INTRANASAL, INHALED AND ORAL CORTICOSTEROIDS AND SIDE EFFECTS IN ASTHMA, RHINITIS AND NASAL POLYPOSIS

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

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‘This thesis is the result of the author’s original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.’

Abstract

Corticosteroids are prescribed as anti-inflammatory agents for the treatment of respiratory disorders including asthma, rhinitis and nasal polyposis. The safety of corticosteroids has been questioned due to a possible increased risk of side effects such as adrenal suppression, osteoporosis, hypertension and diabetes mellitus with increasing corticosteroid dose. The main aim of this study was to assess and quantify the corticosteroid burden and potential side effects across a population of patients with a combination of asthma, rhinitis and/or nasal polyposis. A total of 113 patients were recruited from two specialist clinics in Glasgow.

A high performance liquid chromatographic method for the detection and quantification in plasma of seven commonly prescribed corticosteroids has been developed and validated. The validated analytical method shows good linearity ($r^2 \geq 0.95$), precision (<10 %RSD) and the LOD/LOQ for the analytes were determined to be 0.02 – 0.26 μg mL^{-1} and 0.07 – 0.37 μg mL^{-1} respectively when using UV detection. Using this method only prednisolone could be detected and quantifed in patients samples (0.07 – 0.92 μg mL^{-1}). Using the Spearman rank correlation analysis, no correlation was found between the measured plasma concentration of prednisolone and the oral prednisolone dose taken by the patients.

The research presented here in indicates that no correlation was found between corticosteroid dose and suppression of salivary cortisol or osteoporosis or an increased incidence of diabetes. Intranasal betamethasone showed a statistically significant risk of suppression of salivary cortisol with an increasing dose ($p = 0.044$)

but the dataset is small (n = 22). Clinical data indicated that patients receiving a higher corticosteroid dose (≥1 milligram BDP equivalent daily) exhibited an increase in both systolic and diastolic blood pressure, however, no correlation was found in patients receiving corticosteroid doses of less than 1 milligram BDP equivalent daily.

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Glossary

Abbreviation	Text in full
17-BMP	Beclometasone-17-monopropionate
21-BMP	Beclometasone-21-monopropionate
ACN	Acetonitrile
ACQ	Asthma control questionnaires
ACTH	Adrenocorticotropic hormone
ALP	Alkaline phosphatase
AP	Activator protein
As	Symmetry factor
ATS	American Thoracic Society
$AUC_{0\text{-}24h}$	24 - hour plasma area under curve
BDP	Beclometasone dipropionate
BETA	Betamethasone
BHS	British Hypertension Society
BMD	Bone mineral density
BMI	Body mass index
BOH	Beclometasone
BP	British Pharmacopoeia
BTS	British Thoracic Society
BUD	Budesonide
CFC	Chlorofluorocarbon
CRS	Chronic Rhinosinusitis
CT	Computed tomography
DBP	Diastolic blood pressure
DEXA	Dual Energy X-ray Absorptiometry
DPI	Dry powder inhalers
EDQM	European Directorate for the Quality of Medicines and Healthcare
ENT	Ear, Nose and Throat
EP	European Pharmacopoeia
ERS	European Respiratory Society
ESI	Electrospray ionisation
FACET	Formoterol® and Corticosteroids Establishing Therapy
FDA	Food and Drug Administration
FE_{NO}	Fraction of exhaled nitric oxide
FESS	Functional Endoscopic Sinus Surgery
FEV_1	Forced expiratory volume in 1 second
FP	Fluticasone
GINA	Global Initiative for Asthma
GLUT-4	Glucose transporter 4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOAL	Gaining Optimal Asthma Control
GRAD	Gradient
GRI	Glasgow Royal Infirmary
HFA	Hydrofluoroalkane
HPA	Hypothalamic adrenal suppression
HPLC	High performance liquid chromatography

i.d	Internal diameter
ICAM	Intercellular adhesion molecule
IgE	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
IS	Internal standard
ISO	Isocratic
k’	Capacity factor
kg m^{-2}	Kilogram per square metre
LABA	Long-acting beta-agonist
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LTC	Leukotriene
LTRA	Leukotriene receptor antagonists
m/z	Mass-to-charge ratio
MeOH	Methanol
MF	Mometasone furoate
mg	Milligram
mmHg	Millimeters of mercury
mmol L^{-1}	Millimoles per litre
MHRA	Medicines and Healthcare products Regulatory Authority
MS	Mass spectrometry
N	Theoretical plates
n	Number of samples
NF-kB	Inhibitor of nuclear factor
NHS	National Health Service
NIH	National Institutes of Health
nmol L^{-1}	Nanomoles per litre
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS	National Osteoporosis Society
NP-LC	Normal phase liquid chromatography
P1NP	Procollagen I N-terminal extension peptide
Par.	Particle
PaO_2	Partial pressure of oxygen in the blood
PEFR	Peak expiratory flow rate
PgD	Prostaglandin
pMDI	Pressurised metered dose inhaler
PNIF	Peak nasal inspiratory flow
ppb	Parts per billion
PRED	Prednisolone
RSD	Relative standard deviation
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RBG	Random blood glucose

RIA	Radioimmunoassay
RP-HPLC	Reverse phase high performance liquid chromatography
RP-LC	Reverse phase liquid chromatography
RRF	Relative response factor
RRF_{IS}	Response factor relative to the internal standard
RRF_{UV}	Response factor relative to the UV standard
RRT	Relative retention time
Rs	Resolution
s.d	Standard deviation
S/N	Signal-to- noise ratio
SABA	Short-acting beta-agonist
SBP	Systolic blood pressure
SF-36	Medical Outcomes Study 36-Item Short Form Health Survey
SH	Stobhill Hospital
SIGN	Scottish Intercollegiate Guideline Network
SMART	Symbicort® Maintenance And Reliever Therapy
SNOT-20	Sino Nasal Outcome Test -20 Questionnaire
SNOT-22	Sino Nasal Outcome Test -22 Questionnaire
SPE	Solid-phase extraction
SpO_2	Oxygen saturation
SST	Short synacthen test
TGF	Transforming growth factor
T_H2	T helper cells type 2
THF	tetrahydrofuran
TNF-α	tumor necrosis factor-α
t_R	Retention time
UV	Ultraviolet
v/v	Volume-per-volume
VCAM	Vascular cell adhesion molecule
V_D	Volume of distribution
WHO	World Health Organisation
α	Selectivity factor
λ_{max}	Wavelength at maximal UV absorption

Acknowledgement

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Natrah Amira. They bore me, raised me, supported me, taught me, and loved me. I hope that I can continue to make them proud.

To them I dedicate this thesis.

Chapter 1

Introduction

1.1 Corticosteroids

Corticosteroids mimic the action of cortisol, a natural glucocorticoid hormone, produced by the adrenal cortex in the body. Corticosteroids are given in many ways: oral, inhaled, intranasal or by injection. The purpose of corticosteroid treatment is to obtain disease remission by reducing inflammation and prevent disease exacerbations in the long term. Corticosteroids are not used as a single entity in treating asthma, rhinitis or nasal polyps but are used as the main therapy in asthma (Scottish Intercollegiate Guideline Network (SIGN) / British Thoracic Society (SIGN/BTS, 2008), rhinitis and nasal polyposis (Stanaland, 2004; Bousquet *et al*., 2001).

Corticosteroids exhibit a combination of pharmacological activities both glucocorticoid and mineralcorticoid. The presence of delta (δ)-4, 4-keto-11-beta, 17-alpha (α), 21-trihydroxyl determines the glucocorticoid activity. The structure of cortisol (Figure 1.1) is modified by the addition of a variety of chemical moieties (Table 1.1) to produce the different corticosteroids used in the management of asthma, rhinitis or polyps. The variability in properties of the corticosteroids affects the amount of drug delivered, drug delivery methods and the efficacy on respiratory outcomes.

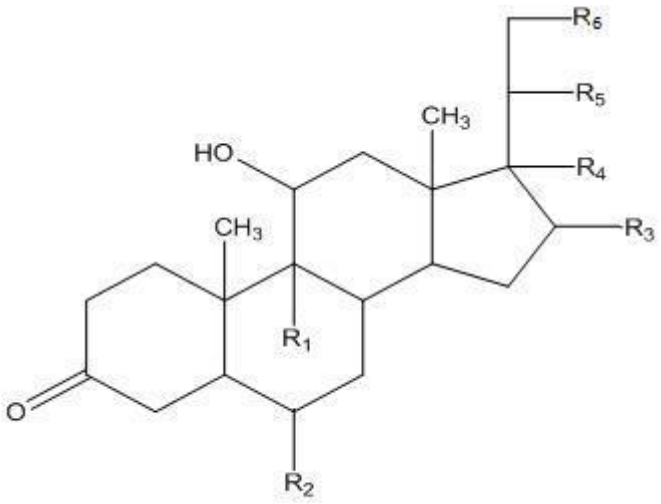
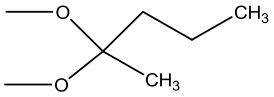
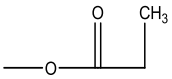
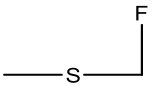
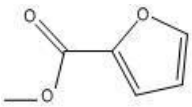
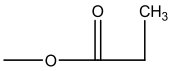
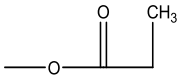


Figure 1.1 The basic corticosteroid structure

Table 1.1 The structure of corticosteroids

Compound	R_1	R_2	R_3	R_4	R_5	R_6
prednisolone	- H	- H_2	- H_2	- OH	= O	-OH
betamethasone	- F	- H_2	- CH_3	- OH	= O	-OH
budesonide	- H	- H_2			= O	-OH
fluticasone propionate	- F	- F	- CH_3		= O	
mometasone furoate	- Cl	- H_2	- CH_3		= O	- Cl
beclometasone dipropionate	- Cl	- H_2	- CH_3		= O	

1.2 Mechanism of action of corticosteroids

Corticosteroids have a direct inhibitory effect on inflammatory mechanisms (Barnes, 2001). They reduce the production of eosinophils, inflammatory mediators and mucus secretion. At a cellular level the corticosteroid molecule binds at the C-terminal of the glucocorticoid receptor, which later alters transcription. This modification results in an increase in protein synthesis. Deacetylation of the histone residue terminates unwinding of DNA. One theory (Nelson, 1999) claimed that corticosteroids have no significant effect on mast cells but inhibit eosinophil release and reduce interleukin 4, 5, 6 and 8 (IL-4, IL-5, IL-6 and IL-8) production. Inhibition of phospholipase A_2 in the lipoxygenase pathway blocks the production of interleukin 1 and 10 (IL-1, IL-10). Expression of the cell adhesion molecule, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) in response of cytokines such as tumour necrosis factor - alpha (TNF-α) are blocked by activation of the glucocorticoid complex. T-cell apoptosis is enhanced by the presence of corticosteroids which reduce the amount of cytokine released. In a similar way, overproduction of mucus is effectively reduced by drug inhibition of mucin glands.

Administration of corticosteroids to a patient affects the natural synthesis of the endogenous steroid, cortisol. The amount of cortisol secreted is decreased as a result of negative feedback to the pituitary adrenal glands. The long term of suppression of cortisol is believed to have a link with increased susceptibility to infection, development of diabetes (Gulliford *et al.*, 2006), cataracts (Cumming *et al.*, 1997), hypothalamic pituitary (HPA) axis suppression (Tayab *et al.*, 2007; Fardon *et al.*,

2004) or corticosteroid-induced osteoporosis (Langhammer *et al*., 2007; Gudbjornsson *et al.*, 2002).

1.3 Formulation of corticosteroids

The ideal drug formulation for inhalation and intranasal application is designed to have maximal therapeutic effect at the bronchial and nasal mucosa surface with minimal side effects.

1.3.1 Inhaled corticosteroids

The ideal particle size for lung deposition of inhaled corticosteroids is less than 5 micrometers and particles of less than 1 micrometer will be exhaled. Particle size and inhaler devices can affect lung deposition (Invernizzi *et al*., 2009; Leach *et al*., 2009; Tarsin *et al.*, 2006; Edsbacker & Johansson, 2006). About 8 – 15 % of the inhaled particles at a size of 2.6 – 4.0 micrometres will be deposited in the lung (Gentile & Skoner., 2010; Leach *et al*., 2009). Any inhaled particles more than 5 micrometers might be deposited at the oropharyngeal region and exhaled (Usmani *et al.*, 2005).

1.3.1.1 Inhaler devices

Several inhaler devices are available for the delivery of medication to patients with asthma; these include pressurised metered dose inhalers (pMDIs), breath actuated pMDIs, dry powder inhalers (DPIs) and nebulisers. The pMDI which was introduced in 1956 is convenient to use and portable but it is reported to have high oropharyngeal deposition. Only 10 – 20 % of the actuated dose will be deposited in

the lung even if good inhaler technique is applied (Newman, 2005). The breath actuated inhaler pMDI is similar to the pMDI in mechanism of drug delivery and automatically delivers medication without the need for good coordination between actuation and inhalation (Price *et al.*, 2003). Lenney *et al.,* (2000) found that despite being widely used only 79 % of the patients using a pMDI showed good inhalation technique compared to 91 % in the group receiving breath actuated pMDI.

A non-pressurised breath actuated inhaler was developed in 1970: these are known as dry powder inhalers (DPI). The DPIs are currently marketed as breath actuated inhalers and consist of both single dose and multi dose devices. Compared to the DPI, the pMDI requires slow and deep breathing while a high inspiratory flow is needed by a patient using a DPI to ensure complete inhalation of the drug (Chrystyn, 2007). When the canister of pMDI is pressed or actuated, the pressurised liquid in the canister escapes through the nozzle and is vaporised as an aerosol. While with a DPI, the inhaler has a different airflow resistance that requires the patient to generate a forceful inspiratory flow to achieve the required dose.

A spacer can facilitate better inhalation with pMDIs (SIGN/BTS, 2008). A spacer improves drug deposition at the inflammatory site and decreases deposition in the oropharynx (Scally *et a*l., 2010; Nair *et al*., 2010; Newman *et al*., 2002). The disadvantages of spacers are that the delivery system is less portable and therefore less convenient to use than a pMDI on its own (Newman *et al*., 2002).

Counselling on the proper method of inhalation should be carried out (SIGN/BTS, 2008) as the proper inhalation technique increases the possibility of good asthma management. Suitable information regarding the inhalers, demonstrating the correct inhalation technique and addressing patient's inquiries regarding the inhaler are part of the healthcare professional's responsibility (Mehuys *et al*., 2008).

Hydrofluoroalkane (HFA) inhalers were developed due to the growing concern regarding chlorofluorocarbon (CFC) propellant inhalers (Busse *et al.*, 2000; Anderson, 2001). Beclometasone dipropionate (BDP) with HFA as the propellant is formulated as a solution compared to a suspension in the CFC propellant (Anderson, 2001; Global Initiative for Asthma (GINA), 2008). The reduced size of the particles in Qvar®, a non-CFC containing pMDI, has shown better lung deposition of BDP compared to CFC-pMDI inhalers (Leach *et al.*, 2002). The two CFC-free BDP inhalers, Clenil® and Qvar® are both licensed for treatment of asthma. The HFA propellant inhaler, Clenil Modulite® is equipotent to the existing CFC-propellant inhalers whereas the other HFA-propellant inhaler Qvar® is prescribed at half the dose of CFC propellant BDP (Busse *et al.*, 2000, SIGN/BTS, 2008).

1.3.2 Intranasal corticosteroids

The thin nasal mucosa is highly vascularised and the drug reaches the target sites (turbinates) directly but only a limited volume of drug (100 – 150 microlitres) can enter the nasal cavity (Bhise *et al*., 2008; Costantino *et al*., 2007). Epithelial cells covering the turbinates contain cilia and goblet cells which secrete mucus. The bioavailability of the drug administered intranasally is affected by the drug's

molecular weight, polarity, pH and partition coefficient (Jadhav *et al*., 2007; Arora *et al*., 2002). Mucociliary clearance in the nose relies on mucus production and cilia function (Boatsman *et al.*, 2006). The alcohol content in the nasal spray might increase the viscosity of mucus.

1.3.3 Oral corticosteroids

Oral corticosteroids are prescribed to patients no longer controlled by a high dose of inhaled corticosteroids or a combination of inhaled corticosteroids with a long-acting beta-agonist (LABA). It takes about 6 – 12 hours after ingestion for oral corticosteroids to reach their maximal effect (Rouster-Stevens *et al*., 2008). The drug is readily absorbed from the gastrointestinal tract following oral administration. The oral corticosteroid is metabolised by the liver and is distributed to the kidneys, skin, lungs and muscle. The systemic bioavailability (70 %) of oral prednisolone is higher than inhaled corticosteroid such as mometasone furoate (MF) and fluticasone propionate (FP) (<1 %).

1.4 Pharmacokinetics

1.4.1 Absorption

The plasma concentrations of the corticosteroids differ according to the route of administration (Tattersfield *et al*., 2004; Daley-Yates *et al.*, 2001). The quantity of corticosteroid reaching the site of action, nose or lung, describes the efficacy of the drugs.

1.4.1.1 Oral administration

Oral prednisolone is absorbed rapidly via the gastrointestinal tract after ingestion. The peak of plasma concentration of prednisolone can be seen 1 – 2 hours after administration.

1.4.1.2 Inhaled corticosteroids

It is known that despite good inhalation technique, a large part of the inhaled dose is swallowed (50 – 90 %) (Chrystyn, 2007; Derendorf *et al.*, 2006). Only 15 – 27 % of the dose inhaled reaches the lung (Chrystyn, 2007; Leach *et al*., 2002; Dales-Yates *et al*., 2001). Corticosteroids are rapidly absorbed throughout the lung by passive diffusion through the plasma membrane in the lung epithelium which is the barrier for absorption of inhaled drugs. It takes 5 – 7 hours (FP) or around 1 hour (budesonide (BUD)) for the drug to be available systemically (van der Brink *et al*., 2008). The swallowed portion is absorbed from the gastrointestinal tract and metabolised by the liver.

1.4.1.3 Intranasal corticosteroids

Good permeation of drug across the cell membrane in the nasal mucosa ensures that the drug reaches the target sites readily. Good permeation depends on the viscoelastic properties of mucus secretion and activity of mucociliary clearance (Pires *et al.*, 2009; Dales-Yates *et al*., 2004; Homer *et al*., 2002). Intranasal drug permeation is increased with slower activity of nasal mucocilliary clearance and decreased mucus production. Destruction of the nasal mucosa, nasal congestion and excessive mucous discharge reduce drug absorption in the nasal mucosa. The

presence of vasoconstrictor agents such as phenylephrine will inhibit intranasal drug absorption (Pires *et al.*, 2009).

The portion of the dose of intranasal corticosteroid which is directly absorbed by the nose and enters the systemic circulation is about 30 % (Derendorf & Meltzer, 2008). Intranasal MF and FP are poorly absorbed into the systemic circulation (<1 %) from the nose due to high lipophilicity (Pires *et al.*, 2009; Allen *et al*., 2007; Nave *et al*., 2007).

In a study by Dales-Yates *et al.*, (2001) on different routes of administration (intravenous, inhaled and intranasal) for BDP, the absorption of the active metabolite of BDP, beclometasone-17-monopropionate was reported as less than 1 % in the nasal mucosa for intranasal BDP and 36 % in the lung when administered by inhalation.

The dosage forms of intranasal corticosteroids, nose drops or nasal spray, may influence the absorption of the drug (Algorta *et al*., 2008; Homer *et al*., 2002; Dales-Yates *et al.*, 2001). The plasma concentration of intranasal FP was found to be eight times lower if instilled by nasal drops compared to a nasal spray (Dales-Yates *et al.*, 2001). On the other hand, Homer *et al*., (2002) demonstrated no correlation between different delivery methods (nasal drops or nasal spray) and plasma concentration of FP following intranasal delivery instead suggesting the administration technique might affect the drug absorption at nasal mucosa.

1.4.2 Distribution

The volume of distribution (V_D) reflects the distribution of drugs in the extra pulmonary tissue and is strongly associated with their lipophilicity. Higher lipophilicity reflects better tissue-drug binding. Only free and unbound drugs are able to bind to tissue and result in pharmacological effects (Winkler *et al.*, 2004; Daley-Yates *et al.*, 2001).

The larger the V_D of the drug, the longer the drug will stay in the peripheral compartment. BDP and its active metabolite, beclometasone-17-monopropionate (17-BMP) have a reported V_D of 20 L and 424 L, respectively (Daley-Yates *et al.*, 2001). The extensive V_D of 17-BMP was calculated based on assumption all the BDP absorbed was metabolised to 17-BMP. The high V_D of FP (318 – 859 L) does not indicate high pharmacological activity as the drug is bound in an inactive form (van den Brink *et al*., 2008; Hughes *et al*., 2008; Singh *et al*., 2003).

1.4.3 Metabolism

Corticosteroids are mainly metabolised by the liver and in the gastrointestinal tract (Derendorf & Meltzer, 2008). FP and MF are metabolised in the liver by the cytochrome P-450 3A4 pathway to the inactivate metabolites, 17β-carboxyfluticasone and 6β-hydroxymometasone respectively (Hughes *et al*., 2008; Sahasranaman *et al*., 2006). These inactive metabolites that enter systemic circulation have less than 1 % of the pharmacological activity of their parent compounds (Nave *et al*., 2007; Sahasranaman *et al*., 2006; Fedorak & Bistritz, 2005). BDP is metabolised in the liver to three metabolites; beclometasone-17-

monopropionate (17-BMP), beclometasone-21-monopropionate (21-BMP), and beclometasone (BOH). The most active metabolite, 17-BMP has extra pulmonary effects and is detected in plasma 2 hours after inhalation (Nave *et al*., 2007; Daley-Yates *et al.*, 2001).

1.4.4 Elimination

BUD and FP are both eliminated by the liver, with a clearance of 55 – 84 L hr^{-1} and 66 – 90 L hr^{-1}, respectively (Hughes *et al*., 2008; Edsbacker & Johansson, 2006; Krisnaswami *et al*., 2005). BDP has a clearance of 150 – 230 L hr^{-1}, which is greater than hepatic blood flow as its metabolites are widely distributed. This explains the extra pulmonary effects of BDP and its metabolites. Corticosteroids and their metabolites are mainly excreted in faeces and only 2 – 10 % is excreted in urine (Martindale, 2009; Hughes *et al*., 2008; Tayab *et al*., 2007; Sahasranaman *et al*., 2006; Teng *et al*., 2003).

A reduced rate of drug clearance will affect the rate and extent of drug accumulation in the body. A longer elimination half life might increase the risk of adverse effects as the drug will accumulate in the body. The elimination half life can differ from the terminal half life as absorption can be a rate limiting factor in measuring the terminal half life (Winkler *et al.*, 2004). The long elimination half life of FP (7.8 – 14.4 hours) is evidence of an extensive distribution of FP and leads to slow elimination of the drug (van den Brink *et al*., 2008; Singh *et al*., 2003; Thorsson *et al*., 2001; Minto *et al*., 2000). The shorter elimination half life (0.5 – 6 hours) of other inhaled corticosteroids (BDP, BUD, MF) is explained by the lower volume of distribution of

these agents (Tronde *et al*., 2008; Lonnebo *et al*., 2007; Sahasranaman *et al*., 2006; Mortimer *et al*., 2006; Thorsson *et al*., 2001; Daley-Yates *et al.*, 2001).

1.5 Pharmacodynamics

1.5.1 Plasma protein binding

Higher plasma protein binding has been reported for MF (98 – 99 %) compared with other corticosteroids, FP (91 – 95 %), BDP (87 %) and BUD (85 – 90 %) (Wu *et al*., 2009; Hochhaus, 2008; Tayab *et al*., 2007; Godfrey *et al*., 2002). A strong relationship between patient cortisol suppression and the degree of plasma protein binding has been proven (Edsbacker & Johansson, 2006; Tayab *et al.*, 2007). Plasma protein binding also explains the duration of drug retention at the target site and its pharmacological activity. Less than 15 % of the drugs in the free unbound form are distributed and bind to the target sites (Edsbacker & Johansson, 2006; Winkler *et al.*, 2004).

1.5.2 Bioavailability

Bioavailability is the fraction of the drug that becomes systemically available after absorption from the lung, or gastrointestinal tract and first pass metabolism of the swallowed portion of the dose (Winkler *et al.*, 2004). Increases in drug concentration in the plasma will increase the risk of side effects (Tayab *et al*., 2007; Whelan *et al*., 2005). For BDP, the parent compound and its active metabolite, 17-BMP, are found in the systemic circulation. Once BDP is inhaled, it is metabolised to 17-BMP in the lung and 36 % of 17-BMP is absorbed from the lung (Dales-Yates *et al.*, 2001). FP and MF showed less drug available systemically (<1 %) from the

nose and lung which reflects complete first pass metabolism (Derendorf & Meltzer, 2008; Sahasranaman *et al*., 2006; Dales-Yates & Baker, 2004). A similar amount of BUD (18 – 25 %) was reported as a total of the swallowed portion and pulmonary bioavailability (Tronde *et al*., 2008; Lonnebo *et al*., 2007; Nave *et al*., 2007).

1.5.3 Glucocorticoid receptor affinity

The efficacy of binding of corticosteroids to glucocorticoid forming glucocorticosteroid complexes depends on the affinity of the drug molecule to the glucocorticoid receptors and drug dissociation (Valotis & Hogger, 2008; Edsbacker & Johansson, 2006; Winkler *et al.*, 2004). Lipophilic drugs are cleared slowly from the target sites which provide better attachment at the site of action. High receptor binding will improve the efficacy of the drug. Fewer conclusions on efficacy can be drawn based on protein binding as binding does not block the activity of the corticosteroid molecule (Winkler *et al.*, 2004).

FP and MF demonstrate similar binding affinity to glucocorticoid receptors (Valotis & Hogger, 2008; Tayab *et al*., 2007; Issar *et al*., 2006; Fardon *et al*., 2004). Valotis & Hogger (2008) believed that the presence of an ester group at position R_4 in corticosteroids, for example FP or BDP, improves its lipophilicity and indirectly affects the binding of the drug to the glucocorticoid receptor. FP demonstrates a faster rate of association with glucocorticoid receptors compared to triamcinolone and BUD (Dalby *et al*., 2009; Valotis & Hogger, 2008; Krishnaswami *et al*., 2005). 17-BMP has a 5-fold higher binding affinity than its parent compound BDP (Valotis & Hogger, 2008; Dales-Yates *et al.*, 2001).

1.5.4 Lipophilicity

Lipophilicity of the drug explains the tendency of the drug to partition into the hydrophobic compartment and aqueous environment. The half life and duration of action of a corticosteroid in the body are dependent on lipophilicity (Winkler *et al.*, 2004). Corticosteroids are naturally lipophilic substances which bind poorly to protein molecules. FP is more lipophilic, has slower absorption from the lung (7 – 8 hours) than BUD (3 – 4 hours) or triamcinolone acetonide (1 hour) (Dalby *et al*., 2009; Baumann *et al*., 2009; Derendorf & Meltzer, 2008; Mortimer *et al*., 2006). The presence of mucus and bronchoconstriction affect the deposition of drug at the target sites. FP is more likely to reach the central airways than peripheral airways and is less soluble in mucus compared to BUD due to its high lipophilicity (Dalby *et al*., 2009; van den Brink *et al*., 2008) and more efficient delivery devices (Dalby *et al*., 2009; Derendorf *et al*., 2006).

The efficacy of intranasal corticosteroids is also correlated with the lipophilicity of the drug. Drug absorption is expected to be greater with an increase in drug lipophilicity. However, highly lipophilic drugs, such as FP, may have limited nasal absorption due to a slower dissociation rate which might favour elimination of the drug from the nasal mucosa by mucociliary clearance (Dales-Yates *et al*., 2004; Brattsand & Miller-Larsson, 2003). The reduced lipophilicity of BUD results in it being more readily partitioned into the mucous than FP (Dalby *et al*., 2009; van den Brink *et al*., 2008; Winkler *et al.*, 2004).

1.5.5 Lipid conjugation

Lipid conjugation occurs between the drug molecules and the fatty acids in lung and nasal mucosa. The presence of an enzyme, lipase, makes the conjugation reversible. The corticosteroids which undergo lipid conjugation are BDP, BUD and FP which all have a hydroxyl group at R_5. The reversible fatty acid formation in BUD helps to retain the drug in the nasal mucosa and prolongs its anti inflammatory effect (van den Brink *et al.*, 2008). A fatty acid BUD ester is formed one hour after inhalation (Algorta *et al*., 2008; Lonnebo *et al*., 2007;Brattsand & Miller-Larsson, 2003) and acts as an intracellular depot which releases the BUD ester to be hydrolysed to active BUD (Brattsand & Miller-Larsson, 2003). The lipid conjugation of BDP, BUD or FP affects the pulmonary and nasal retention time as it takes longer for the drug to be absorbed into the systemic circulation (Algorta *et al*., 2008; Winkler *et al.*, 2004). A longer pulmonary half life and mean residence time may result in fewer adverse effects (Winkler *et al.*, 2004; Edsbacker & Johansson, 2006).

1.6 Corticosteroids available in the United Kingdom

1.6.1 Prednisolone

Prednisolone is actively metabolised by the liver to prednisone. Since, prednisolone is ingested orally, the drug is rapidly absorbed by the gastrointestinal tract and the maximum concentration (C_{max}) can be observed 1 – 2 hours after administration. The pharmacokinetics of prednisolone are not linear and are affected by the prednisolone dose (Xu *et al*., 2007). The elimination half life of prednisolone is 18 – 36 hours and is longer than any of the inhaled corticosteroids (Tiigiame –Saar *et al*., 2010).

In the treatment of acute asthma, high dose inhaled corticosteroids (2400 – 4000 micrograms daily) have been found to be as effective as oral corticosteroids (30 – 40 milligrams) (Belda *et al*., 2007; Fitzgerald *et al*., 2000). Prednisolone is given orally and is as effective as intravenous methylprednisolone in the treatment of acute asthma (Lahn *et al*., 2004).

Oral corticosteroids are introduced in step 5 of SIGN/BTS guideline for management of stable asthma where increasing the dose of inhaled corticosteroids to 2000 micrograms daily is no longer effective in providing asthma control (SIGN/BTS, 2008). A short course (4 to 7 days) of oral prednisone improves control regardless of the previous current dose of inhaled corticosteroids (Ahrens *et al*., 2001). While prolonged treatment with oral prednisone should be avoided if possible, the lowest dose of prednisolone, without jeopardising asthma control, should be used in long term corticosteroid treatment where withdrawal is not possible.

1.6.2 Beclometasone dipropionate (BDP)

The stable pharmacologically active compound, 17 - beclometasone monopropionate (17-BMP) is formed after beclometasone dipropionate (BDP) is hydrolysed by lung tissue (Nave *et al*., 2007; Dales-Yates *et al.*, 2001). BDP is a prodrug which not active in its ingested form and is biotransformed to its active metabolite at the target sites. In an open label study (Dales-Yates *et al.* 2001) BDP was assumed to be completely metabolised to 17-BMP and later to beclometasone (BOH). A quantity of unchanged BDP (319 pg ml^{-1} (300 – 400 pg ml^{-1})) was detected when BDP 1000 micrograms was inhaled compared to no detectable BDP when a dose of 336

microgram was administered intranasally. The major metabolite of BDP, 17-BMP is widely distributed (424 litre) after administration intravenously compared to its parent compound, BDP. Within 24 hours, all BDP is metabolised to BOH in the lung (Nave *et al*., 2007). About 12 – 15 % of BDP is excreted in urine as free and conjugated polar metabolites (Martindale, 2009).

Several double blind, crossover studies have been conducted to measure the difference between hydrofluoroalkane (HFA-BDP) and chlorofluorocarbon (CFC-BDP) in terms of efficacy in patients with mild asthma (Micheletto *et al.*, 2005; Leach *et al*., 2002). Greater improvement in lung function, measured as forced expiratory volume in one second (FEV_1), was observed in the HFA-BDP group compared to the CFC-BDP group. This can be explained by an increase in drug deposition in lung of HFA-BDP (53 %) compared to CFC-BDP (4 %) and CFC-FP (12 – 13 %) (Leach *et al*., 2002). Deposition of corticosteroids delivered by HFA-BDP in the oropharynx was lower (29 – 30 %) compared to CFC-BDP (90 – 94 %) and CFC-FP (72 – 78 %). The dose of inhaled HFA-BDP (Clenil®) was found to be equipotent to half the dose of inhaled CFC-FP (SIGN/ BTS, 2008; Masoli *et al*., 2004; Leach *et al*., 2002). Inhaled CFC-FP significantly improved mean FEV_1 by 0.14 litre and morning peak expiratory flow rate (PEFR) compared to inhaled HFA-BDP at half the dose (Adams *et al*., 2007).

1.6.3 Budesonide (BUD)

Budesonide is rapidly metabolised to 16α-hydroxyprednisolone and 6β-hydroxybudesonide in the liver. The unchanged budesonide has been quantified in

plasma (Harrison, 2003). The parent compound is completely undetectable in urine (Deventer *et al.*, 2006; Fedorak & Bistritz, 2005). BUD is a pharmacologically active drug when inhaled and is composed of two racemic compounds (22-R and 22-S) with the 22-R enantiomer being three times more potent than 22-S (British Pharmacopeia (BP), 2008). The inactive metabolites, 16α-hydroxyprednisolone and 6β-hydroxybudesonide have less receptor affinity than the parent compound and are excreted mainly by the kidney (Fedorak & Bistritz, 2005).

The commonly used fixed dose combination of BUD and the long-acting beta–agonist, formoterol (Symbicort®), is promoted as a successful treatment in managing of chronic asthma (O'Byrne *et al*., 2008; Rabe *et al*., 2006; Lalloo *et al*., 2003; Pauwels *et al*., 1997). In a one year Formoterol and Corticosteroids Establishing Therapy (FACET) study, the fixed dose combination of inhaled BUD (100 – 400 micrograms) and formoterol (12 micrograms) was found to provide better control of lung function compared to inhaled BUD (200 – 800 micrograms) alone in patients with moderate to severe asthma (Pauwels *et al*., 1997). The study clarified that the addition of the LABA, formoterol to a low dose of inhaled BUD significantly improved asthma control as effectively as formoterol added to a high dose of BUD. The lowest number of patients without a severe exacerbation was reported in combination of higher dose of inhaled BUD (800 micrograms) and formoterol followed by higher dose of BUD given alone (800 micrograms) (Pauwels *et al*., 1997; O'Bryne *et al.*, 2008). The definition of an exacerbation in this study takes in an emergency visit or hospitalisation, number of short courses oral corticosteroids and change in PEFR of 20 – 30 % below the baseline (Kuna *et al*., 2007; Rabe *et al*.,

2006). The fixed dose of BUD and formoterol was observed to be a better alternative than increasing the dose of inhaled BUD alone when the patients showed a poor response to low dose inhaled BUD (200 micrograms daily). There was a greater reduction in exacerbation rate in patients with mild to moderate asthma with a fixed combination of BUD and formoterol (O'Byrne *et al.*, 2008; Corren *et al*., 2007; Lalloo *et al*., 2003; O'Byrne *et al.*, 2001; Pauwels *et al*., 1997) and a significantly larger increase in the lung function parameter, PEFR (16.5 % vs 7.3 %, respectively, p = 0.002) (Lalloo *et al*., 2003).

A fixed dose of budesonide/formoterol as maintenance and reliever therapy termed Symbicort® maintenance and reliever therapy (SMART) was introduced as an improved method of using an inhaled corticosteroid/LABA combination as both preventer and reliever therapy. In these studies, a low dose of fixed combination of BUD (100 – 200 micrograms twice daily) and formoterol (4.5 – 9 micrograms twice daily) maintenance therapy was used as the comparator arm (Kuna *et al*., 2007; Rabe *et al*., 2006; O'Byrne *et al*., 2005; Sacchitano *et al*., 2004). SMART prolongs the time between relapse and a greater reduction in hospitalisation (5 – 39 %) was recorded compared to a fixed dose of budesonide and formoterol maintenance therapy (Kuna *et al*., 2007; Rabe *et al*., 2006). Fewer doses of reliever therapy (0.84 – 1.27 inhalations per day) and a reduction in night time awakenings (9.6 – 16 %) were reported in the SMART group compared to traditional dosing including a short-acting beta-agonist (SABA) (Rabe *et al*., 2006; Scicchitano *et al*., 2004). In a large randomised, double blind, multicentre study, 2421 mild to moderate asthmatic patients were randomised to three different groups either SMART at 80/4.5

micrograms twice a day and as required, a fixed dose of budesonide/formoterol (80/4.5 micrograms twice daily) with inhaled SABA and inhaled BUD (320 micrograms twice daily) and inhaled SABA (O'Byrne *et al*., 2005). The SMART group reported a 47 % longer time to relapse compared to the group on a higher dose of BUD whereas there was similar data on time to first severe exacerbation between patients on fixed dose combination of budesonide/formoterol (21 %) and high dose of inhaled budesonide (19 %). A significant result ($p < 0.001$) was observed in a comparison between SMART and a fixed combination of budesonide/formoterol where SMART showed a greater improvement in the morning and evening PEFR, a reduction in night awakenings and provided more symptom free days. In comparison to the FACET study (Pauwels *et al*., 1997) this study showed that SMART not only prolonged the time between relapse but reduced the exacerbation rate in patients with mild to moderate asthma.

1.6.4 Fluticasone propionate (FP)

Fluticasone propionate is a trifluorinated compound and has the highest binding affinity to glucocorticoid receptors (1.0) compared to 17-BMP (0.66) and BUD (0.5) (Wu *et al*., 2009; Winkler *et al*., 2004; Crim *et al*., 2002). FP has a higher association and slower dissociation rate (60 % after 10 hours) from the glucocorticoid receptor compared to other inhaled corticosteroids. The presence of the propionate group at position R_4 and methyl group at position R_3 in its molecular structure are associated with the high potency of FP (Johnson, 1998). FP is almost completely metabolised (90 %) in the liver to 17β-carboxylic acid and the metabolite

has 2000 times less affinity for the target sites at the lung (Nave *et al*., 2007; Dales-Yates *et al*., 2004).

A significant difference in drug absoption in patients with airflow obstruction such as patients with asthma compared with healthy patients has been described (Mortimer *et al*., 2007; Brutsche *et al.*, 2000). The plasma concentration of inhaled FP in patients with asthma was 60 – 62 % lower compared to healthy people. However, patients without airflow obstruction demonstrated similar bioavailability of inhaled BUD to patients with airflow obstruction (Mortimer *et al*., 2007). Due to its high lipophilicity, FP is likely to be deposited in the central airways in patients with airflow obstruction whereas BUD is readily absorbed by the airway tissue in patients with or without airflow limitation (Dalby *et al*., 2009; van der Brink *et al*., 2008). Therefore, BUD is absorbed from the lung to systemic circulation faster than FP since BUD has lower lipophilicity and volume of distribution than FP.

Inhaled FP with doses as low as 200 micrograms daily have proved to be effective in treating mild to moderate asthma (Dahl *et al*., 2010; Boulet *et al*., 2009; Buhl *et al*., 2006; Foresi *et al*., 2005). Several randomised, double blind studies which followed patients for between two weeks and two months (Dahl *et al*., 2010; Ehrs *et al*., 2009; Boulet *et al*., 2009; Buhl *et al*., 2006; Foresi *et al*., 2005) confirmed than inhaled FP significantly improved FEV_1, increased the number of asthma symptom free days and reduced the need for rescue medication compared to placebo as early as two weeks after starting treatment and its benefit stays constant with time. A linear dose-

effect relationship for inhaled FP was reported in patients with moderate to severe asthma (Fardon *et al*., 2004; Bateman *et al*., 2004; Holt *et al*., 2001).

