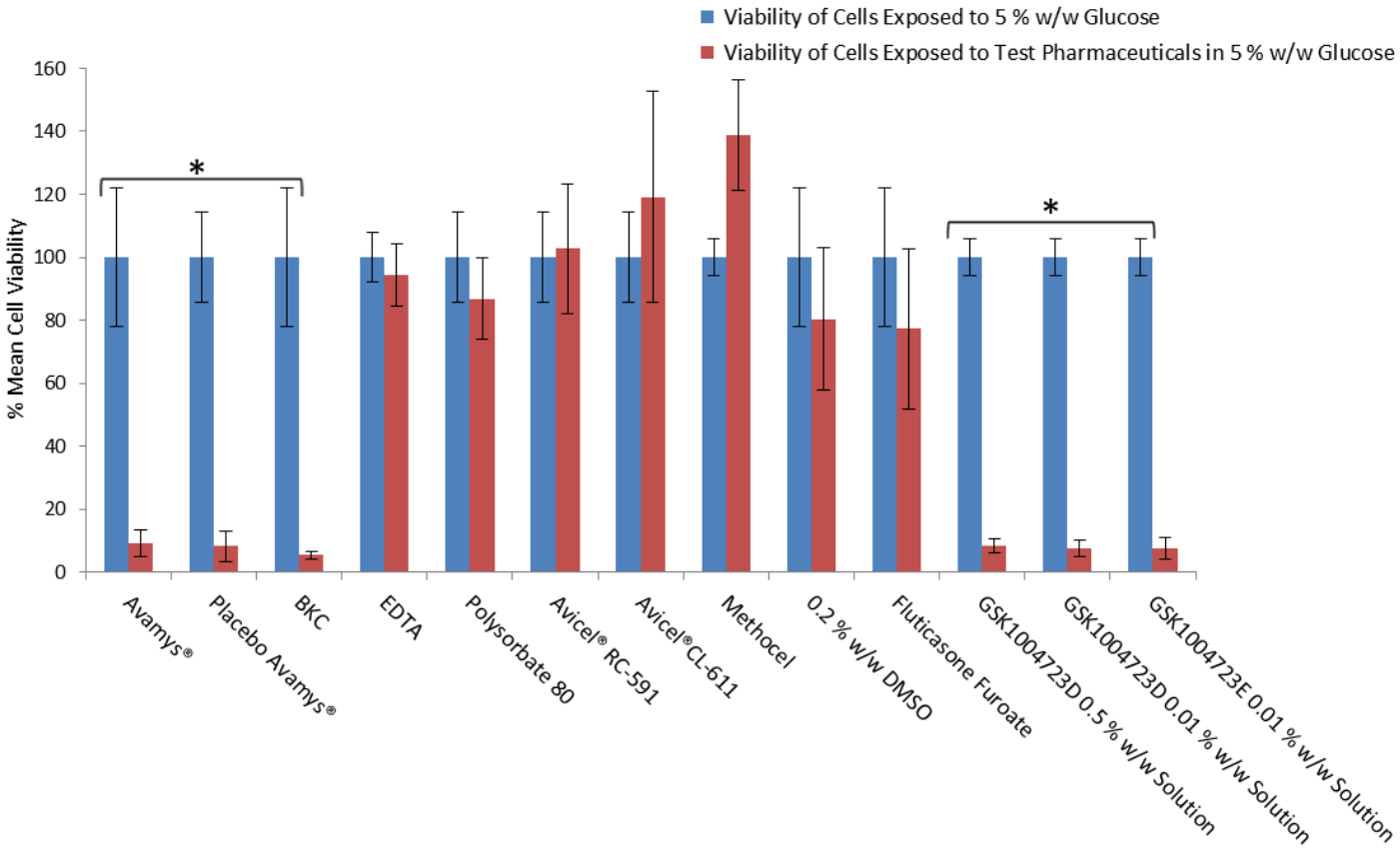


Figure 6.9 Effect of Some IN Pharmaceuticals on the Viability of 16HBE14o- Cells using 5 % w/w Glucose as Reference Treatment.

(mean ± SD, n = 13 to 16 pooled from two cell passages)

* indicates significant responses.

Since BKC is generally used in IN formulation at a concentration of 0.002 - 0.02 % w/v [328], its effect on cell viability was further studied at different concentrations covering this range. As elaborated in figure 6.10, the significant decrease in cell viability ($p < 0.05$) by BKC persisted at lower concentrations.

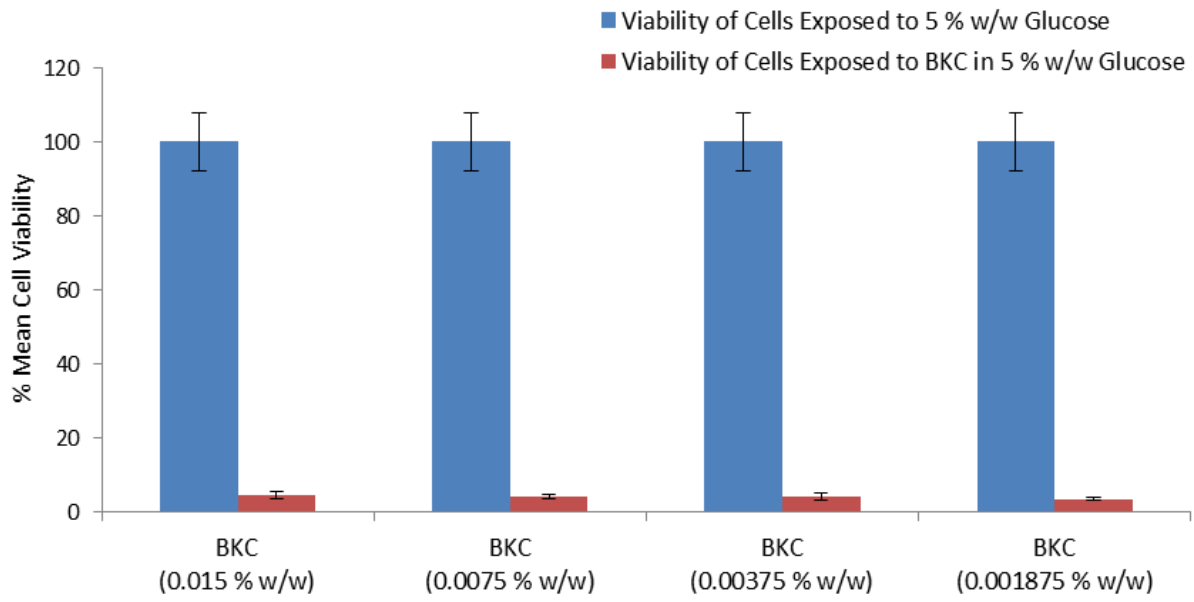


Figure 6.10 Effect of BKC on the Viability of 16HBE14o- Cells.

(mean ± SD, n = 16 pooled from two cell passages)

6.4.2.3 Effect the IN Pharmaceuticals Prepared in Cell Culture Medium on Cell Viability

As illustrated in figure 6.11, potassium sorbate and propylene glycol did not induce significant alteration to the viability of 16HBE14o- cells ($P > 0.05$). However, with the exception of the 5 % w/w concentration ($P > 0.05$), PEG300 induced a significant decrease in cell viability ($P < 0.05$) at all higher concentrations. It can also be inferred from figure 6.11 that the antihistamine APIs, GSK1004723E and GSK1004723D, continued to induce significant decreases in cell viability ($P < 0.05$) while formulated as suspensions, which was comparable to their effects in solution (Figure 6.9). Notably, the placebo suspensions also induced a significant decrease in the 16HBE14o- viability levels ($P < 0.05$) though with clearly less damaging effects than in the presence of the APIs (see section 6.5 for further discussion).