The coadministration of a LABA with an inhaled corticosteroid was suggested when single low dose inhaled corticosteroids no longer provide asthma symptom control. Findings from randomised, double blind trials have demonstrated the efficacy of fluticasone/salmeterol in improving lung function and controlling asthma symptoms compared to salmeterol alone (Nathan *et al*., 2006; Lundback *et al*., 2006) or inhaled FP alone (Schermer *et al*., 2007; Barnes *et al*., 2007; Houghton *et al*., 2007; Bateman *et al*., 2006; Lee *et al*., 2003). The fixed dose of fluticasone/salmeterol (100/50 or 250/50 micrograms daily) provided a greater improvement in FEV_1 (2.6 %) and 1.1 more symptom free days compared to moderate dose inhaled FP (250 – 500 micrograms daily) in a 12-week duration study (Schermer *et al*., 2007). A combination of fixed dose fluticasone/salmeterol (100/50 micrograms twice daily) was found to provide additional lung function benefit compared to fixed dose fluticasone/salmeterol (100/50 micrograms once daily) (Peters *et al*., 2007) or low dose of inhaled FP (100 micrograms twice daily) (Schermer *et al*., 2007; Houghton *et al*., 2007; Bateman *et al*., 2004). The rate of exacerbation was reported to be lower in the fixed dose inhaled fluticasone/salmeterol groups compared to inhaled FP alone at all doses (200 – 1000 micrograms daily) in the Gaining Optimal Asthma Control (GOAL) study (Bateman *et al*., 2004). The percentage of patients with totally controlled asthma (absence of exacerbations, no rescue medication and no asthmatic symptoms in 7 – 8 weeks) was reported to be higher in the fixed dose inhaled fluticasone/salmeterol groups (41 %) compared to inhaled fluticasone alone (28 %)

after a year. The addition of LABA to an inhaled corticosteroid did not improve the sputum eosinophils count or exhaled nitric oxide in the patients studied (Foresi *et al*., 2005; Lee *et al*., 2003).

Several placebo controlled studies of intranasal FP aqueous spray showed a significant improvement in nasal symptom scores in patients with allergic rhinitis (Nair *et al*., 2010; Kaiser *et al*., 2007; Jen *et al*., 2000)or nasal polyposis (Jankowski *et al*., 2009; Aukema *et al*., 2005; Fowler *et al*., 2002). No significant difference was found in comparison to placebo for the ocular symptoms of eye itching and eye redness (Kaiser *et al*., 2007). In two multicentre, randomised, double blind studies regarding patients with nasal polyposis (n = 384) (Jankowski *et al*., 2009; Penttila *et al*., 2000), the difference in nasal symptoms scores between two different doses (200 micrograms daily or 200 micrograms twice daily) of FP seems to support using the higher dosing regimen. The higher dose of FP provided a significant improvement in nasal obstruction, sense of smell and a reduced size of nasal polyp compared to lower doses (100 micrograms once daily) in an eight week study.

1.6.5 Mometasone furoate (MF)

A halogenated corticosteroid, mometasone furoate has recently (2005) become available with an onset of action of seven hours and a plasma half life of 5.8 – 18.4 hours (Small *et al*., 2008; Sahasranaman *et al.*, 2006; Teng *et al*., 2003). It has a chlorine atom at position R_1 compared to fluorine atom in the structure of FP. It undergoes hydroxylation to form 6β-hydroxyl-mometasone furoate and hydrolysis of the furoate ester (Sahasranaman *et al.*, 2006; Tayab *et al.*, 2007). Five metabolites of

MF were found after first pass hepatic metabolism (Sahasranaman *et al.*, 2006). About 0.08 % of the parent compound is excreted in the urine (Sahasranaman *et al.*, 2006; Teng *et al*., 2003). MF has proved to be equipotent to FP (Fardon *et al.*, 2004; Tayab *et al.*, 2007).

Several studies on efficacy of MF in asthma of 8 – 12 weeks duration have been conducted (Tayab *et al.*, 2007; Karpel *et al*., 2007; Sahasranaman *et al.*, 2006; Bensch *et al*., 2006; Karpel *et al*., 2005; Fardon *et al*., 2004;Wardlaw *et al*., 2004). In comparison with placebo, inhaled MF (200 – 400 micrograms daily) significantly improved the patients FEV_1 by 16 – 24 % in patients previously on SABA alone (Bensch *et al*., 2006; Karpel *et al*., 2005). Inhaled MF (800 – 1600 micrograms daily) significantly reduced the use of oral prednisone (49 – 56 %) compared to placebo (14 %) over 12 weeks treatment (Karpel *et al*., 2007). About 58 % of patients in the placebo group increased their oral prednisolone dose and 31 – 39 % patients in the MF treated group stopped oral prednisone altogether. A dose response relationship was observed in low to high dose (400 – 1600 micrograms daily) inhaled MF in patients with mild to moderate asthma (Tayab *et al.*, 2007; Fardon *et al*., 2004). The dose response effect is not seen in severe persistent asthma as a similar improvement in FEV_1 and asthma symptom score was seen when the dose of inhaled MF increased between 800 micrograms daily and 1600 micrograms daily (Karpel *et al.*, 2007).

Loss of smell and nasal obstruction were found to be the most disturbing nasal symptoms identified by the participants in the study, therefore, it would be clinically

important if the treatment improved these symptoms (Meltzer *et al*., 2005). The treatment effect on nasal symptom scores were found to be related to the dosage of intranasal MF (Meltzer *et al.*, 2005; Small *et al*., 2008). The effect of 200 micrograms intranasal MF twice daily resulted in less nasal obstruction (10.9 %) and an improvement in sense of smell (9.6 %) compared to 200 micrograms intranasal MF once daily (8.6 % and 7.8 %, respectively) (Stjarne *et al*., 2006; Small *et al*., 2005). Several placebo controlled trials have proved that MF intranasal spray is effective in reducing nasal polyp size (Stjarne *et al*., 2009; Small *et al*., 2008; Meltzer *et al*., 2005). The bilateral polyp grade scores improved significantly after one month of treatment by 11.4 % compared to baseline and maximum treatment effects, as defined by the study, (28.1 %) were observed after four months of intranasal MF (200 micrograms twice daily) (Small *et al*., 2008).

1.6.6 Betamethasone (BETA)

Intranasal betamethasone sodium phosphate is recommended for the treatment of rhinitis and nasal polyposis. It has half life of more than 36 hours, is very water soluble and has limited first pass metabolism (Findlay *et al*., 1998; Nutting & Page, 1995). These pharmacokinetic properties explain the high systemic bioavailability of BETA. Systemic bioavailability for intranasal BETA was reportedly the highest compared to intranasal FP and MF (<1 %) and intranasal BDP (20 – 30 %) (Fowler *et al*., 2002; Homer *et al*., 1999). The over administration of intranasal BETA may be linked to formulation of intranasal BETA in drops, not spray (Patel *et al*., 2004). Several studies have found a correlation between intranasal BETA and adrenal

suppression (Patel *et al*., 2004; Fowler *et al*., 2002; Homer *et al*., 1999; Findlay *et al*., 1998)

1.6.7 Triamcinolone acetonide

The potent esterified form of triamcinolone which acts as an anti-inflammatory is biologically available after inhalation and is hepatically metabolised to three inactive compounds: 6-beta-hydroxytriamcinolone acetonide, 21-carboxytriamcinolone acetonide and 21-carboxy-6beta-hydroxytriamcinolone acetonide (Martindale, 2009; Hubbard *et al*., 2003). Triamcinolone acetonide has the least affinity for the glucocorticoid receptor and the highest unbound drug fraction (<30 %) compared to FP (10 %), BDP (13 %) and BUD (4 – 12 %) (Wu *et al*., 2009; Winkler *et al.*, 2004; Dales-Yates *et al*., 2001).

Inhaled BUD produced a better quality of life (activity limitation, emotional function, symptoms and response to environmental stimuli) at all times up to 52 weeks when compared to inhaled triamcinolone acetonide ($p < 0.001$) (Weiss *et al*., 2004). The reduction in symptoms and episode free days and improved Medical Outcomes Study 36-Item Short Form Health Survey (SF-36) symptom scores were seen in the BUD treated group compared to the triamcinolone acetonite treated group. No significant difference was noted in improvement of rhinitis symptoms between intranasal triamcinolone acetonide and intranasal MF (Lee *et al.*, 2003) at an equivalent dose but the authors believed a difference in better systemic bioactivity might be seen at higher doses of intranasal MF and intranasal triamcinolone acetonide.

1.7 Problems with corticosteroids

The ideal corticosteroid should be safe with few or minimal adverse effects (Winkler *et al.*, 2004). A higher percentage of free drug indicates better diffusion of the drug through lung tissue to the systemic circulation and thus increases the risk of systemic side effects. Modification of existing corticosteroid formulations produces inhaled corticosteroids which provide less deposition in the oropharynx and a longer retention time in the lung (Winkler *et al.*, 2004; Leach *et al.*, 2002). The safety of corticosteroids has been questioned for years and numerous studies (Algorta *et al*., 2008; Tayab *et al*., 2007; Angeli *et al*., 2006;Ton *et al.*, 2005; Fardon *et al.*, 2004; Benninger *et al.*, 2003) have attempted to clarify this issue. Patients believe that tolerance to steroids will develop with time and this can be reflected in increasing steroid doses (Brutsche *et al.*, 2000). The minimal sufficient dose to control symptoms is the most appropriate way to minimise the risk of systemic side effects.

1.7.1 Hypothalamic adrenal suppression

The hypothalamic adrenal axis is a direct feedback mechanism between the hypothalamus, pituitary glands and adrenal glands. The adrenal cortex which is the outer layer of adrenal glands releases cortisol in response to circulating adrenocorticotropin hormone released by the anterior pituitary glands (Tsigos & Chrousos, 2002). The introduction of corticosteroids into the systemic circulation sends a negative signal to the pituitary gland instructing it to stop releasing adrenocorticotropin hormone. This hormone, which is secreted following positive feedback of corticotrophin hormone on the pituitary, is secreted by the hypothalamus (Marik, 2002). The continuous presence of exogenous cortisol in the form of oral,

inhaled or intranasal corticosteroids might affect this feedback loop which might result in hypothalamic adrenal (HPA) suppression (Benninger *et al.*, 2003). Several studies relating to HPA suppression after administration of corticosteroids have been published (Tayab *et al.*, 2007; Derom *et al*., 2005; Fardon *et al*., 2004; Patel *et al*., 2004**).** The extent of the HPA suppression may be linked with the dose (Tayab *et al.*, 2007; Derom *et al*., 2005) and the duration for which the corticosteroids were taken (Whelan *et al*., 2005).

Several studies have reported a link between intranasal corticosteroids and adrenal suppression (Patel *et al*., 2004; Fowler *et al*., 2002). The association was shown to be strong with intranasal BETA steroids (63 %) compared to intranasal MF (0 – 4 %) (Rosenblut *et al*., 2007; Patel *et al*., 2004; Lee *et al*., 2003; Fowler *et al*., 2002). The intranasal corticosteroid BETA has also been suspected in links to Cushing's syndrome. About 4 % and 63 % of patients treated with intranasal MF (200 micrograms daily) and intranasal BETA (200 micrograms daily) respectively showed adrenal suppression (Patel *et al*., 2004). However, several studies have disagreed with Patel *et al*., (2004) and found intranasal corticosteroids to be safe with no significant adrenal suppression (Sachanandani *et al*., 2009; Algorta *et al*., 2008; Fowler *et al*., 2002; Keith *et al*., 2000). The lack of effect of intranasal FP (400 micrograms daily) on serum cortisol compared to placebo after 8 – 12 weeks treatment proved there is no link between intranasal FP and adrenal suppression (Fowler *et al*., 2002; Keith *et al*., 2000). A similar conclusion was made with studies of 4 weeks duration on safety of intranasal BUD (500 micrograms daily) on chronic

rhinosinusitis and nasal polyposis patients (Sachanandani *et al*., 2009) and in healthy patients (Algorta *et al*., 2008).

In studies of one to six weeks duration it has been reported that the extent of adrenal suppression is correlated with the corticosteroid dose taken by the patients (Tayab *et al*., 2007; Derom *et al*., 2005; Whelan *et al*., 2005; Martin *et al*., 2002; Fardon *et al*., 2004; Affrime *et al*., 2000). In two randomised placebo controlled studies with inhaled FP as the comparator where the dose increased from 500 micrograms twice daily to 1000 micrograms twice daily, the rate of plasma cortisol suppression increased from 29 % to 59 % (Derom *et al*., 2005). The urinary cortisol was reduced by 44 % in patients taking 500 micrograms of inhaled FP daily and by 69 % in patients taking 1000 micrograms of inhaled FP daily. A similar result in plasma cortisol suppression (69 %) was observed as early as one week after starting treatment on high dose inhaled FP (400 micrograms twice daily) (Whelan *et al*., 2005). Few trials have evaluated the extent of adrenal suppression based on comparison between two corticosteroids (Tayab *et al*., 2007; Fardon *et al*., 2004). These two studies compared the dose response relationship of inhaled MF and FP at low (400 – 500 micrograms daily), moderate (800 – 1000 micrograms daily) and high doses (1600 – 2000 micrograms daily). Similar behaviour in cortisol suppression as shown by Derom *et al*., (2005) was observed in patients on moderate to high doses of inhaled MF and FP.

Corticosteroids reduce inflammation by inhibiting secretion of inflammatory mediators. Activation of the corticosteroid-glucocorticoid receptor may increase the

production of anti inflammatory proteins, annexin - 1, secretory leukoprotease inhibitor, interleukin (IL) 10, and the inhibitor of nuclear factor (NF)-kB (Adcock & Lane, 2003; Tsigos & Chrousos, 2002). The interaction between the activated glucocorticoid receptor and the inhibitor of nuclear factor, (NF)-kB, and activator protein (AP) - 1, reduced the cytokine production of IL-1, 2, 3, 4, 5, 6, 9, 11, 12, 13, granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokines (IL - 8, RANTES), nitric oxide synthase and intercellular adhesion molecules (ICAM). When the adrenal cortex no longer produces sufficient cortisol due to the prolonged presence of exogenous corticosteroids, the body becomes more susceptible to infection as the immune system is compromised. Patients suffering from adrenal suppression may complain of continuous lethargy, weakness, anaemia and weight loss. Laboratory findings might suggest hyponatremia, hypokalemia but this might not be specific.

1.7.1.1 Basal adrenal function tests

Several methods have been developed to measure adrenal response after stimulation of the gland by the level of cortisol production throughout the day (Brutsche, 2000; Benninger *et al.*, 2003; Fardon 2004; Derom *et al*., 2005; Tayab *et al*., 2007). A Short Synacthen Test (SST) and morning salivary cortisol have been used to assess the correlation between corticosteroid dose and adrenal suppression (Patel *et al.*, 2004). A low salivary cortisol (less than 5 nmol L^{-1}) was confirmed by SST to be a good indication of adrenal suppression even though none of the patients presented with clinical signs of adrenal suppression. The salivary cortisol test is non invasive and stress free compared to blood collection. Cortisol is secreted in a circadian

rhythm, reaching its peak in the morning. Since corticosteroids suppress the production of endogenous cortisol, it is expected that the salivary cortisol obtained in the morning will reflect the depth of serum cortisol suppression (Dorn *et al*., 2007). The time that the corticosteroid is taken during the day affects the measurements of the HPA function. Frequent blood sampling for analysis of plasma cortisol over 24 hours to create a 24 - hour plasma area under curve ($AUC_{0\text{-}24h}$) profile might create a better plasma cortisol profile for patients on corticosteroids compared to one time sampling (Kaliner, 2006; Martin *et al*., 2002). A cosyntropin test is most commonly used as it can be used at any time of the day and it is simple and inexpensive (Neidert *et al*., 2010; Giordano *et al*., 2008). Amount of cortisol is measured before and after a supraphysiologic dose of ACTH is given to the patients. However this test can be misleading in interpreting adrenal insufficiency due to a variety of cut offs for the adrenal response and false positive responses in patients with suspected secondary adrenal insufficiency (Tanemoto *et al*., 2009; Giordano *et al*., 2008; Klose *et al*., 2007).

1.7.2 Corticosteroid induced osteoporosis

Osteoporosis is defined by the National Institutes of Health (NIH) (2001) as a continuous process of low bone mass and deterioration of bone microarchitecture that leads to fragility and fracture. The density of cancellous bone is decreased with age. The trabecular thickness in the cancellous bone determines the bone strength and resistance to fracture. Women over 60 years of age who have a strong family background of osteoporosis are more likely to have osteoporosis than men (Marshall *et al*., 1996; SIGN, 2003). Patients with one or more fractures have an increased

incidence of future fractures (Kanis, 2002). Corticosteroids may disturb bone homeostasis by affecting the balance of osteoclasts (bone breaking cells) and osteoblasts (bone forming cells) which could then cause osteopenia or osteoporosis. Bone loss is likely to occur in bone with a high cancellous bone to compact bone ratio such as the lumbar spine or neck of femur (Kanis, 2002; Tsugeno *et al*., 2002).

Studies on corticosteroids have reported that prolonged corticosteroid use is associated with bone loss (Sosa *et al*., 2008; de Vries *et al*., 2007; Langhammer *et al*., 2007; Angeli *et al*., 2006; Vestergaard *et al*., 2005; Ton *et al*., 2005; van Staa *et al*., 2000). Bone loss was accelerated in the first 6 – 12 months of treatment with oral corticosteroids but at slower rate afterwards (Saigal *et al*., 2006; van Staa *et al*., 2003), however, there were conflicting results regarding the relationship between the extent of the reduction in bone density and the corticosteroid dose (de Vries *et al*., 2007; Langhammer *et al*., 2007; Angeli *et al*., 2006; Vestergaard *et al*., 2005; van Staa *et al*., 2000). A postmenopausal woman taking oral corticosteroids has a higher risk of developing osteoporosis compared to a premenopausal woman due to reduction in oestrogen which may be linked to bone fragility (Angeli *et al*., 2006; Iwamoto *et al.*, 2004; Ton *et al.*, 2005).

It is widely accepted that oral corticosteroids have a strong link with a reduction in bone density compared to other steroid formulations such as inhaled or intranasal corticosteroids (Sosa *et al*., 2008; Langhammer *et al*., 2007; Vestergaard *et al*., 2005). Patients who received oral corticosteroids at a dose less than 7.5 milligrams daily had a 45 – 50 % increased risk of developing hip fractures compared to 117 –

144 % risk in patients prescribed 7.5 – 15 milligrams of oral corticosteroids daily (de Vries *et al*., 2007; Vestergaard *et al*., 2005). Patients are more likely to develop vertebral fractures (16 %) compared to hip fractures at dose as low as 2.5 micrograms of oral prednisolone equivalent. However, an increase in dose of oral corticosteroids to above 15 milligrams of prednisolone daily is not correlated with a greater risk of hip or vertebral fractures. The hip or vertebral fracture risk tends to be similar between patients on a corticosteroid dose of 7.5 – 15 milligrams of prednisolone equivalent daily and patients on 15 milligrams of prednisolone equivalent daily.

Currently, there is limited published evidence regarding the correlation between intranasal corticosteroids and fracture risk. Intranasal corticosteroids at therapeutic doses (200 micrograms daily) failed to show any association with risk of fracture (Vestergaard *et al*., 2005; Suissa *et al*., 2004; Gazis *et al*., 1999).

Prescribing of inhaled corticosteroids, at a dose of as low as 2.5 milligrams daily of prednisolone equivalent, has been associated with an increase in fracture risk (Vestergaard *et al*., 2005). Patients prescribed a high dose of corticosteroids (greater than 900 micrograms BDP equivalent) are more likely to have an increased fracture risk in shorter term than patients on low dose corticosteroids (Pasaoglu *et al*., 2006; Natsui *et al*., 2006). Use of high dose inhaled corticosteroids (greater than 40 milligrams daily prednisolone equivalent) has been shown to increase the fracture risk within 2 months of treatment (Natsui *et al*., 2006). There is unlikely to be a correlation between an increase in inhaled corticosteroid dose (50 – 800 micrograms

daily) with the reduction in distal BMD (Langhammer *et al*., 2007; Vestergaard *et al*., 2005). Hip fracture is 13 % more likely to occur when the dose of inhaled corticosteroid is more or equivalent to 7.5 micrograms daily oral prednisolone (Vestergaard *et al*., 2005). There is no link suggesting that any inhaled corticosteroid may increase the fracture risk (Vestergaard *et al*., 2005; Suissa *et al*., 2004).

1.7.2.1 Mechanism of corticosteroid induced osteoporosis

The biochemical markers indicative of osteoporosis are alkaline phosphatase, the bone isoenzyme alkaline phosphatase, hydroxyproline, osteocalcin, and the procollagen propeptides of type I collagen (Delmas *et al*., 2000). These biochemical markers may be classified into two groups: bone resorption and bone formation. Bone formation markers such as alkaline phosphatase (ALP) are derived from bone and liver. Other biochemical bone formation markers, osteocalcin and procollagen I N-terminal extension peptide (P1NP) are secreted from osteoclasts and directly correlated with bone formation rate (Talwar & Aloia, 2009). The risk of osteoporosis is associated with a decrease in the bone formation markers. The increase in bone resorption rate in postmenopausal women creates an imbalances in bone remodelling and increases the incidence of vertebral fracture (Kaji *et al*., 2010; Nordin *et al*., 2004). In corticosteroid induced osteoporosis, the osteoclast activity increases rapidly initially with osteoblastogenesis slowing down (Canalis *et al*., 2007; Natsui *et al*., 2006). The apoptosis of mature osteoclasts declines while corticosteroids induce apoptotic effects on osteoblasts. Trabecular thickness decreases with increased osteoblast apoptosis.

Calcium resorption happens due to binding of the osteoclasts to the bone and release of calcium into the blood stream. This process is complimented by the action of osteoblasts releasing collagen. Corticosteroids increase the excretion of calcium by inhibiting the renal reabsorption of calcium which may lead to secondary hyperparathyroidism (Canalis *et al*., 2007; Natsui *et al*., 2006). The resulting decrease in calcium in the body helps to accelerate bone resorption (Kaji *et al*., 2010).

1.7.2.2 Bone density assessment

A Dual Energy X-Ray Absorptimetry (DEXA) scan is suggested to measure the extent of reduction in bone mineral density (BMD) in patients treated with oral corticosteroids but not patients prescribed inhaled corticosteroids (National Osteoporosis Society (NOS, 2008)). Measurement of BMD is recommended by the Global Initiative for Asthma (GINA) guidelines (2008) for patients with asthma treated with oral corticosteroids with a mean dose equal to or greater than 7.5 milligrams prednisolone for 6 months; post menopausal women on 5 milligrams oral prednisolone for three months and any patient with asthma who has history of fracture related to osteoporosis. The preferred sites for the DEXA scan suggested by (NOS, 2008) are at the lumbar spine and the neck of femur. Measurement of BMD at multiple sites does not improve the prognosis compared to measurement of BMD at one site (Kanis *et al*., 2006; Leslie *et al*., 2007). Hip fracture risk assessment is more likely to be accurate when measured at the hip compared to the lumbar spine or distal radius (Kanis, 2002). The World Health Organization (WHO, 2004) and National Osteoporosis Society (NOS, 2008) use BMD to define osteoporosis: a

person with T-score <-2.5 SD, which means a BMD 2.5 standard deviations below that of a young healthy woman (30 years old), is classified as osteoporosis. The BMD is measured as either a T-score or a Z-score. The T-score is the difference in measurement compared to a 30 year old of the same sex (Table 1.2) while the Z-score is the difference compared to a normal healthy subject of the same age and sex. The Z-score may provide a normal BMD for an elderly women as the measurement is based on a reference value of the same age and sex.

Table 1.2 Classification of bone mass density (BMD) and T-score (NOS, 2008; SIGN, 2003)

BMD status	T-score
Normal	Less than 1 standard deviation below the norm
Osteopenia	Between 1 and 2.5 standard deviations below the norm
Osteoporosis	More than 2.5 standard deviations below the norm

BMD measurement should be repeated every 1 to 3 years if the patient is still taking corticosteroids. The disadvantage of the WHO classification (1994) using T-score is that the comparison it is based solely on young Caucasian women (Faulkner, 2005) and ignores other ethnic groups (Curtis *et al*., 2009; Walker *et al*., 2007) and age (Chevallety *et al*., 2008; Gajic-Veljanoski *et al*., 2007).

1.7.3 Corticosteroid induced diabetes

Long term use of prednisolone may expose patients to the risk of developing Type 2 diabetes (SIGN/BTS, 2008). The same conclusion was reached in a three year cohort study with over 30,000 elderly patients prescribed oral corticosteroids (Blackburn *et al.,* 2002). Participants prescribed oral corticosteroids suffered a higher rate of developing diabetes mellitus type 2 compared to patients prescribed inhaled corticosteroids alone (adjusted rate ratio: 2.31 vs 1.03, respectively). The risk of developing type 2 diabetes in patients prescribed oral corticosteroids increased with time (4.3, 7.7 and 11.0 % at 1, 2, and 3 years, respectively). A case control study (Gulliford *et al.,* 2006) recruited patients treated with corticosteroids (oral, inhaled, topical) who were diagnosed with diabetes. This study failed to find any association between corticosteroid therapy and diabetes. Patients prescribed oral corticosteroids tend to have a higher risk of developing diabetes compared to other formulations of corticosteroid.

1.7.4 Corticosteroid induced high blood pressure

Chronic use of corticosteroids increased the patients' susceptibility to Cushingoid symptoms. Several theories on corticosteroid induced hypertension are the renin angiotensin pathway and the mineralcorticoid activity of the corticosteroids (Roy *et al*., 2009; Walkers *et al*., 1994). The corticosteroids may induce an increase in blood pressure through modulating the angiotensin II mediated signalling pathway (Roy *et al*., 2009). The increase in reabsorption of sodium and water and low concentrations of renin might contribute to an increase in blood pressure. The mineralcorticoid properties of corticosteroids might mediate a change in cardiac output or peripheral

vascular resistance which leads to an increase in blood pressure (Walkers & Edwards, 1994).

The use of oral corticosteroids at a dose as low as 7.5 milligrams prednisolone equivalent daily is associated with an increased risk of developing any cardioavascular or cerebrovascular event (adjusted odd ratio = 1.44) and the risk increases when the oral corticosteroid dose increases (Souverein *et al.*, 2004). The study did not look for a correlation between patients on antihypertensive medication and current use of oral corticosteroids.

1.7.5 Other adverse effects

1.7.5.1 Cataracts

The risk of cataracts in patients treated with corticosteroids is well known (Lipworth, 1999). The posterior subcapsular cataracts that result may partially or completely resolve after withdrawal of the drug, or following surgery. The risk of cataracts increases with increasing doses of corticosteroid and long term use (Ernst *et al.*, 2006). A survey of more than 3000 volunteers (Cumming *et al.*, 1997) found a weak link between cataracts and inhaled corticosteroids. Corticosteroid treated patients with a combination of diabetes and hypertension might have a higher risk of developing posterior capsular cataracts. The same correlation between cataracts and corticosteroids was found in a four year population study of patients over 65 years old (Ernst *et al.*, 2006). Coexistence of other risk factors such as diabetes and hypertension were not excluded and age might contribute to the presence of cataracts.

1.7.5.2 Local side effects

The main local side effects of inhaled corticosteroids reported are oral candidiasis, voice harshness, dysphonia and headache (Bousquet *et al*., 2009; Schermer *et al*., 2007; Stjarne *et al*., 2006; Rosenblut *et al*., 2007; Meltzer *et al*., 2002). No significant differences were reported in adverse effects between corticosteroids (Dahl *et al*., 2010; Buhl *et al*., 2006). No correlation was found between the dose of inhaled corticosteroid and the number of adverse effects reported (Foster *et al*., 2006; Hawkins *et al*., 2003).

1.8 Asthma definition

Asthma is defined as "*a chronic inflammatory disorder of the airways in susceptible individuals, inflammatory symptoms are usually associated with widespread but variable airflow obstruction and an increase in airway response to a variety of stimuli*" (SIGN/BTS, 2008). Asthma is reversible either with treatment or spontaneously. Asthma can be classified as either extrinsic or intrinsic (GINA), 2008). Extrinsic asthma is asthma with an identifiable external cause. It is prominent in childhood asthma that may or may not carry on to adulthood. Intrinsic asthma is classified as late onset asthma which occurs at a mature age with no known cause. In practice, asthma is unlikely to be well differentiated as most patients suffer from a mixed type of asthma.

1.8.1 Pathology of asthma

Asthma is predominantly an inflammatory disorder. It is believed that when the airways become sensitive to the presence of the allergens, the bronchial smooth

muscles tighten, the airways constrict as they become irritated, airflow becomes limited and mucus secretion is accumulated (GINA, 2008).

The inflammatory mechanisms of asthma are divided into two phases; immediate and delayed. The immediate phase, which is linked to genetic factors, involves an inflammatory response to allergens and occurs within minutes. Allergens enter the lung and stimulate the secretion of immunoglobulin (lgE) antibodies. This process induces mast cell degranulation which leads to the release of inflammatory mediators including: histamine, leukotriene C_4, D_4 (LTC_4, LTD_4) and prostaglandin (PgD_2) (Bradding *et al*., 2006; Hamid *et al*., 2003). These mediators cause bronchoconstriction and alteration of vascular permeability. T cells produce T helper cells, type 2 (T_H2) cytokines which regulates interleukin (IL)-4, IL-5 and IL-13. IL-3 and IL-4 induces expression of vascular cell adhesion molecule-1(VCAM-1) on endothelial cells. These pro-inflammatory cytokines are involved in bronchial hyperresponsiveness, mucus hypersecretion, airway remodelling and chemoattraction of eosinophils (Fixman *et al*., 2007; Bradding *et al*., 2006; Hamid *et al*., 2003). At this stage, the patient may complain of one or more symptoms including shortness of breath, wheeze, cough or excessive mucus secretion. More inflammatory mediators are then released from the mast cells which contribute to airway hyper-responsiveness (GINA, 2008).

In the delayed phase reaction, along with bronchoconstriction, there is significant mucus production due to goblet cell activation leading to blocked airways which results in an acute asthma attack (Fixman *et al*., 2007; Meltzer *et al*., 2004). This

phase starts within four to six hours of introduction of the allergen to the lung. The release of proinflammatory cytokines (IL-4, IL-5 and IL-13), tumor necrosis factor-α (TNF-α) is synthesised in abundance by mast cells (Bradding *et al*., 2006; Hamid *et al*., 2003). These cytokines are responsible for the production of eosinophils. In bone marrow, the eosinophil production and differentiation is regulated by IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Lemanske & Busse, 2010). The eosinophils migrate to the lung and release a number of proinflammatory cytokines (IL-1, IL-6, TNF-α), immunoregulatory cytokines (IL-2, IL-4), eosinophils growth factors (IL-3, Il-5) transforming growth factor (TGF)-α, TGF-β and chemokine known as Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) which leads the prolonged survival of the eosinophils, airway wall remodelling and increased expression of adhesion molecule (Lemanske & Busse, 2010; Fixman *et al*., 2007). At this stage, the symptoms may last up to 24 hours.

1.8.2 Pharmacological management of asthma

Both a non pharmacological and pharmacological approach is recommended for treating patients with asthma. Weight reduction for obese people (body mass index (BMI) $\geq$ 30), less exposure to house mites and stopping smoking are some of the non-drug interventions proven to control the symptoms of asthma (SIGN/BTS, 2008).

The management of asthma involves three groups of medications: relievers; preventers and/or controllers. A reliever, usually a short acting beta agonist,

alleviates the symptoms quickly; a preventer, usually an inhaled corticosteroid, reduces the symptoms, preserves lung function and reduces the possibility of exacerbations in future and a controller, usually a long acting beta agonist, works as an adjunctive therapy to an inhaled corticosteroid. Pharmacological treatment in asthma can be divided into two groups of patients: patients with stable asthma and patients with acute exacerbation asthma.

1.8.2.1 Stable asthma

The aim of treating patients with stable asthma is to control the disease by abolishing symptoms during the day, reducing sleep disturbance due to coughing, reducing wheezing, preserving lung function and reducing the number of exacerbations (SIGN/BTS, 2008).

Corticosteroids act as anti-inflammatory agents and are recommended for all patients with mild to severe persistent asthma. Inhaled corticosteroids are used alone or in combination with long acting beta agonists in the treatment of asthma. The use of inhaled corticosteroids does not eliminate the need for inhaled short acting beta agonist (SABA) as bronchodilator. SABAs are used for treatment of the symptoms of asthma rather than treating the underlying inflammatory mechanism of asthma (Petanjek *et al.*, 2007). Inhaled corticosteroids at dose at a low of 200 micrograms daily chlorofluorocarbon (CFC) containing beclometasone equivalent reduce the asthma symptom score, improve pulmonary function measured by FEV_1, or PEFR and increase asthma symptom free days compared to placebo (Busse *et al*., 2008;

Pauwels *et al*., 2003), inhaled SABA (Haahtela *et al*., 2009; Papi *et al*., 2007) or oral leukotriene receptor antagonists (LTRA) (Boushey *et al*., 2005).

The long acting beta agonists (LABA) are suggested as first line add on therapy to inhaled corticosteroids when a low dose of inhaled corticosteroid (equal to 400 micrograms daily BDP equivalent) fails to provide asthma control. A combination of inhaled LABA/corticosteroid is proven to significantly reduce the exacerbation rate, improve pulmonary function and asthma control days and reduce asthma related night awakenings compared to increasing the dose of inhaled corticosteroid (O' Bryne *et al*., 2008; Schermer *et al*., 2007; Barnes *et al*., 2007; Houghton *et al*., 2007; Bateman *et al*., 2006; Foresi *et al*., 2005; Lee *et al*., 2003). A study (Barnes *et al*., 2002) revealed that the actions of corticosteroids and LABA are complimentary. Inhaled corticosteroids increase the expression of beta receptors through activated glucocorticoid receptors, and thus reduce the risk of desensitisation due to chronic use of LABA on beta receptor (Barnes, 2002). When the patient's asthma is still uncontrolled, further inventions of increasing the dose of inhaled corticosteroid up to 800 micrograms BDP equivalent daily should be considered (Currie *et al*., 2005). The LABA should be discontinued if it does not give any benefit to the patient.

The dosage of inhaled corticosteroid can be increased up to 2000 micrograms twice a day of CFC containing BDP or equivalent when no response to a combination of inhaled LABA and corticosteroid is achieved (SIGN/BTS, 2008). The addition of two or more controller medications to the preventer might be useful in preserving lung function. A trial of a LTRA, montelukast or zafirlukast, or oral theophylline to

current inhaled corticosteroid is considered at this stage when there is poor response to a high dose of inhaled corticosteroid (Barnes *et al*., 2007; Price *et al*., 2006; Jayaram *et al.*, 2005).

The continuous use of oral corticosteroids might be suitable in patients with uncontrolled asthma symptoms with high dose of corticosteroids (greater than or equal to 2000 micrograms daily BDP equivalent). Monitoring of blood pressure, blood sugar and bone density are recommended for patients on long term oral corticosteroid therapy as the patient may develop steroid related side-effects such as diabetes, cataracts, or steroid-induced osteoporosis (Langhammer *et al*., 2007; Vestergaard *et al*., 2005; Gulliford *et al.*, 2006; Gudbjornsson *et al.*, 2002).

The use of high dose inhaled or oral corticosteroids should be tapered to the lowest dose while maintaining asthma control. The randomised, double blind, parallel group studies (Boulet *et al*., 2009; Foresi *et al*., 2005; Hawkins *et al*., 2003) which were conducted in a group of mild to moderate asthmatic patients found that initiating corticosteroids at a high dose (1000 – 2000 micrograms BDP equivalent daily) to gain asthma control and then reducing the dose to the minimum dose of inhaled corticosteroid is possible. Stopping inhaled corticosteroid treatment after gaining the asthma control is not an option as the patient might loose the benefit of treatment and the FEV_1, PEFR, sputum eosinophils may be reversed to baseline (Foresi *et al*., 2005).

1.8.2.2 Acute exacerbations of asthma

Severe acute asthma is defined as a patient with an episode of continuous shortness of breath, inability to complete one sentence in one breath, poor respiratory effort that can be quantified by one or combination of the following: the measurement of lung function, peak expiratory flow rate (PEFR) of less than 50 % predicted, oxygen saturation (SpO_2) of less than 92 % or a respiratory rate of more than 25 breaths a minute (SIGN/BTS, 2008). A patient with PEFR between 50 – 75 % but who is able to finish complete sentences and does not have any of the other characteristics of acute severe asthma is classified as presenting with a moderate exacerbation of asthma. A patient with poor respiratory effort and a PEFR less than 33 % predicted, a partial pressure of oxygen in the blood (PaO_2) of less than 8 kiloPascals (kPa) and an SpO_2 of less than 92 % is defined as life threatening asthma. The most common presentations of patients with life threatening asthma are altered consciousness, exhaustion, arrhythmia, hypotension, cyanosis and silent chest.

The patient is given treatment of oxygen to achieve an SpO_2 of 94 – 98 % to treat hypoxaemia (Rodrigo *et al*., 2003; Inwald *et al*., 2001). The dose of oxygen therapy should be tailored individually as the patient's response to oxygen therapy may be variable as hypercapnia is more likely in patients with severe asthma receiving 100 % oxygen.

A SABA given by inhalation repeatedly using pMDI with large volume spacer (Boonsawat *et al*., 2003) or by nebulisation driven by high flow oxygen is recommended in the treatment of life threatening asthma. Nebulised SABA is not

given using compressed air as this might worsen hypoxaemia as a result of oxygen desaturation (Inwald *et al*., 2001). The non selective anticholinergic, nebulised ipratropium bromide (500 micrograms every 4 – 6 hours) should be used in combination with nebulised SABA where partial response to the SABA is observed (Gelb *et al,* 2008; Talib *et al*., 2002; Rodrigo & Rodrigo, 2002).

Oral prednisolone at dose of 40 – 50 milligrams is prescribed at least for five days starting as soon as possible. Intramuscular methylprednisolone at 160 milligrams is preferred when swallowing is compromised (Lahn *et al*., 2004). Rowe *et al.,* (2006) believed than magnesium sulphate given intravenously might be beneficial in treating patients with an acute exacerbation asthma. A combination of inhaled magnesium sulphate with inhaled salbutamol is unlikely to give any additional effect compared to inhaled salbutamol alone (Rowe & Camargo, 2008). The use of antibiotics, nebulised furosemide or a combination of helium and oxygen (heliox) are not recommended for treating an acute exacerbation of asthma. Introduction of heliox along with nebulised SABA failed to demonstrate that heliox is equal or more effective than oxygen driven nebulised SABA (Dhuper *et al*., 2006; Kress *et al*., 2002).

1.9 Rhinitis

Rhinitis is an inflammatory response to inhalation of allergens such as grass pollen, house dust mite or animal dander. Rhinitis differs from asthma as the condition is considered to be an upper respiratory disease whereas asthma is a chronic inflammatory response in the lower airways (Table 1.3) (Bousquet *et al.*, 2003; Meltzer *et al.*, 2004). About 40 – 60 % of patients with rhinitis present with a co-diagnosis of asthma (Bousquet *et al.*, 2003; Meltzer *et al.*, 2004; Bugiani *et al.*, 2005). Rhinitis might be under diagnosed as it is not a life threatening disease (Bauchau & Durham, 2004). Allergic rhinitis may be seasonal or perennial (Table 1.4). Nasal congestion, nasal obstruction, conjunctival irritation and watery nasal secretions are the main symptoms that patients with rhinitis exhibit. Properly treated rhinitis reduces the risk of an exacerbation of asthma (Bousquet *et al.*, 2003).

Table 1.3 Comparison of asthma and rhinitis (Bousquet *et al.*, 2003; Meltzer *et al.*, 2004)

Properties	**Rhinitis**	**Asthma**
Definition	Inflammation of the lining in the nose; triggered by pollen	Inflammation of the airways
Inflammation site	Upper airway	Lower airway
Symptoms	Runny nose, itchy nose, sneezing, blocked nose, watery and inflamed eyes	Breathlessness, wheezing

Table 1.4 Comparison of seasonal and perennial rhinitis (adapted from: Meltzer, 2004; Bousquet *et al.*, 2003)

Parameters	**Seasonal rhinitis**	**Perennial rhinitis**
Definition	May occur with repetitive and predictable seasonal symptoms.	Symptoms present all year round
Causes	Pollen, moulds	Dust mites, moulds, animal allergens
Symptoms		
Obstruction	Variable	Predominant
Secretion	Watery	Postnasal drip
Sneezing	Common	Variable
Loss of smell	Variable	Common
Chronic sinusitis	Occasionally	Frequent
Eye problems	Itchy and watery eyes	Less intense

1.9.1 Pathology of rhinitis

Rhinitis has two continuous stages: early phase and late phase. The presence of allergens to the IgE that binds to mast cells leads to mast cell degranulation. The inflammatory mediators, histamine, phospholipases, leukotrienes and prostaglandins, are synthesised and released as a result of mast cells degranulation. Patients with rhinitis may complain of sneezing, itching and mucous secretion when histamine acts on the H_1 receptors which activate the parasympathetic response. Parasympathetic modulators, such as acetylcholine, catecholamines, peptides and nitric oxide, are released in higher concentrations and cause smooth muscle relaxation and an increase in vascular permeability which causes a watery nose.

In the late phase response which occurs up to six hours after the onset of the immediate phase, mast cells are more prominent. Histamine is released as a product of the basophiles rather than mast cells. T helpers 2 (T_H2) enter the mucosa and promote release of interleukins, activate eosinophils and neutrophils and increase lgE production (Hansen *et al.*, 2004). The introduction of IgE to eosinophils causes cell apoptosis and promotes positive feedback to the inflammatory mechanism. This causes increased basophile degranulation and saturation at the IgE receptors. Several biomolecules called intercellular adhesion molecules 1 and 2 (ICAM-1, ICAM-2) and vascular cell adhesion molecule 1 (VCAM-1) are activated by the release of pro-inflammatory mediators. The concentration of nitric oxide is expected to be high due to activation of eosinophils which promote vasodilation. Excessive amounts of nitric oxide inhibit the kinins which might exacerbate rhinitis (Hansen *et al.*, 2004). This leads to nasal airway hyper-responsiveness and chronic nasal obstruction, thus, complaints of a blocked nose and excessive mucus are common in this phase.

1.9.2 Pharmacological management of rhinitis

The current treatments for rhinitis are detailed in Table 1.5. The two major classes of pharmacological treatment for rhinitis are oral antihistamines and intranasal corticosteroids (Bousquet *et al.*, 2003) although nasal decongestants, antimuscarinics and mast cells stabilisers may also be used.

Table 1.5 Pharmacological management of rhinitis

Drug class	Mechanism of action	Symptoms relieved	Example
Corticosteroid	Inhibit the inflammatory mediators from mast cells and decrease kinins, leukotrienes, prostaglandins.	Sneezing, rhinorrhea, itching, congestion	Beclometasone, Budesonide, Fluticasone
Antihistamine	Inhibit the binding of histamine to the receptor sites.	Sneezing, rhinorrhea, itching, do not relieve congestion	Loratadine, Desloratadine; Diphenhydramine
Decongestant	Acts as a sympathomimetic agent which causes constriction of blood vessel to reduce the blood flow. Acts within 5 – 10 minutes might last up to 1 hour.	Congestion	Oxymetazoline, Xylometazoline
Anticholinergic	Acts by inhibiting the parasympathetic stimulation which causes less vasoconstriction but no effect on the sensory receptors.	Rhinorrhea	Ipratropium bromide
Mast cell stabiliser	Blocks histamine degranulation and eosinophil secretion. Might block Ca^{2+} channel in mast cells	Sneezing, rhinorrhea, itching, congestion	Sodium cromoglycate; Nedocromil

1.9.3 Corticosteroids in rhinitis

Corticosteroids are the first choice treatment for rhinitis (Houchaus *et al*., 2008; Bousquet *et al*., 2001; Kaszuba *et al*., 2001). Intranasal BETA, MF, FP and BUD are the intranasal corticosteroids most commonly prescribed. In a study on the efficacy of intranasal corticosteroids in adults with perennial allergic rhinitis, two intranasal

corticosteroids, intranasal MF and FP were given in equal doses: 200 micrograms daily. With an equal number of patients in both groups, no significant difference was found in the efficacy in gaining control over the rhinitis symptoms of running nose, blocked nose, or sneezing (Gupta & Gupta, 2004). Equipotency was also reported between intranasal MF and FP at a higher dose of 800 micrograms three times daily (Dales-Yates *et al.*, 2004). There was reduction in 24 hour urinary cortisol and serum cortisol in both groups but it was not statistically significant when compared to placebo.

When the benefits of intranasal corticosteroids were judged in a one to one comparison to oral antihistamines (Pinar *et al*., 2008; Saengpanich *et al*., 2003; Kaszuba *et al.*, 2001) patients in the corticosteroid treated group showed lower symptom scores in all aspects measured including sleep, nasal symptoms, and quality of life, which indicated that the patients had better control of their symptoms when using intranasal corticosteroids compared to oral antihistamines. All patients suffered from seasonal allergic rhinitis in these studies. The dose of intranasal corticosteroid used in these studies was 200 micrograms daily against oral antihistamine at 5 – 10 milligrams daily.

1.10 Nasal Polyposis

About 2 % of the UK population suffer from nasal polyps, a subtype of rhinosinusitis defined as '*inflammation of the nose and paranasal sinuses'*. Rhinosinusitis links rhinitis and sinusitis which is then classified into four groups: acute (bacterial) rhinosinusitis, chronic rhinosinusitis (CRS) without polyps, CRS with polyps and

allergic fungal sinusitis (Gendy *et al.*, 2007). It is almost impossible to differentiate between chronic rhinosinusitis and nasal polyps (Benninger *et al.*, 2003). Polyposis is described as a bilateral, localised oedema endoscopically visualised in the middle meatus of the nasal cavity (Fokkens *et al.*, 2005). A physical examination will typically reveal grape-like tissue at the sinus line. Allergy, asthma, family history and sensitivity to aspirin are factors that have been linked to nasal polyposis (Rinia *et al.*, 2007). Nasal polyposis, which can be classified into four stages (Table 1.6), is more common in men than women (Busaba *et al.*, 2008).

Table 1.6 The stages of nasal polyposis (Fokkens *et al.*, 2005)

Stage	Description
Stage 0	Normal
Stage 1	Single focus disease (involved middle meatus) Anatomic abnormalities
Stage 2	Bilateral ethmoidal disease with involvement of one dependent sinus
Stage 3	Bilateral ethmoidal disease with involvement of two or more dependent sinuses of each side
Stage 4	Diffuse sinonasal polyposis

1.10.1 Pathology of nasal polyposis

The pathophysiology of nasal polyposis is unknown and is based on three theories; Bernstein; Vasamotor; and Epithelia Rupture Theory (Kirtsreesakul, 2005). Polyps can block the mucus drainage resulting in mucus stagnating in the sinus cavities. The accumulation of mucus can lead to infection. In Bernstein's theory, nasal polyps are formed as a result of turbulent flow of air commonly at the lateral wall of the nose and ulceration occurs. This changes the flow in the sodium / potassium channel

which leads to an increased concentration of sodium and reduced water absorption in the cell. The polyps become enlarged as more water gets absorbed. On the other hand, the vasomotor imbalance theory applies to non atopic rhinitis where swelling is much more likely to occur due to gravitational force. The theory of epithelial rupture is based on epithelia ruptured from the nasal mucosal lining which then forms nasal polyps.

1.10.2 Pharmacological management of nasal polyposis

The pharmacological approach to management of nasal polyps is mainly based on the objectives of: minimising the size of nasal polyps; preventing the recurrence and exacerbation of nasal polyps; restoring respiratory function; and restoring the quality of life for the patient. Corticosteroid based preparations are the first line treatment delivered in the form of nasal sprays escalating to oral administration based on the grading of the disease (Penttila *et al.*, 2000). In two placebo-controlled studies, (Small *et al.*, 2005; Penttila *et al.*, 2000) increasing the dose and frequency of intranasal corticosteroid was unlikely to increase the adverse effects. In two randomised controlled trials, (Alobid *et al*., 2006; Hissaria *et al*., 2006) the patients with severe nasal polyposis were given oral prednisone 30 – 50 milligrams. Greater improvement was observed in nasal symptoms scores of nasal obstruction and sense of smell and nasal polyp size score after 14 days treatment with prednisolone in comparison with the placebo group. There was no difference in improvement of nasal symptoms, size of nasal polyps or eosinophil count between nasal polyposis with or without atopy. The use of oral corticosteroids in nasal polyposis is not

recommended on daily basis since there is high incidence of adverse effects related to oral prednisolone in long term use.

Several investigations regarding microbial causes for nasal polyposis suggest an alternative option for treatment: the use of antibiotics (Fokkens *et al.*, 2005; Ragab *et al*., 2004; Bucher *et al*., 2003; Dolor *et al*., 2001). Patients were randomised to treatment with either oral antibiotics or a combination of oral antibiotics and intranasal corticosteroids and followed up at 10, 21 and 56 days (Dolor *et al*., 2001). No significant difference in nasal symptom score was seen. Bucher *et al*., (2003) and Williamson *et al*., (2007) also found that antibiotics are not effective in treating nasal symptoms. Surgery or polypectomy is advised if patients with nasal polyposis show no response to inhaled or oral corticosteroids (Schalek *et al.*, 2009; Fokkens *et al.*, 2005). There are no trials which report on the effectiveness of mucolytics or decongestants in the treatment of nasal polyposis.

1.10.3 Corticosteroids in nasal polyposis

Corticosteroids in the form of nasal sprays or oral tablets may shrink or eliminate polyps (Nonaka *et al*., 2010; Jankowski *et al*., 2009; Small *et al*., 2008; Alobid *et al*., 2006; Stjarne *et al*., 2006; Hissaria *et al*., 2006; Aukema *et al*., 2005; Keith *et al*., 2000). Intranasal FP at 400 micrograms daily significantly reduced the need for endoscopic sinus surgery, improved the nasal symptoms scores of nasal blockage, rhinorrhea, and loss of smell after 12 weeks compared to placebo (Aukema *et al*., 2005; Keith *et al*., 2000). A similar observation was found in patients treated with intranasal corticosteroids at dose of 200 – 400 micrograms daily for 4 – 8 months

(Jankowski *et al*., 2009; Stjarne *et al*., 2006). Significant improvements in nasal obstruction, anterior rhinorrhea and nasal drip were observed at higher doses of intranasal corticosteroid, 400 micrograms daily, compared to lower doses of 200 micrograms daily 1 month after treatment initiation (Jankowski *et al*., 2009; Stjarne *et al*., 2006). No significant difference was found in nasal symptoms and peak nasal inspiratory flow (PNIF) between intranasal corticosteroids, 200 micrograms daily and 400 micrograms daily at the end of a 8 month study (Jankowski *et al*., 2009). Oral corticosteroids are needed if polyps block the airways or cause frequent sinus infections (Alobid *et al*., 2006; Hissaria *et al*., 2006). Intranasal corticosteroids are recommended for use after polypectomy or endoscopic surgery (Fokkens *et al*., 2005). A randomised, double blind, placebo-controlled study which reviewed patients one year after polypectomy found intranasal FP at a dose of 800 – 1600 micrograms daily failed to reduce the recurrence of nasal polyps (Dijkstra *et al*., 2004).

1.11 Aim

The aim of this thesis is to assess and quantify the burden of steroid therapy and the prevalence of systemic effects in patients attending two clinical sites: the Ear, Nose and Throat clinic, Glasgow Royal Infirmary (GRI) and the Problem Asthma clinic, Stobhill Hospital.

1.12 Objectives

1. To develop and validate an analytical method for quantifying multiple oral, inhaled and nasal corticosteroids in plasma.
2. To use the method developed to determine the absorption of intranasal and inhaled corticosteroids in patients with asthma, rhinitis or nasal polyps.
2. To identify the relationship between the dose of inhaled and intranasal corticosteroids and systemic effects.
3. To compare data from patients on oral, intranasal or inhaled corticosteroids and when used in combination.

Chapter 2

Method Development

2.1 Detection of corticosteroids in biological fluids

The separation of multiple metabolites and exogenous corticosteroids in human biological samples requires specific analytical methods that are capable of measuring very low concentrations (pg mL^{-1}) of these analytes. Many methods have been developed for the determination of single corticosteroids in biological fluids; BETA (Fu *et al*., 2010; Xiong *et al*., 2009; Zou *et al*., 2008; Allen *et al*., 2007; Pereira *et al.*, 2005; Fluri *et al.*, 2001); FP (Mascher *et al*., 2008; Carter & Carpa, 2008; Li *et al.*, 2001; Krishnaswami *et al.*, 2000; Laugher *et al.*, 1999; Mistry *et al.*, 1997); BUD (Qu *et al.*, 2007; Deventer *et al.*, 2006; Wang & Hochhaus, 2004; Hou *et al.*, 2001; Hochhaus *et al.*, 1998); MF (Sahasranaman *et al.*, 2005) and PRED (Chen *et al*., 2009; Zhang *et al*., 2006). Separation of two or more corticosteroids in biological fluids have also been studied since the combination of different coricosteroids by different administration routes are common in disease management ;Touber *et al*., 2007 (PRED, BETA, BUD); Taylor *et al.*, 2004 (BDP, FP, BETA, BUD, PRED); Cirimele *et al*., 2000 (PRED, BETA); Teng *et al.*, 2001 (PRED, BDP, FP, BUD); Tang *et al.*, 2001 (PRED, BETA) and Foe *et al.*, 1998 (BDP, 17-BMP). Currently, there is a lack of analytical studies relating to the quantification of a combination of corticosteroids prescribed to patients with respiratory tract disorders.

2.2 Sample preparation

Pre-treatment is an important step when preparing a biological sample for analysis. The analytes of interest should be isolated from any biological interferants, such as proteins, lipids, sugars or salts, before analysis by an appropriate instrumental method. There are several pre-treatment methods for extraction of corticosteroids

from biological fluids including: protein precipitation; solid-phase extraction (SPE) and liquid-liquid extraction (LLE). Protein precipitation is a simple pre-treatment method whereby proteins are removed from the biological media before analysis (Panusa *et al*., 2010; Polson *et al*., 2003). Protein precipitation is used before SPE and LLE to provide cleaner samples and improve analyte response during analysis compared to extraction by SPE or LLE alone (Qu *et al.*, 2007; Sahasranaman *et al.*, 2005; Samtani *et al*., 2005; Wang & Hochhaus, 2004; Taylor *et al*., 2004; Wang & Hochhaus, 2004; Krishnaswami *et al.*, 2000).

Several studies of the analysis of corticosteroids have proposed solid-phase extraction (SPE) (Carter & Capka, 2008; Qu *et al.*, 2007; Allen *et al*., 2007; Sahasranaman *et al.*, 2005; Peng *et al*., 2005; Pereira *et al.*, 2005; Wang & Hochhaus, 2004; Li *et al.*, 2001; Laugher *et al.*, 1999; Li *et al.*, 1996) as the extraction procedure of choice for corticosteroids present in plasma though liquid-liquid extraction (LLE) has also been reported as a suitable clean up procedure (Chen *et al*., 2009; Ionita *et al*., 2009; Zhang *et al*., 2006; Samtani *et al*., 2005; Teng *et al.*, 2001; Krishnaswami *et al.*, 2000; Foe *et al.*, 1998). Similar results for corticosteroid recovery from plasma were found in these studies either using solid-phase extraction (76 – 107 %) or liquid-liquid extraction (76 – 106 %). Liquid-liquid extraction has been the most frequently used technique for the isolation of corticosteroids from urine (Tolgyesi *et al*., 2010; Zou *et al*., 2008; Touber *et al*., 2007; Zhang *et al*., 2006; Deventer *et al.*, 2006; Taylor *et al*., 2004; Fluri *et al.*, 2001; Tang *et al.*, 2001) with corticosteroid recovery of 76 – 111 % compared to solid-phase extraction of 66 – 85 % (Andersen *et al.*, 2008; Vazquez *et al.*, 2005; Peng *et al*., 2005; Pujos *et al.*, 2005).

2.2.1 Protein precipitation

Protein precipitation is a method used to remove undesirable biomolecules, which may interfere with an analytical method, from biological samples. Several studies suggest adding 4 – 5 % phosphoric acid (Qu *et al*., 2007; Samtani *et al*., 2005; Polson *et al*., 2003), acetonitrile (Panusa *et al*., 2010; Ionita *et al*., 2009; Janzen *et al*., 2008; Polson *et al*., 2003) or acetate buffer (Jung *et al*., 2010) to plasma for removal of proteins from the biological samples. The mixture is then centrifuged to separate the precipitated solid protein and the filtrate is then analysed. Protein precipitation is a poorer technique than SPE in corticosteroid sample pre-treatment as it creates problems with chromatography, including an increase in background noise, and poor peak shape, due to the high percentage of solvents used. The presence of phospholipids and other contaminants unable to be removed by protein precipitation in the sample may lead to poor levels of recovery of 16 – 75 % (Qu *et al.*, 2007; Kristopher *et al.*, 2009; Zhang *et al*., 2006; Polson *et al*., 2003).

2.2.2 Solid-phase extraction (SPE)

Solid-phase extraction is used to extract an analyte from a liquid sample. Like protein precipitation, this process removes biological contaminants that may block the column and cause back pressure in the HPLC system, as a result (Huck & Bonn, 2000; Hennion, 1999). SPE is an environmentally friendly method as it uses small quantities of solvents, is subject to low variability, is efficient and reproducible, and generally, has greater recovery than LLE (Xie *et al*., 2007; Dufresne *et al.*, 2001).

Bonded silica non-polar phases (C_8, C_{18}) are designed for moderately polar and non polar analytes while polymeric sorbents based styrene–divinylbenzene copolymers (polyamide) are useful for polar analytes and are stable over a wide pH range of 1 – 14 (Hennion, 1999). Both types of sorbents, polymeric and silica-based, need to be pre-conditioned before loading the sample. The characteristics of the sample matrix, including solubility, the polarity of the analyte relative to the sorbents, presence of functional groups, determine the type of SPE sorbent which should be used. For neutral hydrophobic compounds such corticosteroids which are highly soluble in organic solvents rather than water, reverse-phase SPE sorbents, C_8, C_{18}, are the best option (Xiong *et al*., 2009).

Several authors (Andersen *et al*., 2008; Carter & Capka, 2008; Qu *et al.*, 2007; Sahasranaman *et al.*, 2005; Wang & Hochhaus, 2004; Li *et al.*, 2001; Krishnaswami *et al.*, 2000; Cirimele *et al.*, 2000; Laugher *et al.*, 1999; Li *et al.*, 1996) recommend solid-phase extraction as a highly efficient and sensitive method for isolation of corticosteroids from biological samples. A C_{18}, non-polar hydrophobic sorbent, appears to be the best choice for corticosteroid analysis as it has been used in many published studies (Qu *et al.*, 2007; Sahasranaman *et al.*, 2005; Li *et al.*, 2001; Krishnaswami *et al.*, 2000; Cirimele *et al.*, 2000; Laugher *et al.*, 1999; Li *et al.*, 1996).

There are four stages to a typical solid-phase extraction (Huck & Bonn, 2000) (Figure 2.1) these are preconditioning, sample loading, sample washing, and elution of analytes.

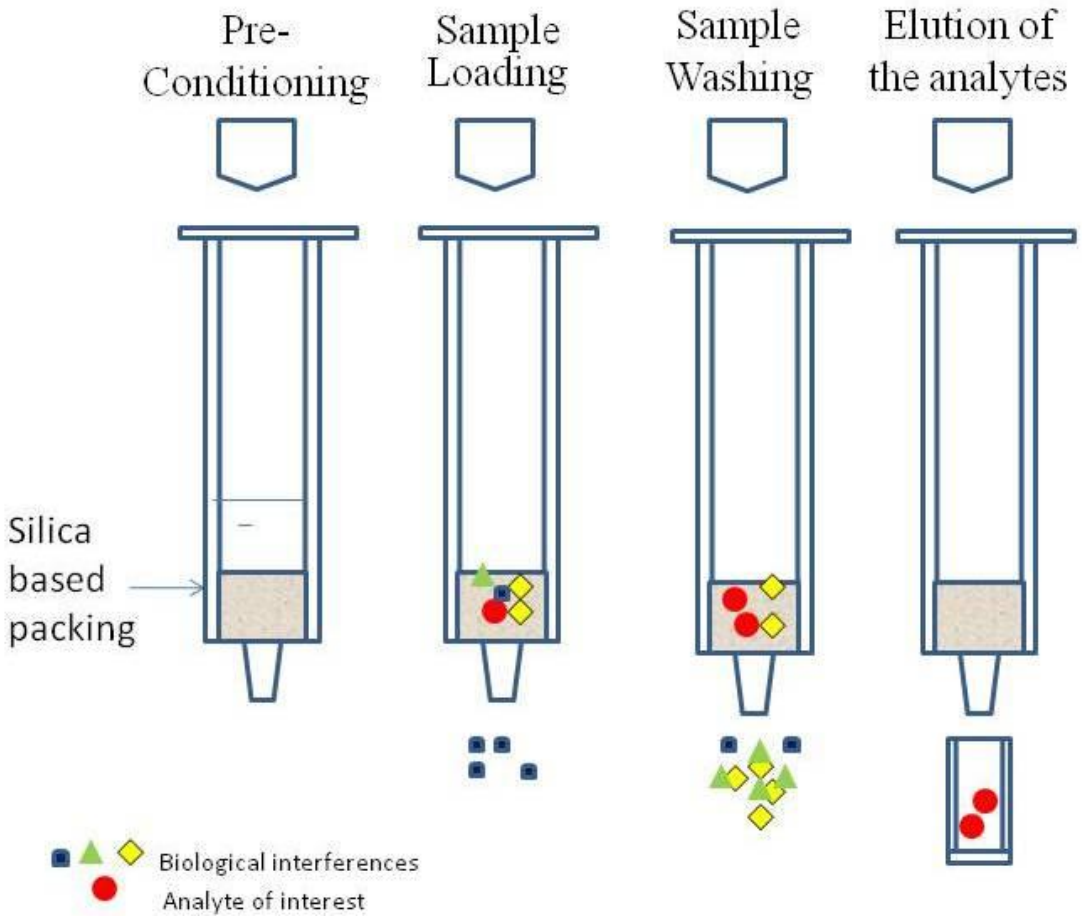


Figure 2.1 Solid-phase Extraction

2.2.2.1 Pre-Conditioning

The reversed phase SPE sorbents are conditioned with an organic solvent followed by aqueous solvents (Tolgyesi *et al*., 2010; Frerichs & Tornatore, 2004). The introduction of solvents wet the sorbent for efficient interaction with the sample. After conditioning, the sorbents are left to equilibrate for 1 – 2 minutes. The equilibriation time should not be longer than this as the sorbents tend to dry out leading to sorbent inefficiency.

2.2.2.2 Sample Loading

The sample is dissolved in an appropriate solvent before it is introduced onto the SPE cartridge. The sorbents can hold a total mass of 5 % of the sorbent mass: 5 mg in 100 mg sorbent (Phenomenex, http://www.phenomenex.com; accessed on 17 September 2010). In some cases, ethanol is added to the plasma before extraction to precipitate the proteins and minimise their interference (Qu *et al.*, 2007; Sahasranaman *et al.*, 2005; Wang & Hochhaus, 2004; Krishnaswami *et al.*, 2000). Some studies describe dilution of plasma samples with water before loading to reduce sample viscosity and improve recovery of the analytes (Carter & Capka, 2008; Peng *et al*., 2005).

2.2.2.3 Sample Washing

The loaded sample is washed with a solvent that will wash away the sample impurities but not the analyte of interest. The interferants are washed away and the analytes of interest are retained in the sorbent before eluting.

2.2.2.4 Elution of the Analytes

A strong non-polar solvent is passed through the column to dissociate the analytes of interest from the SPE sorbent. The eluted fractions are collected and either analysed directly or concentrated by evaporation and then reconstituted in an appropriate solvent or mobile phase and then analysed.

2.2.3 Liquid-liquid extraction (LLE)

Solvent extraction or liquid-liquid extraction separates compounds based on differences in partition coefficient between two immiscible liquids (Watson, 2005). Liquid-liquid extraction, compared to solid-phase extraction, has been proven to be as effective as SPE in isolating corticosteroids from plasma (Chen *et al*., 2009; Lee *et al*., 2009; Ionita *et al*., 2009; Zhang *et al*., 2006; Pereira *et al.*, 2005; Teng *et al.*, 2001; Hochhaus *et al.*, 1998) but more effective than SPE in extracting corticosteroids in urine samples (Tolgyesi *et al*., 2010; Andersen *et al.*, 2008; Zou *et al*., 2008; Touber *et al*., 2007; Deventer *et al.*, 2006; Tang *et al.*, 2001; Fluri *et al.*, 2001). LLE involves extraction of the analytes from the sample which is dissolved in an appropriate solvent, usually aqueous, and another immiscible solvent. The analytes are extracted into the organic liquid phase and isolated after separation and evaporation to dryness. The residue is then reconstituted with a suitable solvent or mobile phase before analysis.

2.3 Methods utilised in the quantification of corticosteroids

The detection of corticosteroids in biological samples has been reported using several analytical methods. The most common is liquid chromatography (LC) coupled with one of a variety of detection methods including: ultraviolet spectroscopy (UV) (Lin *et al*., 2009; Zhang *et al*., 2006; Assi *et al*., 2006; Hou *et al.*, 2001; Teng *et al.*, 2001); mass spectrometry (MS) (Chen *et al*., 2009; Streel *et al*., 2009; Li *et al*., 2008; Qu *et al.*, 2007; Deventer *et al.*, 2006; Pereira *et al.*, 2005; Sahasranaman *et al.*, 2005; Taylor *et al.*, 2004; Wang & Hochhaus, 2004; Li *et al.*, 2001; Tang *et al.*, 2001; Fluri *et al.*, 2001; Laugher *et al.*, 1999; Krishnaswami *et al.*, 2000; Cirimele *et*

al., 2000); radioimmunoassay (RIA) (Nair *et al*., 2009; Baid *et al*., 2007; Fardon *et al*., 2004; Hochhaus *et al*., 1998) or nuclear magnetic resonance (NMR) (Lin *et al*., 2009; Naikwade & Bajaj, 2008). Pujas *et al.,* (2005) stated that chromatographic techniques, coupled with UV, MS or NMR detection, were preferred over radioimmunoassay as RIA lacks specificity.

Chromatography is defined as "*distribution of molecules between two immiscible liquid phases, stationary phase (SP) and mobile phase (MP)*" (Watson, 2005). The separation mechanism is based on the polar properties of the mobile phase. The presence of Van der Waals interactions, hydrogen-bonding and dipole-dipole attractions determine the solubility properties of the stationary phase (Watson, 2005). If the stationary phase is hydrophilic and the mobile phase is non-polar this is termed normal phase liquid chromatography (NP-LC). The concept of normal phase is less common than reverse phase liquid chromatography (RP-LC) where the stationary phase is hydrophobic and the mobile phase is hydrophilic. The concept of "*like dissolves like*" is applicable in both types of chromatography.

Literature reveals that reverse phase liquid chromatography (RP-LC) has been employed in the analysis of corticosteroids in patients taking single therapy (Xiong *et al*., 2009; Zou *et al*., 2008; Qu *et al.*, 2007; Deventer *et al.*, 2006; Sahasranaman *et al.*, 2005; Pereira *et al.*, 2005; Hou *et al.*, 2001; Fluri *et al.*, 2001; Krishnaswami *et al.*, 2000; Laugher *et al.*, 1999; Hochhaus *et al.*, 1998) but few studies have described the analysis of samples from patients receiving combination therapy (Ionita *et al*., 2009; Mascher *et al*., 2008; Touber *et al*., 2007; Wang & Hochhaus,

2004; Taylor *et al.*, 2004; Tang *et al.*, 2001; Li *et al.*, 2001). Therefore, there is a need to establish a simple robust and accurate HPLC method for the analysis of corticosteroids in patients taking combination therapy. HPLC analysis involves two phases; the stationary phase and the mobile phase.

2.3.1 Stationary phase

Reverse phase liquid chromatography (RP-LC) is defined a process where the analyte binds to hydrophobic stationary phase in a polar solvent (Dong, 2006). Hydrocarbon bonded stationary phases C_8 (*n*-octyl) (Teng *et al.*, 2001) and C_{18} (*n*-octyldecyl) (Tolgyesi *et al*., 2010; Kaur *et al*., 2010; Li *et al*., 2008; Mascher *et al*., 2008; Zou *et al*., 2008; Naikwade & Bajaj, 2008; Zhang *et al*., 2006; Hashem & Jira, 2004) are commonly used in RP-LC although the same stationary phases obtained from different suppliers can produce slightly different selectivity (Fu *et al*., 2010; Xiong *et al*., 2008; Foe *et al.*, 1998). The lipophilic alkyl chain is chemically bonded to a silanol group attached to the silica support surface. In reverse phase chromatography, the amount of analyte taken up by the stationary phase is proportional to the carbon load of the stationary phase. Experiments have shown that the higher the carbon load of the stationary phase, the more lipophilic it will be and thus a more non-polar mobile phase, one with a higher proportion of organic modifier, needs to be used to elute the analytes of interest (Xiong *et al*., 2009; Streel *et al.*, 2009).

2.3.2 Mobile phase

Non polar solvents such as methanol (Naikwade & Bajaj, 2008; Zou *et al*., 2008; Carvalho *et al*., 2008; Zhang *et al*., 2006; Sahasranaman *et al.*, 2005; Wang & Hochhaus, 2004; Teng *et al.*, 2001;Tang *et al.*, 2001; Krishnaswami *et al*., 2000; Laugher *et al.*, 1999; Hochhaus *et al.*, 1998) or acetonitrile (Xiong *et al*., 2009; Mascher *et al*., 2008; Qu *et al.*, 2007; Deventer *et al.*, 2006; Pereira *et al.*, 2005; Taylor *et al.*, 2004; Fluri *et al.*, 2001; Hou *et al.*, 200) are preferred as the HPLC mobile phase in combination with water since these organic solvents are miscible with water and keep it at a neutral pH. The more polar the eluent, the longer the time it takes for the analyte of interest to elute (Li *et al*., 2008; Teng *et al*., 2001; Watson, 2005). Ionisation may not be necessary in the analysis of neutral compounds such as corticosteroids. Some studies have shown that the addition of an acidic buffer, such as formic acid, or acetic acid (pH 2.4 – 5.8), is useful to aid protonation when using liquid chromatography-mass spectrometry (LC-MS) to determine the concentration of corticosteroids (Tolgyesi *et al*., 2010; Panusa *et al*., 2010; Ionita *et al*., 2009; Fu *et al*., 2010; Mascher *et al*., 2008; Touber *et al*., 2007; Qu *et al.*, 2007; Deventer *et al.*, 2006; Pereira *et al.*, 2005; Wang & Hochhaus, 2004).

Isocratic elution is defined as a constant ratio of mobile phase constituents throughout the analysis whereas gradient elution allows the concentration of the mobile phase to change over time and results in a faster elution of the analytes (Schellinger & Carr, 2006). Gradient elution of corticosteroids has been quoted in several papers (Tolgyesi *et al*., 2010; Xiong *et al*., 2009; Lin *et al*., 2009; Mascher *et al*., 2008; Touber *et al.*, 2007; Qu *et al.*, 2007; Deventer *et al.*, 2006; Peng *et al*.,

2005; Taylor *et al.*, 2004; Fluri *et al.*, 2001; Cirimele *et al.*, 2000; Tang *et al.*, 2000; Mistry *et al.*, 1997) although others (Panusa *et al*., 2010; Ionita *et al*., 2009; Li *et al*., 2008; Zhang *et al*., 2006; Sahasranaman *et al.*, 2005; Pereira *et al.*, 2005; Samtani *et al*., 2005; Pujas, 2004; Wang & Hochhaus, 2004; Teng *et al.*, 2001; Hou *et al.*, 2001; Krishnaswami *et al.*, 2000; Laugher *et al.*, 1999; Foe *et al.*, 1998; Hochhaus *et al.*, 1998; Li *et al.*, 1996) prefered to use isocratic elution. Similar resolution to isocratic elution with improved selectivity can be obtained using a gradient elution method (Schellinger & Carr, 2006).

2.3.3 Internal and external standards

The internal standard is a measured quantity of a known substance added to sample solutions and is used to increase precision and account for any loss of analyte during sample preparation, or variation in injection volume or detector response which may be subject to change with time. The internal standard should be pure, stable and readily available; chemically similar to the analyte; elute close to the analyte of interest and not react or interact with any other components in the sample. There are three types of internal standard. Isotopically labelled internal standards such as d_4-cortisol, d_5-testosterone, d_7-triamcinolone acetonide (Tolgyesi *et al*., 2010; Panusa *et al*., 2009; Ionita *et al*., 2009; Mascher *et al*., 2008; Carter & Capka, 2008; Ding *et al*., 2008; Qu *et al.*, 2007; Sahasranaman *et al.*, 2005; Taylor *et al.*, 2004; Cirimele *et al.*, 2000; Tang *et al.*, 2000; Krishnaswami *et al.*, 2000; Laugher *et al.*, 1999; Li *et al.*, 1996); structurally-related analogues such as betamethasone (Deventer *et al.*, 2006), fluticasone propionate (Wang & Hochhaus, 2004), beclometasone dipropionate (Zou *et al*., 2008), triamcinolone acetonide (Vasquez *et al*., 2005), flumethasone (Gao *et*

al., 2009; Frerichs & Tornatore, 2004), prednisolone (Glowka *et al.*, 2006) and dexamethasone acetate (Teng *et al.*, 2001) or structurally unrelated compounds such as chloramphenicol (Pereira *et al.*, 2005), tetramethylsilane (Naikwade & Bajaj, 2008) and fluoxymesterone (Fluri *et al.*, 2001) have all been used. It is also common to use more than one internal standard when there are multiple analytes of interest (Touber *et al*., 2007; Taylor *et al.*, 2004). Some researchers (Kaur *et al.*, 2010; Fu *et al*., 2010; Xiong *et al*., 2009; Zhang *et al*., 2006; Hou *et al.*, 2001; Mistry *et al.*, 1997) explored the concept of an analytical method using external standardisation in determination of corticosteroids. Using external standardisation, Xiong *et al.*, (2009), Fu *et al*., (2010) and Zhang *et al.*, (2006) showed a range of recovery of corticosteroids between 92 – 102 % (percentage relative standard deviation (% RSD) = 0.3 – 0.6) and limit of quantification (LOQ) of 3 ng mL^{-1} (Zhang *et al.*, 2006) and LOQ of 500 ng mL^{-1} (Fu *et al*., 2010; Xiong *et al*., 2009). Similar recoveries from biological fluids were reported in corticosteroid analysis in the presence of isotopically labelled corticosteroids as internal standard including d_4-cortisol (Ding *et al*., 2009); $^2H_7$-cortisone (Ionita *et al*., 2009); d_2 - methylprednisolone (Panusa *et al*., 2010) but with poorer precision (% RSD = 2.4 – 10.3) and a lower LOQ (0.5 - 11 ng mL^{-1}).

2.3.4 Detector

Detectors must be sensitive enough to detect the analytes of interest as they elute from the column. Previous studies (Chen *et al*., 2009; Streel *et al*., 2009; Lin *et al*., 2009; Li *et al*., 2008; Zhang *et al*., 2006; Assi *et al*., 2006; Deventer *et al*., 2006; Sahasranaman *et al.*, 2005; Pereira *et al.*, 2005; Pujas, 2005; Hashem & Jira, 2004;

Wang & Hochhaus, 2004) have used ultraviolet (UV) and mass spectrometry as detectors. Mass spectrometer detection was proven to be more sensitive than UV detection with an LOQ of 5 – 50 pg mL^{-1} and 3 – 200 pg mL^{-1}, respectively.

There are two types of UV detectors; fixed wavelength detectors and multiwavelength detectors. The fixed wavelength detector operates at a single wavelength whereas the multiwavelength detector selects a single wavelength or a range of wavelengths and can monitor several analytes at different wavelength simultaneously. Several authors have described UV detection for quantifying; betamethasone (Kaur *et al*., 2010); budesonide (Naikwade & Bajaj, 2008; Assi *et al*., 2005); prednisolone (Zhang *et al*., 2006); prednisolone and dexamethasone (Hashem & Jira, 2004); and mometasone (Teng *et al.*, 2001) in both solution and biological samples. UV detection was performed using a multiwavelength detector, photo diode array detector in these studies.

2.3.4.1 Wavelength of detection

A suitable UV wavelength for detection in HPLC analysis of corticosteroids is important to minimise the interference. UV measures the light absorbed by the sample and the intensity of light varies at different wavelengths. The optimal wavelength of detection is determined by the maximal absorption by the analyte on the UV spectrum. In several studies (Kaur *et al*., 2010, Naikwade & Bajaj, 2008, Zhang *et al*., 2006, Hashem & Jira, 2004, Teng *et al.*, 2001), the UV detector used was operated at a variable wavelength of 240 – 254 nm with the limit of detection (LOD) and LOQ at 25 – 40 ng $mL^{-1}$and 100 - 1000 ng mL^{-1}, respectively.

2.4 Experimental

2.4.1 Materials

2.4.1.1 Chemicals and reagents

Beclometasone dipropionate (99 % purity), betamethasone (98 % purity), prednisolone (99 % purity) and budesonide (99 % purity) were purchased from Sigma-Aldrich (Dorset, UK). Fluticasone dipropionate (99 % purity) and mometasone furoate were obtained from the European Directorate for the Quality of Medicines and Healthcare (EDQM, Strasbourg, France). Beclometasone-17-monopropionate was gifted from the European Directorate for the Quality of Medicines and Healthcare (EDQM, Strasbourg, France). HPLC grade methanol and acetonitrile were obtained from Fisher Scientific (Leicestershire, UK). Ethyl acetate, formic acid and heptane (laboratory reagent grade) were purchased from Sigma-Aldrich (Dorset, UK).

2.4.1.2 Stationary phases and solid-phase extraction cartridges

Four stationary phases were evaluated in the development of a reverse phase high performance liquid chromatography (RP-HPLC) method (Table 2.1).

Table 2.1 Properties of columns used in the analysis

Column	Name	Dimensions	Internal diameter (i.d)	Particle size (par.)	% carbon load	Manufacturer
A	Ultrasphere C_8	150 mm	4.6 mm	5 µm	10.0	Beckman Coulter[a]
B	ACE C_{18}	150 mm	4.6 mm	5 µm	15.5	Agilent Technologies[b]
C	Hypersil C_{18}	100 mm	4.6 mm	5 µm	11.0	Thermo[c]
D	Kromasil C_{18}	250 mm	4.6 mm	5 µm	19.0	HiChrom[d]

[a]Buckinghamshire, UK, [b]Santa Clara CA, USA, [c]Lancashire, UK, [d]Berkshire, UK

The columns were fitted with a Phenomenex C_{18} guard (4 × 3.0 mm (i.d)) column, (Macclesfield, UK) and maintained at 25 °C using a Hewlett Packard model 1100 column heater.

Solid-phase extraction (SPE) cartridges, Strata-C_{18} 55 µm (part no: 8B-S001-EBJ) were purchased from Phenomenex (Macclesfield, UK).

2.4.1.3 Instrumentation

Analyses were conducted on a high performance liquid chromatography (HPLC) instrument consisting of a Hewlett Packard (HP) series 1100 Binary Pump and sample processor linked to a HP Variable Wavelength UV detector. The system control and data analysis were controlled by ChemStation software (version 1.1) (Agilent Technologies, Santa Clara CA, USA). Samples were analysed in HPLC screw vials (2 mL (volume)) with 0.15 mL conical inserts (Kinesis, Cambridgeshire,

UK). The analytical conditions evaluated during development are described (Table 2.2).

Table 2.2 High Performance Liquid Chromatography conditions. (a) isocratic programme and (b) gradient programme

(a) Isocratic programme

Method	Time (min)	% organic solvent	Flow rate (mL min^{-1})	Temp (°C)
Column A : C_8 Ultrasphere (150 x 4.6 mm, 5 µm)				
A1	60	59	1.5	22 ± 2
Column B : C_{18} ACE (150 x 4.6 mm, 5 µm)				
ISO B1	20	48[a]	1.5	22 ± 2
ISO B2	60	59	1.5	22 ± 2
Column C : C_{18} Hypersil BDS (100 mm x 4.6 mm, 5 µm)				
ISO C1	30	59	1	22 ± 2
ISO C2	80	55	1	22 ± 2
Column D : C_{18} Kromasil (250 mm x 4.6 mm, 5 µm)				
ISO D1	150	59	1	22 ± 2
ISO D2	120	62	1	22 ± 2

(b) Gradient programme

Method	Time (min)	% organic solvent	Flow rate (mL min^{-1})	Temp (ºC)
Column B : C_{18} ACE (150 x 4.6 mm, 5 µm)				
GRAD B3	0	55	1.5	22 ± 2
	6	55		
	8	59		
	22	61		
	35	70		
	40	55		
GRAD B4	0	55	1.5	22 ± 2
	6	55		
	8	59		
	45	61		
	50	70		
	50	55		
Column C : C_{18} Hypersil BDS (100 mm x 4.6 mm, 5 µm)				
GRAD C3	0	50	1	22 ± 2
	25	50		
	65	55		
	75	57		
	80	50		
GRAD C4	0	50	1	22 ± 2
	25	50		
	50	55		
	75	57		
	85	59		
	90	50		
Column D : C_{18} Kromasil (250 mm x 4.6 mm, 5 µm)				
GRAD D3	0	62	1	25
	60	62		
	80	80		
	85	62		
GRAD D4	0	57	1	25
	55	57		
	70	62		
	87	80		
	100	57		
	110	57		

Mobile phase composition: organic solvent is methanol unless stated; [a] acetonitrile
ISO = isocratic programme; GRAD = gradient programme
*The UV wavelength was set at 239 nm

Liquid Chromatography – Mass Spectrometry (LC-MS) analysis was performed on a Thermoquest system consisting of an Agilent 1100 Autosampler linked to a binary

pump and Finnigan LCQ Deca Mass Spectrometer. The system was controlled by Xcalibur software (version 2.0) installed in Hewlett Packard computer linked to the system.