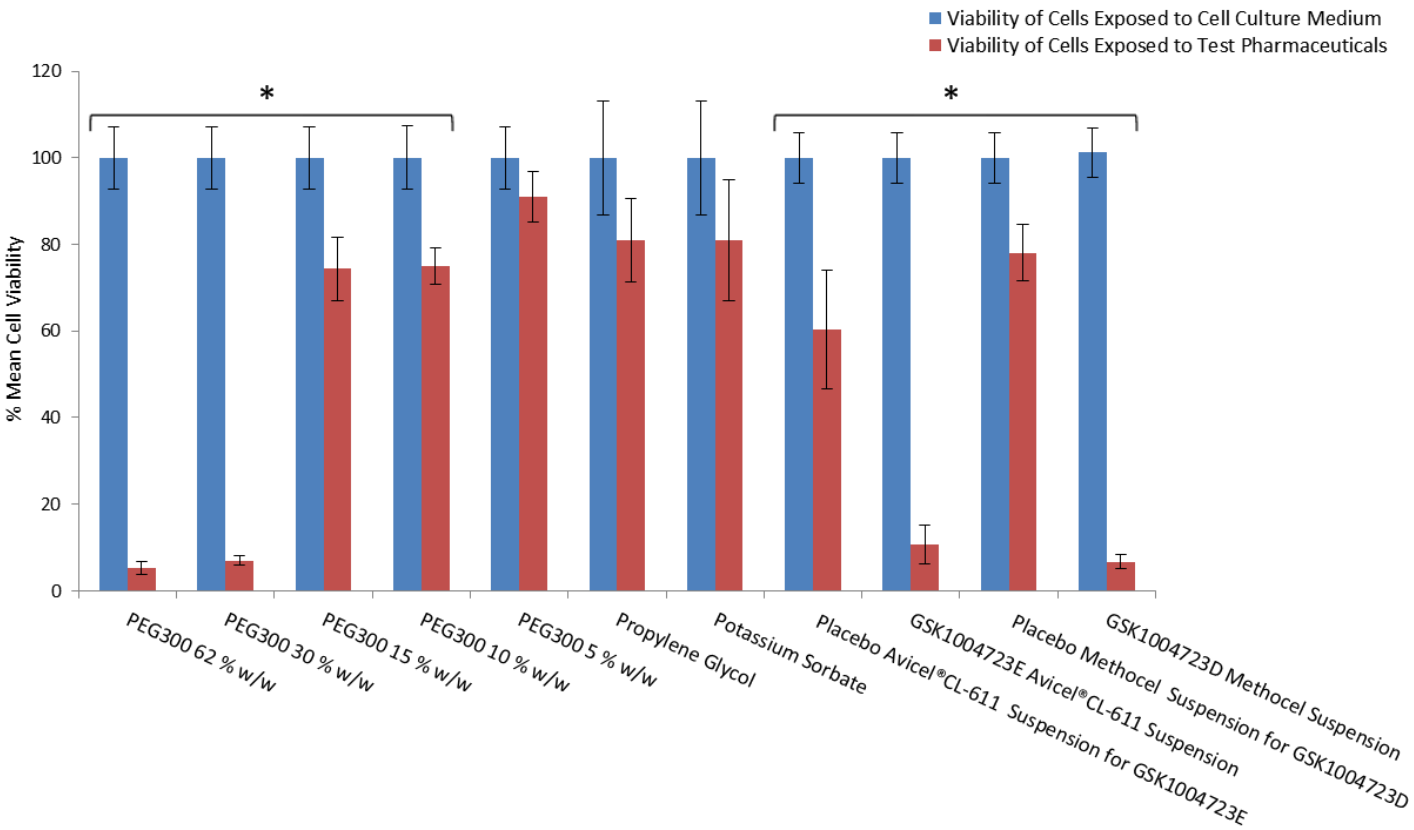


Figure 6.11 Effect of Some IN Pharmaceuticals on the Viability of 16HBE14o- Cells using Cell Culture Medium as the Reference Treatment.

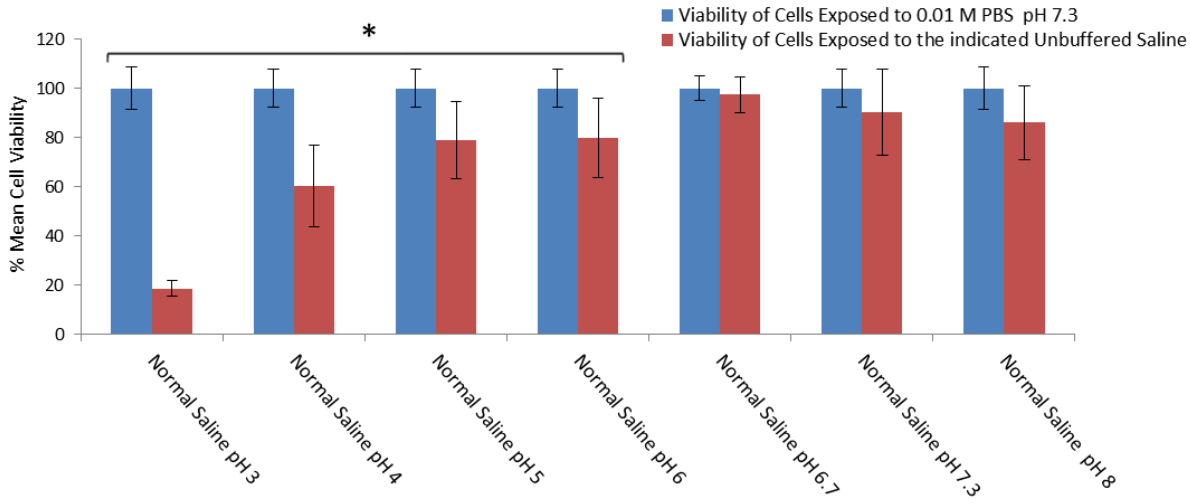
(mean ± SD, n = 13 to 16 pooled from two cell passages)

* indicates significant responses.

6.4.2.4 Effect of pH and Various IN Buffers on Cell Viability

Extreme pH 3 and 4 elicited a significant decrease to cell viability as demonstrated in figure 6.12 (a) and (b). Meanwhile, although pHs 5 and 6 significantly lowered the cell viability levels when tested using unbuffered normal saline solutions ($p < 0.05$, figure 6.12 (a)), it showed hardly any effect on cell viability when maintained using citrate buffer pH 5 and phosphate buffer pH 6 ($p > 0.05$, figure 6.12 (b)). At pHs 6.7 and 7.3, no significant effect on cell viability was observed ($p > 0.05$) following exposure to normal saline solutions at these pHs (Figure 6.12 (a)) as compared to phosphate buffered saline (PBS) pH 7.3. Nevertheless, citrate buffer at pHs 6 and 7.3 elicited a significant decrease in cell viability ($p < 0.05$, figure 6.12 (b)) indicating a toxic effect inherent to citrate ions. Meanwhile, pH 8 caused non-significant decrease in cell viability when tested as normal saline solution ($p > 0.05$, figure 6.12 (a)), although the effect became significant when the pH 8 was maintained by phosphate buffer ($p < 0.05$, figure 6.12 (b)).

(a)



(b)

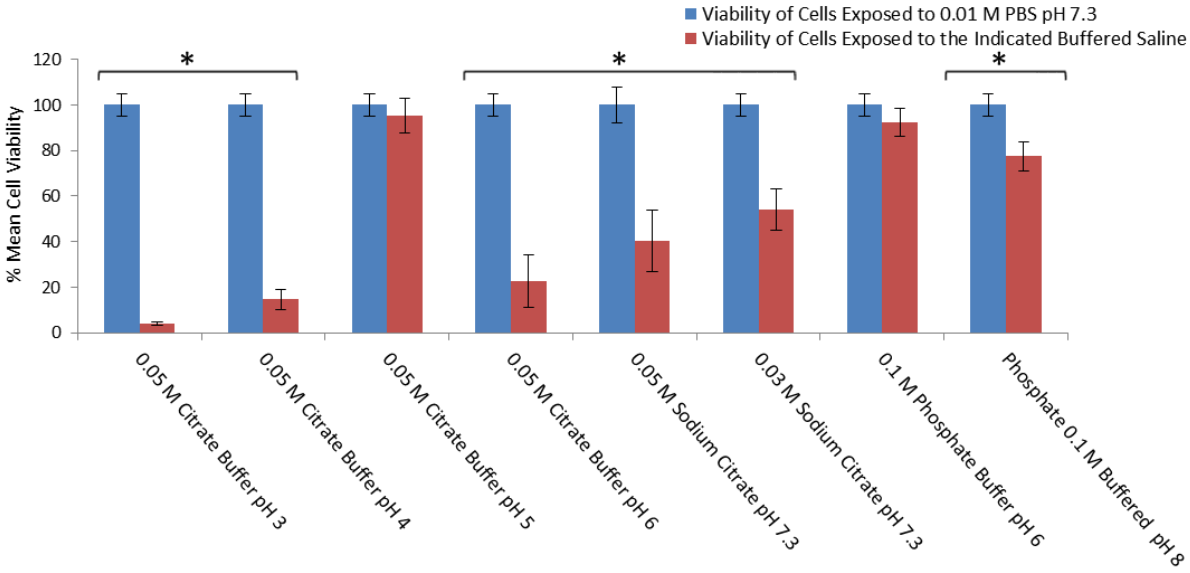


Figure 6.12 Effect of pH on the Viability of 16HBE14o- Cells.

(a) The effect of unbuffered normal saline (0.9 % w/v sodium chloride) at different pH on cell viability. (b)The effect of citrate and phosphate buffers at different pH on cell viability. (mean ± SD, n = 15 to 16 pooled from two cell passages)

* indicates significant responses.

6.4.2.5 Summary of Findings

The pH and osmolality values of the preparations studied here, as measured before being used in experiments, are listed below in table 6.2 along with their observed effects on the viability of 16HBE14o- cells.

Table 6.2 The pH, Osmolality and Observed Effects of the Studied Preparations.