Ultraviolet absorbance spectra were measured using a Unicam UV Visible Spectrometer UV 300 (Thermo Spectronic, Cambridge).

All weights (twice) were measured on a calibrated six figure analytical weighing balance GR-200-EC (A & R instruments, Japan). Gilson pipettes with a range of 20 µl – 1000 µl were used to handle solutions with a volume of less than 1 mL. Vacuette Lithium Heparin coated blood collection tubes (6 mL) were obtained from Greiner BioOne (Gloucestershire). Blood samples were filtered with a Millipore® Syringe Driven Filter Unit 0.22 µm, 33 mm (Cork, Ireland). Blood samples were centrifuged using a Heraeus® Labofuge 400 centrifuge (Buckinghamshire, UK). Water was purified with a Milli-Q system (Millipore, France) and filtered using 0.45 µm nylon Whatman membrane filter (Fisher Scientific, Leicestershire, UK). Microcentrifuge tubes (1.5 mL) were purchased from Fisherbrand (Leicestershire, UK). A sample concentrator (Techne) and dB-3 Dri block heater were linked to a liquid nitrogen cylinder for heating and evaporating samples to dryness at 37 ºC.

2.4.1.4 Mobile phases

The mobile phases were prepared daily by mixing the measured volume of organic solvents and the aqueous solvents. The mobile phases were degassed through a 0.45µm Whatman nylon membrane filter (Fisher Scientific, Leicestershire, UK).

2.4.2 Methods

2.4.2.1 Stock solution and standards

Stock Solution A – Prednisolone (1 mg mL^{-1})

Prednisolone (10 mg) was weighed accurately into a 10 mL volumetric flask and diluted to volume with HPLC grade methanol to give a solution containing prednisolone (1 mg mL^{-1}).

Stock Solution B – Budesonide (1 mg mL^{-1})

Budesonide (10 mg) was weighed accurately into a 10 mL volumetric flask and diluted to volume with HPLC grade methanol to give a solution containing budesonide (1 mg mL^{-1}).

Stock Solution C – Beclometasone-17-monopropionate (1 mg mL^{-1})

Beclometasone-17-monopropionate (10 mg) was weighed accurately into a 10 mL volumetric flask and diluted to volume with HPLC grade methanol to give a solution containing beclometasone-17-monopropionate (1 mg mL^{-1}).

Stock Solution D – Fluticasone (1 mg mL^{-1})

Fluticasone (10 mg) was weighed accurately into a 10 mL volumetric flask and diluted to volume with HPLC grade methanol to give a solution containing fluticasone (1 mg mL^{-1}).

Stock Solution E – Beclometasone dipropionate (1 mg mL^{-1})

Beclometasone dipropionate (10 mg) was weighed accurately into a 10 mL volumetric flask and diluted to volume with HPLC grade methanol to give a solution containing beclometasone dipropionate (1 mg mL^{-1}).

Stock Solution F – Betamethasone (1 mg mL^{-1})

Betamethasone (10 mg) was weighed accurately into a 10 mL volumetric flask and diluted to volume with HPLC grade methanol to give a solution containing betamethasone (1 mg mL^{-1}).

Reference Solution F – Betamethasone (100 µg mL^{-1})

One mL from stock solutions F measured by a Gilson pipette, was added to 10 mL volumetric flask and the solution was made up to 10 mL with mobile phase [methanol-water (57:43 v/v)].

Internal standard solution F1 – Betamethasone (2.5 µg mL^{-1})

One mL of the stock solution of F (1 mg mL^{-1}) was added to 100 mL volumetric flask and the solution was made up to 100 mL with mobile phase [methanol-water

(57:43 v/v)]. Five mL of this solution was added to 20 mL volumetric flask and the solution was made up to 20 mL with mobile phase [methanol-water (57:43 v/v)].

Stock Solution G – Mometasone (1 mg mL^{-1})

Mometasone (10 mg) was weighed accurately into a 10 mL volumetric flask and diluted to volume with HPLC grade methanol to give a solution containing mometasone (1 mg mL^{-1}).

Reference Solution G – Mometasone (100 µg mL^{-1})

One mL from stock solutions G measured by a Gilson pipette, was added to 10 mL volumetric flask and the solution was made up to 10 mL with mobile phase [methanol-water (57:43 v/v)].

Internal standard solution G1 – Mometasone (2.5 µg mL^{-1})

One mL of the stock solution of G (1 mg mL^{-1}) was added to 100 mL volumetric flask and the solution was made up to 100 mL with mobile phase [methanol-water (57:43 v/v)]. Five mL of this solution was added to 20 mL volumetric flask and the solution was made up to 20 mL with mobile phase [methanol-water (57:43 v/v)].

Reference Solution A – Corticosteroid mixture (100 µg mL^{-1})

One mL from each stock solution A – E measured by a Gilson pipette, was added to 10 mL volumetric flask and the solution was made up to 10 mL with mobile phase [methanol-water (57:43 v/v)].

Reference Solution B – Corticosteroid mixture (20 µg mL^{-1})

One mL from Reference Solution A was added to 5 mL volumetric flask and the solution was made up to 5 mL with mobile phase [methanol-water (57:43 v/v)].

Reference Solution C – Corticosteroid mixture (10 µg mL^{-1})

One mL from Reference Solution A was added to 10 mL volumetric flask and the solution was made up to 10 mL with mobile phase [methanol-water (57:43 v/v)].

Reference Solution D – Corticosteroid mixture (5 µg mL^{-1})

One mL from Reference Solution A was added to 20 mL volumetric flask and the solution was made up to 20 mL with mobile phase [methanol-water (57:43 v/v)].

Calibration standards solution of all analytes (except betamethasone and mometasone)

A series of calibration standard solutions containing 10 µg mL^{-1}, 5 µg mL^{-1}, 2.5 µg mL^{-1}, 1 µg mL^{-1}and 0.5 µg mL^{-1}of mixture of corticosteroids were prepared from Reference Solution A and labelled as Reference Solution A1 – A5 (Table 2.3). Each concentration was diluted to the final volume with mobile phase [methanol-water (57:43 v/v)]. In each of the calibration standard solutions prepared, an internal standard solution, betamethasone or mometasone, with a final concentration of 0.5 µg mL^{-1} was added. The solutions were protected from light.

Table 2.3 An example of the final concentration for preparation of calibration standard solutions (0.5 µg mL^{-1} – 10 µg mL^{-1}) from Reference Standard A

Analyte	Reference solution					
	A	**A1**	**A2**	**A3**	**A4**	**A5**
	100 µg mL^{-1}	**10 µg mL^{-1}**	**5 µg mL^{-1}**	**2.5 µg mL^{-1}**	**1 µg mL^{-1}**	**0.5 µg mL^{-1}**
Prednisolone	101	10.1	5.05	2.53	1.01	0.51
Budesonide	101	10.1	5.05	2.53	1.01	0.51
Beclometasone-17-monopropionate	100	10.0	5.00	2.50	1.00	0.50
Fluticasone	103	10.3	5.15	2.58	1.03	0.52
Beclometasone Dipropionate	103	10.3	5.15	2.58	1.03	0.52

Calibration standard solution of betamethasone

A series of calibration standard solutions containing 10 µg mL^{-1}, 5 µg mL^{-1}, 2.5 µg mL^{-1}, 1 µg $mL^{-1}$and 0.5 µg mL^{-1} of betamethasone were prepared from Reference Solution F and labelled as Reference Solution F1 – F5 (Table 2.4). Each concentration was diluted to final volume with the mobile phase [methanol-water (57:43 v/v)]. In each of the calibration standard solutions prepared, an internal standard solution, mometasone, with a final concentration of 0.5 µg mL^{-1} was added. The solutions were protected from light.

Table 2.4 An example of the final concentration for preparation of calibration standard solutions (0.5 µg mL^{-1} – 10 µg mL^{-1}) from Reference Standard F

Analyte	Reference solution					
	F	**F1**	**F2**	**F3**	**F4**	**F5**
	100 µg mL^{-1}	**10 µg mL^{-1}**	**5 µg mL^{-1}**	**2.5 µg mL^{-1}**	**1 µg mL^{-1}**	**0.5 µg mL^{-1}**
Betamethasone	100	10.0	5.00	2.50	1.00	0.50

Calibration standard solution of mometasone

A series of calibration standard solutions containing 10 µg mL^{-1}, 5 µg mL^{-1}, 2.5 µg mL^{-1}, 1 µg $mL^{-1}$and 0.5 µg mL^{-1} of mometasone were prepared from Reference Solution G and labelled as Reference Solution G1 – G5 (Table 2.5). Each concentration was diluted to final volume with the mobile phase [methanol-water (57:43 v/v)]. In each of the calibration standard solutions prepared, an internal standard solution, betamethasone, with a final concentration of 0.5 µg mL^{-1} was added. The solutions were protected from light.

Table 2.5 An example of the final concentration for preparation of calibration standard solutions (0.5 µg mL^{-1} – 10 µg mL^{-1}) from Reference Standard G

Analyte	Reference solution					
	G	**G1**	**G2**	**G3**	**G4**	**G5**
	100 µg mL^{-1}	**10 µg mL^{-1}**	**5 µg mL^{-1}**	**2.5 µg mL^{-1}**	**1 µg mL^{-1}**	**0.5 µg mL^{-1}**
Mometasone	106	10.6	5.30	2.65	1.33	0.67

Solutions for Ultraviolet analysis

Four mg of each substance was weighed on an analytical balance and a series of corticosteroid standards were prepared at a concentration of 4 mg in a 100 mL volumetric flask in methanol and were labelled UV Stock Solution (Table 2.6). Each corticosteroid solution (except beclometasone-17-monopropionate) was diluted with mobile phase [methanol-water (57:43 v/v)] to a final concentration of 2 mg in 100 mL. Beclometasone-17-monopropionate was measured at the initial concentration of 4 mg in 100 mL. Prednisolone was further diluted to 1 mg in 100 mL (Table 2.6). The solutions were stored at room temperature and protected from light.

Table 2.6 Actual concentration of UV stock solutions of each analyte for ultraviolet analysis

Analyte	UV Stock solution (mg/100 mL)	UV standard solution (mg/100 mL)
Prednisolone	4.4	1.1
Betamethasone	4	2.0
Budesonide	4.2	2.1
Beclometasone-17-monopropionate	4.8	4.8
Mometasone	4.6	2.3
Fluticasone	4.4	2.2
Beclometasone Dipropionate	4.2	2.1

2.4.2.2 Preparation of samples for analysis of corticosteroids

Plasma

Blank plasma

One unit of fresh frozen plasma was obtained from the Scottish National Blood Transfusion Service (SNBTS) every month for 18 months.

Blank plasma solution

Blank plasma (900 µl) was transferred, using a Gilson pipette, into a vial and mixed with 100 µl of mobile phase [methanol-water (57:43 v/v)].

Corticosteroid plasma solution (20 µg mL^{-1})

Blank plasma (700 µl) was spiked with 200 µl of Reference Solution A and 100 µl of the internal standard solution (F1 or G1).

Corticosteroid plasma solution (10 µg mL^{-1})

Blank plasma (800 µl) was spiked with 100 µl of Reference Solution A and 100 µl of the internal standard solution (F1 or G1).

Corticosteroid plasma solution (5 µg mL^{-1})

Blank plasma (650 µl) was spiked with 250 µl of Reference Solution B and 100 µl of the internal standard solution (F1 or G1).

Corticosteroid plasma solution (2.5 µg mL^{-1})

Blank plasma (650 µl) was spiked with 250 µl of Reference Solution C and 100 µl of the internal standard solution (F1 or G1).

Corticosteroid plasma solution (1 µg mL^{-1})

Blank plasma (800 µl) was spiked with 100 µl of Reference Solution C and 100 µl of the internal standard solution (F1 or G1).

Corticosteroid plasma solution (0.5 µg mL^{-1})

Blank plasma (800 µl) was spiked with 100 µl of Reference Solution D and 100 µl of the internal standard solution (F1 or G1).

2.4.2.3 High Performance Liquid Chromatography method development

Selection wavelength of detection

A solution of each corticosteroid was scanned individually using the UV spectrophotometer from 200 – 600 nm to ascertain the maximal absorbance of the

substance. The Beer-Lambert law was used to calculate the optimal analyte concentration to run for ultraviolet analysis based on the specific absorbance value, A (1 %, 1 cm). For beclometasone-17-monopropionate, the Woodward – Fieser Rules (W-F-R) were used to calculate its expected UV wavelength.

2.4.2.4 Extraction method optimisation

Blank plasma was spiked with an appropriate concentration of the corticosteroid mixture. Three methods were considered for extraction of the corticosteroids from plasma.

Liquid-liquid extraction

Ethyl acetate (250 µL) was added to a blank plasma solution (250 µL) in a separating funnel. The solution was shaken for one minute and the two layers were allowed to separate over 5 minutes. The ethyl acetate layer was removed and the plasma sample was extracted a second time with ethyl acetate (250 µL). The combined ethyl acetate fractions were concentrated to dryness under a nitrogen stream at 37ºC. The residue was dissolved in 500 µL of mobile phase [methanol-water (57:43 v/v)], shaken for 30 seconds and filtered before analysis.

Solid-phase extraction using Strata C_{18} cartridge

SPE Method 1 (Li *et al.*, 2001)

The solid-phase extraction (SPE) Strata C_{18} cartridge (3mL, 100 mg) was preconditioned with equal volumes of methanol and water (3 mL each) which was allowed to elute through under vacuum at a flow rate of 1 drop/second. There was a brief equilibration phase between the preconditioning and loading of sample. The

spiked plasma (1 mL) was introduced onto the cartridges. The sample was allowed to elute through the cartridges at a flow rate of 1 drop/second. The loaded sample was then washed with 3 mL of a mixture of methanol and water (25:75 v/v), followed by 3 mL of water. The cartridge was then washed with 2 mL of ethyl acetate and heptane (2:98 v/v). The cartridge was dried for 15 minutes under vacuum. The vacuum was disconnected and the HPLC vials (2 mL) were positioned aligned to the cartridges. The eluate was collected with 2 mL of ethyl acetate: heptane (35:65 v/v). The solvent was evaporated to dryness under a nitrogen stream at 37 ºC. The residue was dissolved in 100 µL of mobile phase [methanol-water (57:43 v/v)] before analysis.

SPE Method 2

The solid-phase extraction (SPE) Strata C_{18} cartridge (3mL, 100 mg) was preconditioned with 3 mL of methanol followed by 3 mL of water and allowed to drip through under vacuum at a flow rate of 1 drop/second. There was a brief equilibration phase between the preconditioning and loading of the sample. The spiked plasma (1 mL) was loaded onto the preconditioned cartridge. The samples were allowed to pass through the cartridges at a flow rate of 1 drop/second. The cartridge was left for 150 seconds under vacuum before the next step. The spiked plasma was washed with 1 mL water and 1 mL of a mixture of methanol-water (10:90 v/v). The vacuum was disconnected and the HPLC vials (2 mL) were positioned aligned to the cartridges. The cartridge was eluted with 0.5 mL of solvent [methanol-water (80:20 v/v)] under vacuum into the HPLC vials and this solution was analysed directly.

2.5 Results and Discussion

2.5.1 Optimisation of the chromatographic method for the analysis of corticosteroids

2.5.1.1 Selection of wavelength of detection

The stock solutions of the individual corticosteroids were prepared at a concentration of 4 mg in 100 mL. The concentration of stock solution was calculated based on the Beer Lambert Law (Equation 1 and 2).

Absorbance according to the Beer Lambert Law is defined as follows,

$A = \text{Log } I_o/I_t = \varepsilon bc$ - Equation 1

A = absorbance = amount of light absorbed by the sample
I_o = intensity of incident radiation
I_t = intensity of transmitted radiation
ε = molar extinction coefficient i.e. absorbance of a 1 M solution of the analyte
b = pathlength of the cell in cm (usually 1 cm)
c = concentration of analyte in moles per litre

In pharmaceutical products, the equation can be simplified to (Watson, 2005):

$A = C \times A\ (1\ \%, 1\ cm)$ - Equation 2

A = Amount of light absorbed by the sample
C = Concentration of sample in g mL^{-1}
A (1 %, 1 cm) = Specific absorbance

The value for A (1 %, 1 cm) were obtained from British Pharmacopoeia (BP, 2008) and Clarke's Analysis (2004). According to Beer Lambert Law, the UV absorbance of light by the sample is proportional to the analyte concentration. The optimal UV absorbance range is between 0.5 – 1.5. The absorbance scale is logarithmic where any absorbance above 1.5 should be retested since the absorbance unit is less sensitive to differences in analyte concentration above this concentration. The stock

concentration was later diluted to 2 mg in 100 mL except for beclometasone-17-monopropionate (17-BMP).

At a concentration of 2 mg in 100 mL solution, prednisolone (PRED) showed a higher UV absorbance (A (1 %, 1cm) = 415; A = 1.889) than the other analytes. The reported absorbance showed that the solution of prednisolone was too concentrated. The prednisolone (PRED) solution was later diluted to 1 mg in 100 mL and the reported measured absorbance (A) was 0.980 which was within the acceptable range.

At a concentration of 2 mg in 100 mL solution, the absorbance of 17-BMP was of a lower absorbance than the acceptable range (A = 0.3568) and the solution was reported as too diluted. The 17-BMP was later prepared in a more concentrated solution (4 mg in 100 mL). The solution containing 17-BMP at 4 mg in 100 mL reported gave an absorbance (A) 0.517 and was within the accepted range. The choice of UV detection wavelength was based on the maximum absorbance shown by the UV absorbing chromophores of the analytes being examined. From the overlaid UV-spectra (Figure 2.2) the samples showed the maximal absorbance in the range of 239 – 248 nm. These are compared to the British Pharmacopoeia (BP, 2008) recommendation of the optimal UV wavelength (Table 2.7).

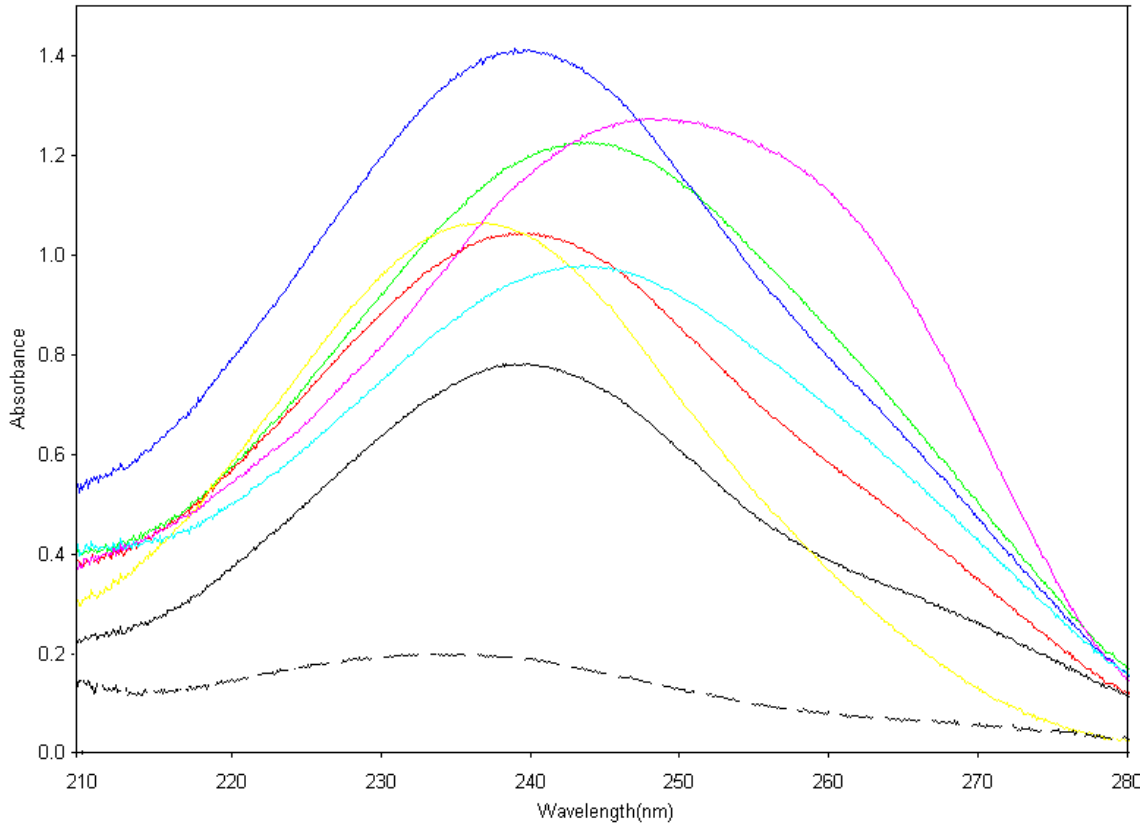


Figure 2.2 Overlay UV Absorption spectra of the mixture of the eight corticosteroids under investigation

___ BDP (2 mg in 100 mL)
___ BUD (2 mg in 100 mL)
___ FP (2 mg in 100 mL)
_ _ 17-BMP (4 mg in 100 mL)
___ BETA (2 mg in 100 mL)
___ PRED (1 mg in 100 mL)
___ dexamethasone acetate (2 mg in 100 mL)
___ MF (2 mg in 100 mL)

Table 2.7 Comparison between reported and measured maximal absorbance values and wavelength spectra of each analyte

Analyte	Conc. (mg in 100 mL)	Specific absorbance (A 1 %/1 cm)	Maxima Absorbance		UV wavelength (nm)	
			Reported	Measured	Reported	Measured
PRED	1.1	415[c,d]	0.830[a]	0.980	240 - 254[c]	244
BETA	2.0	395[c]	0.790[a]	1.045	241[c]	240
BUD	2.1	n/a	n/a	1.228	240[c]	244
17-BMP	4.8	n/a	n/a	0.517	242[b]	237
MF	2.3	481[c]	0.962[a]	1.275	254[c]	248
FP	2.2	n/a	n/a	1.066	239[c]	237
BDP	2.1	292[d]	0.584[a]	0.784	239[c]	239

*Conc = concentration; n/a = not available in the monograph
[a]According to Beer-Lambert law
[b]According to Woodward-Fieser Rules
[c]BP, 2008
[d]Clarke's Analysis, 2004

From the overlay UV spectra, the maximal UV absorption (λ_{max}) of the corticosteroids can be grouped into two wavelengths range: BETA, 17-BMP, FP, and BDP at 237 -240 nm and PRED, BUD and MF at 244 - 248 nm. Since there was no UV multiwavelength detector available, a single UV wavelength detector was used in this study. The HPLC analysis of the corticosteroid mixture was then undertaken at two UV wavelengths of 239 nm and 246 nm based on the two groups described earlier to determine if a single wavelength could be used for future analyses (Table 2.8).

Table 2.8 Chromatographic data of the analytes in the corticosteroid mixture (20 µg mL^{-1}) at two different wavelengths obtained using C_{18} Kromasil; gradient mobile phase (method GRAD D4); Flow rate: 1.0 mL min^{-1}. UV: 239 nm

Analyte	Ultraviolet wavelength (nm)	Retention time, t_R (min)	Peak area	RRF $_{(PRED)}$ ±
PRED	239	11.69	569.298	1.00
	246	11.29	725.026	1.00
BETA	239	17.65	861.877	1.51
	246	16.87	942.547	1.30
BUDE	239	53.15,55.68*	499.595	0.88
	246	50.05,52.42*	521.699	0.72
17-BMP	239	64.09	708.327	1.24
	246	60.486	726.927	1.00
MF	239	77.91	988.609	1.74
	246	76.23	1391.237	1.92
FP	239	79.49	1177.961	2.07
	246	78.16	1257.408	1.73
BDP	239	89.25	616.780	1.08
	246	88.80	769.733	1.06

* BUD eluted as two isomer peaks.
±RRF = Relative Response Factor in relative to PRED

The UV relative response factor (RRF_{UV}) of each analyte was determined based on the absorbance of each analyte compared to PRED (Table 2.8). The RRF is defined as the response of the analyte of interest in relative to the reference analyte under the same analytical conditions (Equation 3). The relative response factor (RRF) is calculated to correct for differences in analyte response with an RRF value of 1 defined as optimal.

$$\text{The Relative Response Factor (RRF)} = \frac{\text{Peak Area}_{analyte}}{\text{Peak Area}_{reference}} \quad \text{- Equation 3}$$

Where $\text{Peak Area}_{analyte}$ is the peak area of the analyte of interest
$\text{Peak Area}_{reference}$ is the peak area of the reference analyte

The calculated RRF is then used to determine the corrected peak area for the corresponding analyte (Equation 4).

$$\text{Peak Area}_{Corrected} = \text{RRF} \times \text{Peak Area}_{Analyte} \quad \text{- Equation 4}$$

Where $\text{Peak Area}_{analyte}$ is the peak area of the analyte of interest
$\text{Peak Area}_{Corrected}$ is the peak area of the analyte of interest after corrected using relative response factor

Based on the similar response shown by the corticosteroids at UV wavelength 239 nm and 246 nm, it can be summarised that both wavelengths would be suitable for the study. In conclusion, a UV wavelength of 239 nm was selected for the analysis of corticosteroids in this study along with calculated RRF_{UV} to correct the peak area of each analyte measured during the HPLC analysis.

2.5.1.2 Selection of mobile phase and stationary phase

Development of the HPLC method for analysis of the corticosteroids in this study was performed under reverse phase condition with a mobile phase composed of methanol and water or acetonitrile and water. Four different internal standards were examined during the study. Dexamethasone acetate was selected initially as the internal standard along with column A as suggested by Teng *et al*., (2001). The internal standard later changed since the dexamethasone acetate degraded to dexamethasone and did not meet the requirements of internal standard. A second of internal standard, dexamethasone (≥ 98 %) from Sigma, was evaluated but it was

discarded since it elutes at a similar retention time as one of the analytes of interest, BETA. As a result, betamethasone (BETA) and mometasone (MF) were used as alternative internal standards in the study. The selection of the internal standard is discussed in later in this thesis.

Column A – C_8 Ultrasphere (150 x 4.6 mm (i.d), 5 µm (par.))

The analysis of the corticosteroid mixture using an Ultrasphere C_8 column (method A1) was based on the analytical method reported by Teng *et al*., (2001). This group reported quantification of PRED, BUD, MF, FP and BDP in plasma samples by HPLC. The mobile phase used was methanol:water (59:41 v/v) with a flow rate 1.5 mL min^{-1} and UV detector set at 239 nm. Under these conditions, the separation of corticosteroids was inefficient as four of the components co-eluted: BUD and 17-BMP co-eluted at t_R = 17.186 min and MF and FP co-eluted at t_R = 23.661 min (Figure 2.3). Thus, Column A, C_8 Ultrasphere (150 x 4.6 mm (i.d), 5 µm (par.)) was discarded due to incomplete separation of the analytes in the analysis.

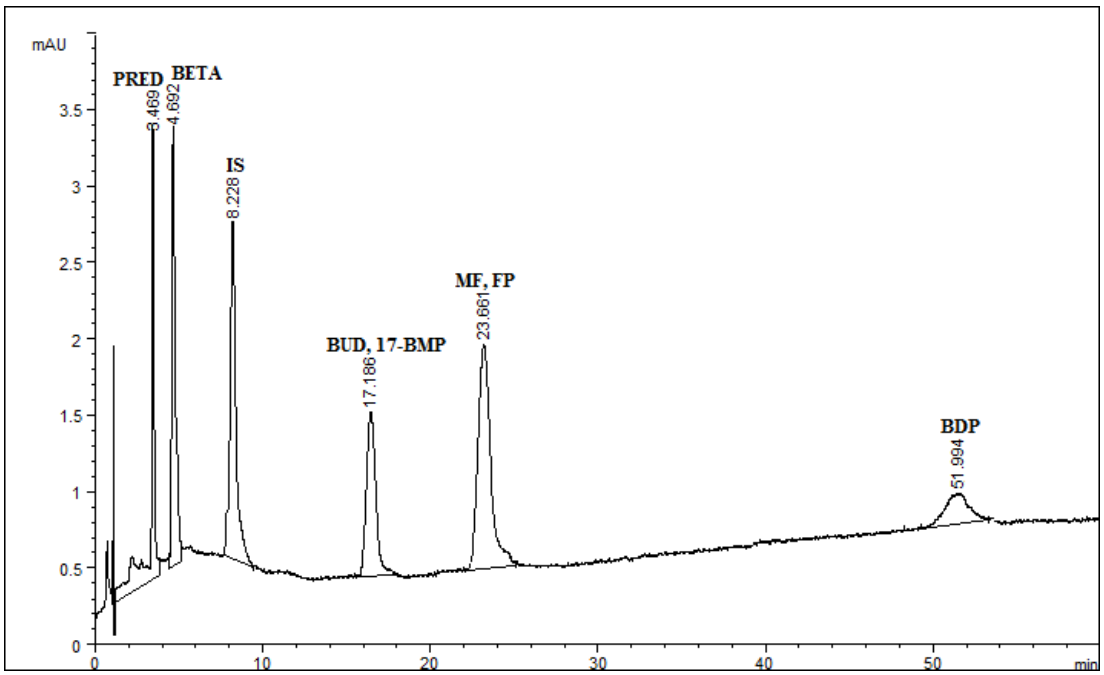


Figure 2.3 Representative chromatogram of a spiked solution (Reference Solution A5) containing corticosteroids (0.5 µg mL^{-1}) using C_8 Ultrasphere 150 x 4.6 mm (i.d), 5 µm (par.); isocratic mobile phase [methanol-water (59:41 v/v)]; Flow rate: 1.5 mL min^{-1}. Dexamethasone acetate was used as the internal standard (IS)

After the C_8 Ultrasphere column was discarded, several C_{18} columns were examined for use in this study. A stationary phase with a higher carbon load, C_{18}, is known to provide better selectivity for hydrophobic compounds than the C_8 column (Dolan *et al*., 2000). The C_{18} column has better hydrophobic retention than a C_8 column thus it helps to improve the resolution of the compounds in the mixture. It is also expected that the C_{18} column would result in a longer analysis time compared to the C_8 column.

Column B – C_{18} ACE (150 x 4.6 mm (i.d), 5 µm (par.))

A C_{18} ACE column (150 x 4.6 mm (i.d), 5 µm (par.)) was employed with a flow rate of 1.5 mL min^{-1} and the temperature maintained at 22 ± 2 °C. A C_{18} column is more

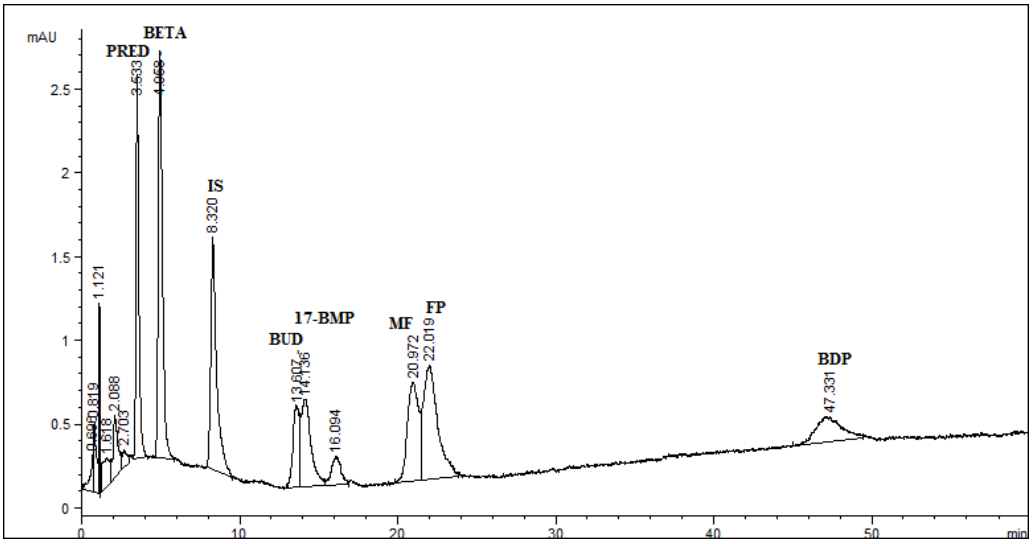
lipophilic than a C_8 column as there is more carbon present in the C_{18} column and thus it promises a better separation of hydrophobic compounds such as corticosteroids. Two different mobile phases, a mixture of methanol-water (method ISO B1) or acetonitrile-water (method ISO B2) were used in an attempt to separate the mixture of corticosteroids using the C_{18} ACE column (Table 2.9). The percentage of organic solvent used in the experiments started with methanol-water (59:41 v/v). This initial concentration was selected based on the previous HPLC conditions stated by Teng *et al.,* (2001). A different proportion of acetonitrile and water (48:52 v/v) was tried along with the mixture of methanol-water (59:41 v/v). This ratio was chosen based on the comparison of the eluent strength between two water miscible mobile phases: methanol (MeOH) and acetonitrile (ACN) based on Dolan's rule (Watson, 2005).

Table 2.9 The proportions of HPLC mobile phase for spiked solution containing corticosteroids (40 µg mL^{-1}) performed on column C_{18} ACE (150 x 4.6 mm (i.d), 5 µm (par.)); Flow rate of 1.5 mL min^{-1}

Mobile phase	**Composition (A:B)**	**Ratio (v/v)$^{\pm}$**
ISO B1	Methanol : water	59:41
ISO B2	Acetonitrile : water	48:52

$^{\pm}$organic solvent:water ratio; v/v = volume - per-volume

(a)



(b)

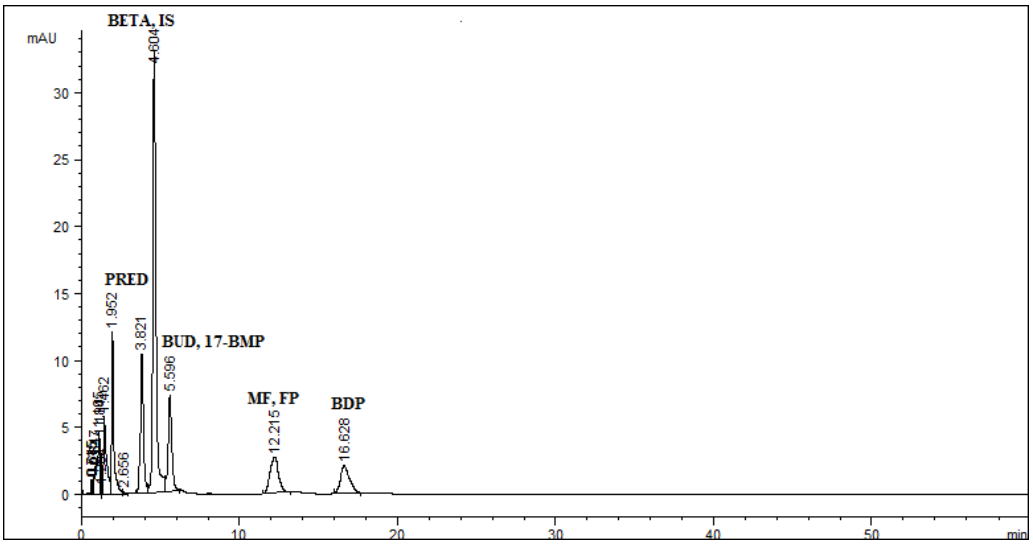


Figure 2.4 Representative chromatograms of the spiked solution containing corticosteroids (40 µg mL^{-1}) performed on C_{18} ACE column with mobile phase (a) ISO B1 and (b) ISO B2. Flow rate: 1.5 mL min^{-1}. Dexamethasone acetate was used as the internal standard

In these chromatograms the baseline was rising between 20 and 60 minutes in Figure 2.4 (a) compared to a stable baseline in Figure 2.4(b). The drifting baseline occurs when using methanol-water (method ISO B1) (Figure 2.4 (a)) due to methanol having a higher background absorbance than acetonitrile. Based on these

chromatograms, methanol-water (method ISO B1) provided better separation of the two critical pairs than acetonitrile-water (method ISO B2) even though four components of the sample still co-eluted; BUD and 17-BMP and MF and FP. To ensure complete baseline separation of the all compounds, the resolution (Rs) should be calculated. Resolution is defined as the separation between two peaks in terms of the peak width (Equation 5) and should be equal to or greater than 1.5 for baseline separation (BP, 2008).

Resolution, Rs, is defined as,

$$Rs = \frac{1.18\,(t_{R2}-t_{R1})}{(\text{Peak width}_{\text{peak 1(0.5)}} + \text{Peak width}_{\text{peak 2(0.5)}})} \qquad \text{- Equation 5}$$

where Rs is the resolution
t_{R1} is retention time of the first peak
t_{R2} is retention time of the peak after first peak
Peak width $_{\text{peak 1(0.5)}}$ is the width of peak 1 at half peak height
Peak width $_{\text{peak 2(0.5)}}$ is the width of peak 2 at half peak height

The resolutions for all compounds used in the current experiment are shown in Table 2.10. From these calculations it can be seen that the resolution of two critical pairs, between BUD and 17-BMP and between MF and FP were below 1.5 in both mobile phases. The resolution of these critical pairs was better with methanol-water (method ISO B1) than with acetonitrile-water (method ISO B2). The resolution between the two critical pairs; BUD and 17-BMP and MF and FP were reported as Rs = 0.80 and 0.94, respectively with methanol-water as mobile phase compared to 0 with acetonitrile-water as the mobile phase.

Table 2.10 Resolution of the compounds performed on column B, C_{18} ACE column with mobile phase (a) methanol-water (ISO B1) and (b) acetonitrile-water (ISO B2). Flow rate: 1.5 mL min^{-1}

Compound	**Resolution, Rs**	
	Methanol:water (59:41 v/v) ISO B1	**Acetonitrile:water (48:62 v/v) ISO B2**
PRED	-	-
BETA	5.67	4.25
IS$^{\infty}$	9.69	0
BUD	11.59	4.75
17-BMP	0.80	0
MF	7.76	15.47
FP	0.94	0
BDP	14.36	6.94

$^{\infty}$Dexamethasone acetate was used as the internal standard (IS).

While the capacity factor, k' helps to measure the retention of each analyte relative to the retention time of the unretained compound (Equation 6) (BP, 2008). The longer the analyte is retained, the greater the capacity factor. The optimum k' should be between 2 and 10 (Watson, 2005). This optimal range helps to keep the peak of the analytes away from the dead volume and improves selectivity.

The value of k' or capacity factor is defined as:

$$k' = \frac{t_R - t_o}{t_o} \quad \text{- Equation 6}$$

where k' is the capacity factor
t_R is retention time of the peak of interest
t_o is the time taken for an unretained molecule to pass through the void volume.

Analysis with the isocratic method ISO 2a on column B, C_{18} ACE (150 x 4.6 mm (i.d), 5 µm (par.)) with a flow rate of 1.5 mL min^{-1} provided the optimal retention factor (k') for the first three eluting compounds (PRED, BETA, IS) (Table 2.11). BDP showed the greatest retention (k' = 41.22) and a broad peak as the retention time increased.

Table 2.11 The capacity factor of a spiked solution containing corticosteroids (40 µg mL^{-1}) performed on column B, C_{18} ACE (150 x 4.6 mm (i.d), 5 µm (par.)) with isocratic mobile phase, ISO B1 [methanol-water (59:41 v/v)]

Compound	Capacity factor, k'
PRED	2.41
BETA	3.42
IS	6.42
BUD	11.13
17-BMP	11.61
MF	17.71
FP	18.64
BDP	41.22

* The initial retention time, t_0 was 1.121 min

The order of elution was identical for both methods but the retention times were different. Based on the better resolution of the critical pair of analytes MF and FP, with method ISO B1 [methanol-water (59:41 v/v)] compared to method ISO B2

[acetonitrile-water (48:62 v/v)], methanol-water was considered to be the most suitable mobile phase for further method optimisation.

In isocratic elution using a mobile phase of methanol-water (59:41 v/v), the two critical pairs of BUD and 17-BMP and MF and FP started to resolve as four separate peaks using the C_{18} ACE (150 x 4.6 mm (i.d), 5 µm (par.)) column though the retention time is long (47.33 mins). This prompted consideration of gradient elution to optimise separation of the analytes and lower the overall analysis time.

A gradient programme, GRAD B3 employing methanol-water was performed on the C_{18} ACE column (Table 2.12), however, this method (GRAD B3) failed to separate the critical pair (MF and FP) (Rs = 0.95) despite baseline separation of BUD and 17-BMP (Rs = 3.64) (Figure 2.5). The peak at 7.561 min was later identified as an impurity of the IS (dexamethasone acetate). The length of the gradient method was increased from 40 to 55 minutes (GRAD B4) to improve the resolution of overlapping peaks but failed to separate MF and FP.

Table 2.12 Gradient programme used with C_{18} ACE column in the chromatographic analysis of a spiked solution containing corticosteroids (40 µg mL^{-1})

Method	Flow rate (mL min^{-1})	Temp (ºC)	Time (min)	% MeOH	% water
GRAD B3	1.5	22 ± 2	0	55	45
			6	55	45
			8	59	41
			22	61	39
			35	70	30
			40	55	45
GRAD B4	1.5	22 ± 2	0	55	45
			6	55	45
			8	59	41
			45	61	39
			50	70	30
			55	55	45

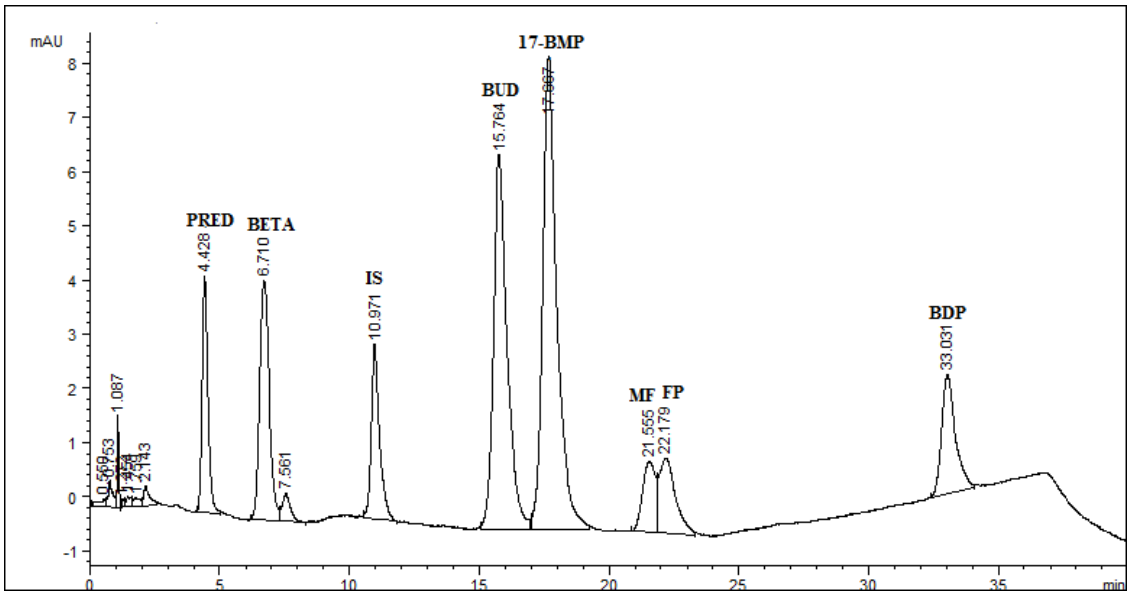


Figure 2.5 Representative chromatogram of a spiked solution containing corticosteroids (40 µg mL^{-1}) performed on C_{18} ACE column with gradient mobile phase (methanol-water) (method GRAD B3). Dexamethasone acetate was used as the internal standard (IS)

Thus, due to incomplete baseline resolution between the compounds of interest, MF and FP (Rs = 0.95) (method GRAD B3), the C_{18} ACE column (150 mm x 4.6 mm

(i.d), 5 µm (par.)) was discarded as the appropriate HPLC column for the analysis of corticosteroids in plasma.

Column C – C_{18} Hypersil BDS (100 mm x 4.6 mm (i.d), 5 µm (par.))

Two different lengths of C_{18} column were tested after the C_{18} ACE column failed to show complete separation of the analytes: C_{18} Hypersil BDS (100 mm x 4.6 mm (i.d), 5 µm (par.)) and C_{18} Kromasil (250 mm x 4.6 mm (i.d), 5 µm (par.)), (Column D). The C_{18} Hypersil BDS is known to have moderate silanol activity due to low metal contamination on the surface of the stationary phase and this property helps to improve peak shape of basic analytes. Initially, the C_{18} Hypersil BDS was tried using the same isocratic mobile phase composition [methanol-water (59:41 v/v)] (method ISO C1) as method ISO B1 using C_{18} ACE. The flow rate was changed from 1.5 mL min^{-1} to 1 mL min^{-1} and the temperature maintained at 22 ± 2 °C. The column using methanol-water (59:41 v/v) succeed in separating BUD and 17-BMP (Rs = 2.29) but still lacked of complete separation of MF and FP (Rs = 1.47) (Figure 2.6). PRED and BETA were observed at 2.64 min and 3.37 min which might cause the problem of coelution with biological interferences in plasma which elute at 0 to 4 minutes in a typical HPLC analysis.

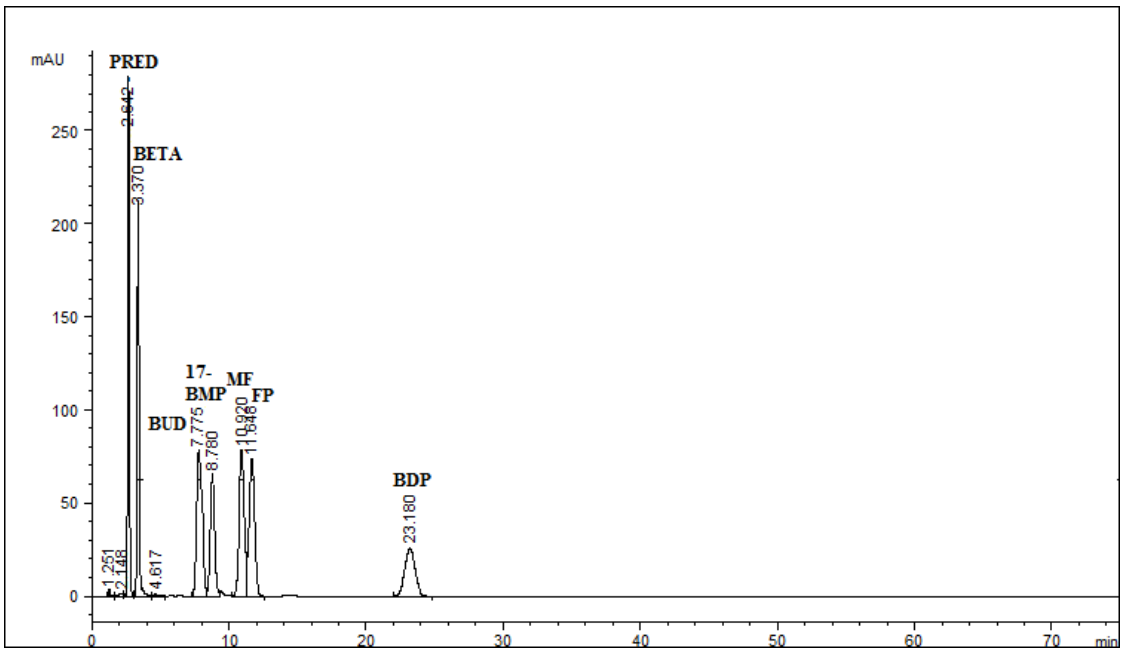


Figure 2.6 Representative chromatogram of spiked solution corticosteroids (40 µg mL^{-1}) performed on C_{18} Hypersil BDS column with isocratic mobile phase [methanol-water (59:41 v/v)] (ISO C1)

The method ISO C1 for C_{18} Hypersil BDS was later improved to ISO C2 using the isocratic methanol-water (55:45 v/v) with a flow rate of 1 mL min^{-1} with the aim to avoid early elution of the first two compounds (PRED and BETA) but this failed.

The isocratic method (ISO C2) for C_{18} Hypersil BDS was modified to the gradient method GRAD C3 starting with methanol-water at 50:50 v/v (Table 2.13).

Table 2.13 Gradient method used with C_{18} Hypersil BDS column in the chromatographic analysis of a spiked solution containing corticosteroids (40 µg mL^{-1})

Method	Flow rate (mL min^{-1})	Temp (ºC)	Time (min)	% MeOH	% water
GRAD C3	1	22 ± 2	0	50	50
			25	50	50
			65	55	45
			75	57	43
			80	50	50
GRAD C4	1	22 ± 2	0	50	50
			25	50	50
			50	55	45
			75	57	43
			85	59	41
			90	50	50

Using method GRAD C3 and the C_{18} Hypersil BDS, the first two early eluting compounds, PRED and BETA eluted at 5.38 min and 8.59 min, respectively, which is outside the range of biological interferences (0 – 4 min). BUD was observed as a split peak using method GRAD C3 (Figure 2.7). The split peak of BUD was identified as the two epimers of BUD, 22-R BUD and 22-S BUD, which have similar anti-inflammatory properties (BP, 2008). However, the method GRAD C3 failed to improve the resolution between MF and FP. Further improvement in the gradient mobile phase in method GRAD C3 with the C_{18} Hypersil BDS known as method GRAD C4 (Table 2.13) showed baseline separation for all compounds but lacked separation of critical pair, MF and FP.

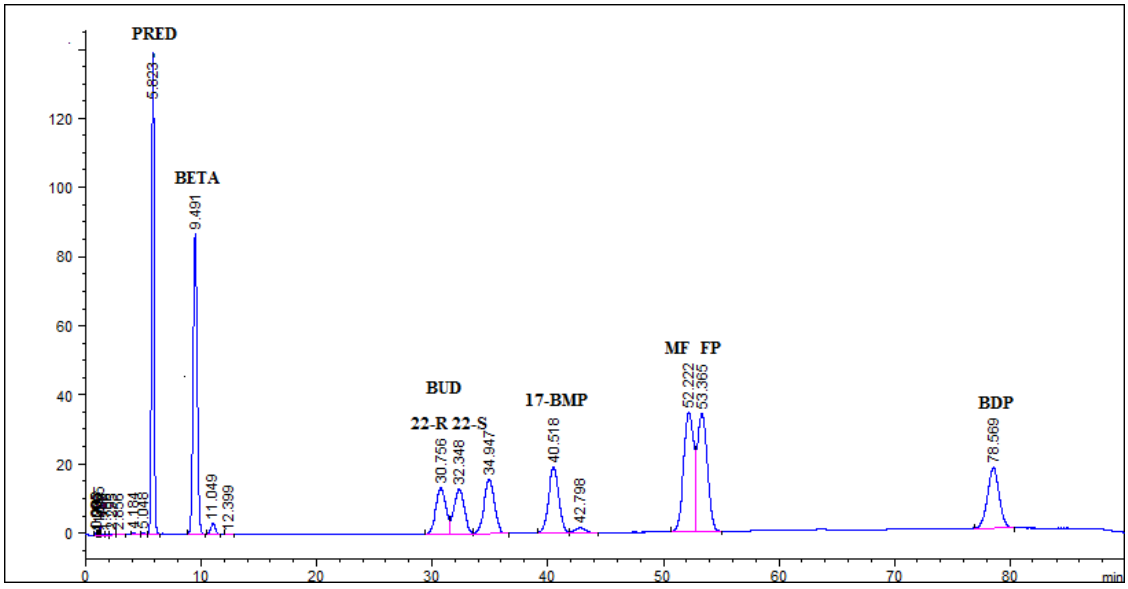


Figure 2.7 Representative chromatogram of a spiked solution containing corticosteroids (40 µg mL^{-1}) performed on C_{18} Hypersil BDS column with gradient mobile phase (methanol-water) (method GRAD C3)

It was observed (Figure 2.7) in the chromatogram of the corticosteroid mixture run using method GRAD C4 that there were unknown peaks at t_R = 34.94 min. The unknown peaks were identified as degradants of BDP after individual analysis of a BDP run using method GRAD C4 (Figure 2.8). Based on this BDP degrades into two unknown compounds at t_R = 35.44 min and t_R = 41.06 min.

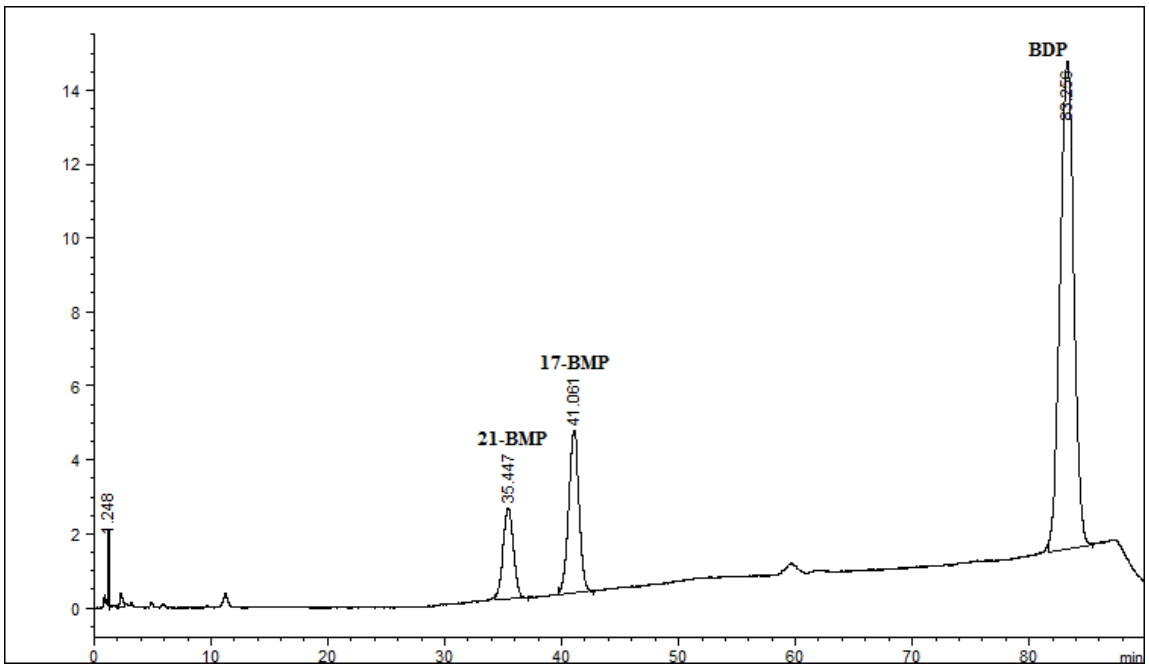


Figure 2.8 Representative chromatogram of BDP (40 µg mL^{-1}) performed on the C_{18} Hypersil column with the gradient mobile phase (methanol-water) (method GRAD C4)

This indicated the instability of BDP in the solvent when stored at room temperature and exposed to light.

The analysis was repeated on LC-MS using the C_{18} Hypersil column (100 x 4.6 mm (i.d), 5 µm (par.)) with an isocratic mobile phase [methanol-0.1 % formic acid in water (62:38 v/v)] (Figure 2.9). The mass spectrometer (MS) detector settings were.

Ion source	: Electrospray ionisation (ESI) mode
Polarity	: Positive
Capillary temperature	: 220 °C
Sheath gas flow	: 60 psi
Auxillary valve flow	: 20 psi
Source voltage	: 4.5 kV

Based on these results (Figure 2.9), the mass spectrum showed that the first degradant peak with retention time of 7.64 min had a *m/z* of *465.05* and the second degradant with a retention time of 6.95 min had a *m/z* of *465.04*. Thus the peaks were identified as beclometasone-17-monopropionate (17-BMP) and beclometasone-21-monopropionate (21-BMP).

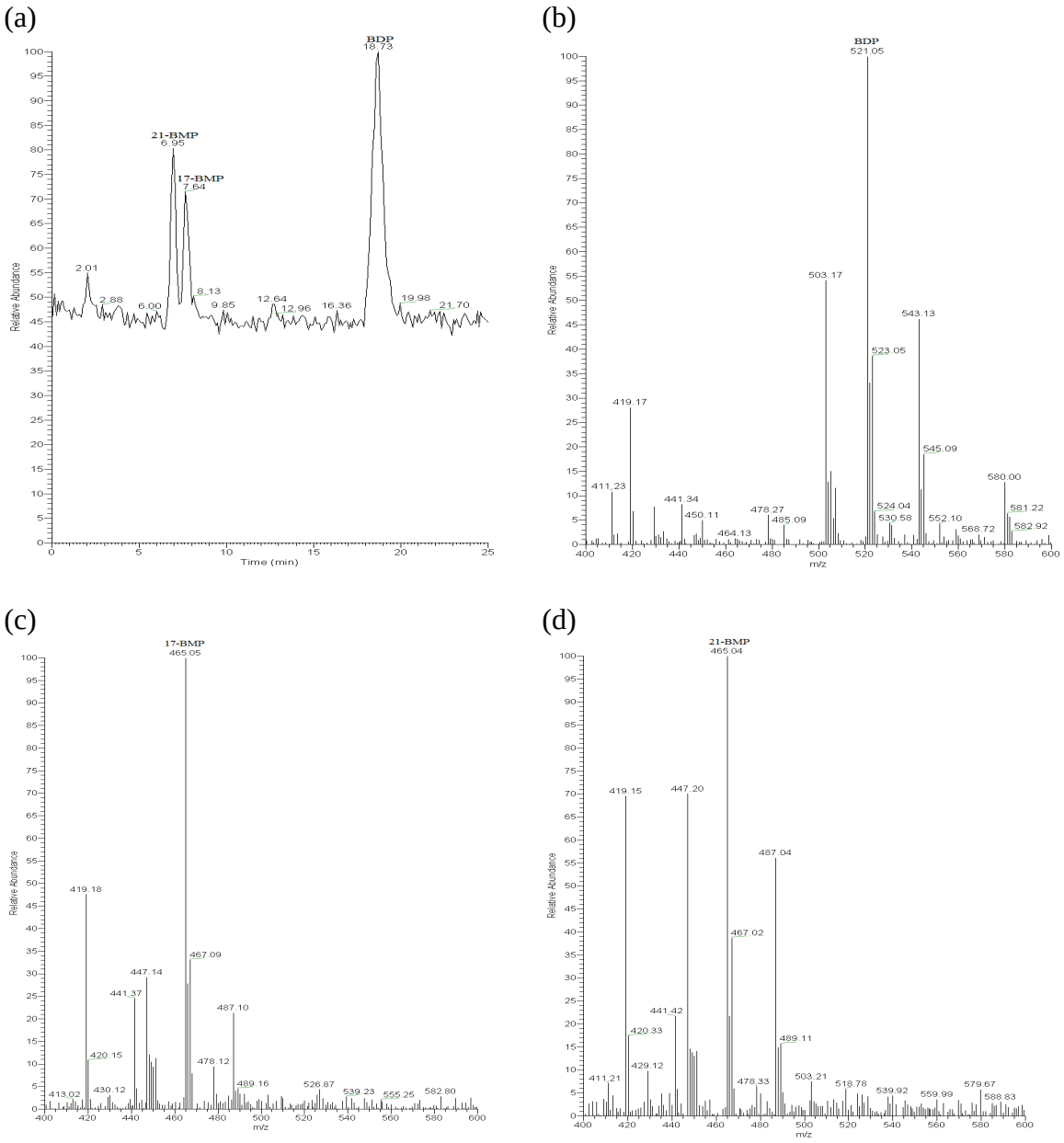


Figure 2.9 Representative chromatographic data of BDP and its degradants using the C_{18} Hypersil column (100 x 4.6 mm (i.d), 5 µm (par.)); [methanol – 0.1 % formic acid in water (62:38 v/v)]; Flow rate of 1.0 mL min^{-1}. (a) Chromatogram of beclometasone dipropionate (t_R = 18.73 min) with its degradation product at t_R = 6.95 min and 7.64 min. Mass spectra of the (b) BDP; (c) 17-BMP and (d) 21-BMP

Since, BDP was proven to degrade in the presence of laboratory lighting and at room temperature, therefore, the BDP stock solution was freshly prepared every week and

protected from light to reduce risk of photodegradation (Kaur *et al*., 2010; Lin *et al*., 2009).

Since the C_{18} Hypersil BDS did not meet the minimum required resolution for separation of the analytes in the mixtures the C_{18} Hypersil BDS was excluded from further analysis.

Column D – C_{18} Kromasil (250 mm x 4.6 mm (i.d), 5 µm (par.))

Using a longer column is believed to solve the resolution problem with increase in the number of theoretical plates but it may result in a longer analysis time (Dong, 2006). After failing to separate MF and FP using the C_{18} (100 – 150 mm) column, a 250 mm C_{18} Kromasil was chosen as it has low silanol activity which ensures column efficiency and it is a longer column to optimise the resolution. Isocratic elution using the mobile phase [methanol-water (59:41 v/v)] (method ISO D1) with a flow rate of 1 mL min^{-1} and temperature of 25 °C; was tested and led to successful baseline separation of all components though the analysis time was long at 150 mins. The method was modified using the computer simulation program, Dry Lab® software (Molnar-Institute, Berlin, Germany) to optimise the resolution of the analytes and avoid the long analytical time (150 minutes).

According to the Dry Lab® software, the optimum isocratic mobile phase was methanol-water (62:38 v/v). Using this isocratic mobile phase [methanol-water (62:38 v/v)] provided separation of the two critical pairs and resulted in BDP eluting at 102 min but the BUD appeared as one peak (Figure 2.10). As BUD is a

distereoisomer there should be two peaks therefore this method did not show complete resolution of the peaks.

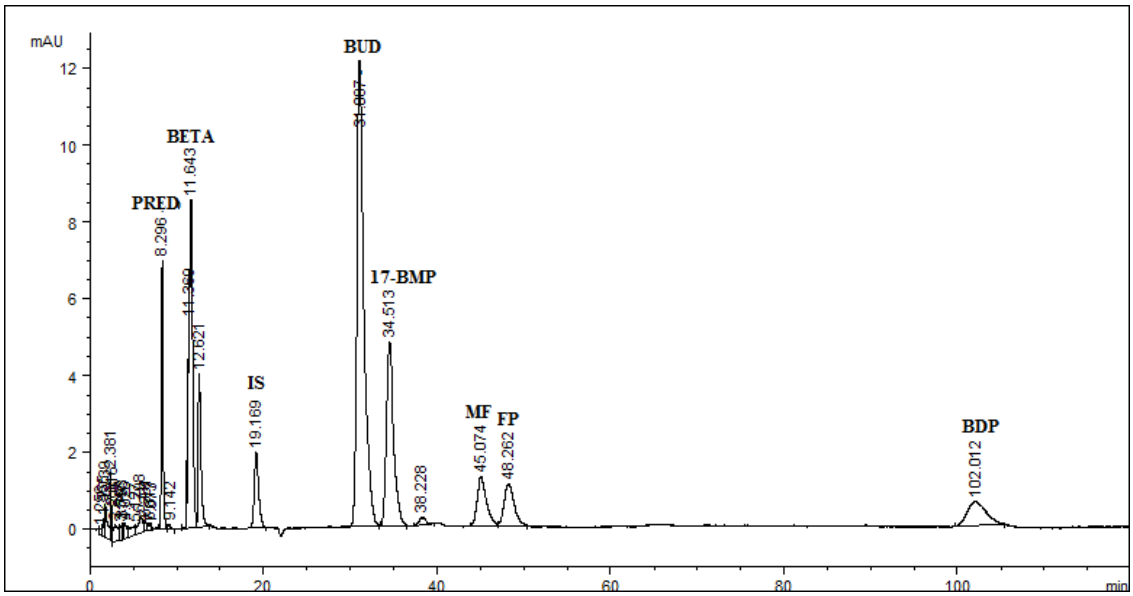


Figure 2.10 Representative chromatogram of a spiked solution containing corticosteroids (40 µg mL^{-1}) performed on the C_{18} Kromasil column with an isocratic mobile phase [(methanol-water (62:38)] (method ISO D2). Flow rate: 1 mL min^{-1}. UV detector: 239 nm. Dexamethasone acetate was the IS

To reduce the long HPLC running times and to achieve baseline resolution on all analytes particularly the epimers of BUD, the isocratic method was optimised. Employing the gradient programme (GRAD D3) (Table 2.14) which started with methanol-water (62:38 v/v) using the C_{18} Kromasil column which ran for 85 minutes promising results with mild separation of the budesonide epimers (Rs = 0.67) was achieved while other analytes showed complete baseline separation.

Table 2.14 Gradient programme used with the C_{18} Kromasil column in the chromatographic analysis of spiked solution containing corticosteroids (40 µg mL^{-1})

Method	Flow rate (mL min^{-1})	Temp (ºC)	Time (min)	% MeOH	% water
GRAD D3	1	25	0	62	38
			60	62	38
			80	80	20
			85	62	38

GRAD = gradient; MeOH = methanol

The British Pharmacopoeia (BP) and the European Pharmacopoeia (EP) report a requirement for resolution of the budesonide (BUD) epimers to be equal to or more than 1.5, that the number of theoretical plates from the epimer R (first peak) should be at least 4000 and the symmetry factor of the same peak should be less than 1.5 for the analytical method for budesonide to be accepted (BP, 2008; EP, 2008). The chromatographic procedure outlined by the BP (2008) was an HPLC column with the dimensions 120 x 4.6 mm, 5 µm and acetonitrile-phosphate buffer (pH 3.2) (32:68 v/v) as the mobile phase. Reduction of the initial composition of the mobile phase, methanol-water (62:38) to methanol-water (57:43) in the gradient programme GRAD D4 (Table 2.15) resulted in baseline separation for all the components including the epimers of BUD (Figure 2.11). The resolution between the critical pair, MF and FP, was greater than 1.5 (Rs = 3.58) using the C_{18} Kromasil column which indicated optimal separation of the analytes. The presence of two symmetrical peaks at t_R = 55.19 and t_R = 57.81 minutes was explained by the epimeric mixture of budesonide. Budesonide eluted as two identical peaks with resolution (Rs) = 2.15 which indicated baseline separation and adequately meets the other requirements of number of theoretical plates and the symmetry factor (As) of epimer R: the apparent number of

theoretical plates (N) of 43050 and As = 0.9214. The peak at t_R = 60.68 is a degradant of BDP, 21- BMP. The peak at t_R = 73.82 and t_R = 20.51 minutes were not identified during this study.

Table 2.15 Gradient programme used with the C_{18} Hypersil BDS column in the chromatographic analysis of a spiked solution containing corticosteroids (40 µg mL^{-1})

Method	Flow rate (mL min^{-1})	Temp (ºC)	Time (min)	% MeOH	% water
GRAD D4	1	25	0	57	43
			55	57	43
			70	62	38
			87	80	20
			100	57	43
			110	57	43

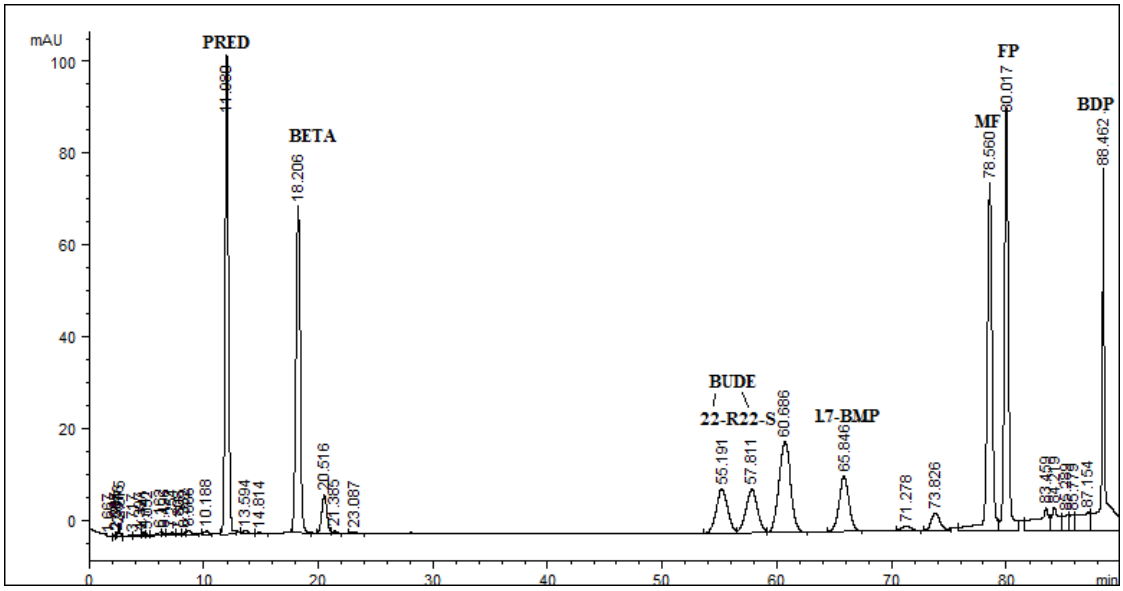


Figure 2.11 Representative chromatogram of a spiked solution containing corticosteroids (40 µg mL^{-1}) performed on the C_{18} Kromasil column with the gradient mobile phase (methanol-water) (method GRAD D4)

The programme, GRAD D4 (C_{18} Kromasil (250 x 4.6 mm (i.d), 5µm (par.)); gradient mobile phase using methanol-water at 25 °C with a flow rate of 1 mL $min^{-1}$and the

UV detector set at 239 nm was chosen to be the most appropriate HPLC method for the analysis of a corticosteroid mixture in solution. A guard column with a similar bonded phase as the stationary phase was connected between the analytical column and the injection valve to preserve the column life.

2.5.1.3 Selection of internal standard

Four compounds were evaluated as possible internal standards for HPLC analysis of corticosteroids in biological fluids.

2.5.1.3.1 Dexamethasone acetate

For a substance to be chosen as an internal standard, it should elute near to the analyte of interest, be stable and be distinguishable from the other analytes. Several studies have used a structural analogue as the internal standard for quantification of corticosteroids. These structural analogues have included betamethasone (Deventer *et al.*, 2006), fluticasone propionate (Wang & Hochhaus, 2004), an epimer of budesonide acetate (Li *et al.*, 1997) and dexamethasone acetate (Teng *et al.*, 2001). Dexamethasone acetate was adopted as the initial internal standard since this was the internal standard used in the method developed by Teng *et al.,* (2001) which was chosen as the starting point for method development. Dexamethasone acetate is available in high purity (99 %) from Sigma Aldrich. During the chromatographic run on the Kromasil C_{18} column (250 x 4.6 mm, 5 µm) with an isocratic mobile phase [methanol-water (62:38 v/v)] (method ISO D2), dexamethasone-21-acetate was shown to elute at t_R = 29.83 min (Figure 2.12). An unknown peak at t_R = 12.27 min in the chromatogram was later characterised as a degradation product,

dexamethasone based on the mass spectra (*m/z* of *393.62*) following LC - MS analysis (Figure 2.13).

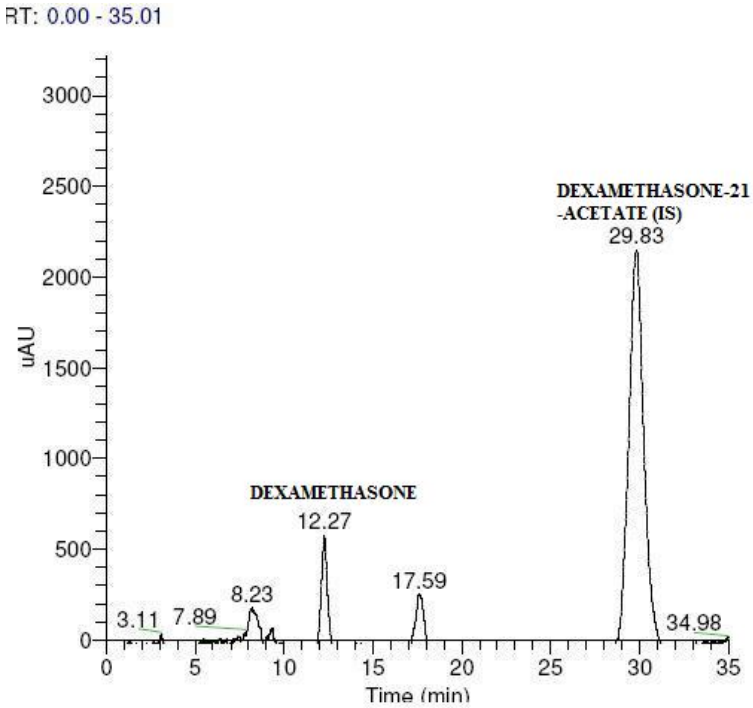


Figure 2.12 Representative chromatogram of a solution spiked with dexamethasone-21-acetate which eluted at t_R = 29.8 min and dexamethasone which eluted at t_R = 12.2 min using a Kromasil C_{18} column (250 x 4.6 mm (i.d), 5 µm (par.)) at 25 °C with an isocratic mobile phase [methanol-water (62:38 v/v)] (method ISO 4b); Flow rate: 1 mL min^{-1}

The mass spectrometry (MS) detector settings were as follows:

Ion source	: Electrospray ionisation (ESI) mode
Polarity	: Positive
Capillary temperature	: 220 °C
Sheath gas flow	: 60 psi
Auxillary valve flow	: 20 psi
Source voltage	: 4.5 kV

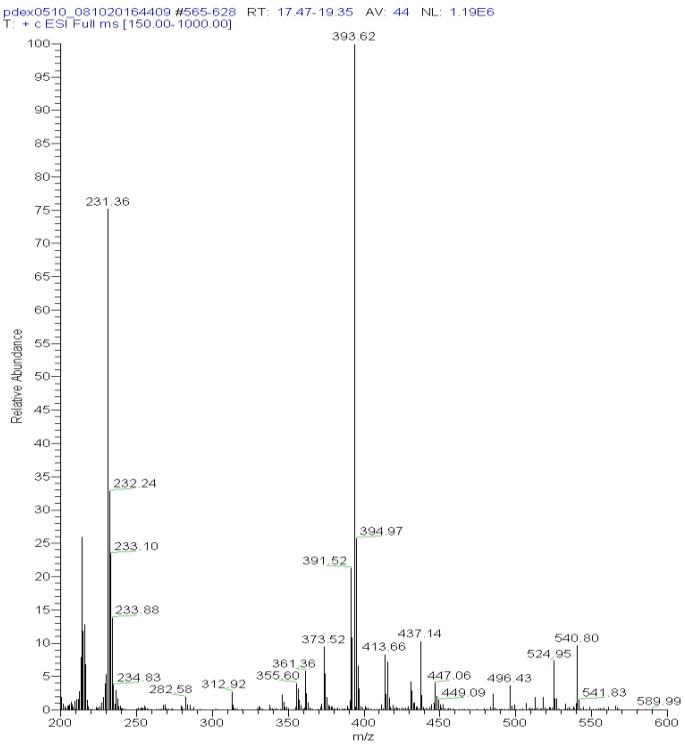


Figure 2.13 Representative mass spectra of the dexamethasone-21-acetate degradation product dexamethasone *(m/z 393.62)*

In less than seven days, dexamethasone acetate degraded by 30 – 40 % to dexamethasone (Figure 2.14) this was shown by a gradual increase in peak area of dexamethasone over time (Figure 2.15).

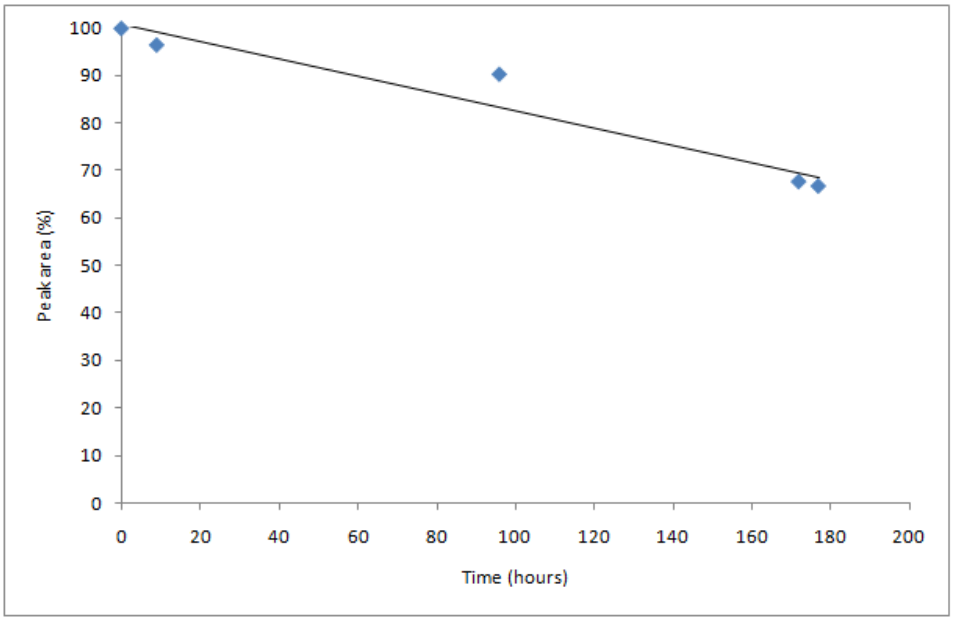


Figure 2.14 The degradation of dexamethasone-21-acetate over time using a Kromasil C_{18} column (250 x 4.6 mm (i.d), 5 µm (par.)) at 25 °C with an isocratic mobile phase [methanol-water (62:38 v/v)] (method ISO D2)

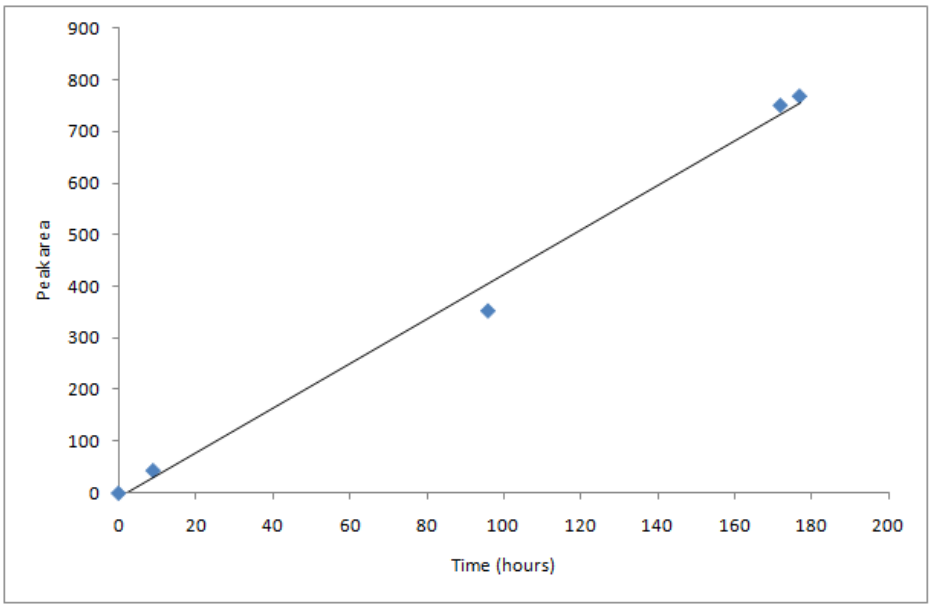


Figure 2.15 The peak area of dexamethasone, the degradant of dexamethasone-21-acetate using the Kromasil C_{18} column (250 x 4.6 mm (i.d), 5 µm (par.)) at 25 °C with an isocratic mobile phase [methanol-water (62:38 v/v)] (method ISO D2)

The instability of dexamethasone-21-acetate in solution at ambient lab temperature was considered to be a disadvantage and due to the poor stability dexamethasone-21-acetate was discarded as an internal standard.