The Test Preparation	pH	Osmolality (mOsmol/kg)	Effect on the 16HBE14o- cell viability
Glucose solution (5 % w/w)	6.26 to 7.30 (Interday variability)	296 to 300 (Interday variability)	Non-significant decrease
Avamys®/ Placebo Avamys®	5.89	313	Significant decrease
BKC (0.015 % w/w in 5 % w/w glucose solution)	6.70	300	Significant decrease
EDTA (0.015 % w/w in 5 % w/w glucose solution)	6.50	293	Non-significant decrease
Avicel® RC-591 (1.5 % w/w in 5 % w/w glucose solution)	6.11	314	Non-significant decrease
Avicel® CL-611 (2.4 % w/w in 5 % w/w glucose solution)	6.85	636	Non-significant decrease
Methocel™ (1.0 % w/w in 5 % w/w glucose solution)	7.40	342	Non-significant decrease
Polysorbate 80 (0.025 % w/w in 5 % w/w glucose solution)	6.80	302	Non-significant decrease
Potassium sorbate /propylene glycol (0.3 % w/w /1.5 % w/w in cell culture medium respectively)	8.16	570	Non-significant decrease
Propylene glycol (1.5 % w/w in cell culture medium)	8.26	508	Non-significant decrease
PEG 300 (62 % w/w in cell culture medium)	8.00	Over-range	Significant decrease
PEG 300 (30 % w/w in cell culture medium)	7.88	Over-range	Significant decrease
PEG 300 (15 % w/w in cell culture medium)	7.93	1285	Significant decrease

PEG 300 (10 % w/w in cell culture medium)	7.96	832	Significant decrease
PEG 300 (5 % w/w in cell culture medium)	8.00	519	Non-significant decrease
DMSO (0.2 % w/w in 5 % w/w glucose solution)	6.36	333	Non-significant decrease
Fluticasone furoate (4µg/ml in 5 % w/w glucose/ 0.2% w/w DMSO solution]	7.50	300	Non-significant decrease
GSK1004723D (0.01 % w/w in 5 % w/w glucose solution)	7.36	302	Significant decrease
GSK1004723D (0.5 % w/w in 5 % w/w glucose solution)	6.56	292	Significant decrease
GSK1004723E (0.01 % w/w in 5 % w/w glucose/ 0.2 % w/w DMSO solution)	5.96	331	Significant decrease
GSK1004723D Methocel™ suspension	6.30	342 (estimated)	Significant decrease
Placebo Methocel™ suspension for GSK1004723D	6.70	342 (estimated)	Significant decrease
GSK1004723E Avicel® CL-611 suspension	6.06	336 (estimated)	Significant decrease
Placebo Avicel® CL-611 suspension for GSK1004723E	6.80	336 (estimated)	Significant decrease
Saline citrate buffer (0.05 M) pH 3	3.00	305	Significant decrease
Saline citrate buffer (0.05 M) pH 4	4.00	300	Significant decrease
Saline citrate buffer (0.05 M) pH 5	5.00	309	Non-significant decrease
Saline citrate buffer (0.05 M) pH 6	5.99	326	Significant decrease

Saline sodium citrate (0.03 M) pH 7.3	7.30	303	Significant decrease
Saline sodium citrate (0.05 M) pH 7.3	7.30	297	Significant decrease
Phosphate buffered (0.01 M) saline (PBS) pH 7.3	7.30	301	Non-significant decrease*
Saline Phosphate buffer (0.1 M) pH 6	5.99	313	Non-significant decrease
Saline Phosphate buffer (0.1 M) pH 8	8.03	312	Significant decrease
Normal saline pH 3.0	2.98	310	Significant decrease
Normal saline pH 4.0	3.99	310	Significant decrease
Normal saline pH 5.0	5.40	316	Significant decrease
Normal saline pH 6.0	6.40	351	Significant decrease
Normal saline pH 6.7	6.70	293	Non-significant decrease
Normal saline pH 7.3	7.30	308	Non-significant decrease
Normal saline pH 8.0	8.30	326	Non-significant decrease

* This was using PBS as reference treatment (RT) to enable the data to be compared with that from different buffers, for which PBS was used as RT.

6.5 Discussion and Conclusion

An MTT assay was validated at the beginning of this part of the work that was aimed at screening the toxicity of the IN pharmaceuticals to human airway epithelium. Initially, cell exposure to MTT was carried out by adding 10 µl MTT solution (5 mg/ml in culture medium), with no change of medium or wash step, to the supernatant of the wells containing cultured 16HBE14o- cells in 100 µl culture medium. This method proved inappropriate because it was not simulating the disturbing manipulations of the actual cytotoxicity experiments, in which the medium had to be removed from the culture wells for the cells to be exposed to test agents and to MTT, which was therefore associated with the removal of any non-adherent cells. The former described method, however, was associated the presence of non-adherent cells resulting in linear calibration curves (an increase in absorbance corresponding to an increase in cell number) being observed over cell seeding densities beyond those expected to form a confluent attached cell layer (Figure 6.1) and where the curve was expected to deviate from linearity. This theory was confirmed by observing these viable non-adherent cells, with distinct purple formazan colour, pelleting loosely in the bottom of the culture wells after careful aspiration of the MTT-containing medium in the former described method, which indicated their contribution to the absorbance and thus to the extended linear range of the assay. Since this situation could not be implemented in the actual cytotoxicity experiments due to the inevitable removal of non-adherent cells, it was therefore decided to aspirate the cell culture medium and wash the cultured cells with sterile PBS prior to exposing the cells to any solutions in the subsequent assay validation and cytotoxicity work, which thus ensured the removal of any non-adherent cells. Moreover, a wash step following the removal of test solutions proved critical when exposures involved acidic conditions (reported to interfere with the MTT assay and therefore needed to be washed away [327]). This step was hence later introduced. It is also worth indicating that chelating agents

disrupted the attachment of the cells to plate surfaces and therefore a plate centrifugation step was introduced to preceed aspirating any solutions off the plates. This needed to be for at least 10 minutes as shorter programmes proved inefficient in achieving zero cell count in the supernatant. Meanwhile, the preparation of MTT solution in PBS instead of culture medium was explored. However, this slightly compromised the assay sensitivity so was not taken any further (data not shown).

Cell seeding densities higher than or equal $50x10^3$ cells/well demonstrated deviations from linearity (Figure 6.5). It therefore appeared that 16HBE14o- cells attained confluent-monolayer somewhere in the range of $2.5x10^4$ cells/well and $5.0x10^4$ cells/well. Beyond $50x10^3$ cells/well, cells were observed to stack on top of each other to form a pellet at the bottom of the wells. Consistently, growing in multilayers was documented for 16HBE14o- cells [245, 313] and this justified why calibration curves of absorbance against cell seeding density did not plateau out at cell densities beyond confluent monolayer formation, this plateau that occurs when the culture surface is unable to retain any cell additions required to increase formazan production. A $2.5x10^4$ cells/well seeding density, i.e. $0.78x10^5$ cells/cm^2, was deemed optimum for this study as it achieved good assay sensitivity while falling within the linear range of the assay where formazan formation was proportional to the number of viable cells. Interestingly, 16HBE14o- cells were cultured at a seeding density of $4.3x10^5$ cells/cm^2 for an MTT toxicity screening study although it was at much earlier passage number (passages 65-75) than what was used here (passages 91-99) [326].

Among the MTT assay variables that were optimised here, the analysis wavelength was studied. Although it is widely accepted that the absorbance maximum of formazan is 560 nm [329], the absorption spectrum showed a broad peak around 560 nm [329], this wavelength that was also lacking on some plate readers. We therefore chose to compare measuring at 540 nm to that at 595 nm to find the better wavelength, which proved 540 nm to attain higher assay

sensitivity (Figure 6.2 and 6.3). Similarly, DMSO proved efficient as formazan solvent, which is consistent with Twentyman et al's report [330] that DMSO was the quickest solvent to achieve full solubilisation of formazan with minimal background noise. DMSO demonstrated substantially less background noise than acidified IPA, which was linked to the latter's ability to precipitate proteins in any residual medium in the wells [329, 330]. This explains the higher background noise associated with acidified IPA in the presence of 16HBE14o- cells and/or MTT solution in culture medium (contains 10 % serum). It was therefore evident that, should acidified IPA be used as a solvent for formazan, the blank wells must be seeded with cells and exposed to all assay manipulations except the MTT solution. Meanwhile, the blank wells in the current study were seeded despite employing DMSO as formazan solvent to enable the detection of any background noise arising from an interaction between a test agent and the cells. Notably, the MTT dye itself minimally contributed to the background noise with acidified IPA although it was more detectable with DMSO (Figure 6.6(b)). Nevertheless, this was considered negligible as the MTT would be used up by the cells in the real experiments upon being converted to formazan and only a residual amount would remain in the wells.

The approaches to emptying the 96-well plates of liquid during the MTT assay, either by aspiration of liquids or by inversion (flicking) of plates followed by blot drying, demonstrated no distinct advantage for one technique over the other (Figure 6.3). Liquid aspiration was adopted as it was more efficient in removing test solutions with higher viscosity. In addition, it was less likely to disturb cells pelleted to the bottom of the wells after exposure to chelating agents such as EDTA.

The effect of the IN pharmaceuticals on the viability of the 16HBE14o- cells was investigated using the optimised MTT assay. The choice of the reference treatment (RT) was prompted by the formulations of Avamys® (Table 4.1) and GSK1004723D-Methocel™ suspension (see table 6.1), being 5 % w/w glucose-based formulations, and by the fact that two types of

Avicel® were insoluble in culture medium. The excipients were thus assessed at their working formulation concentrations (table 6.1) in 5% w/w glucose matrix to simulate their actual formulation vehicle. Notably, Avicel®CL611 formulations were non-glucose-based but the 5 % w/w glucose solution was a good vehicle for its assessment due to the Avicel®CL611 solubility issues in culture medium. The 5 % w/w glucose also had the advantage of physiological pH and isotonicity (see table 6.2 for the pH and osmolality data of all the pharmaceuticals assessed in this section). Interestingly, this physiologic solution (5 % w/w glucose) elicited a decrease in cell viability, which was not significantly different to viability in culture medium ($p > 0.05$). It was, however, a noticeable decrease and can be attributed to the lack of essential inorganic ions in this solution.

Our studies revealed Avamys® and Avamys® placebo to significantly reduce cell viability ($P < 0.05$). Studying the effect of all excipients in the Avamys® formulation, it appeared that BKC was responsible for the deleterious effects observed with Avamys® and its placebo as it caused a similar decline in cell viability (Figure 6.9) at various dilutions (Figure 6.10). This was consistent with various investigations that reported the cytotoxic effects of BKC [125, 127-133, 317, 331]. All the other excipients of Avamys® appeared to be innocuous at their working formulation concentrations. These included EDTA, Avicel® RC-591 and polysorbate 80. In agreement, polysorbate 80 was demonstrated as one of the least toxic surfactants [127, 132, 133], although its concentration-dependent cytotoxicity was well documented [139, 318, 319]. Moreover, EDTA was previously shown to be tolerated at low concentration (0.01 % w/v) [130] although its cytotoxic effects became evident at high concentrations [134, 135, 320, 321]. Similarly to Avicel® RC591 , Avicel® CL-611 and Methocel™ elicited no alteration to 16HBE14o- cell viability. It is therefore our finding that these cellulose derivatives were well-tolerated by an airway cellular model, which agrees with the reported innocuous nature of cellulose [325]. It is worth noting that Avicel CL-611 produced hypertonic dispersions at its formulation concentration

(see table 6.2), however hypertonic preparations (636 mOsmol/kg) also appeared to be innocuous to the 16HBE14o- cells. This was consistent with Forbes et al.'s finding that a 545 mOsm solution did not alter the MTT conversion in 16HBE14o- cells [326]. Nevertheless, it has recently been reported that MTT conversion was compromised in Calu-3 cells by hypertonic solutions (osmolarity > 390 mOsm) [139]. Research is therefore warranted to deduce the effect of such solutions on airway epithelium.

The IN corticosteroid, fluticasone furoate (FF), tended to non-significantly decrease the 16HBE14o- cell viability as measured by the MTT conversion, however this decrease was observed in the presence of DMSO, which by itself tended to elicit the same decrease. Further work might give a better insight to the cellular effects of this compound. Meanwhile, GSK1004723, a novel H1/H3 antagonist in phase II clinical trials [332, 333], demonstrated a significant toxic effect to the 16HBE14o- cells ($P < 0.05$) that is being reported here for the first time. This was observed with both the dihydrochloride (the D-form) and the naphthalene (the E-form) salts regardless of the physical form of the formulation (both the solution (Figure 6.9) and the suspension (Figure 6.11) induced significant decline in cell viability). It is worth noting that the E-form suspension was not glucose-based (according to GSK (see table 6.2)), which prompted using culture medium as RT for both suspensions and their placebos. This presumably lay behind the low cell viability observed with the placebo suspensions, which lacked any essential inorganic ions, as well as any source of energy to the cells (e.g. glucose) in case of the placebo Avicel®CL611 suspension, creating clearly less favourable conditions for the cells as compared to culture medium.

Potassium sorbate, in the presence of propylene glycol as a co-solvent, was the preservative of the BKC-free formulations studied here, and these were all non-glucose-based. Its effect on cell viability was thus assessed using culture medium as RT, which was also used as the vehicle to prepare potassium sorbate and propylene glycol. In contrast to BKC, potassium

sorbate as well as its co-solvent propylene glycol were found to be innocuous to the 16HBE14o- cells, which was consistent with Ho et al's findings observed in primary human nasal epithelial cells [131]. It is worth noting that observations of nasal irritation caused by potassium sorbate in nasal mucosa of rats [108, 109] were not reported in a human or mammalian airway model.

PEG300 is considered to be a universal solvent whose use in animal studies of intranasal absorption at concentrations reaching up to 40 % w/w has been reported to increase the solubility of some APIs [155, 334-336]. Apart from Costantino's notion of PEG300-induced low intranasal irritation in humans and Vetter's report of the PEG300 half maximal inhibitory concentration (IC50) of human cilia being higher than other approved solubilisers, there is barely any report on its effect on the respiratory mucosa using any model [6, 120, 337]. Here, PEG300 has demonstrated a concentration-dependent toxic effect on 16HBE14o- cells causing more than 90 % decline in viability at concentrations ≥ 30 % w/w while appearing to be well tolerated at 5 % w/w concentration. The 62% w/w PEG300 formulation suggested by GSK was thus extremely toxic. It is not clear though whether that toxic effect was due to the immensely hypertonic solutions produced by PEG300 (see table 6.2) or whether it is something intrinsic to the compound.

Effect of pH as an important formulation variable was investigated here using PBS as the RT. Normal saline solutions adjusted to different pHs demonstrated the pH range that was well-tolerated by the 16HBE14o- cells to be 6.7-8 (Figure 6.12 (a)). Interestingly, Forbes et al. reported pH 7, 7.3, 8, and 9 (using pH-adjusted HBSS) to be well-tolerated by the 16HBE14o- cells whereas pH 6 induced a significant compromise to their viability [326]. This agreed with our findings using normal saline solutions. On the other hand, Calu-3 cells were demonstrated to maintain their viability level only at pH 6-7 [139]. It is worth noting, however, that the human nasal pH at different sites of the nasal cavity was reported to be in the range

of 5.17 to 8.13 (an average of 6.3) [23] and 5.5 to 6.5 in another report [22]. The pH at the nasal epithelium, however, is 7.3 [14, 25].

Bearing in mind that the normal saline solutions used here at different pHs were not buffered and that they were incubated at 5 % CO_2 : 95 % air atmosphere, it was speculated that the measured pH values of these solutions might have decreased during incubation with the cells. This was confirmed by studying the effect of buffered solutions covering the pH range 3-8 on the viability of 16HBE14o- cells, which revealed the well-tolerated range to be 5 to 7.3 (Figure 6.12 (b)). This range agreed with the normal pH range of the human nose as indicated above. Meanwhile, as seen in figure 6.12 (b), citrate buffer pH 6 and sodium citrate pH 7.3 induced a significant decline in the 16HBE14o- cell viability ($P < 0.05$). Citrate buffers generally cover pH range 3-6 [338] (saline sodium citrate is used at pH 7.3). Hence, pHs 6 and 7.3 lie at the top of the range that could be maintained by these buffers. Therefore, according to the Henderson-Hasselbalch equation ($pH = pK_a + ([A^-]/[HA])$, where [HA] is the molar concentration of the undissociated weak acid (citric acid here), $[A^-]$ is the molar concentration of this acid's conjugate base (citrate ion here) and pK_a is $-\log K_a$ where K_a is the acid dissociation constant), a higher pH is attained by an increase in the concentration of citrate ion relative to citric acid. Citrate ion was previously reported to be cytotoxic [322-324, 338], which plausibly justified the observed decline in cell viability at the upper end of the pH range covered by citrate buffer. Decreasing the concentration of sodium citrate in saline sodium citrate solution (pH 7.3) from 0.05 M to 0.03 M slightly improved cell viability.

In conclusion, most of the IN pharmaceuticals studied here appeared to be safe to use on the airway epithelium. Nevertheless, our findings agreed on the deleterious effects of BKC that has often been reported using *in-vitro* models. The data here also raised concerns over the use of high concentrations of PEG300 as a solvent and the potential effects of the antihistamine GSK1004723.