2.5.1.3.2 Dexamethasone

Since the internal standard should share similar physicochemical properties with the other corticosteroids of interest, be stable and should not be present in the original sample, dexamethasone, one of degradation products of dexamethasone acetate was chosen as the next option for the internal standard in this study. In the HPLC analysis performed on the Kromasil C_{18} column (250 x 4.6 mm, 5µm) using the isocratic mobile phase, methanol-water (62:38) (method ISO D4), dexamethasone eluted at the same time as betamethasone (Figure 2.16). This is explained by similarities in the chemical structures of dexamethasone and betamethasone. Dexamethasone is an epimer of betamethasone with only difference in stereochemical configuration of the methyl group at position C-16 (Figure 2.17). An epimer is a diastereoisomer that differs in configuration at one stereogenic centre. Diastereoisomers are isomers that are not mirror images of each other and have more than one chiral centre. A similar result was found in using methanol-water as the mobile phase in research carried out by Santos-Montes *et al.*, (1994).

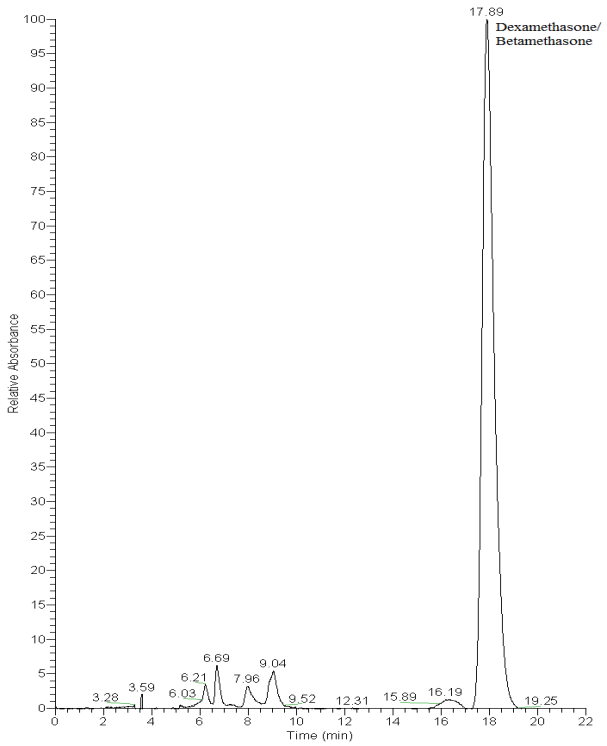


Figure 2.16 Representative chromatogram of dexamethasone and betamethasone which coeluted at t_R = 17.89 min. The analysis was run on a C_{18} Kromasil column (250 x 4.6 mm (i.d), 5 µm (par.)) with an isocratic mobile phase [methanol-water (62:38 v/v)]

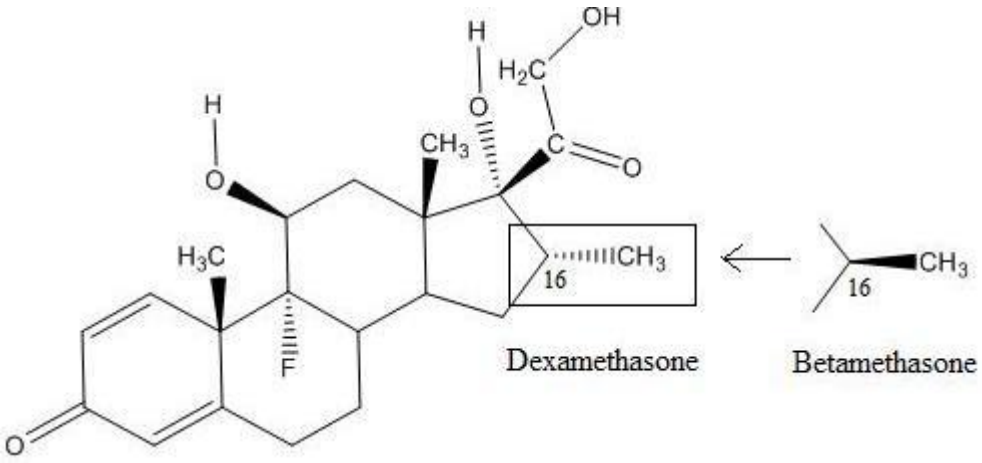


Figure 2.17 Dexamethasone and betamethasone share a similar chemical structure: the only difference is at position C-16 (as shown)

Many studies (Taylor *et al.*, 2004; Arthur *et al.*, 2004; Van den Hauwe *et al.*, 2001) have used mass spectrometry detection to distinguish between betamethasone and

dexamethasone while Luo *et al.,* (2005) used a isocratic buffered mobile phase [acetonitrile – water - formic acid (95:5:0.5 v/v)] to improve separation of the two epimers. Fu *et al.,* (2010) used a different approach by increasing the column temperature to 50 – 55 °C to separate the betamethasone, dexamethasone and related substances on a C_{18} ACE column with a gradient mobile phase consisting of 0.1 % methanesulfonic acid in an aqueous solution and a mixture of *tert*-butanol and 1,4-dioxane (7:93 v/v). Excellent separation of these epimers was also achieved using HPLC-UV by changing methanol-water to tetrahydrofuran (THF)-water (Xiao *et al.*, 2008; Santos-Montes *et al.,* 1994). We did not investigate substituting THF for methanol to optimise the separation in this study as THF may damage the HPLC column by forming peroxides with air and extracting additives by interacting with plastics used for tubing, seals and filters.

Thus, it was decided that dexamethasone was not a suitable internal standard for this corticosteroid analysis since it co-elutes with one of the analytes of interest and therefore does not meet the requirements of an internal standard.

2.5.1.3.3 Mometasone and Betamethasone

After screening of the two corticosteroids (dexamethasone and dexamethasone -21-acetate) as an internal standard, it was concluded that it was difficult in a limited time to obtain a proper structural analogue as an internal standard which yielded a completely resolved peak, mimics the analyte in any preparation steps and is present at the same concentration as the measured analyte. Based on the substance availability and time constraints, two of the analytes used in the study were used as

internal standards. It is common to use more than one internal standard when there are multiple analytes of interest (Taylor *et al.*, 2004).

Both the corticosteroids chosen, betamethasone and mometasone, could be present in the samples in this study depending on what the patient had been prescribed by the physician. After screening and considering the clinical data, none of the patients were prescribed both of these corticosteroids. Betamethasone has a retention time of 17 min while the retention time of mometasone is 78 minutes using the Kromasil C_{18} column (250 x 4.6 mm (i.d), 5 µm (par.)) with a gradient mobile phase (method GRAD D4). Both proposed internal standards elute near to the compounds of interest (Table 2.16).

Table 2.16 The relative response factor (RRF_{IS}) of Reference Solution A1 spiked with the internal standards at concentration of 0.5 µg mL^{-1}

Internal standard*	**Relative Response Factor (RRF)**						
	PRED	**BETA**	**BUD**	**17-BMP**	**MF**	**FP**	**BDP**
Betamethasone (BETA)	0.6685	1.0000	0.3591	0.4933	0.2292	0.2377	0.1708
Mometasone (MF)	1.4442	1.2703	0.5486	0.7534	1.0000	0.5960	0.5711

Thus, betamethasone and mometasone both meet the requirements of an internal standard for this study. The choice of internal standard to be added to the plasma in the analysis depends on the corticosteroids taken by the patients

2.5.2 Analysis of plasma samples

2.5.2.1 Liquid –liquid extraction (LLE)

Liquid-liquid extraction of a plasma sample spiked with the corticosteroid mixture (5 µg mL^{-1} – 40 µg mL^{-1}) was carried out in triplicate using the final method as described. The plasma samples were extracted into ethyl acetate twice and the combined extracts concentrated and then reconstituted in mobile phase before analysis by HPLC. The mean recovery of corticosteroids was expressed as a percentage with relative standard deviation (% RSD) (Table 2.17).

Table 2.17 Recovery of corticosteroids from plasma using liquid–liquid extraction (n = 3).HPLC conditions: C_{18} Kromasil column (250 x 4.6 mm (i.d), 5 µm (par.)); gradient mobile phase GRAD D4; detector wavelength: 239 nm; flow rate: 1 mL min^{-1}

Analyte	**Mean Recovery (% RSD)**			
	40 ug mL^{-1}	**20 ug mL^{-1}**	**10 ug mL^{-1}**	**5 ug mL^{-1}**
PRED	88.46 (4.49)	66.99 (9.99)	77.17 (4.18)	43.48 (8.49)
BETA	89.10 (3.68)	79.72 (9.61)	83.36 (2.22)	42.21 (12.37)
BUD	86.16 (3.93)	68.39 (11.05)	69.78 (4.33)	26.58 (15.82)
MF	67.28 (8.54)	58.94 (16.66)	77.84 (3.77)	50.20 (12.78)
FP	83.66 (4.92)	92.95 (3.78)	78.69 (2.34)	37.15 (11.53)
BDP	86.63 (39.42)	101.53 (11.42)	114.84 (5.59)	40.20 (7.03)

No endogenous interference from human plasma was observed after extraction. The mean extraction efficiency of each corticosteroid varied from 26.58 – 114.84 %. The precision of the procedure varied over the concentration range and gave an RSD of (≤16 %) in most cases except for BDP (39.4 %) at the highest concentration. The recovery of 17-BMP was not calculated since 17-BMP is a degradation product of BDP thus this might result in overestimation of the 17-BMP concentration. However, it might be possible to measure the recovery of 17-BMP in an individual analysis of this analyte. Based on these findings, LLE was ruled out as the sample clean up method for this study due to high variability in recovery.

2.5.2.2 Solid-phase extraction (SPE)

2.5.2.2.1 SPE Method 1

Only three corticosteroids, PRED, BETA and BUD, were analysed using SPE method 1. The plasma samples were loaded onto a Strata C_{18} cartridge and washed twice with two different solvent before extraction from the cartridge by a combination of ethyl acetate-heptane (35:65 v/v). The sample was later concentrated and reconstituted with the mobile phase before HPLC analysis. The Strata C_{18} cartridge showed an extraction efficiencie of 83 – 162 % after sample preparation using SPE method 1 (Table 2.18). The recovery BUD which is greater than 100 % indicates that SPE method 1 may be introducing contaminants or extracting interferants along with the analytes of interest.

Table 2.18 Mean recovery (%) of corticosteroids from human plasma after SPE method 1 at 40 µg mL^{-1} with ethyl acetate: heptane (35:65) as the eluting solvent. HPLC condition: C_{18} Kromasil column (250 x 4.6 mm (i.d), 5 µm (par.)); gradient mobile phase GRAD D4; detector wavelength: 239 nm; flow rate: 1 mL min^{-1}

Analyte	**Recovery (% RSD)**
	40 µg mL^{-1} (n = 4)
PRED	82.79 (5.91)
BETA	90.90 (5.31)
BUD	161.91 (0.11)

Based on these results, SPE Method 1 (Li *et al.*, 2001) was excluded as the sample pre-treatment method for corticosteroids in plasma since it showed unreasonable recovery.

2.5.2.2.2 SPE Method 2

An alternative sample clean up method using a combination of methanol and water as the main extraction solvent was investigated. Corticosteroids are known to be sparingly soluble in alcohol and insoluble in water (BP, 2008). The plasma was loaded onto the Strata C_{18} cartridge at a flow rate of 1 drop/second. The flow rate was carefully monitored as it has been reported that extraction efficiency is affected by flow rate (Palma *et al*., 2002). Additionally the effect of "*drying time*" was investigated and it was determined that if the packing bed was dried for an extra of 15 seconds, the recovery of the corticosteroids from plasma was increased by 10 % or more (Table 2.19). Based on these results, a drying period of exactly 150 seconds was optimal in achieving required recoveries. The precision (% RSD) in this experiment was not calculated since there only two runs per analyte (n = 2).

Table 2.19 The extraction efficiency of solid-phase extraction (SPE method 2) at different drying times after loading step. HPLC conditions: C_{18} Kromasil column (250 x 4.6 mm (i.d), 5 µm (par.)); gradient mobile phase GRAD D4; detector wavelength:239 nm; flow rate: 1 mL min^{-1}

Analyte	Drying time (seconds)[±]	
	135*	**150***
PRED	96.92	106.58
BETA	95.65	106.64
BUD	71.55	96.42

[±]Corticosteroid plasma solution (20 µg mL^{-1})
* The results are reported as mean % recovery (n = 2)

Since corticosteroids are known to be poor miscibility with water, a solvent containing a high aqueous percentage was believed to elute all polar impurities but not the target compounds. Water was used as the first washing step followed by a subsequent washing with a combination of methanol-water (1:9 ratio). Two wash steps are recommended with the first wash to remove the fraction of polar analytes that are less retained to the sorbents and the second wash to discard any relatively polar steroids that are partly miscible in alcohol without removing the desired analytes. A higher percentage of methanol was then used [methanol-water (80:20)] to elute the target analytes from the cartridge while retaining compounds that are less polar than the corticosteroids on the cartridge. A solution containing high percentage of methanol has been used by a number of groups as the final elution stage (Qu *et al.*, 2007; Cirimele *et al.*, 2000; Laugher *et al.*, 1999) to ensure all the retained analytes were removed from the sorbents (Cho *et al.*, 2009; Li *et al.*, 1996).

Qu *et al.,* (2007) suggested several combinations of methanol-water as the elution solvent to optimise analyte extraction recovery. Three different mixtures of methanol-water been employed as the elution solvent in SPE method 2. The extraction recovery of the corticosteroids from plasma with series of elution solvent ratio was determined (Table 2.20). Based on these results, a ratio of methanol in water (8:2) was selected as the elution solvent.

Table 2.20 The extraction efficiency of solid-phase extraction with different elution solvent ratios. HPLC conditions: C_{18} Kromasil column (250 x 4.6 mm (i.d), 5 µm (par.)); gradient mobile phase GRAD D4; detector wavelength: 239 nm; flow rate: 1 mL min^{-1}

Analyte	**Ratio of elution solvent**[±γ]		
	60:40	**80:20**	**90:10**
PRED	96.27	106.58	125.85
BETA	86.68	106.64	125.27
BUD	19.21	96.42	126.97

[±]Corticosteroid plasma solution (20 µg mL^{-1})
[γ]Elution solvent was a mixture of methanol and water represented as methanol:water (A:B)
*The result is reported as percentage recovery (n = 2)

This result indicated that this modified SPE method which was rapid and required minimal volumes of organic solvents, was suitable for the extraction of corticosteroids from plasma. Thus SPE method 2 was used to extract all the analytes of interest from plasma spiked with internal standard.

Table 2.21 The mean recovery (%) of corticosteroids from plasma spiked with internal standard. HPLC conditions: C_{18} Kromasil column (250 x 4.6 mm (i.d), 5 µm (par.)); gradient mobile phase GRAD D4; detector wavelength: 239 nm; flow rate: 1 mL min^{-1}

Internal standard	Mean recovery (% RSD)[±]						
	PRED	BETA	BUD		17-BMP	MF	FP	BDP
			22-R	22-S				
Betamethasone	85.16 (3.18)	-	90.57 (3.15)		76.70 (3.54)	75.62 (3.33)	91.30 (1.52)	68.31 (0.60)
Mometasone	92.82 (0.79)	102.73 (1.93)	104.18 (1.62)		50.72 (1.53)	-	98.23 (2.18)	45.40 (3.22)

[±]Corticosteroid plasma solution (20 µg mL^{-1})

From these results (Table 2.21), it can be seen that the SPE method 2 succeed in extracting the all the corticosteroids from the plasma with good precision. This SPE method was chosen to be the sample preparation method for this study. The reliability of this sample clean-up method was later evaluated based on the validation parameters recommended by FDA (2001).

2.6 Analytical method validation

Validation of a method is crucial to ensure that the chosen protocol is reliable, robust and repeatable for use by other analysts. The validation parameters of the extraction procedure were performed based on Food and Drug Administration (FDA) Guidance for Industry: Bioanalytical Method Validation (2001) and the chromanalytical method was validated in accordance with the British Pharmacopoeia (BP) (2008).

2.6.1 Resolution

As previously discussed, resolution calculates the separation between two peaks based on the retention time and peak width. The resolution (Rs) value should be equal to or greater than 1.5 to ensure the baseline resolution is achieved (BP, 2008). Baseline resolution was obtained in all corticosteroids (Table 2.22) which indicates complete separation of all the compounds.

Table 2.22 Chromatographic data obtained for Reference solution A1 containing a corticosteroid mixture (10 µg mL^{-1}) spiked with internal standard (betamethasone or mometasone) (0.5 µg mL^{-1})

Internal standard	Resolution (Rs)							
	PRED	BETA	BUD		17-BMP	MF	FP	BDP
			22-R	22-S				
Betamethasone	-	7.51	24.82	1.24	4.78	9.88	1.49	10.89
Mometasone	-	7.39	20.27	0.97	3.14	8.30	1.73	12.87

2.6.2 Accuracy

Accuracy measures the closeness of the measured value to the predicted value. This was measured by applying the analytical procedure to a known concentration of corticosteroid and the result was expressed in terms of recovery. Recovery is defined as the percentage of the measured value against expected value. Recovery was measured by comparing the amount of analyte extracted from the sample to the known amount of analyte in the standard and is also known as the extraction efficiency when expressed as a percentage. The analysis was performed in triplicate at five different concentrations of corticosteroid in plasma (0.5, 1, 2.5, 5 and 10 µg mL^{-1}) on three occasions (n = 9). A percentage of recovery of 100 ± 20 % was accepted as an accurate method (FDA, 2001).

Based on the peak area ratio of the corticosteroid extracted from the human plasma to the internal standard and the known concentration of the analyte, the percentage recovery of each analyte was calculated (Table 2.23 and 2.24) for both of the internal standards. The results refer to the average of six replicates for each concentration. The mean extraction efficiency of each corticosteroid from plasma varied between 23 % for BDP to 94 % for PRED. The recovery of BUD was lower when betamethasone was used as the internal standard (59 – 72 %) compared to mometasone (65 – 102 %) as the internal standard.

Table 2.23 The mean recovery (%) of a series of corticosteroid concentrations from plasma spiked with betamethasone (0.5 µg mL^{-1}) as the internal standard

Analytes	**Mean Recovery (% RSD)**				
	10 µg mL^{-1}	**5 µg mL^{-1}**	**2.5 µg mL^{-1}**	**1 µg mL^{-1}**	**0.5 µg mL^{-1}**
PRED	77.29 (2.88)	70.08 (3.93)	94.55 (2.80)	89.01 (4.19)	83.91 (1.02)
BUD	59.20 (3.01)	60.02 (4.41)	72.46 (7.43)	64.71 (6.56)	65.03 (8.87)
MF	48.27 (4.95)	53.02 (5.24)	63.45 (2.02)	47.84 (9.88)	54.47 (3.04)
FP	58.52 (3.86)	59.45 (5.57)	74.67 (2.28)	63.61 (6.47)	64.22 (4.26)
BDP	43.84 (6.38)	36.55 (3.73)	45.67 (6.24)	23.76 (5.59)	31.75 (6.74)

Table 2.24 The mean recovery (%) of a series of corticosteroid concentrations from plasma spiked with mometasone (0.5 µg mL^{-1}) as the internal standard

Analytes	Mean Recovery (% RSD)				
	10 µg mL^{-1}	**5 µg mL^{-1}**	**2.5 µg mL^{-1}**	**1 µg mL^{-1}**	**0.5 µg mL^{-1}**
PRED	80.88 (2.02)	77.16 (9.03)	77.54 (7.07)	96.42 (7.43)	85.57 (3.79)
BETA	65.44 (1.68)	60.50 (8.99)	57.91 (7.05)	77.41 (6.52)	62.85 (3.22)
BUD	75.99 (7.72)	80.85 (9.52)	65.39 (6.18)	102.35 (7.96)	78.35 (7.72)
FP	68.51 (2.59)	72.64 (9.45)	61.08 (6.87)	78.95 (9.01)	70.94 (7.59)
BDP	39.99 (2.33)	42.68 (6.22)	33.87 (7.96)	42.06 (8.19)	39.41 (6.71)

2.6.3 Precision

Precision is commonly expressed as a percentage of relative standard deviation (% RSD). Precision was measured based on the repeatability in a single analytical run (FDA, 2001). In repeatability or intraday precision, the plasma sample was prepared at different concentrations (0.5, 1, 2.5, 5 and 10 µg mL^{-1}) with six replicates of each run (n = 6) using the chosen analytical procedure. An upper limit of 10.0 % was set for the RSD.

The precision of the corticosteroid assay (Table 2.25 and 2.26) was based on six injections of the same sample. All corticosteroids showed an acceptable precision with an RSD ≤ 10.0 % regardless of internal standard and lower than the RSD of less than 15 % recommended by the FDA (2001).

Table 2.25 The precision (% RSD) of a series of corticosteroid concentrations from plasma spiked with betamethasone (0.5 µg mL^{-1}) as the internal standard

Precision (% RSD)*	Analytes				
	PRED	**BUD**	**MF**	**FP**	**BDP**
10 µg mL^{-1}	2.88	3.01	4.95	3.86	6.38
5 µg mL^{-1}	3.93	4.41	5.24	5.57	3.73
2.5 µg mL^{-1}	2.80	7.43	2.02	2.28	6.24
1 µg mL^{-1}	4.19	6.56	9.88	6.47	5.59
0.5 µg mL^{-1}	1.02	8.87	3.04	4.26	6.74

Table 2.26 The precision (% RSD) of a series of corticosteroid concentrations from plasma spiked with mometasone (0.5 µg mL^{-1}) as the internal standard

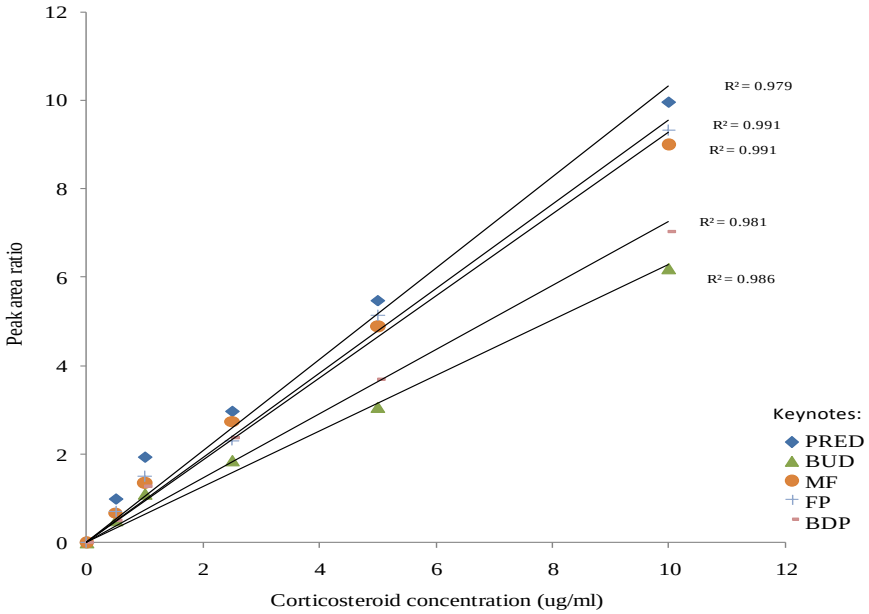
Precision (% RSD)*	Analytes				
	PRED	**BETA**	**BUD**	**FP**	**BDP**
10 µg mL^{-1}	2.02	1.68	7.72	2.59	2.33
5 µg mL^{-1}	9.03	8.99	9.52	9.45	6.22
2.5 µg mL^{-1}	7.07	7.05	6.18	6.87	7.96
1 µg mL^{-1}	7.43	6.52	7.96	9.01	8.19
0.5 µg mL^{-1}	3.79	3.22	7.72	7.59	6.71

2.6.4 Linearity

Linearity is explained by separate dilution of the stock solution of the corticosteroid mixture. Calculation of the regression line measures the linearity between the peak area of the sample assays and sample concentration. For this purpose, a calibration standard of the corticosteroid mixture at different concentrations (0.5 – 10 µg mL^{-1}) was measured. The calibration curve was drawn for the series of concentrations from 0 - 10 µg mL^{-1} with a fixed quantity (0.5 µg mL^{-1}) of the internal standard in the mobile phase [methanol-water (57:43 v/v)]. The ratio of the peak area of analyte to that of the internal standard was calculated. The linear regression using the least

square fit where the y-axis represents the peak area ratio of the corticosteroid to internal standard and x-axis represents the concentration of corticosteroid were calculated (Figure 2.18). The method showed consistent linearity for all corticosteroids ($r^2 \geq 0.95$) in the range of 0 – 10 µg mL^{-1}.

(a)



(b)

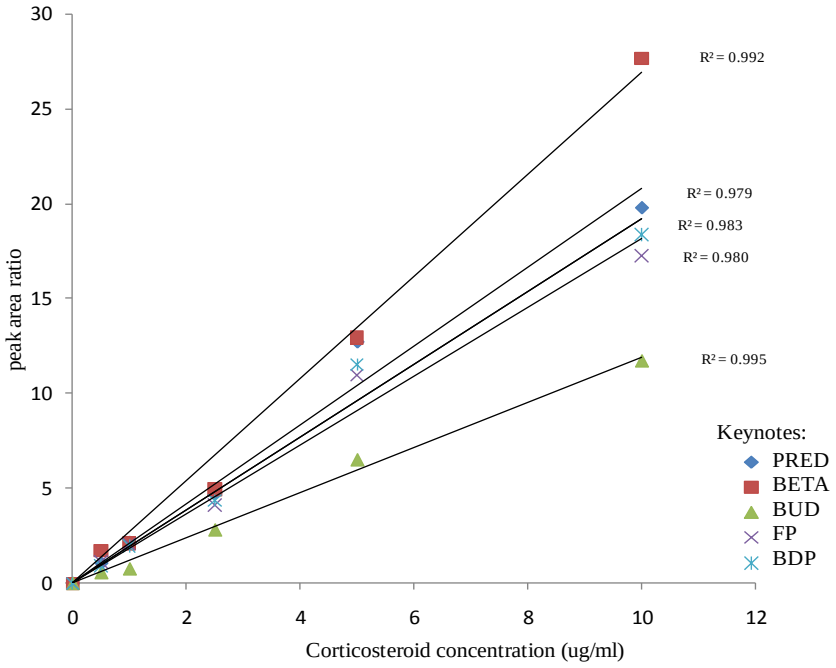


Figure 2.18 Calibration curves of spiked solutions (Reference solution A1 – A5) containing the corticosteroid mixture (0.5 – 10 µg mL^{-1}) spiked with (a) betamethasone (0.5 µg mL^{-1}); (b) mometasone (0.5 µg mL^{-1}) as the internal standard

2.6.5 Asymmetry Factor

The asymmetry factor, also known as the tailing factor, measures the symmetry of the peak of interest. A symmetry factor of 1.0 shows perfect symmetry and Gaussian peaks. The asymmetry factors of the analytes of interest had to be between 0.8 - 1.5 to be acceptable (BP, 2008). All the compounds studied were within the range suggested by the BP (2008) (Table 2.27) thus leading to the conclusion of no peak tailing or fronting for any of the analytes.

Table 2.27 Asymmetry factor of each analyte spiked with the internal standard (0.5 µg mL^{-1})

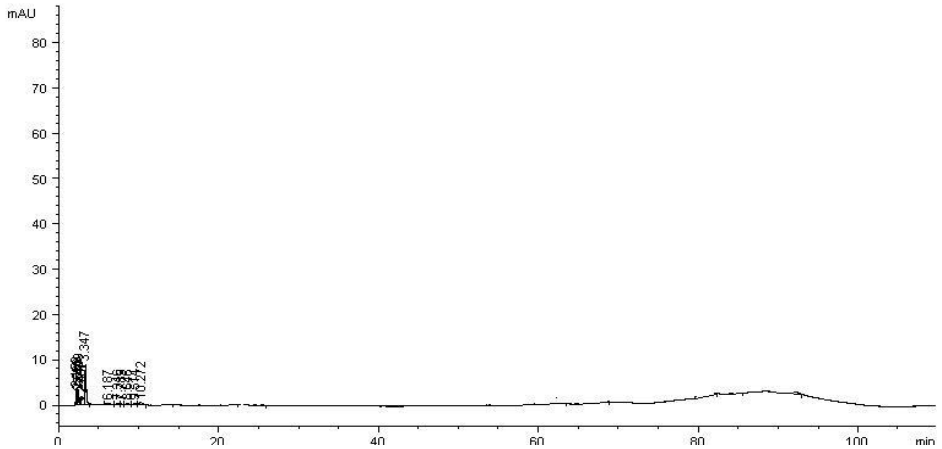
Internal standard	Asymmetry factor (As)±							
	PRED	BETA	BUD		17-BMP	MF	FP	BDP
			22-R	22-S				
Betamethasone	1.16	-	1.18	1.14	1.36	1.34	1.21	1.02
Mometasone	1.18	1.19	1.24	0.94	1.42	-	1.06	1.06

± Reference solution A1spiked with internal standard

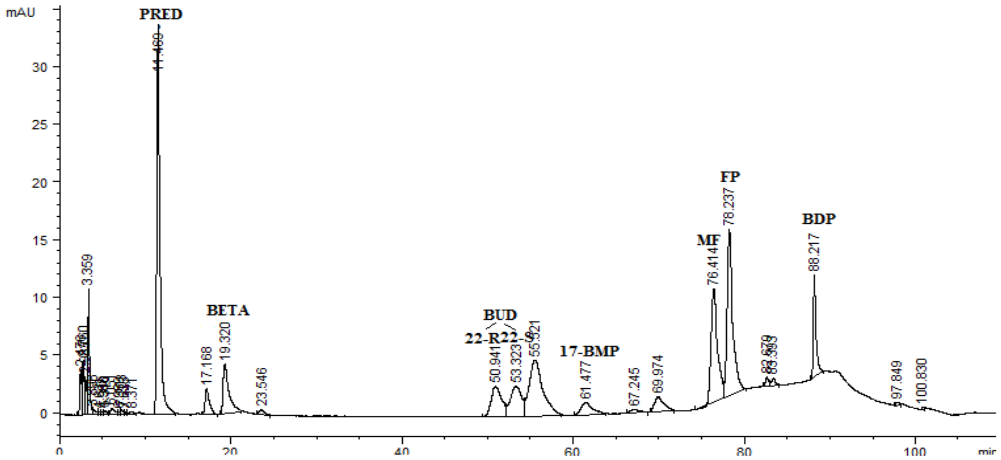
2.6.6 Specificity

Specificity is defined as the extent of the analytical method to identify the analyte of interest compared to possible interference from the instrument, solvents and the sample itself. Specificity was confirmed by comparing the chromatogram of the corticosteroid mixture at 10 µg mL^{-1} spiked with either internal standard to a chromatogram obtained from blank plasma (Figure 2.19). No endogenous interference of human plasma were observed at any retention time using the described method. As can be seen, the eight peaks did not co-elute in either chromatogram (Figure 2.19) which confirmed specificity.

(a)



(bi)



(bii)

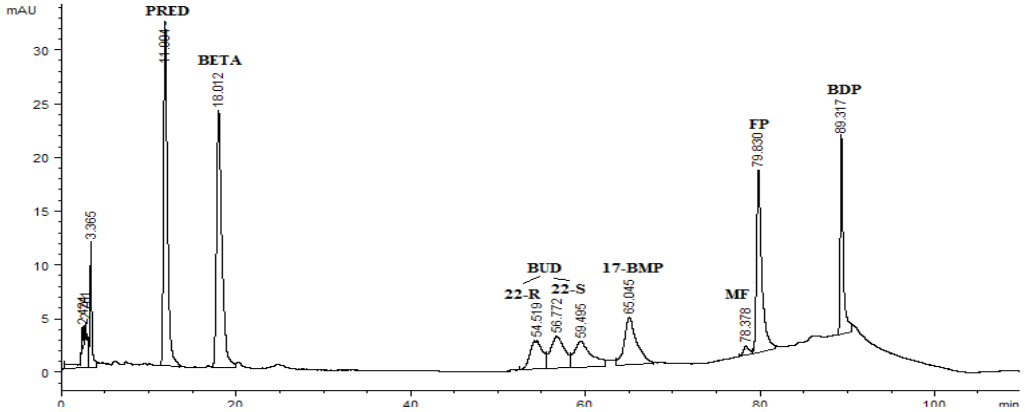


Figure 2.19 Representative chromatograms for (a) blank plasma; (b) plasma spiked with 10 µg mL^{-1} corticosteroid mixture with the internal standard (0.5 ug mL^{-1}) (bi) betamethasone; or (bii) mometasone

It was observed (Figure 2.19) that the sample pretreatment SPE method 2 for analysing corticosteroids in human plasma sample was necessary since it eliminated interference from both the plasma and the reagent. Without sample pretreatment, the biological interference might affect the method sensitivity and clog the HPLC column.

2.6.7 Selectivity

Selectivity is defined as the extent to which an analytical method can determine particular analytes in a complex mixture without interference from other components. Selectivity can be explained by the relative retention time (RRT), relative response factor (RRF), capacity factor (k') and selectivity factor (α).

2.6.7.1 Relative retention time (RRT)

The retention time is affected by the flow rate. A relative retention time (RRT) less than 1 is defined as the compound eluting earlier than the internal standard (Equation 7).

$$RRT = \frac{t_{R2}}{t_{R1}} \qquad \text{- Equation 7}$$

where RRT is the relative retention time
t_{R1} is retention time of the peak 1
t_{R2} is retention time of the peak eluted after the peak 1

The elution order (Table 2.28) shows that the first internal standard, betamethasone eluted as the second peak whereas the second internal standard, mometasone eluted as the sixth peak in the chromatogram.

Table 2.28 Chromatographic data obtained for a spiked solution of Reference solution A1 with the internal standards, betamethasone or mometasone (0.5 µg mL^{-1})

Analytes		Relative retention time (RRT)*	
		Betamethasone	**Mometasone**
PRED		0.67	0.15
BETA		1.00	0.22
BUD	22-R	2.91	0.65
	22-S	3.03	0.68
17-BMP		3.62	0.86
MF		4.27	1.00
FP		4.39	1.03
BDP		5.00	1.16

*RRT = retention time relative to the internal standard

2.6.7.2 Relative response factor (RRF)

The relative response factor for ultraviolet absorbance (RRF_{UV}) was calculated for each analyte using PRED as the reference analyte. The RRF_{UV} acts as a correction factor for the measured peak area during the analysis. The relative response factor was later calculated in relative to the internal standard used (Table 2.29).

Table 2.29 Chromatographic data obtained for a spiked solution of Reference solution A1 with the internal standards, betamethasone or mometasone (0.5 µg mL^{-1})

Analytes	Relative response factor (RRF_{IS})*	
	Betamethasone	**Mometasone**
PRED	0.6685	1.4442
BETA	1.0000	1.2703
BUD 22-R 22-S	0.3591	0.5486
17-BMP	0.4933	0.7534
MF	0.2292	1.0000
FP	0.2377	0.5960
BDP	0.1708	0.5711

*RRF_{IS} = response factor relative to the internal standard

2.6.7.3 Capacity factor (k)

The capacity factor measures the time the analyte was retained in the stationary phase relative to the time it resided in the mobile phase. Only PRED and BETA are within the optimum capacity factor of k' = 2 – 10 (Table 2.30 and 2.31).

Table 2.30 Chromatographic data obtained for a spiked solution of Reference solution A1 with the internal standard betamethasone (0.5 µg mL^{-1})

Analytes		t_o	t_R	k	α
PRED		2.349	11.616	3.95	-
BETA		2.349	17.434	6.42	1.67
BUD	22-R	2.349	50.725	20.59	3.21
	22-S	2.349	52.811	21.48	1.04
17-BMP		2.349	63.111	24.78	1.14
MF		2.349	74.358	30.66	1.24
FP		2.349	76.533	31.58	1.03
BDP		2.349	87.201	36.12	1.14

* k = capacity factor; α = selectivity factor

Table 2.31 Chromatographic data obtained for a spiked solution of Reference solution A1 with the internal standard mometasone (0.5 µg mL^{-1})

Analytes		t_o	t_R	k	α
PRED		2.379	11.295	3.75	-
BETA		2.379	16.764	6.05	1.61
BUD	22-R	2.379	49.441	19.78	3.27
	22-S	2.379	51.853	20.80	1.05
17-BMP		2.379	65.404	24.30	1.26
MF		2.379	76.051	30.97	1.27
FP		2.379	77.953	31.77	1.03
BDP		2.379	88.277	36.11	1.14

* k = capacity factor; α = selectivity factor

2.6.7.4 Selectivity (α)

Selectivity is the ratio between the capacity factor of two adjacent peaks (Equation 8).

$$\alpha = k'_2 / k'_1 \qquad \text{- Equation 8}$$

where k is a measure of the time the analytes reside in the stationary phases compared to time spent in mobile phases.
k'_1 is the first analyte
k'_2 is the analyte after k_1

The efficiency of separation of analytes is affected by the number of theoretical plates of the column (Equation 8). The greater the number of the theoretical plates, the better the resolution of the analytes.

2.6.8 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection is defined as the lowest concentration of analyte that can be measured but is unable to be quantified using the analytical method assigned. The limit of detection (LOD) should be based on a signal-to-noise (S/N) ratio of 3:1 (BP, 2008). The signal-to-noise ratio is calculated using Equation 9:

$$S/N = 2H/h \qquad \text{- Equation 9}$$

where S/N is the signal to noise ratio
H is the height of the peak corresponding to the component concerned in the chromatogram
h is the range of the background noise in a chromatogram obtained after injection or application of a blank

The limit of quantification is defined as the lowest concentration of the analyte that can be measured using the analytical method with acceptable accuracy and precision. The limit of quantification (LOQ) should be based on a signal-to-noise ratio of 10:1 (BP, 2008).

The corticosteroid mixtures of 10 µg mL^{-1} spiked with the internal standards (0.5 µg mL^{-1}) were used to assess the sensitivity of the method, based on signal intensity. Peak heights of the individual corticosteroids were measured from the chromatograms and the noise was taken from the blank mobile phase chromatogram (BP, 2008). The signal-to-noise ratio was then calculated for each of the analytes. The LOD varied from 0.02 µg mL^{-1} for PRED to 0.2 µg mL^{-1} for BUD, at a signal-to-noise ratio of 3. The LOQ was highest with BUD (0.6 - 0.8 µg mL^{-1}) and the lowest quantifiable concentration was found with PRED at 0.07 µg mL^{-1} (Table 2.32 and 2.33).

Table 2.32 Limit of detection and limit of quantification (LOD and LOQ) determined from Reference solution A1 spiked with the internal standard, betamethasone (0.5 µg mL^{-1})

Analytes		**S/N**	**LOD (µg mL^{-1})**	**LOQ (µg mL^{-1})**
PRED		2669.89	0.0234	0.0779
BUD	22-R	280.09	0.2035	0.6783
	22-S	280.09		
17-BMP		537.92	0.1115	0.3718
MF		1025.72	0.0591	0.1969
FP		1188.68	0.0530	0.1767
BDP		1064.04	0.0291	0.0969

Table 2.33 Limit of detection and limit of quantification (LOD and LOQ) determined from Reference solution A1 spiked with the internal standard, mometasone (0.5 µg mL^{-1})

Analytes		S/N	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
PRED		2798.16	0.0217	0.0722
BETA		2821.99	0.0213	0.0709
BUD	22-R	231.32	0.2620	0.8733
	22-S	231.32		
17-BMP		468.28	0.1281	0.4271
FP		1307.74	0.0473	0.1575
BDP		2790.45	0.0221	0.0738

The assay developed are less sensitive than the published methods which have managed to detect between 0.25 – 300 pg mL^{-1} (Qu *et al.*, 2007; Sahasranaman *et al.*, 2005; Pereira *et al.*, 2005; Taylor *et al.*, 2004; Wang & Hochhaus, 2004; Li *et al.*, 2001; Krishnaswami *et al.*, 2000; Laugher *et al.*, 1999; Hochhaus *et al.*, 1998). All these studies used liquid chromatography mass spectrometery (LC-MS) detection rather than UV detection. The study findings are similar to the results obtained by Teng *et al.*, (2001) who used UV detection and reported an LOQ for MF of 0.2 µg mL^{-1}. The LC-MS method was not investigated further as it involved equipment that is not readily available in all laboratories.

The validation parameters are summarised in Table 2.34 and Table 2.35.