Chapter 7

Discussion and Conclusions

7.1 Overview

The study presented here aimed to investigate the effect of a number of representative intranasal formulations on the respiratory mucociliary function in ovine tracheal *ex-vivo* models. *In-vitro* studies of the formulation effects on the viability of human airway epithelium were also conducted here using the human bronchial epithelial cell line, 16HBE14o-. The formulation effects on mucociliary clearance were studied on three major mucociliary components, namely mucin secretion, CBF and MTR. The first two components were assessed in sheep tracheal epithelial explants cultured at an air-liquid interface on collagen-coated nitrocellulose membranes, whereas MTR was assessed on whole sheep tracheae. This holistic approach of studying various components of the mucociliary escalator in the same species offered a valuable addition to knowledge.

Three tasks needed to be achieved before starting the actual assessment of the formulations. Firstly, nitrocellulose membranes were to be prepared and validated to demonstrate appropriate permeability criteria and optical clarity, which were essential for culturing ovine explants and microscopically measuring CBF, respectively. Secondly, it was necessary to have a mucin standard to construct calibration curves for the ELLA. This was prepared from crude human sputum using CsCl isopycnic density gradient ultracentrifugation. Lastly, the ELLA required a considerable amount of validation work to render it suitable for its purpose, which had to be repeated a few times as the lectin product specifications kept changing.

The ELLA was revealed here to be not fit for the purpose of mucin determination in the presence of many pharmaceuticals, which was an early finding of this study. This finding presented a hurdle to the progress of the study though, which prompted the development of a sandwich version of the ELLA. The latter proved fit for purpose, which enabled studying the

effect of pharmaceuticals on mucin secretion yielding some novel findings as discussed below.

The study then progressed as planned with the exception that the CBF work was precluded by technical issues with the microscopy system, which resulted in the discontinuation of this part of the project.

Table 7.1 summarises the findings of the current study regarding the effects of the studied IN pharmaceuticals on mucin secretion, mucociliary transport rate (MTR) and respiratory cell viability.

Table 7.1 The Effects of the Studied IN Pharmaceuticals on CBF, Mucin secretion, MTR and Cell Viability.

The Formulation/Excipient/API	The Effect on				
	CBF	Mucin Secretion	MTR		Cell viability
			When instilled	When sprayed	
Glucose (5 % w/w)	↓	-	↓	↓	↓
BKC (0.015 % w/w)	-	↑*	↓*	↓*	↓*
EDTA (0.015 % w/w)	-	↑	↓*	↓˘	↓
Potassium sorbate (PS) /propylene glycol (PG) (0.3 /1.5 % w/w)		↑*	↓	↓	↓
Propylene glycol (PG) (1.5 % w/w)	-	↑*	↓ (deduced from combined effect of PS+PG)		↓
PEG 300 (5 % w/w)	-	-	-		↓
Polysorbate 80 (0.025 % w/w)	-	↑*	↓*	↑	↓
Avicel® RC-591 (1.5 % w/w)	-	↓	-	↓*	↑
Methocel™ (1.0 % w/w)	-	↑*	↓*	↓*	↑
Avamys®	-	-	↑*˘	-	↓*
Fluticasone furoate (4 µg/ml in 0.2 % w/w DMSO)	-	↑	↓	↓*˘	↓
DMSO (0.2 % w/w)	-	↓˘	↓*	↓˘	↓
GSK1004723D (0.01 % w/w solution)	-	↑	-	↓*	↓*
GSK1004723D (0.5 % w/w solution)	-	-	-	↓*	↓*
GSK1004723E (0.01 % w/w in 0.2 % w/w DMSO solution)	-	↑*	-	↓*	↓*
GSK1004723D (0.5 % w/w) suspension	-	-	-		↓*
GSK1004723E (0.5 % w/w) suspension	-	-	-		↓*
Saline citrate buffer (0.05 M) pH 3,4,6	-	-	-		↓*
Saline citrate buffer (0.05 M) pH 5	-	-	-		↓
Saline sodium citrate (0.03 M and 0.05 M) pH 7.3	-	-	-		↓*
Saline phosphate buffer (0.1 M) pH 6	-	-	-		↓

Phosphate buffered saline (0.01 M) pH 7.3	-	-	-	↓
Saline phosphate buffer (0.1 M) pH 8	-	-	-	↓*
pH 4.78 (un-buffered)	↓*˘	-	-	↓* (deduced from data on normal saline pH 4 and 5)
pH 5.8 (un-buffered)	↓	-	-	-
pH 7.16 (un-buffered)	↓	-	-	↓ (deduced from data on normal saline pH 6.7 and 7.3)

↓ = non-significant decrease, ↑ = non-significant increase, ↑* = significant increase, ↓* = significant decrease, ˘ = Data from small sample size.

7.2 Discussion

Benzalkonium Chloride (BKC)

Whether the commonly used and historically effective preservative, BKC, is safe to use in IN preparations is the subject of some debate in the public domain, although the toxic effects of BKC in the eye [98, 128-130, 339, 340] and its ability to induce bronchospasm[100, 281, 341, 342] in the airways have been established. It was the finding of this study that BKC, a quaternary ammonium (cationic) detergent, triggered a significant increase in ovine mucin secretion. In addition, it irreversibly halted the ovine MTR when instilled onto the respiratory mucosa and significantly decreased the 16HBE14o- cell viability, which was consistent with various *in-vitro* studies reporting BKC as detrimental to the mucociliary escalator [89, 110, 114, 116, 122-124] and the respiratory epithelium [125, 131, 132, 317], respectively. This data, therefore, appears to support the argument against the use of BKC in IN formulations [28, 106-108, 317]. Nevertheless, it was also seen here that the BKC-induced halt in the ovine MTR was substantially ameliorated when a nasal delivery system was used to apply BKC onto the ovine mucosa. Although it was still shown to significantly decrease MTR overall, MTR was not affected in 50 % of cases while a reversible effect was seen in the others. This indicated that the dilution over a large surface area in the presence of the protective mucous blanket contributed to a neutralisation of the detrimental effects of BKC on the respiratory epithelium. This observation therefore flags up the important role of the dose-per-surface area as a factor in assessing the safety of pharmaceuticals, and hence the importance of *in-vivo* investigations [33]. In light of the relatively large surface area of the human nasal cavity, which is lined with mucus, and the dilution effect of the nasal secretions, this data therefore explained the lack of established evidence on the deleterious effects of BKC *in-vivo*[102, 103, 105, 118, 121] and provided a better understanding of the *in-vivo*/ *in-vitro* discrepancy of data on the nasal safety of BKC. It is

worth noting that Bernstein et al. [107] and Riechelmann et al. [102] previously speculated the dilution of BKC to lie behind this data conflict, which was made evident here in this study in agreement with Berg et al.'s and Stanley et al.'s observations [104, 119](Berg et al. demonstrated a decrease in BKC concentration to correlate with an increase in the number of human respiratory mucosal fragments that retained viability after BKC exposure. Stanley et al. observed ciliotoxic effects following *in-vitro* exposure of human ciliated nasal epithelium to BKC although *in-vivo* exposure did not adversely affect mucociliary clearance nor CBF). Further, Verret et al. thoroughly reviewed the *in-vivo* protective mechanisms that appeared to have contributed to the *in-vivo*/ *in-vitro* disparity of results on the IN safety of BKC [94]. These included mucous secretions and mucociliary clearance as mechanical barriers that cause dilution of the BKC concentration and reduce exposure time. In addition, various inactivating components of the mucous secretions, such as surfactants that could possibly neutralise BKC, were included.

EDTA

The chelator EDTA aids stability and preservation of pharmaceutical formulations [116, 343]. Its presence in detergents and disinfectants was recently reported to correlate with some respiratory diseases (occupational asthma and/or rhinitis) [112] and to induce bronchospasm in the airways [281]. EDTA was also established to elicit a dose-dependent cytotoxicity in a variety of cell types [134, 135, 320, 321, 344], although this was observed to be minimal at a concentration of 0.01 % w/v [130]. In the current study, EDTA was studied in a respiratory cellular model for the first time; EDTA (0.015 % w/w) tended to decrease the viability of 16HBE14o- cells although this effect was not significant. In addition, EDTA also showed a tendency to increase ovine mucin secretion; again this effect was not significant. It is possible that these results indicate an element of cell defense against this compound at the concentration used (0.015 % w/w), which might be greater at higher concentrations of EDTA.

Importantly, the pharmaceutical concentration range of EDTA is 0.01 to 0.1 % w/v [328] and EDTA has been shown to elicit no[114]-to-mild[113, 115] ciliotoxicity, using concentrations as high as 0.1 % w/v, in chicken embryo tracheae, in which the mucus layer was conceptually negligible although the model was recently shown to exhibit mucous lining [345]. Stanley et al. also showed EDTA (100 µg/ml, which was equivalent to ~ 0.01 % w/v) to cause no ciliotoxicity to human ciliated cells *in-vitro* [119], where mucus protection was minimal. Meanwhile, Batts et al. reported EDTA (0.1 % w/v) to compromise MTR on frog palate [116] although they, among others, found it innocuous to human mucociliary clearance *in-vivo* [117-119]. These findings agreed with the observed EDTA-induced decrease in ovine MTR here, which appeared to be neutralized by applying EDTA using a nasal delivery system. This latter finding again supports the role of dilution and decreased contact time in ameliorating the effect of the compound.