Table 2.34 Validation parameters for the corticosteroid mixture with betamethasone (0.5 µg mL^{-1}) as the internal standard

Parameters	**Compound**						
	PRED	**BUD**		**17-BMP**	**MF**	**FP**	**BDP**
		22-R	**22-S**				
Weight of the analyte (micrograms)§	10.1	10.1		10.0	10.6	10.3	10.3
Retention time (t_R)(min)§	11.616	50.725	52.811	63.111	74.358	76.533	87.201
RRT (min)§	0.67	2.91	3.03	3.62	4.27	4.39	5.00
Resolution (Rs)§	-	24.82	1.24	4.78	9.88	1.49	10.89
Selectivity, α§	-	3.21	1.04	1.14	1.24	1.03	1.14
Asymmetry Factor (As)§	1.16	1.18	1.14	1.36	1.34	1.21	1.02
S/N§	2669.89	280.09	280.09	537.92	1025.72	1188.68	1064.04
LOD (µg mL^{-1})	0.0234	0.2035		0.1115	0.0591	0.0530	0.0291
LOQ (µg mL^{-1})	0.0779	0.6783		0.3718	0.1969	0.1767	0.0969
RRF	0.6685	0.3591		0.4933	0.2292	0.2377	0.1708
Linearity (R^2)	0.979	0.986		0.985	0.991	0.991	0.981
Precision (% RSD)*							
10 µg mL^{-1}	2.88	3.01			4.95	3.86	6.38
5 µg mL^{-1}	3.93	4.41			5.24	5.57	3.73
2.5 µg mL^{-1}	2.80	7.43			2.02	2.28	6.24
1 µg mL^{-1}	4.19	6.56			9.88	6.47	5.59
0.5 µg mL^{-1}	1.02	8.87			3.04	4.26	6.74
Mean Recovery (%)*							
10 µg mL^{-1}	77.29	59.20			48.27	58.52	43.84
5 µg mL^{-1}	70.08	60.02			53.02	59.45	36.55
2.5 µg mL^{-1}	94.55	72.46			63.45	74.67	45.67
1 µg mL^{-1}	89.01	64.71			47.84	63.61	23.76
0.5 µg mL^{-1}	83.91	65.03			54.47	64.22	31.75
Precision (% RSD)*							
10 µg mL^{-1}	2.88	3.01			4.95	3.86	6.38
5 µg mL^{-1}	3.93	4.41			5.24	5.57	3.73
2.5 µg mL^{-1}	2.80	7.43			2.02	2.28	6.24
1 µg mL^{-1}	4.19	6.56			9.88	6.47	5.59
0.5 µg mL^{-1}	1.02	8.87			3.04	4.26	6.74

RRT = relative retention time; RRF = relative response factor; S/N = signal to noise ratio; RSD = Relative standard deviation; LOQ = limit of quantification; LOD = limit of detection.
* In six replicates (n = 6), § The data was obtained from Reference solution A1.

Table 2.35 Validation parameters for the corticosteroid mixture with mometasone (0.5 µg mL^{-1}) as the internal standard

Parameters	**Compound**						
	PRED	**BETA**	**BUD**		**17-BMP**	**FP**	**BDP**
			22-R	**22-S**			
Weight of the analyte (micrograms)§	10.1	10.0	10.1		10.0	10.3	10.3
Retention time (t_R)(min)§	11.295	16.764	49.441	51.853	65.404	77.953	88.277
RRT (min)§	0.15	0.22	0.65	0.68	0.86	1.03	1.16
Resolution (Rs)§	-	7.39	20.27	0.97	3.14	1.73	12.87
Selectivity, α§	-	1.61	3.27	1.05	1.26	1.03	1.14
Asymmetry Factor(As)§	1.18	1.19	1.24	0.94	1.42	1.06	1.06
S/N§	2798.16	2821.99	231.32	231.32	468.28	1307.74	2790.45
LOD (µg mL^{-1})	0.0217	0.0213	0.2620		0.1281	0.0473	0.0221
LOQ (µg mL^{-1})	0.0722	0.0709	0.8733		0.4271	0.1575	0.0738
RRF	1.4442	1.2703	0.5486		0.7534	0.5960	0.5711
Linearity (R^2)	0.979	0.992	0.995		0.999	0.980	0.983
Precision (% RSD)*							
10 µg mL^{-1}	2.02	1.68	7.72			2.59	2.33
5 µg mL^{-1}	9.03	8.99	9.52			9.45	6.22
2.5 µg mL^{-1}	7.07	7.05	6.18			6.87	7.96
1 µg mL^{-1}	7.43	6.52	7.96			9.01	8.19
0.5 µg mL^{-1}	3.79	3.22	7.72			7.59	6.71
Mean Recovery (%)*							
10 µg mL^{-1}	80.88	65.44	75.99			68.51	39.99
5 µg mL^{-1}	77.16	60.50	80.85			72.64	42.68
2.5 µg mL^{-1}	77.54	57.91	65.39			61.08	33.87
1 µg mL^{-1}	96.42	77.41	102.35			78.95	42.06
0.5 µg mL^{-1}	85.57	62.85	78.35			70.94	39.41
Precision (% RSD)*							
10 µg mL^{-1}	2.02	1.68	7.72			2.59	2.33
5 µg mL^{-1}	9.03	8.99	9.52			9.45	6.22
2.5 µg mL^{-1}	7.07	7.05	6.18			6.87	7.96
1 µg mL^{-1}	7.43	6.52	7.96			9.01	8.19
0.5 µg mL^{-1}	3.79	3.22	7.72			7.59	6.71

RRT = relative retention time; RRF = relative response factor; S/N = signal to noise ratio; RSD = Relative standard deviation; LOQ = limit of quantification; LOD = limit of detection.
*In six replicates (n = 6), § The data was obtained from Reference solution A1.

2.7 Summary of the method development study

The initial HPLC method described by Teng *et al.*, (2001) who used an Ultrasphere C_8 column with an isocratic mobile phase of methanol-water (59:41 v/v) at 248 nm was not suitable.

In our hands, the chromatographic conditions recommended by Teng *et al.,* (2001) resulted in a runtime of 60 min with four of the components co-eluting as two peaks; of BUD and 17-BMP and MF and FP. Changing the carbon load of the column from C_8 to C_{18} was successful in resolving the eight components but with a significantly long analysis time (110 mins) with PRED eluting close to the zone (0 – 4 min) where biological interferants may cause problems with detection and quantification. Optimisation of the stationary and mobile phase and employing a gradient programme ensured of elution of PRED at about 11 mins and all the components were separated in a total runtime of 110 mins, resulting in the final method (Table 2.36):

Table 2.36 Analytical method method for the analysis of corticosteroids in plasma

Procedure	**Summary method**		
Column	C_{18} Kromasil (250 x 4.6 mm, 5µm)		
Injection volume	20 µl		
Mobile phase	Time (min)	% Methanol	% Water
	0	57	43
	55	57	43
	70	62	38
	87	80	20
	100	57	43
	110	57	43
Flow rate	1 mL min^{-1}		
Detector	239 nm		
Temperature	25 °C		

Dexamethasone acetate was employed in this study based on the method used by Teng *et al*., (2001). Dexamethasone acetate was later considered as inadequate as it degraded with time. Dexamethasone, the degradation product of dexamethasone acetate was later employed and was stable throughout analysis. The compound was found to co-elute with betamethasone (BETA), which is one of the analytes of interest. The problem of a suitable internal standard was solved by using two internal standards, mometasone (MF) and betamethasone (BETA) instead of one.

The plasma samples were cleaned by solid-phase extraction to allow quantification of the corticosteroids using the analytical method described above and the sample showed no biological interference during the analysis. The developed method of SPE was validated over the range of 0.5 - 10 µg mL^{-1}. The method was sensitive

enough to detect a concentration of 0.2 µg mL^{-1} for BUD and the minimum quantifiable concentration is as low as 0.07 µg mL^{-1} for PRED. The linearity was tested from 0.5 – 10 µg mL^{-1}. Both accuracy and precision throughout the concentration range (0.5 – 10 µg mL^{-1}) were acceptable resulting in the final method for sample extraction (SPE method 2) (Table 2.37).

Table 2.37 Sample extraction method for the analysis of corticosteroids in plasma

Procedure	**Summary method**
SPE cartridge	Strata C_{18}
Conditioning	3 mL methanol followed by 3 mL water
Loading	1 mL plasma spiked with 0.5 µg mL^{-1} internal standard (betamethasone or mometasone). The cartridge is left for 150 seconds.
Wash	1 mL water followed by 1 mL of methanol-water (10:90 v/v)
Elute	0.5 mL mixture of methanol-water (80:20 v/v) into 2 mL HPLC vial.

*SPE = solid-phase extraction

Chapter 3

Analysis of Clinical Data

3.1 Introduction

Corticosteroids are generally considered as safe and effective medication for the treatment of inflammatory diseases (Winkler *et al.*, 2004), however, several studies have found that some systemic side effects of corticosteroids which are observed are related to both dose and duration of use (Algorta *et al*., 2008; Tayab *et al*., 2007; Kaliner, 2006; Angeli *et al*., 2006; Derom *et al.*, 2005; Ton *et al.*, 2005; Fardon *et al.*, 2004; Benninger *et al.*, 2003). In this chapter, the efficacy and safety of the use of single or combination corticosteroids in patients with asthma, rhinitis or nasal polyposis was assessed using various clinical markers such as symptom questionnaires, salivary cortisol, blood pressure, blood glucose, nasal polyp grade, nasal endoscopy score and Dual Energy X-ray Absorptiometry (DEXA).

3.1.1 Markers of disease

3.1.1.1 Sino Nasal Outcome Test (SNOT-22)

SNOT-22 is a routine self-administered, 22 question multiple choice questionnaire where the patient scores their symptoms between 0 and 5; a score 0 indicates that the symptoms pose no problem to the individual and a score of 5 indicates that the symptoms pose a very severe problem to the individual (Appendix 3.1). The SNOT-22 score helps to measure the impact of rhinitis, chronic rhinosinusitis (CRS) with or without polyps on daily life (Hopkins *et al*., 2009; Moghaddasi *et al*., 2009) and the response of these conditions to corticosteroid treatment (Pinar *et al*., 2008; Dales-Yates *et al.*, 2004; Gupta & Gupta, 2004). SNOT-22 is an improved version of the SNOT-20 questionnaire with additional questions relating to nasal blockage and loss of the individual's sense of smell (Gillett *et al*., 2009; Hopkins *et al*., 2009). The five

most important descriptors reported by patients with chronic rhinosinusitis (CRS) in the SNOT-22 test are nasal obstruction, loss of smell or taste, dizziness, post nasal discharge and a runny nose (Pynnonen *et al*., 2009; Guilemany *et al*., 2009; Browne *et al*., 2007; Small *et al.*, 2005; Bousquet *et al*., 2003). The SNOT-22 scores taken both before and after nasal surgery are used to measure the success of surgery. Following Functional Endoscopic Sinus Surgery (FESS) for chronic rhinosinusitis patients reported an improvement in post operative SNOT-22 score (7 – 27) compared to the preoperative SNOT-22 score (41 – 53) ($p < 0.001$) (Hopkins *et al*., 2009; Moghaddasi *et al*., 2009).

3.1.1.2 Asthma Control Questionnaires (ACQ)

This self administered questionnaire consists of six questions answered on a 7 point scale (0 is no symptoms; 6 is very severe symptoms) where the overall score is the mean of the responses (Appendix 3.2). It measures asthma control based on the severity of morning and night-time asthma symptoms, frequency of bronchodilator inhaler use, bronchoconstriction and daily activity limitation due to asthma symptoms in the past week (Juniper *et al*., 2006). The current ACQ has one less question relating to the percent Forced Expiratory Volume in 1 second (% FEV_1) than the 7-item ACQ used in the Gaining Optimal Asthma Control (GOAL) study (Juniper *et al*., 2006; Bateman *et al*., 2004) and recommended by SIGN/BTS guideline (SIGN/BTS, 2008). A cut off ACQ score of 0.75 has been assigned to draw the line between well controlled and poorly controlled asthma with well controlled asthma defined as fewer than two days in a week with ACQ score greater than 1, no night time awakenings, exacerbations or hospitalisation and minimal use

of a beta agonist as a bronchodilator (up to 4 occasions in two days) (Juniper *et al*., 2006). The ACQ showed a decrease in patients treated with inhaled corticosteroids (Kuna *et al*., 2007; Buhl *et al*., 2006; Bensch *et al*., 2005; Bateman *et al*., 2004).

3.1.1.3 Fraction of Exhaled Nitric Oxide (FE_{NO})

The measurement of the fraction of exhaled nitric oxide (FE_{NO}) is a simple, non specific and non invasive marker of airways inflammation. The inducible enzyme calcium dependent nitric oxide synthase (NOS) generates production of nitric oxide (NO) by the enzymatic conversion of *L*-arginine to *L*-citrulline on the airway epithelium of the bronchial wall (Berry *et al*., 2005; Gelb *et al*., 2004). The FE_{NO} has been found to be higher (27 - 43 parts per billion (ppb)) in moderate to severe asthmatic patients (% FEV_1 of < 80 % predicted) compared to healthy patients (12 - 16 ppb) (Oh *et al*., 2008; Travers *et al*., 2007; Berry *et al*., 2005; Gelb *et al*., 2004). The FE_{NO} was also found to be significantly reduced in patients with asthma treated with either inhaled corticosteroids (Prieto *et al*., 2009; Hahn *et al*., 2007; Smith *et al*., 2005; Boushey *et al*., 2005; Gelb *et al*., 2004) or oral corticosteroids (Berry *et al*., 2005). The improvement in the measured FE_{NO} in patients treated with corticosteroids was seen in those patients exhibiting a combination of moderate to severe asthma and a high FE_{NO} (> 47 ppb) (Schneider *et al*., 2009) or in patients with chronic cough of a duration of greater than or equal to 8 weeks (Oh *et al*., 2008; Hahn *et al*., 2007).

FE_{NO} is less useful in measuring corticosteroid response in patients with mild asthma with an FEV_1 of greater or equal to 80 % predicted with a low FE_{NO} (less than 30

ppb) (Shaw *et al*., 2007; Travers *et al*., 2007; Boushey *et al*., 2005; Berry *et al*., 2005; Smith *et al*., 2005). Cigarette smoking decreases the FE_{NO} due to the presence of nitric oxide in tobacco smoke (Travers *et al*., 2007; Kostikas *et al*., 2007; McSharry *et al*., 2005).

3.1.1.4 Nasal endoscopy score and nasal polyp grade

Nasal endoscopy score staging was modified from the Lund-Kennedy score (Jones, 2002). Any sign of turbinate swelling, presence of secretions, either pus or blood, or the presence of polyps was assessed using a rigid nasal endoscope. The size of polyp in the nasal cavity was measured on a 4 point scale where grade 0 is no polyp present and grade 3 is the presence of polyp reaching the lower and upper turbinate and causing total nasal obstruction. The nasal endoscopy score is relevant to the diagnosis of rhinitis and polyps (Guilemany *et al*., 2009; Hens *et al*., 2008; Polzehl *et al*., 2006; Boari & Castro-Junior, 2005) but is less sensitive in measuring the effect of treatment on the disease progression (Stjarne *et al*., 2006). There is a significant correlation between the nasal endoscopy score and the computed tomography (CT) scan result in diagnosing chronic rhinosinusitis (CRS) but the use of nasal endoscopy is preferred to a CT scan in diagnosing CRS as CT scanning has limitations in distinguishing between oedema and mucous (Robinson *et al*., 2005; Boari & Castro Junior, 2005).

3.1.2 Markers of adrenal suppression

Hypothalamic adrenal suppression (HPA) axis suppression can be detected in several ways such as salivary cortisol (Lederbogen *et al*., 2010; Jerjes *et al*., 2006; Gallagher

et al., 2006; Groschl & Rauh, 2006; Strazdins *et al*., 2005; Jacobs *et al*., 2005)**,** plasma cortisol (Sachanandani *et al*., 2009; Lonnebo *et al*., 2007; Tayab *et al*., 2007; Derom *et al*., 2005; Fardon *et al*., 2004; Lee *et al*., 2003) and urinary cortisol (Rosenblut *et al*., 2007; Tayab *et al*., 2007; Whelan *et al*., 2005; Fardon *et al*., 2004).

3.1.2.1 Salivary cortisol

Saliva collection offers a safe, noninvasive and stress free test for measurement of cortisol in the body. The results of the salivary cortisol test are often reliable except in the outpatient setting which requires a strict standardised protocol and patient training (Jacobs *et al*., 2005; Broderick *et al*., 2004). In a non controlled daily situation, the cortisol collection and measurement of concentration might be affected by non compliance to the instructions given, cross reactivity of the immunoassay and a socially disturbed environment (Jacobs *et al*., 2005). The morning salivary cortisol has been used to determine HPA axis suppression in patients treated with corticosteroids (Lederbogen *et al*., 2010; Patel *et al*., 2004) compared to midnight salivary collection, which is more reliable in assessing prolonged hypercortisolism in conditions such as Cushings Syndrome (Nunes *et al*., 2009; Yaneva *et al*., 2004).

The passive method for collecting saliva, also known as spitting without stimulant into the collection tube, is a simpler and better way to collect saliva than using an active spitting method such as the Salivette® with cotton or polyester (Groschl & Rauh, 2006; Strazdins *et al*., 2005) or the Salivette® with citric acid (Gallagher *et al*., 2006). Passive salivary collection of cortisol is reported to correlate well with plasma cortisol measurements ($R^2 = 0.724$, $p < 0.001$) (Gallagher *et al*., 2006).

3.1.3 Markers of osteoporosis

Dual Energy X-ray Absorptiometry (DEXA) is commonly used to measure bone density and the measurement is presented as bone mineral density (BMD), T-score and Z-score (Kaji *et al*., 2010; Natsui *et al*., 2006; Ton *et al.*, 2005; Campbell *et al*., 2004). The T-score and Z-score report the bone mass density in comparison with population values. For the T-score, the comparison is to a 30 year old healthy woman (Faulkner, 2005) and for the Z-score the comparison is corrected for age and sex (Salem *et al*., 2010; Kaji *et al*., 2009). In studies (Sosa *et al*., 2008; Natsui *et al*., 2006) the bone mineral density (BMD) at the lumbar spine (0.74 – 1.08 g cm^{-3}) and femoral neck (0.57 – 0.84 g cm^{-3}) were reported to be significantly lower in patients on high dose corticosteroids equal or greater than 7.5 milligrams of prednisolone equivalent compared to the BMD at the lumbar spine (0.7 – 1.11 g cm^{-3}) and femoral neck (0.59 – 0.84 g cm^{-3}) of the control group. The T-score was used to confirm the severity of the BMD reduction classifying the patients as having either osteopenia or osteoporosis based on the WHO definition (Table 3.1) (Salem *et al*., 2010; Sosa *et al*., 2008).

Table 3.1 Classification of bone mass density (BMD) and T-score (NOS, 2008; SIGN, 2003).

BMD status	T-score
Normal	Less than 1 standard deviation below the norm
Osteopenia	Between 1 and 2.5 standard deviations below the norm
Osteoporosis	More than 2.5 standard deviations below the norm

Several studies (Suman *et al*., 2010; Salem *et al*., 2010; Kaji *et al*., 2009; Hayashi *et al*., 2008; Chevallety *et al*., 2008) have found that the Z-score is more relevant in identifying the risk of osteoporosis compared to the T-score in patients treated with corticosteroids aged over 50 years. However, some studies (Haeck *et al*., 2009; Angeli *et al*., 2006; Ton *et al.,* 2005) have shown that there is no correlation between the corticosteroid dose and a reduction in BMD.

3.2 Aim

The aim of the clinical study was to investigate the relationship between different delivery methods of corticosteroids (inhaled, intranasal and oral), steroid burden and markers of disease progression and the salivary cortisol in patients with the respiratory diseases of asthma, rhinitis, and/or nasal polyposis.

3.3 Objectives

1. To quantify the absorption of inhaled and intranasal corticosteroids in patients with asthma, rhinitis and/ or nasal polyps.
2. To identify the side effects related to the intranasal and inhaled corticosteroids
3. To determine if there is any association between the corticosteroid burden in patients with asthma, rhinitis and/ or nasal polyps with the known side effects of corticosteroids
4. To determine if there is a correlation between the extent of nasal polyposis and the absorption of corticosteroids.

3.4 Methods

3.4.1 Study design

This study was conducted over the period of October 2008 to June 2010 in two out-patient clinics in Greater Glasgow and Clyde Health Board using questionnaires (Appendix 3.1, 3.2 and 3.5) to collect patient specific data about their corticosteroid use and a variety of direct and indirect methods to identify disease markers. The study was categorised as an observational, longitudinal cohort study.

3.4.2 Study population

3.4.2.1 Clinical sites

Patients aged between 18 and 70 years with a diagnosis of rhinitis, chronic rhinosinusitis with or without nasal polyposis and/or asthma were enrolled from two clinical sites: the Ear, Nose and Throat (ENT) clinic, Glasgow Royal Infirmary (GRI) and the Problem Asthma clinic, Stobhill Hospital (SH).

3.4.2.2 Selection of participants

Patients were recruited based on the inclusion and exclusion criteria (Table 3.2). Subjects who met the inclusion criteria were eligible to enrol for the study. Potential patients were approached and informed about the purpose of the study by the researcher and were informed that their participation in the study was voluntary and they were free to withdraw from the study at any time. Patients were given a patient information leaflet and informed written consent was obtained from the study participants in the presence of the medical staff.

Table 3.2 Inclusion and exclusion criteria for the clinical study

Inclusion criteria	**Exclusion criteria**
Patients aged 18-79 years old	Patient receiving high dose oral corticosteroids for other medical conditions
Patients treated for asthma and/or rhinitis and/or nasal polyps at outpatient clinic GRI and SH	Patient unwilling to participate in the study
Patients on inhaled or intranasal corticosteroids or on combination of inhaled and intranasal corticosteroids	Patient has a nasal tumour
New patients to be prescribed inhaled or intranasal corticosteroids	Patient has a nasal deformity
Patient provides consent in writing	

3.4.2.3 Process of recruitment

Patients were recruited and enrolled in the study following a flow diagram (Figure 3.1) which was available in the clinic to remind the medical and nursing staff.

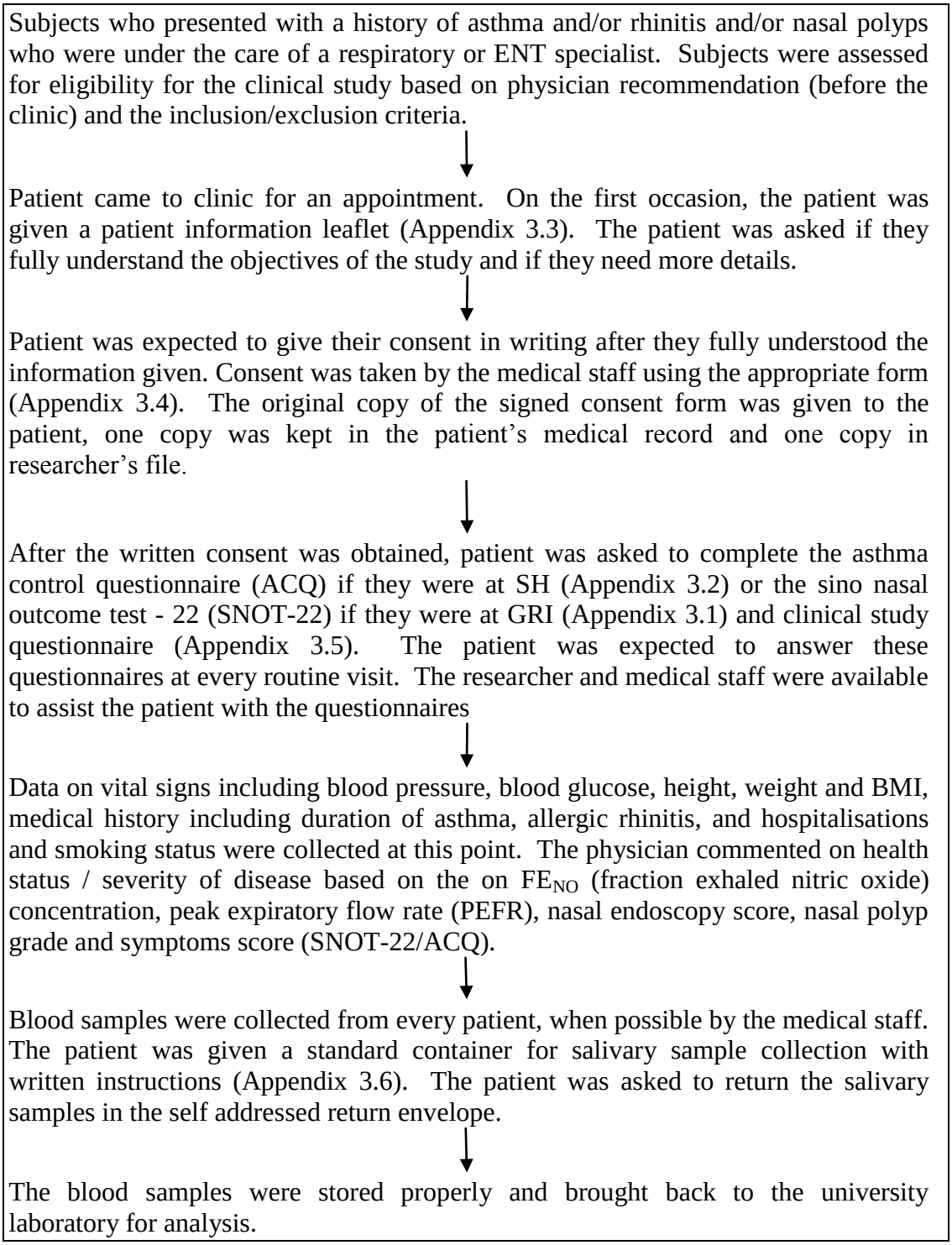


Figure 3.1 Flow chart of patient recruitment, enrolment and continuation in the clinical study.

3.4.3 Ethical considerations

Ethical permission was obtained from the North Glasgow Universities National Health Service (NHS) Trust Research Ethics Committee. Approval for the clinical study was also received from the Research Ethics Committee of the University of Strathclyde.

3.4.4 Data collection

3.4.4.1 Questionnaires

3.4.4.1.1 Sino Nasal Outcome Test (SNOT-22)

Patients attending the ENT clinic, at GRI were asked to complete a validated multiple choice questionnaire: the Sino Nasal Outcome Test-22 (SNOT-22) (Appendix 3.1) at each routine visit to the clinic. The 22 items in the questionnaire were assessed on a 6 point scale. The total score from the 22 items was calculated by summation and recorded. The total score of SNOT-22 is in a range of 0 to 110.

3.4.4.1.2 Asthma Control Questionnaire (ACQ)

Patients attending the Problem Asthma clinic at SH were asked to complete a validated questionnaire: the Asthma Control Questionnaire (ACQ) (Appendix 3.2) at each routine visit. The total score from the 6 items was added and divided by six to obtain the overall score of ACQ.

3.4.4.1.3 Clinical Study Questionnaire

A questionnaire was designed for the clinical study to assess the patient's steroid burden and general health. The clinical study questionnaire (Appendix 3.5) used a

combination of multiple choice questions and closed questions. The questionnaire contained questions about age, sex, ethnicity, daily corticosteroid dose by oral, inhaled and/or intranasal route, availability of a steroid card, smoking and alcohol habits, bone health and daily medication for other health conditions.

3.4.4.2 Assessment of disease progression

3.4.4.2.1 Collection of salivary cortisol

Verbal and written instructions on how to obtain a salivary cortisol (Appendix 3.6) were given to the patients along with two five millilitre plastic bottles for sample collection. Patients were asked to collect their saliva upon waking in the morning before brushing their teeth, eating, drinking or taking any medicines. The saliva bottles were labelled with patients' details: name, home address and hospital number. The patient was asked to return the salivary cortisol to the clinic either by post using the pre-labelled envelope or by hand at their next clinic visit. The collected saliva was sent to the biochemistry laboratory for analysis. The data are reported in nanomoles per litre (nmol L^{-1}).

3.4.4.2.2 Plasma analysis

Venous blood samples were collected by the medical staff at every clinic visit, when possible. The subjects were seated in upright posture when blood samples were taken. A heparin coated blood collection tube (Greiner Bio One, Gloucestershire) was used for blood collection. Plasma was collected after centrifugation of the blood sample using a Heraeus® Labofuge 400 centrifuge (Buckinghamshire, UK) at 3500 *x*

g for 15 minutes. The plasma samples were stored at -20 °C until required for analysis.

The analyte (corticosteroid) in plasma was extracted using solid-phase extraction, and subsequently analysed by High Performance Liquid Chromatography (HPLC). The analytical method used was that developed and described in Chapter 2 (Table 3.3 and 3.4).

Table 3.3 Sample extraction method for the analysis of corticosteroids in plasma

Procedure	**Summary method**
SPE cartridge	Strata C_{18}
Conditioning	3 mL methanol followed by 3 mL water
Loading	1 mL plasma spiked with 0.5 µg mL^{-1} internal standard (betamethasone or mometasone). The cartridge is left for 150 seconds.
Wash	1 mL water followed by 1 mL mixture of methanol-water (10:90, v/v)
Elute	0.5 mL mixture of methanol-water (80:20, v/v) into 2 mL HPLC vials

*SPE = solid-phase extraction

Table 3.4 Analytical method for the analysis of corticosteroids in plasma

Procedure	**Summary method**		
Column	C_{18} Kromasil (250 x 4.6 mm i.d, 5µm particle size)		
Injection volume	20 µL		
Mobile phase	Time (min)	% Methanol	% Water
	0	57	43
	55	57	43
	70	62	38
	87	80	20
	100	57	43
	110	57	43
Flow rate	1 mL min^{-1}		
Detector	UV, 239 nm		
Temperature	25 °C		

3.4.4.2.3 Nasal polyp grade

Patients with polyps were graded according to the nasal endoscopy score. The polyps were scored for each nasal cavity on a 4-point scale (Table 3.5). Data on nasal polyp grade is only available from the ENT clinic at GRI.

Table 3.5 Nasal polyp grade

Polyp grade	Explanation
Grade nil	No polyps
Grade A	Polyps in the middle meatus; not reaching below the inferior border of the middle turbinate
Grade B	Polyps reaching below the inferior border of the middle turbinate but not the inferior border of the inferior turbinate
Grade C	Large polyps reaching to or below the inferior border of the inferior turbinate or polyps medial to the middle turbinate

3.4.4.2.4 Dual Energy X-ray Absorptiometry (DEXA) scan

The results of any DEXA scan available in patient's medical records within the last three years were recorded.

3.4.4.2.5 Measurement of blood pressure

A seated blood pressure was measured using an automatic upper arm blood pressure monitor (Welch Allyn® Spot Vital Signs Monitor). Information on any antihypertensive medication prescribed was determined by any documentation on antihypertensive treatment in the medical records and clinical study questionnaire.

3.4.4.2.6 Measurement of blood glucose

A random blood glucose concentration was measured at every clinic visit using the Precision Xceed Pro® Blood Glucose test kit regardless of the patient having eaten or drunk beforehand. The information on antidiabetic medication was determined from

any documentation on antidiabetic treatment in the medical records or from the clinical study questionnaire. Patients with random blood glucose (RBG) greater than or equal to 11 mmol L^{-1} and with no reported antidiabetic treatment were referred for further diagnostic testing for suspected diabetes mellitus.

3.4.4.2.7 Fraction exhaled nitric oxide (FE_{NO})

Exhaled nitric oxide was measured using a hand held chemiluminescence analyser, NIOX MINO® Airway Inflammation monitor (Aerocrine AB). The patient was asked to take a deep inspiration and then exhale slowly and continuously through the mouthpiece as recommended by the American Thoracic Society (ATS) and European Respiratory Society (ERS) task force (2005).

3.4.5 Data analysis

The data were analysed using Minitab15® statistical package. Descriptive data are presented in mean ± standard deviation (s.d) or median (interquartile range, IQR). Pearson product-moment correlation coefficient and Spearman's rank correlation coefficient tests were used to determine the correlation between continuous variables. A nonparametric test, Mann-Whitney U-test was used for hypothesis testing of group difference. Comparison between data on the first visit and all visits was compared using the Fisher transformation r-to-z analysis. The difference was considered significant if p was less than 0.05.

3.5 Results

3.5.1 Demographics

A total of 113 patients from the ENT clinic at Glasgow Royal Infirmary (GRI) and the Problem Asthma Clinic at Stobhill Hospital (SH) were enrolled in the clinical study (Table 3.6). The age and weight distribution were similar in the groups with asthma, rhinitis and combined disease. More than one third of the participants (45/113 (39.8 %)) had chronic rhinosinusitis (CRS) and nasal polyposis and asthma.

Table 3.6 Baseline demographics of the study participants

Variable	ENT clinic (GRI)	Problem asthma clinic (SH)	Attended both clinics	Total
Number of participants	73	31	9	113
No of visits	140	107	12	259
Age, years (median, IQR)	56 (43 – 63)	52 (44 – 61)	47 (41 – 50)	54 (43 – 62)
BMI (kg m^{-2}) (mean ± s.d)	27.1 ± 4.8	29.3 ± 7.2	26.5 ± 4.2	27.6 ± 5.6
Sex				
Male	39	11	5	55
Race				
Caucasian	72	31	9	112
Asian	1	-	-	1
Diagnosis				
Rhinitis only	4	-	-	4
CRS only	2	-	-	2
CRS with polyps only	27	-	-	27
Rhinitis and anatomical abnormality	3	1	-	4
Rhinitis and asthma	4	-	3	7
CRS with polyps and asthma	33	6	6	45
Asthma only	-	24	-	24

3.5.2 Clinical findings and effects of corticosteroids

3.5.2.1 Salivary cortisol

A total of 155 saliva samples from 77 patients were collected during the study. The measured salivary cortisol was analysed against the calculated corticosteroid burden for each patient (Figure 3.2). Pearson's correlation analysis showed that the correlation between salivary cortisol and daily corticosteroid burden was poor and not statistically significant (r = - 0.155; p = 0.054).

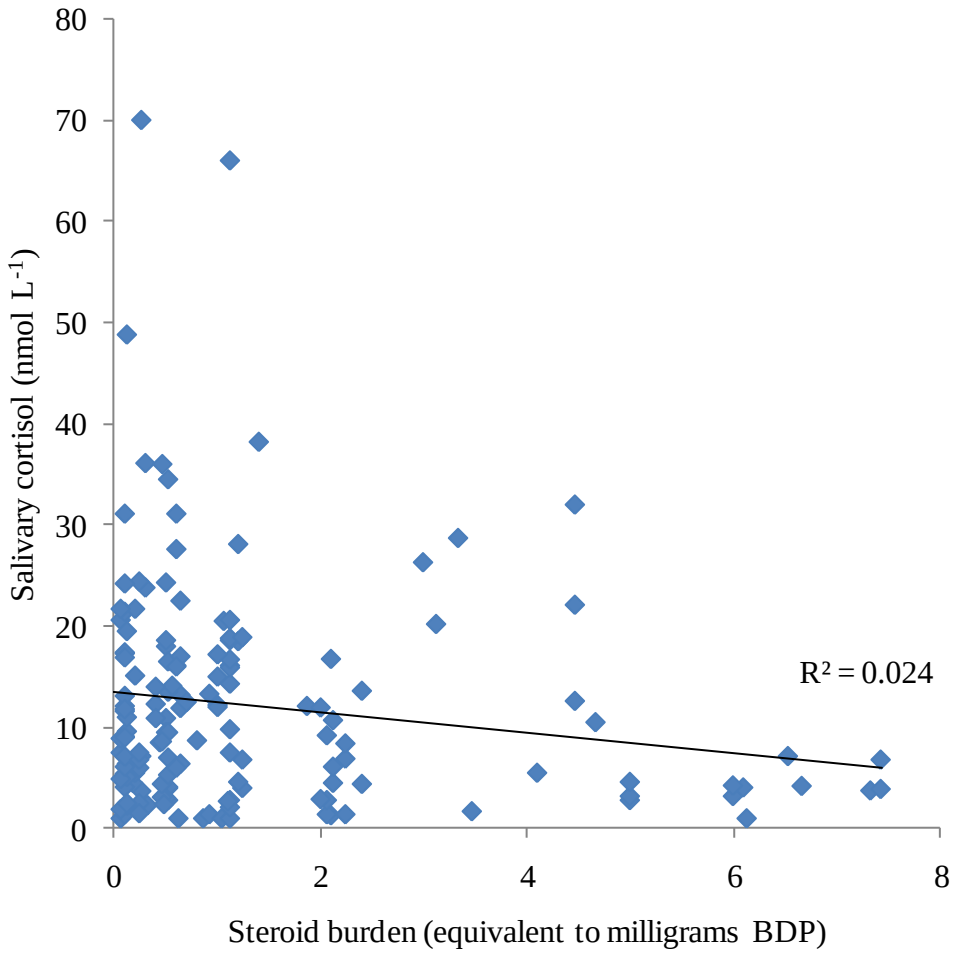


Figure 3.2 Correlation between the measured salivary cortisol and daily corticosteroid burden (n = 155)

The reference range for salivary cortisol is 5 – 25 nmol L^{-1} (Angus *et al*., 1992). From the 155 salivary cortisol measurements, 48 (30.9 %) samples showed a salivary cortisol below the reference range which indicates the possibility of adrenal suppression. Of these samples twenty-three (47.9 %) were from patients taking a corticosteroid dose less than 1 milligram of BDP equivalent daily. A corticosteroid burden less than 1 milligram BDP equivalent is considered to lack toxicity (Medicines and Healthcare products Regulatory Authority (MHRA), 2006). The depression in salivary cortisol compared to corticosteroid burden measured between patients on a corticosteroid dose of less than 1 milligram and patients on a corticosteroid dose equal or greater than 1 milligrams ratio (BDP equivalent daily) (0.9:1) was not statistically significant ($p = 0.236$). Based on this analysis, patients on a corticosteroid dose less than 1 milligram BDP equivalent daily have a similar risk of corticosteroid induced adrenal suppression to patients on a corticosteroid dose equal to or greater than 1 milligram BDP equivalent daily.

Patients were grouped based on the route of administration of their corticosteroid therapy (Figure 3.3). Eighty-five (54.8 %) of the salivary cortisol measurements were from patients on a combination of inhaled and intranasal corticosteroid with 27 salivary cortisol samples (17.4 %) from patients on intranasal therapy alone. Only 11 salivary cortisol samples (7.1 %) were taken from patients on the combination of three different route of administration: oral, inhaled and intranasal. No significant differences were observed in the salivary cortisol measured between any of the groups.

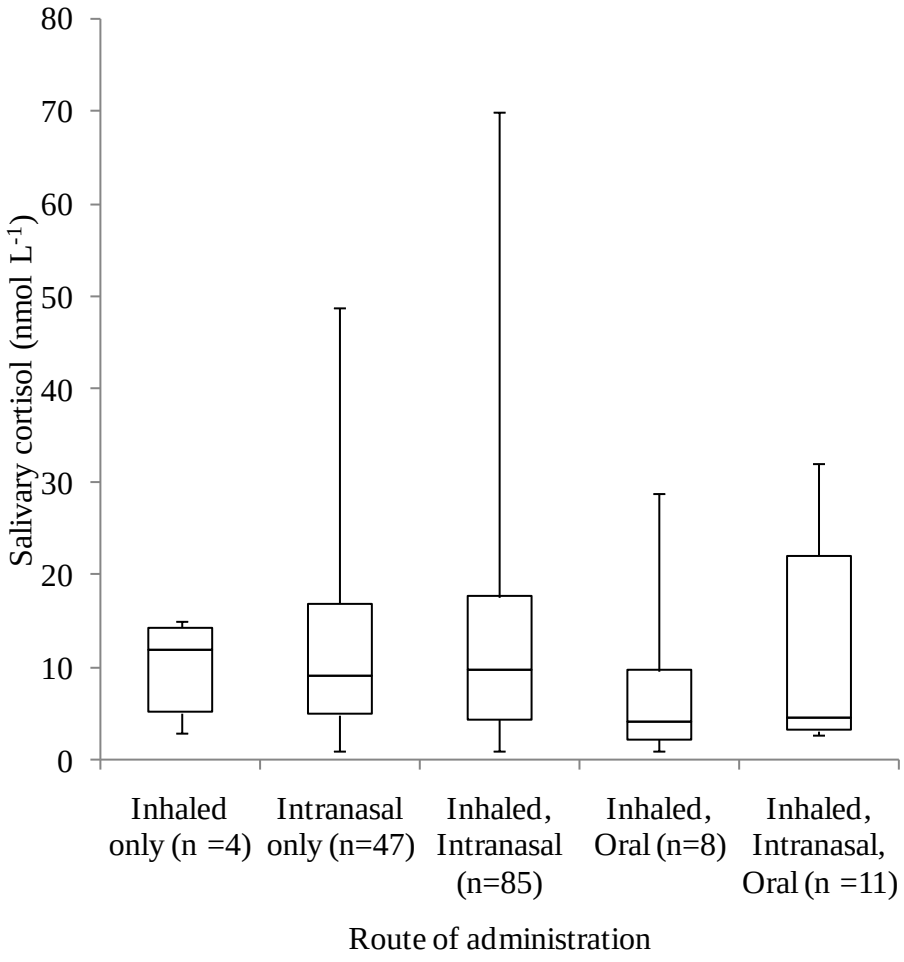


Figure 3.3 The salivary cortisol according to administration route of corticosteroids.