Potassium Sorbate (PS)

The preservative, PS, is often referred to as a potential respiratory irritant based on studies in rats [108, 109]. This might be related to the PS-induced increase in ovine mucin secretion that was observed in the current study. Interestingly, PS was not toxic to 16HBE14o- cells, which agreed with the findings of Ho et al. in primary human nasal epithelial cells [131]. Moreover, PS was shown here for the first time to illicit no significant alteration to the ovine MTR, which indicated that the effect on mucin secretion was too subtle to affect the mucociliary escalator. In addition, PS was reported not to be ciliotoxic in models that were minimally protected with mucus such as cultured human-nasal and rabbit-tracheal mucosae [89, 110, 111]. As such, PS appeared to be a safer alternative to BKC, which, however, remains the preservative of choice for IN formulations in the pharmaceutical industry. This can probably be attributed to the stability and solubility issues of sorbates [343], which have precluded PS from attaining this status.

Propylene Glycol (PG)

The solubilizer, PG, has not been associated with general toxicity [346], reproductive toxicity [347] or cytotoxicity [348] and has been reported to induce only minimal and reversible ciliotoxicity [120]. PG was reported to be among the least cytotoxic solubilizers, used in IN formulations, when applied to human airway cellular models [120, 139], which was consistent with its non-significant effect on the viability of 16 HBE14o- cells in the current study. Moreover, PG did not affect ovine MTR, a novel finding, despite causing a significant increase in ovine mucin secretion. It is assumed that this increase in mucus secretion was too subtle to affect ovine MTR although it might be linked to the PG-induced respiratory irritation that has been reported in response to occupational exposure [349]. It is worth noting that PG has surfactant properties [348] and some surfactants were seen to enhance clearability of airway secretions in cystic fibrosis, neonatal distress syndrome and stable chronic bronchitis [350-352] and to improve MTR in cystic fibrosis [353] as well as healthy models [354, 355]. The PG-induced increase in mucin secretion that was seen in the current study might therefore be linked to its surface active properties despite being too subtle to impact on MTR here.

PEG300

The solubilizer, PEG300, was noted to cause mild IN irritation in humans [6] although, in the current study, it appeared innocuous to the 16HBE14o- cells at the concentration of 5 % w/w. PEG300 was also reported to have a higher human ciliary half-maximal-inhibitory-concentration (IC50) than other commonly used IN solubilizers [120]. Further work is therefore warranted to investigate the effect of PEG300 on MTR and mucin secretion with prospects of demonstrating acceptable respiratory safety.

Polysorbate 80

The surfactant, polysorbate 80, has been reported to induce neither general [346] nor reproductive toxicity [347] and to cause minimal ocular toxicity [98, 127, 356]. It has also been reported to be one of the least cytotoxic surfactants [132, 133] despite its established concentration-dependent cytotoxicity [139, 318, 319, 357] and ciliotoxicity [136-138]. In the current study, polysorbate 80, at its working formulation concentration (0.025 % w/w), was shown to be tolerated by the respiratory epithelium and mucociliary clearance with some novel observations being reported here. It did not significantly affect the viability of 16HBE14o- cells or ovine MTR when applied as nasal spray although MTR was significantly impaired when the polysorbate 80 solution was instilled on to the epithelial surface. Polysorbate 80, however, significantly increased ovine mucin secretion, which correlated well with the impaired MTR following application by instillation whereas its effect was presumably too subtle to significantly affect MTR following application as a spray. Notably, a small tolerable increase in mucin secretion is expected to stimulate mucociliary clearance whereas excessive accumulations of mucins signals tissue defence reaction or damage [357], which often negatively impacts on MTR as in many respiratory diseases, e.g. COPD, and in smokers [162], and as was seen here following exposure to BKC and instilled polysorbate-80. This postulation has recently been experimentally confirmed by Liu et al., who elucidated that the mucus load auto-regulates MC [39, 358]. Interestingly, ovine MTR tended to improve in two out of three tracheae (refer to figure 5.15 (b)) following application of polysorbate 80 as a spray, which despite not being significant might correlate with a small increase in mucin secretion by polysorbate 80. This tendency is also consistent with the surfactant-induced enhancement of airway secretion clearability and MTR as discussed above with PG.

Cellulose Polymers

In the current study, the viscosity enhancers, Avicel® and Methocel™, were shown to be innocuous to the respiratory epithelium as they did not decrease the viability of 16HBE14o- cells. The polymers of Avicel® and Methocel™ sodium carboxymethylcellulose (Na-CMC) and hydroxypropyl methyl cellulose (HPMC) respectively, were also shown to be well-tolerated by Calu-3 cells [139], which supports the results reported here. However, in the current study, both compounds were also observed to compromise ovine MTR. This supports the intended role of viscosity enhancers in IN formulations, which is to increase the nasal residence time and therefore the effectiveness of these formulations [33]. This inverse relationship between MTR and viscosity has been well-documented for cellulose derivatives such as Na-CMC and HPMC [117, 142-147, 149]. Further, these compounds have been observed to elicit a partially-reversible reduction of CBF [148, 150], which supported their effect on MTR. These observations were most likely attributed to mechanical interference with the beating cilia particularly with the lack of evidence on any associated histological damage to the ciliary structures [150]. Nevertheless, it was the finding of this study that Methocel™ significantly increased ovine mucin secretion although it was not clear whether this increase contributed to the effect of Methocel™ on ovine MTR or not.

Fluticasone furoate (FF)

The effect of FF on the mucociliary escalator was investigated here for the first time whilst also studying its effect on an airway epithelial cell line. As reviewed by Verret et al. and recently observed by Fokkens et al. in human subjects, nasal steroids generally prove innocuous to the nasal epithelium even after long term exposures [94, 289]. This agreed with the preserved viability of the 16HBE14o- cells in the current study following exposure to FF and of ocular epithelial cells following exposure to betamethasone in the studies of Wang et al.

and Ayaki et al. [359, 360]. Interestingly, corticosteroids were recently shown to demonstrate concentration dependent toxicity to human mesenchymal stem cells [361]. Meanwhile, MTR and CBF were also shown to tolerate steroidal compounds [89, 92, 94, 103, 297, 309] although high concentrations of prednisolone interfered with MTR [297]. FF did not significantly alter ovine MTR here despite being solubilised in 0.2 % DMSO, which itself caused a significant compromise to the ovine MTR. This therefore raises the question of whether FF exerted a protective effect or probably induced some improvement in ovine MTR that was neutralised by the presence of DMSO. The latter assumption agrees with the observation of Naclerio et al. regarding a small improvement in MTR following two weeks treatment with nasal budesonide, except that their observation was in perennial allergic rhinitis patients rather than healthy subjects [92]. However, Oliveira-Braga et al. reported no alteration to murine MTR following systemic exposure of normal subjects to prednisone although a decrease in murine mucus transportability on frog palate was observed [297]. Notably, the tendency for FF to increase ovine mucin secretion, despite being non-significant, can possibly explain this observation of Oliveira-Braga et al. as well as that of Mcgregor et al. of potentiated mucous secretion in the nasal lavage fluid of healthy human subjects following three-week exposure to nasal steroids [290]. Paradoxically, it has often been noted that these compounds have no direct effect on mucin production/secretion processes and that their effectiveness in hypersecretory conditions emerged from blocking the inflammatory process and mediators that stimulate mucin secretion [246, 286-288]. Research is, therefore, warranted to establish a better understanding of the effect of steroids on mucin secretion in health and disease as well as to illustrate the long-term effects of these agents on MTR.

GSK1004723

The novel H/H3 antihistamine that completed some phase II studies [332, 333, 362] was revealed here be cytotoxic to 16HBE14o- cells. This effect was elicited by both salt-forms of the drug

(GSK1004723E and GSK1004723D) at both of the concentrations suggested by GSK (0.01 % w/w and 0.5 % w/w) and regardless of the physical properties of the formulation (solution or suspension). This observation probably correlated well with the initial nasal discomfort associated with this API reported in clinical trials [332]. GSK1004723 was also detrimental to ovine MTR and increased mucin secretion. Nevertheless, in light of the BKC model whose *in-vitro* detrimental effects appear to get neutralised *in-vivo*, GSK1004723 still has prospects of proving innocuous to mucociliary clearance *in-vivo* in the presence of the various protection barriers such as mucus, surface area of the nasal cavity and dilution by nasal secretions.

pH

The nasal mucosal pH (5.5-6.5) [17, 22, 23] has also been identified as a nasal protection barrier as reviewed by Verret et al. [94]. The slightly acidic environment of the nose favours one form (dissociated or un-dissociated) of weakly acidic/basic compounds to the other, which would protect against toxicity of the less favoured entities. For instance, BKC is a quaternary ammonium salt and hence acidic environments favour its un-dissociated form, which enhances preservation and has been noted to attenuate toxicity [94]. Meanwhile, IN formulation pH is a variable that has often been studied for its impact on nasal physiology. Washington et al. demonstrated no significant alteration to human nasal MTR *in-vivo* following IN exposure to formulations in the pH range of 5.0-7.2 [23], which was in agreement with the lack of significant alteration to the ovine tracheal CBF observed here following exposure to pH 5.8, 6.26 and 7.16. Moreover, the viability of 16HBE14o- cells here was also preserved over the pH range 5.0-7.3. Nevertheless, human bronchial CBF was previously reported to change significantly at pH < 7-7.5 [167, 168]. Human airway CBF (bronchial, bronchiolar and nasal), studied in culture models with minimal mucous protection, generally appeared to better tolerate alkaline (up to pH 10.5 [167-169]) rather than acidic pH, which appeared confounding in

the nasal area due the slightly acidic physiological pH in the nose. This also disagreed with the decline in the 16HBE14o- cell viability at pH 8 that was observed here.

Buffers

Potential issues with the IN use of citrate buffer have also been raised in the current study, which were linked to both extreme pH and the chelation properties of citric acid/citrate molecules [363]. Low pH values of pH 2-4 were detrimental to the viability of 16HBE14o- cells, which was also lowered significantly at physiological pH 6-7.3 owing to the enhanced chelation properties of citrate ions at higher pH values. Consistently, a recent study has demonstrated that the citrate chelation capacity decreases ~10 fold for each pH unit decrease from pH 6 to pH 3 [364]. Paradoxically, the chelator EDTA was found to be innocuous to the viability of 16HBE14o- cells in the current study despite its higher calcium binding capacity compared to citrate [363]. Nevertheless, the examined concentration for EDTA (0.015 % w/w i.e. ~ 0.0004 M; an off-shelf formulation concentration, e.g. Avamys®; See table 4.1) was much lower than that investigated for citrate buffer (0.05 M although the higher concentration 0.1 M (~ 1.48 % w/w sodium citrate plus 0.96 % w/w citric acid anhydrous at pH 4.7 as per the BKC-free FF formulation by GSK; See table 4.1) is an approved buffering concentration [328]). Future work therefore might investigate any impact of citrate chelation on mucociliary clearance. Meanwhile, citrate buffer appeared to be well tolerated around pH 5, where the pH of most citrate buffered marketed formulations revolve, e.g. Nasarel®, AllerNaze®,and Nascobal® [27].

It is evident that surfactants; such as BKC and polysorbate 80, and chelators; such as EDTA and citrate buffer, constitute the most potentially hazardous groups of pharmaceuticals in IN formulations due to possible interference with cytoplasmic membranes [130, 139, 357, 365]. Ho et al. demonstrated BKC to cause cell membrane lysis of human nasal epithelial cells *in-vitro* [131]

whereas EDTA, citric acid and polysorbate 80 have been investigated as absorption enhancers based on their potential effects on cell membranes[121, 136, 282, 364, 366].

7.3 Conclusion

This *in-vitro* study revealed some commonly used IN pharmaceuticals to be well-tolerated by the ovine mucociliary defence system whilst indicating others to require further *in-vitro* and/or *in-vivo* studies to establish their IN safety. It also illustrated that, despite the proven value of *in-vitro* investigations in the safety screening of pharmaceuticals, *in-vivo* studies continue to be indispensable [33]. The *in-vivo* protective mechanisms such as mucous secretions and the surface area at the site of action shouldn't be overlooked in assessing the safety of pharmaceuticals [104, 116], which was demonstrated here by the attenuated significance of the BKC-induced damage to ovine MTR via the effect of dilution. Further, since IN formulations are life-time treatments for chronic conditions such as allergic rhinitis, research is warranted to investigate the long term effects of debatable compounds such as BKC as well as compounds associated with concentration-dependant effects such as EDTA. Moreover, such chronic conditions were often associated with altered properties of important *in-vivo* protective mechanisms [104], namely the mucus blanket and the rate of mucociliary clearance, which flags up the importance of future *in-vivo* studies of potentially hazardous compounds in the intended group of patients.

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Appendices

Appendix 1 – Ethics Application Form

University of Brighton
Faculty of Science and Engineering
School of Pharmacy and Biomolecular Sciences

Application form for ethical approval of research projects

Project title: Effect of formulation variables on the uptake and availability of drugs used in the treatment of allergic rhinitis.

Investigator name(s): Marwa Ayoub

Name of Supervisor (s): Alison Lansley, Mike Lethem

Location of the work: University of Brighton

Timescale: 3 years

Course (if applicable): MPhil/PhD

Details of Funding (if applicable): University of Brighton and GlaxoSmithKline (GSK)

Please send the completed form and any associated documents to pabs.ethics@brighton.ac.uk

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This form should be checked carefully for typographical and grammatical errors. Incomplete or badly presented forms will be returned. Supervisors of student projects have a responsibility to ensure that the guidance is followed and that applications are properly presented.

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1. Introduction
Brief introduction to background and rationale for study; for ***undergraduate projects*** *review of key articles supporting the research study – maximum 500 words, no more than 6 refs.*

Allergic rhinitis tends to be treated with two groups of drugs; steroids and/or anti-histamines. The aim of the study is to look at the effect of these drugs and other ingredients present in the formulated medicine on mucus secretion.

Epithelial tissue from sheep tracheae (obtained from a local abattoir) is cultured on permeable membranes and the secretion of mucin by the goblet cells of the epithelium is measured using an enzyme-linked lectin assay (ELLA) (Clancy, S.M., et al, 2004, *Endothelin-1 inhibits mucin secretion from ovine airway epithelial goblet cells.* Am. J. Respir. Cell Mol. Biol. 31:663-671). In order to quantify the amount of mucin secreted a mucin standard is required. Ideally this should be of human origin and needs to come from people suffering from conditions causing an over production of mucus since the healthy lung does not produce enough for collection.

Ethical approval is required for the collection of a small number of sputum samples. This could be as few as one sample from a single participant but would not be expected to exceed five samples from one or more participants. Mucins will be purified from the human sputum according to the method of Carlstedt et al (1983) (Carlstedt, I., et al., 1983, *Isolation and characterization of human cervical-mucus glycoproteins.* Biochem J. 211:13-22.).

2. Purpose of the study
The aims of the study or the hypothesis to be tested should be clearly stated.

The aim of the project is to look at the effect of steroid and anti-histamine drugs and other ingredients present in the formulated medicine on mucus secretion. We aim to test the hypothesis that neither group of drugs or other formulation variables increases mucin secretion.

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3. Methods

Details of the study design and how it is to be conducted should be included. Describe the number and length of meetings/attendances required of the participants. Studies involving survey questionnaires or structured interviews should include a copy of the questionnaire or interview questions. Other studies should include a copy of leaflets giving information for participants and the consent form.

It is envisaged that members of the research team will attend one or more meetings of the patient support group 'Breatheasy' to explain the requirements of the study and to recruit participants. There are a number of such support groups locally which meet once per month. It is possible that sputum samples could be collected from the participants or that the participants could deliver them to the University of Brighton. This will be decided on an individual basis to allow for the greatest convenience to the participants (see Information sheet and consent form for participants).

The sputum sample (approximately10-30 g) will be expectorated by the participant(s) into an appropriate container with lid (which will be supplied by the research team) and kept cool (ideally refrigerated at 4°C) until it can be collected or delivered.

Once the sample has been brought to the laboratory it will be processed immediately.

The sample will be weighed, added to 10 volumes of solubilisation buffer (10 mM phosphate buffer, pH 6.5, containing 6 M guanidinium chloride, 5 mM disodium ethylenediaminetetraacetic acid (EDTA) and 1 mM phenylmethylsulphonyl fluoride). This will be stirred gently at 4°C for 18 hours and then centrifuged at 10,000 x g at 4°C for 1 hour to remove insoluble material from the solution. Insoluble material, which will include any cellular debris, will be discarded and destroyed by incineration. Caesium chloride and 10 mM phosphate buffer, pH 6.5 will be added to the soluble material so that the final concentration of guanidinium chloride will be 4 M and the density of the solution will be 1.39 g ml^{-1}.

The solution will be loaded into Beckman polyallomer 'quick – seal' tubes and centrifuged at 120,000 x g and 15 °C for 65 hours. Each tube will then be pierced at the bottom with a needle and 1 ml fractions of the solution will be collected from each tube with the same fractions from different tubes being combined.

The density of each fraction will be found by weighing a 300 µl sample which will then be assayed for sialic acid, protein and DNA. The fractions containing a peak of sialic acid with densities of 1.4 – 1.5 g ml^{-1}, will be pooled and dialysed against several changes of 10 mM phosphate buffer (pH 6.5) containing 0.2 M guanidinium chloride and 5 mM disodium EDTA. Caesium chloride and 10 mM phosphate buffer (pH 6.5) containing 0.2 M guanidinium chloride and 5 mM disodium EDTA, will be added to the dialysed solution using the above equation, so that the final density will be 1.5 g ml^{-1}.

The solution will be reloaded into Beckman polyallomer 'quick – seal' tubes and centrifuged at 120,000 x g and 15 °C for a further 65 hours. The tubes will be fractionated again and analysed for density, sialic acid, protein and DNA. The fractions containing a peak of sialic acid with densities of 1.45 – 1.55 g ml^{-1} will be pooled and dialysed for 24 hours against three changes of distilled H_2O. The final mucin concentration of the preparation will be determined by measuring the dry weight of six 100 µl aliquots. An appropriate volume of distilled H_2O will be added to the preparation so that the final concentration of mucin in the solution will be 3 mg ml^{-1}. This will be stored in a 50 ml tube at 4 °C and used as the mucin standard in further work.

4. Participants

Details of putative participants should be described here, including the number, age and gender of subjects and specific inclusion/exclusion criteria. In addition, give details of the recruitment strategy to be used.

Participants will be suffering from chronic obstructive pulmonary disease or other respiratory disease characterised by an over production of mucus. They can be either male or female and will be over 18 years of age. It should be possible to obtain enough sputum from a single participant but it is not envisaged that more than five would be required.

In order to recruit participants, members of the research team will attend meetings of the support group ' Breatheasy', there are a number of these groups based locally which meet once a month. We are already in contact with one such group and hope to make presentation at their January meeting. The study will be explained to the members of the support group and interested participants recruited on to the study. The attached 'Information Sheet' will be given to each potential participant to provide sufficient information to permit an informed decision to be made concerning whether they wish to participate or not. In addition, any further questions will be answered by the member of the research team present at the meeting.

5. Confidentiality and anonymity

Describe any issues relating to confidentiality; state how will data be stored and who will have access to the data; state how anonymity of the participants in reports and publications will be assured.

Once the sample(s) are obtained the name(s) of the participant(s) will be converted into a code to preserve their anonymity. The names of the participants will not be used in reports or publications.

Records will be kept of the following:
the names of the participants and their code,
when the material was acquired and where from,
what has been consented to
The uses to which the material is put whilst in use at the University of Brighton
Any processes applied to it
The time, place, method of and reason for disposal of the material.

This would enable any participant to trace the material provided in future years should they wish to do so.

Currently, procedures are being developed within PABS for the storage of such information. Once these have been finalised the information will be stored in this manner. Prior to this the information will be held electronically and as a hard copy by the supervisors of the project. The participant, investigator and supervisors will have access to the information.

6. Data analysis

Quantitative studies should detail which statistical tests will be employed, and qualitative studies should detail the approach that will be used for data analysis.

The mucin content of the purified sputum sample will be quantified by measuring the dry weight of six 100 µl aliquots. The mucin sample will then serve as a standard to permit the quantification of mucin secretion from epithelial explants from sheep tracheae using an enzyme-linked lectin-binding assay. This will enable the effect of the steroid and anti-histamine drugs on mucin secretion to be investigated. Statistical tests, such as analysis of variance, will be used to compare the effects of the drugs. Statistical test will not be used in the quantification of the mucin content of the sample.

7. Risk assessment
Please give details of any potential risks for both participants and researcher(s) and indicate how they will be addressed and managed.

Participants: There are no risks to participants

Researchers: There is a risk that researchers could be exposed to samples of sputum contaminated with human pathogens. To minimise the chance of infection of the researchers, the researchers will wear protective laboratory coats, safety glasses and gloves and work within a Class 2 safety cabinet when handling the sample. The sample will remain enclosed at all times except when solutions are being added to it.

8. Ethical issues raised by this study
Please think carefully about the ethical issues that are relevant to your study and give details here.

The sputum sample(s) required for this study are coughed up by the participant and would otherwise be discarded. No invasive operation will be involved in obtaining the sample therefore the participant will not experience any discomfort or pain. Any human cells containing the participant's DNA that might be present within the sample will be removed from the sample within 24 hours and destroyed by incineration therefore there is no potential to clone the participant should the expertise be available. A record of the participant's sample will be kept to ensure that the participant will be able to discover what the sample was used for. The anonymity of participant will be maintained should the results of the study be published.

9. Potential benefits to volunteers
Describe any potential benefits to individual volunteers - if there are no benefits to the individual participants this should be stated. Details of any compensation/reimbursment of expenses should be entered here.

No obvious benefits beyond the knowledge of making a contribution to the advancement of science.

Should the participants prefer to deliver their samples to the University then their travelling expenses will be reimbursed.

10. Environment
Give details of any anticipated negative effects on the environment and any steps taken to manage them. If there are no anticipated effects, this should be stated.

No anticipated negative effects on the environment.

11. Further information

Please use this section for any information not covered above, that you would like to give to the Ethics Committee.

The sample(s) are exempt under the Human Tissue Act because they will be processed within 24 hours of their receipt from the participant(s).

Please confirm that at the end of the project all data will be handed to the supervisor/principal investigator for storage according to School and University governance requirements (normally 10 years). In addition to paper records, electronic data will be removed from personal computers to disc and handed to the supervisor.

X *please tick*

This application is accompanied by :-

Please tick, as appropriate:

- **X Participant information sheet**
- **X Participant consent form**
- ☐ **Questionnaire**
- ☐ **The letter of information that will accompany the questionnaire**
- ☐ **Draft interview questions**
- ☐ **Advertising material**

Appendix 2 – Ethics Approval

22nd December 2009

PABSREC APPLICATION 0905

Project title: "Effect of formulation variables on the uptake and availability of drugs used in the treatment of allergic rhinitis."

Investigator name(s): Marwa Ayoub

Name of Supervisor (s): Alison Lansley, Mike Lethem

The School Ethics Committee has approved the above application.

Please ensure that records of tissue processing and disposal are kept in such a way as to allow the Principal Investigator to make them available for inspection purposes.

Yours sincerely,

Dr Anne Jackson

Chair, School of Pharmacy and Biomolecular Sciences Research Ethics Committee.

Appendix 3 – Ethics Information Sheet for Participants

Information sheet

Thank you for considering to participate in this study. If you decide to get involved you will be required to provide us with a sample of your 'coughed up' sputum. We will give you a container, with a lid for you to cough your sample into. You will be able to take this container home with you if you would prefer to provide a sputum sample in the privacy of your home. Once you have provided a sample, we would like you to store the container with your sample in it in a cool place (preferably a fridge) until we can collect it. We can come to your home to collect the sample, you can bring it to the University of Brighton at Moulsecoomb or we can collect it at a support meeting of Breatheasy. Please let us know which you would prefer. Ideally, the sputum sample should be produced no earlier than 24 hours before collection because we would like the sample to be as fresh as possible.

Once we have your sputum sample we will take it to our laboratory where we will weigh it. We will then add a special solution to it and stir it for 18 hours to help liquefy it. Once in a liquid form we will purify the sample so that all that remains are the long molecules that give the mucus its gel-like properties. These are called mucins. Anything other than the mucin molecules will be discarded and incinerated.

In our study we are interested in how certain drugs, for example drugs used to treat hay fever, affect mucin secretion. We need the mucin molecules from your sample to help us to do this.

We hope to publish the results of the study in the scientific literature but your name will not appear in any publication. If you would like a copy of any publications then please let us know.

Your participation in the study is entirely voluntary and you will be free to withdraw at any time.

Thank you for taking the time to read this sheet and to consider participating in the study.

Contacts at the University of Brighton

Dr Alison Lansley 01273 644542 (a.lansley@brighton.ac.uk)
Dr Mike Lethem 01273 642046 (m.i.lethem@brighton.ac.uk)

Appendix 4 – Ethics Consent Form

I, ……..... (please print name)

consent to provide a sample of sputum which will be purified by researchers at the University of Brighton and used to investigate the effects of drugs used to treat hay fever, on mucus secretion in the airways.

…… (sign name) …… (date)

Please indicate below how you would like your sample to be collected/delivered by circling your preferred option.

A) I would like my sputum sample to be collected from the following address. Please ring (01273 644542) when your sample is ready for collection:

   House number or name ……

   Street name ……..

   Town ……..

   Post code ……..

   Contact number ……..

   Email address ……

B) I would like to bring my sputum sample to the University of Brighton
   Please ring before bringing the samples (01273 644542) to check that somebody is available to receive them. Please deliver to the reception area of Cockcroft Building, University of Brighton, Lewes Road, Brighton, BN2 4GJ. One of the researchers will come down to collect the sample from you.

C) I would like my sample to be collected at a meeting of the support group – Breatheasy.
   Please indicate the date of the meeting when the sample should be collected.

   Date ……..

Please indicate whether you would like to be sent any literature resulting from the study. YES/NO

Thank you