Of the 155 salivary cortisol samples, 19 (12.3 %) were from patients on oral corticosteroids with only two samples from patients on an oral corticosteroid dose less than 7.5 milligrams prednisolone equivalent daily (Figure 3.4). Even though the median salivary cortisol in patients taking oral corticosteroids (4.3 nmol L^{-1}) was lower than in patients not prescribed oral corticosteroids (9.5 nmol L^{-1}), the difference was not statistically significant (Mann Whitney U-test; $p = 0.149$).

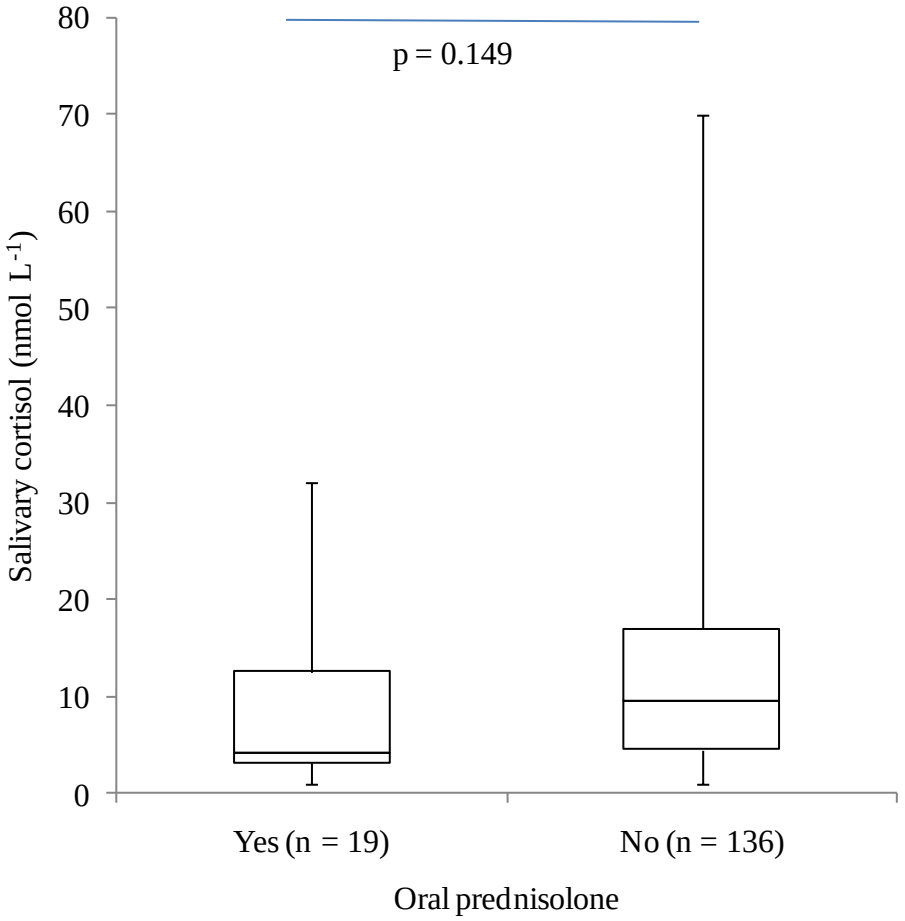


Figure 3.4 Comparison between salivary cortisol from patients on oral corticosteroids and patients not on oral corticosteroids

In a subset of patients prescribed intranasal corticosteroids alone (n = 47), 22 samples were from patients taking intranasal betamethasone (BETA), 19 taking intranasal mometasone (MF), 5 taking intranasal beclometasone dipropionate (BDP) and one prescribed intranasal fluticasone (FP) (Table 3.7). A similar range of corticosteroid doses was observed in the groups. Statistically significant differences in measured salivary cortisol were observed between patients prescribed intranasal BETA, 3.1 (4.2 – 10.7) nmol $L^{-1}$and intranasal MF, 5.9 (5.8 – 8.4) nmol L^{-1} (Mann Whitney U-test; p = 0.044). No statistically significant differences were noted

between patients prescribed intranasal MF or intranasal BETA compared with intranasal BDP. Intranasal FP was not included in the statistical analysis as only one salivary cortisol sample was available.

Table 3.7 Subset of patients prescribed intranasal corticosteroids alone (n = 47).

Parameters (Median (IQR))	**Intranasal corticosteroids**			
	BETA (n = 22)	**MF (n = 19)**	**BDP (n = 5)**	**FP (n = 1)**
Corticosteroid burden (micrograms)	120 (60 – 240)	100 (100 – 400)	240 (150 – 400)	120
Salivary cortisol (nmol L^{-1})	3.1 (4.2 – 10.7)*	5.9 (5.8 – 8.4)*	6.3 (6 – 15.8)	2.5

*Statistically significant difference between intranasal BETA and intranasal MF (p = 0.044)

In a subset of patients treated with both inhaled and intranasal corticosteroids (n = 85) (Table 3.8), the combination of any inhaled corticosteroid (beclometasone dipropionate (BDP), fluticasone (FP) or budesonide (BUD)) with intranasal BETA showed a lower median then the combination of any inhaled corticosteroid with any other intranasal corticosteroid. However, the difference was not statistically significant.

Table 3.8 Subset of patients treated with inhaled and intranasal (n = 85).

Inhaled (Median (IQR))	**Intranasal corticosteroids***			
	BETA	**MF**	**BDP**	**BUD**
BDP	7.2 (1 – 8.6)	13.6 (7.5 – 19)	12.5	17.2
FP	7.5 (4.4 – 16.7)	14.6 (10.2 -16.6)	9.8 (7.3 – 12.6)	-
BUD	7.6 (2.5 – 11.3)	9 (6.6 – 14.1)	7.5 (5.1 – 9.8)	36

* The salivary cortisol is expressed in nmol L^{-1}.

No analyses were carried out of patients prescribed inhaled corticosteroid alone as the number of salivary samples was too small (n = 4).

A subset of 79 patients was studied to investigate the correlation between measured salivary cortisol and nasal polyp grade. The data on nasal polyp grade were available only from the ENT clinic at GRI. Physical examination and nasal endoscopy revealed that of the 79 patients recruited from the ENT clinic at GRI, only 10 patients (12.6 %) presented with no nasal polyposis. Twenty eight patients (35.4 %) presented with nasal polyp grade A, 31 (39.2 %) patients with nasal polyp grade B and 10 (12.6 %) patients with nasal polyp grade C. The median (interquartile range) measured salivary cortisol for patients with nasal polyp grade A was 8.9 (4 – 17.4) nmol L^{-1}, for nasal polyp grade B was 8.5 (5.1 – 13.8), and for nasal polyp grade C was 18 (12.2 – 9.2) nmol L^{-1} (Figure 3.5). Patients without nasal polyps were reported to have a salivary cortisol of 9.1 (3.3 – 11.6) nmol L^{-1} which is similar to patients with nasal polyp grade A or nasal polyp grade B. Using the Mann-Whitney U-test, there was a statistically significant difference in the salivary cortisol in patients with nasal polyp grade C compared to patients with nasal polyp grade A (p =

0.049), nasal polyp B (p = 0.012) but there was no statistically significant difference when compared to the salivary cortisol in patients with no polyps (p = 0.066).

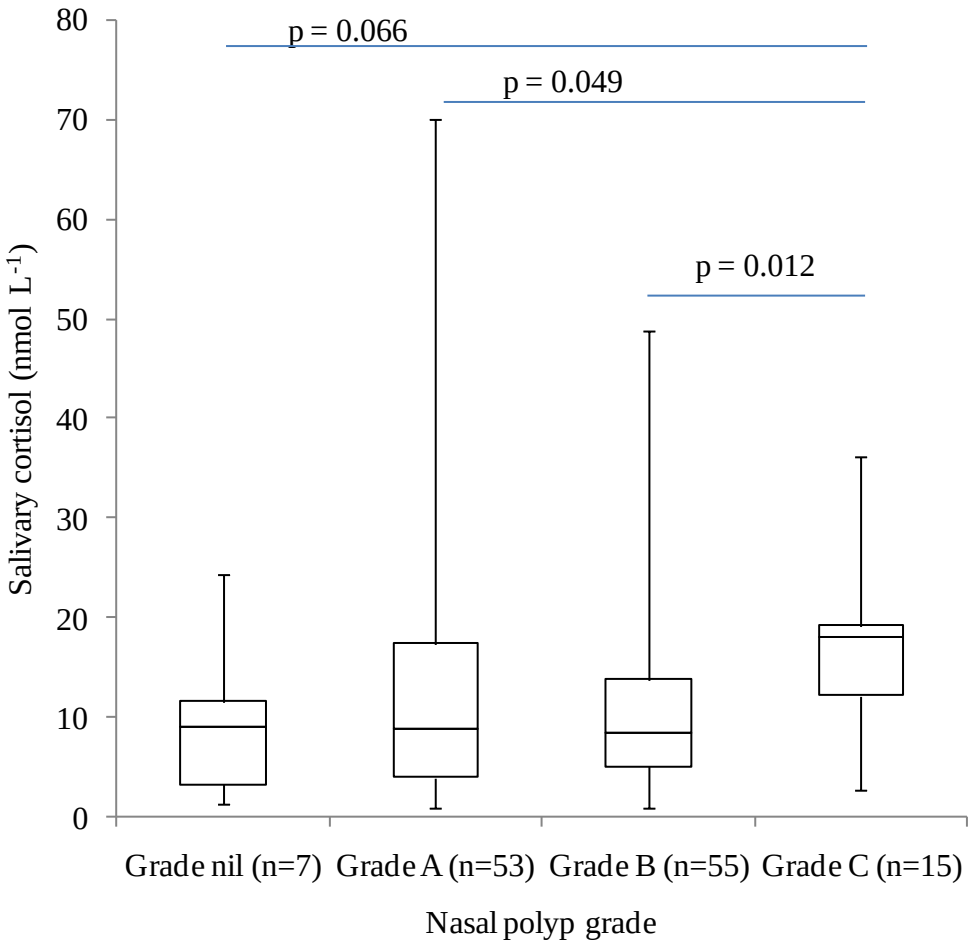


Figure 3.5 Correlation between salivary cortisol and nasal polyp grade in patients attending the GRI clinic (n = 130). The p value is in comparison to polyp grade C

3.5.2.2 Measurement of plasma concentration of corticosteroids

Using the analytical method developed it was possible to quantify prednisolone in 23 plasma samples (n = 23). The method developed was not sensitive enough to quantify any of the inhaled or intranasal corticosteroids. The measured prednisolone concentration was plotted against the patients daily prednisolone dose (Figure 3.6).

Using Pearson's correlation analysis, a statistically significant correlation was found between the plasma concentration of prednisolone and the daily prednisolone dose ($r = 0.604$; $p = 0.002$). Spearman rank correlation analysis was performed to test if the relationship was influenced by outliers. The Spearman correlation analysis found that the correlation was not statistically significant between the plasma concentration of prednisolone and the daily prednisolone dose ($r = 0.27$; $p = 0.217$). Therefore, based on Spearman rank correlation analysis, the previous correlation calculated by Pearson's correlation analysis was influenced by the outliers. It can be concluded that the plasma concentration of prednisolone measured using the developed method has no correlation to the oral prednisolone dose taken by the patients.

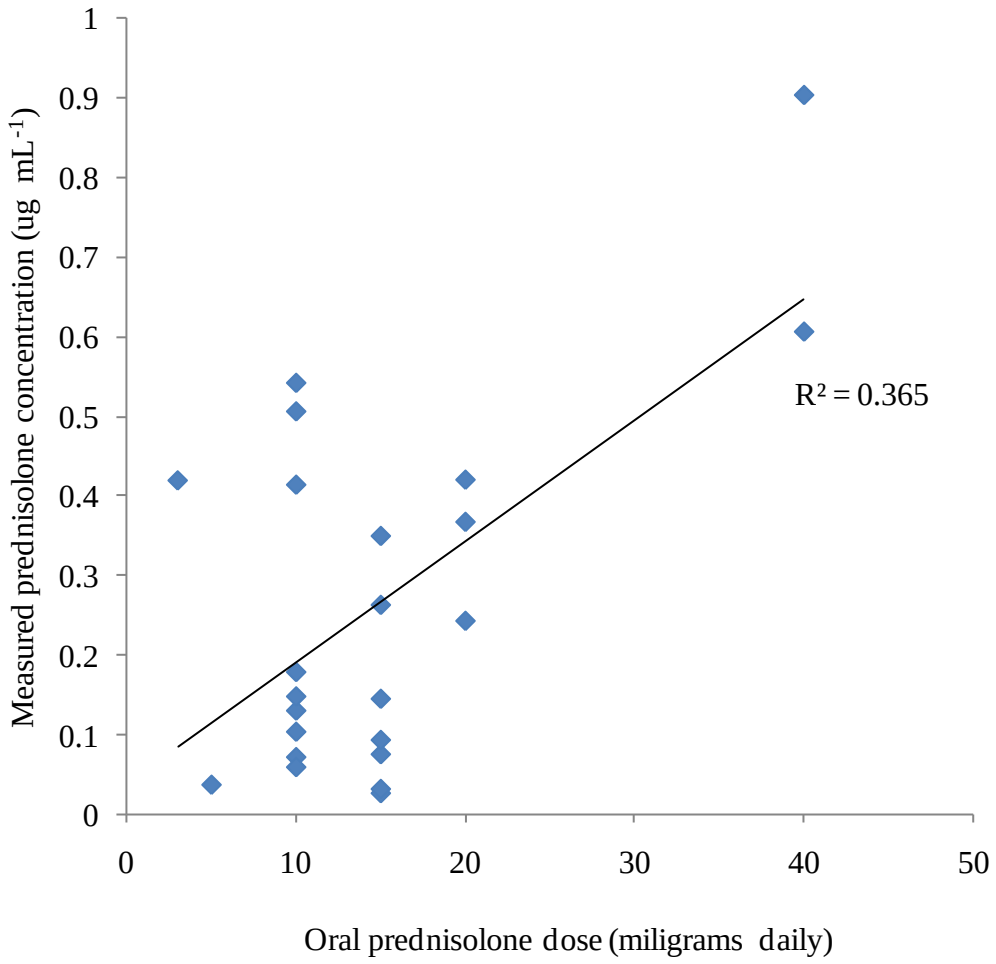


Figure 3.6 Correlation between plasma concentration of prednisolone and daily prednisolone dose (n = 23)

A similar conclusion was observed between the measured prednisolone concentration plotted against steroid burden (Figure 3.7). Using Spearman rank correlation analysis, the correlation calculated by Pearson correlation analysis ($r = 0.507$; $p = 0.014$) was influenced by the outliers. The Spearman rank correlation analysis found statistically non significant correlation between plasma concentration of prednisolone and the steroid burden ($r = 0.32$; $p = 0.134$).

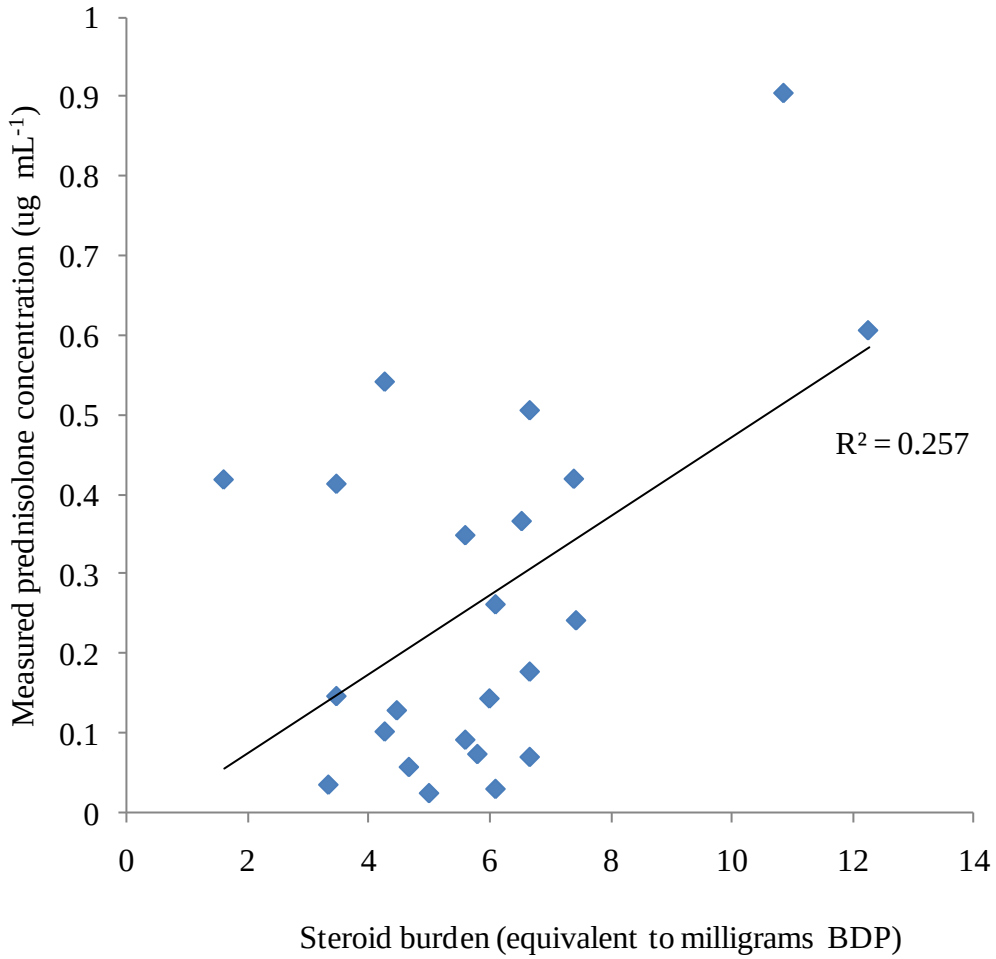


Figure 3.7 Correlation between plasma concentration of prednisolone and corticosteroid burden (n = 23)

The measured salivary cortisol was investigated for a relationship with the plasma concentration of prednisolone (Figure 3.8) and no correlation was observed (Pearson's correlation analysis (n = 10; r = 0.201; p = 0.577)).

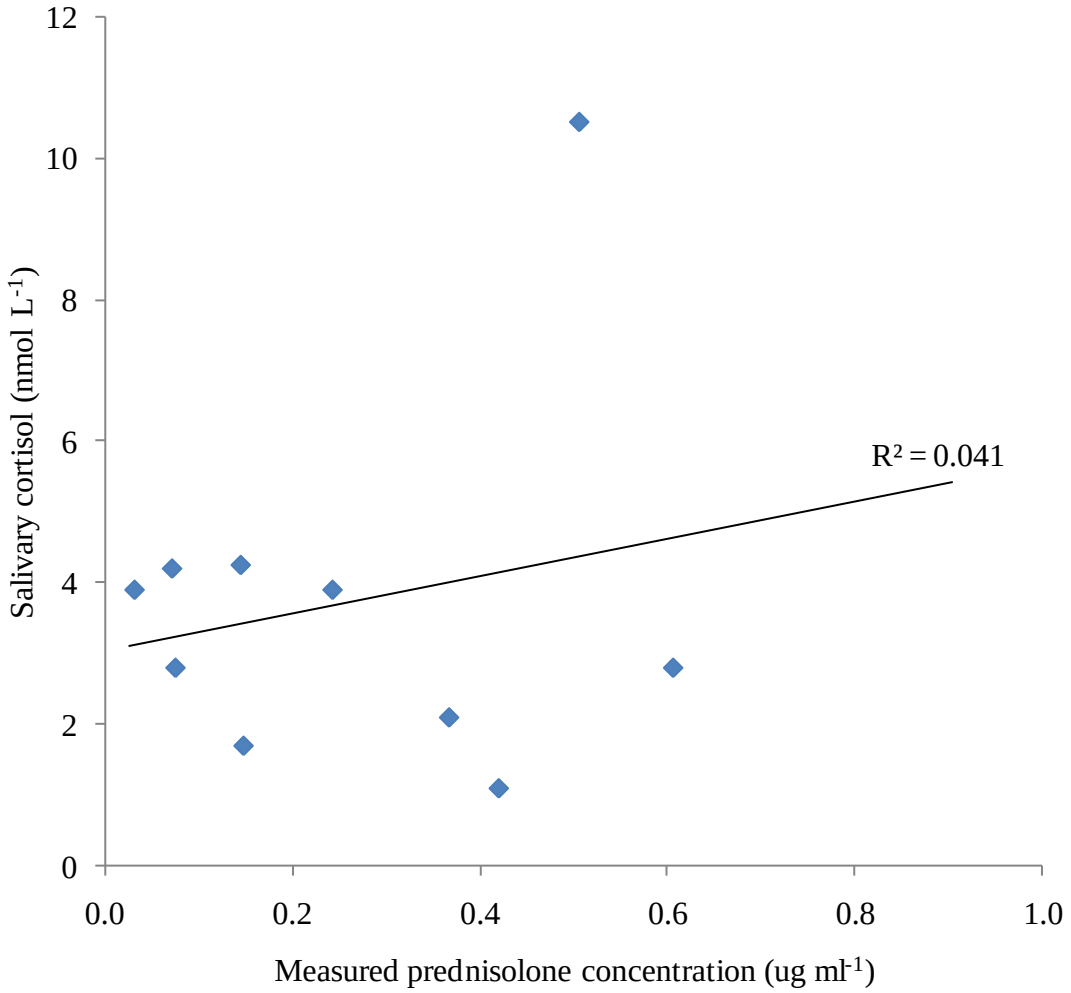


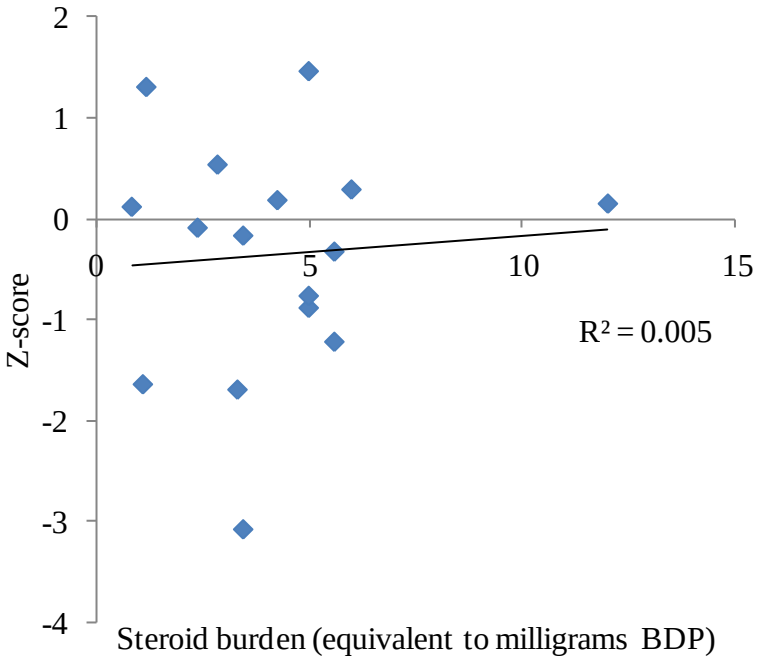
Figure 3.8 Correlation between salivary cortisol and plasma concentration of prednisolone (n = 10)

3.5.2.3 Correlation between corticosteroid dose and the risk of osteoporosis

Among the 113 patients recruited, only 16 (14.2 %) patients had their bone mineral density (BMD) measured by Dual Energy X-ray Absorptiometry (DEXA) scan. Thirteen patients who had a DEXA scan were prescribed daily oral corticosteroids with eleven patients prescribed oral prednisolone equal to or greater than 5 milligrams daily. Four patients were on bone protection medication. The Z-score and T-score were used to compare the measured BMD to healthy age-gender-

matched population BMD with the daily corticosteroid burden (Figure 3.9, 3.10 and 3.11). Using Pearson's correlation analysis, no significant correlations were found between corticosteroid burden and lumbar spine Z-score ($r = 0.073$; $p = 0.786$); femoral neck Z-score ($r = 0.041$; $p = 0.878$) and total hip Z-score ($r = 0.211$; $p = 0.433$). Similar observation were found between corticosteroid burden and lumbar spine T-score ($r = 0.110$; $p = 0.681$); femoral neck T-score ($r = 0.105$; $p = 0.704$) and total hip T-score ($r = 0.245$; $p = 0.361$).

(a)



(b)

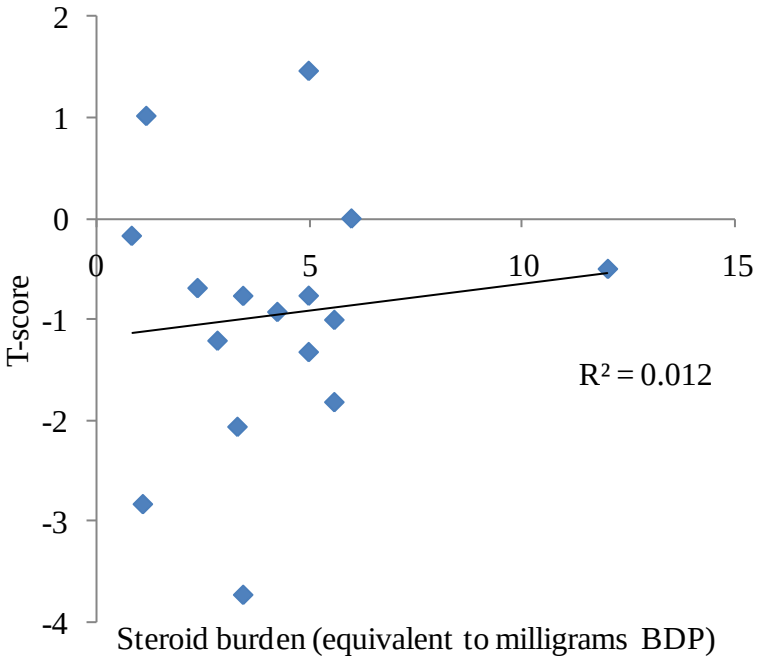
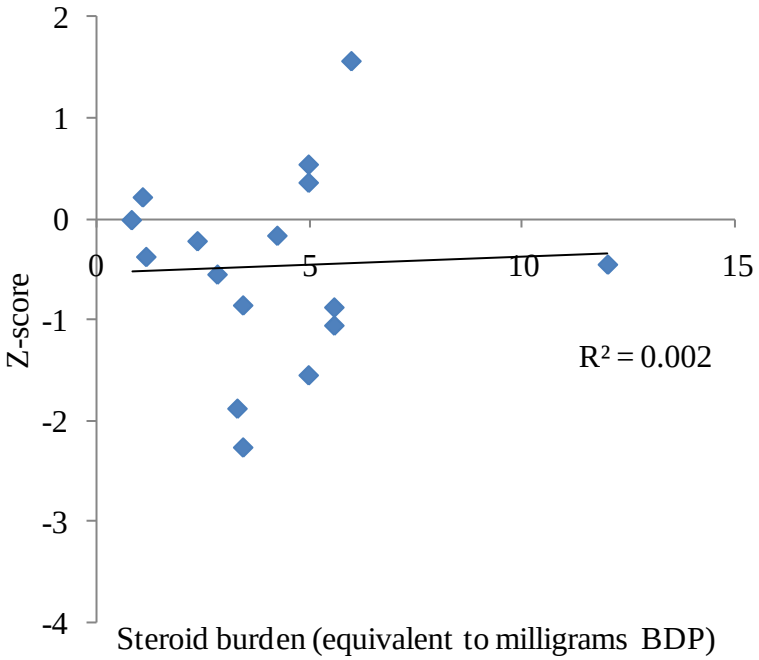


Figure 3.9 Correlation between lumbar spine (a) Z-score and (b) T-score with daily corticosteroid burden (n = 16) (p = 0.786)

(a)



(b)

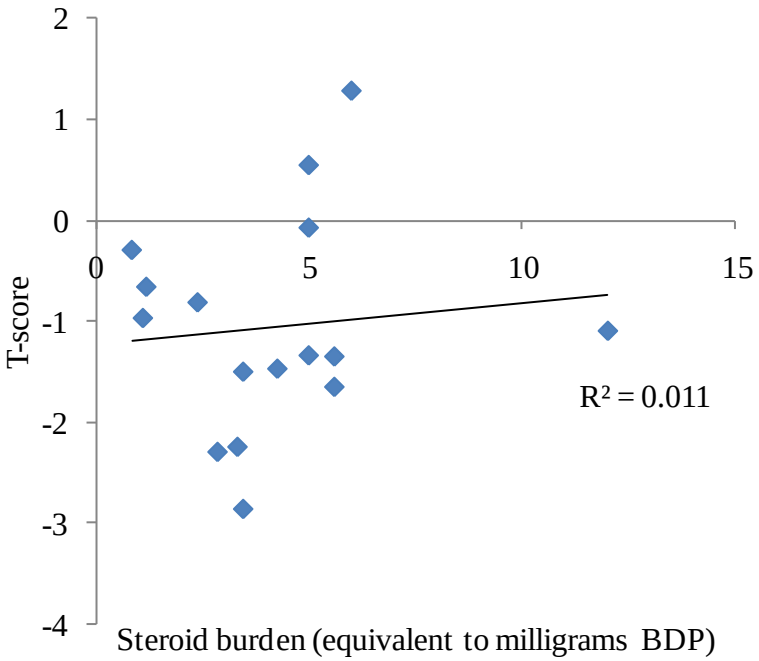
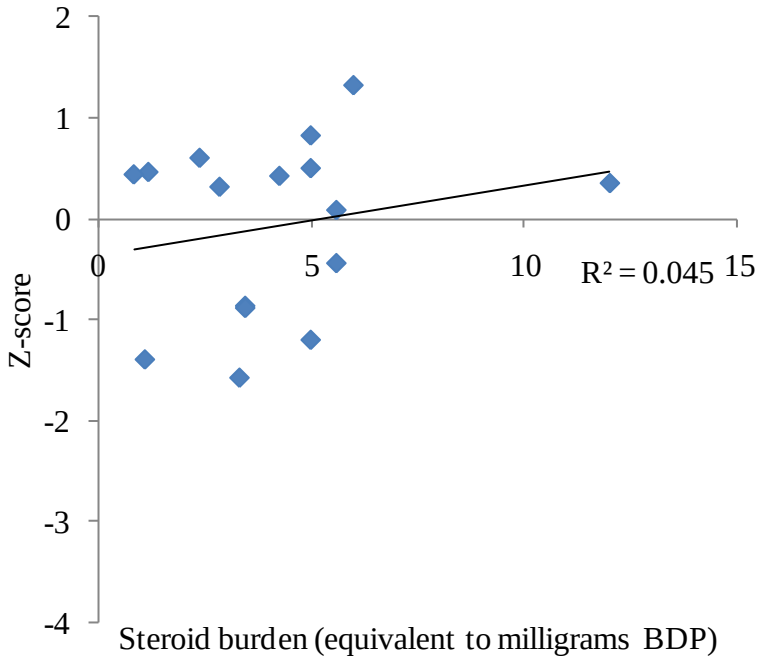


Figure 3.10 Correlation between femoral neck (a) Z-score and (b) T-score with daily corticosteroid burden (n = 16) (p = 0.878)

(a)



(b)

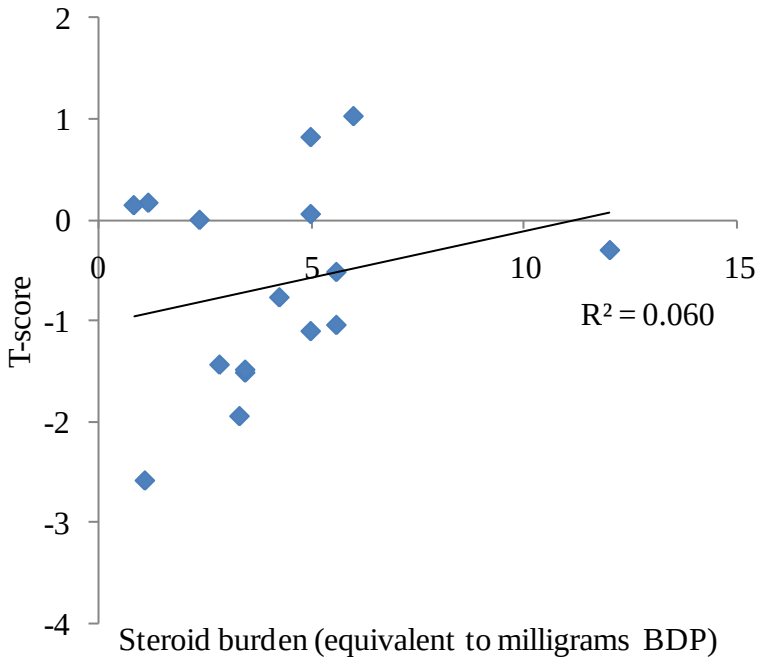


Figure 3.11 Correlation between total hip (a) Z-score and (b) T-score with daily corticosteroid burden (n = 16) (p = 0.433)

The mean (± s.d) of the Z-score was – 0.36 (±1.12) for the lumbar spine, -0.47 (±0.93) for the femoral neck and -0.07 (±0.84) for the total hip. When a Z-score of less than – 2 standard deviations (s.d.) is defined as a low BMD for the matched age

(National Osteoporosis Foundation (NOF), 2008), only 2 patients presented with a low BMD at the lumbar spine and femoral neck but not at the total hip measurement. Out of 16 patients, two of these patients had a recorded lumbar spine T-score below -2 s.d and were thus categorised as osteoporotic and five patients were considered as osteopenic. No significant differences in Z-score or T-score were observed between these 3 measurements sites (lumbar spine, femoral neck and total hip) suggesting the reduction in bone density in one site is at a similar rate to the other sites in corticosteroid treated patients.

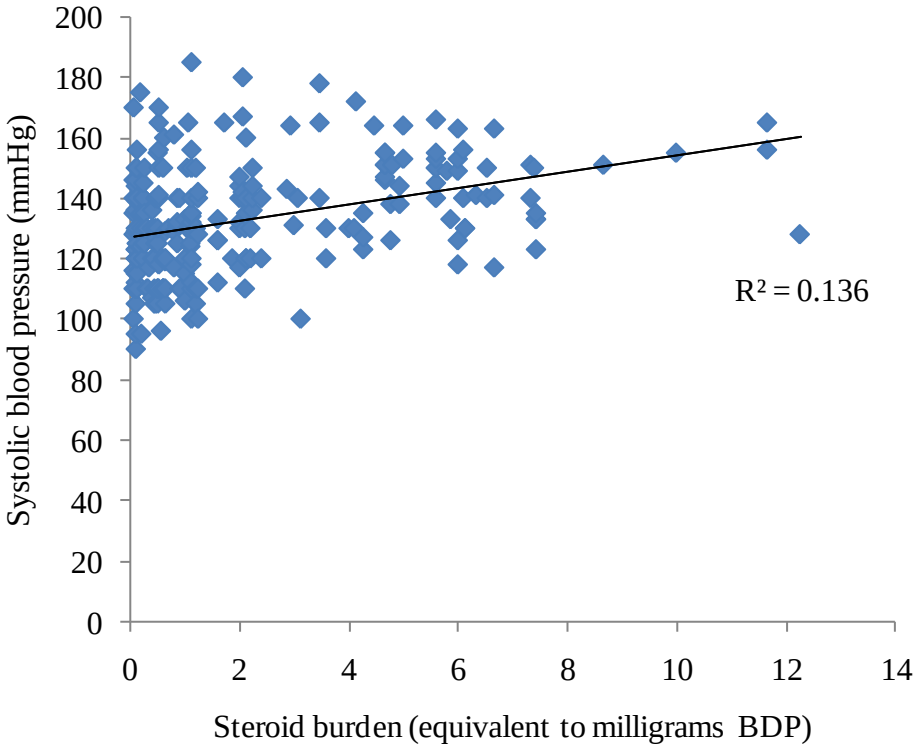
Of the 113 patients, 16 (14.2 %) were prescribed oral corticosteroids for more than 3 months which should have resulted referral for BMD measurements using DEXA (NOS, 2008) but only 12 patients underwent a DEXA scan. Due to the low number of DEXA scans (n = 16) reported within the recruited patients, the effect of corticosteroids on the bone health in this clinical study population was inconclusive.

3.5.2.4 Hypertension

Blood pressure measurements were recorded at every clinic visit where possible. From the population of 113 patients, a total of 259 blood pressure measurements were available. Of the 113 patients included in the study, fourteen patients (12.4 %) reported being prescribed antihypertensive medications. Thirty five patients (31 %) were considered to be categorised as hypertensive based on the British Hypertension Society (BHS) (Williams *et al*., 2004) guideline which defines a systolic blood pressure of greater than or equal to 140 mmHg and/or a diastolic blood pressure of greater than or equal to 90 mmHg as hypertension.

The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were plotted against the daily corticosteroid burden (Figure 3.12, 3.13 and 3.14). For all patient measurements (n = 259), the Pearson's correlation analysis found a positive correlation for SBP (r = 0.369) and DBP (n = 259; r = 0.462) with corticosteroid burden (Figure 3.12). Both correlations were poor but statistically significant ($p < 0.0001$). Further analysis with the Spearman rank correlation analysis showed a similar correlation for SBP (r = 0.37; $p < 0.0001$) and DBP (r = 0.45; $p < 0.0001$) suggesting the correlation did not happen by chance nor was it affected by the outliers.

(a)



(b)

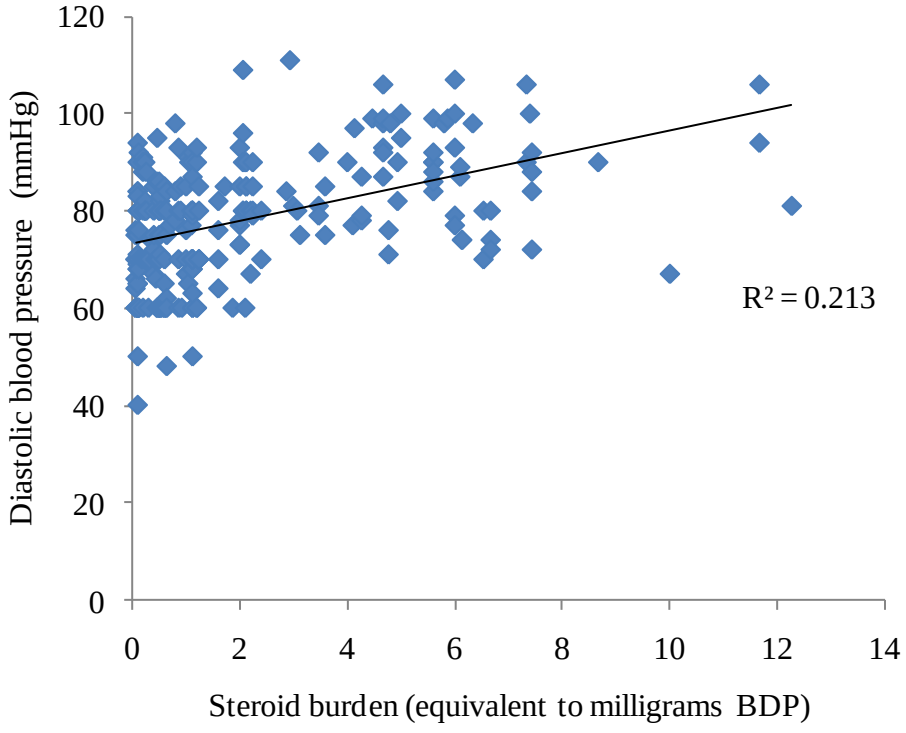
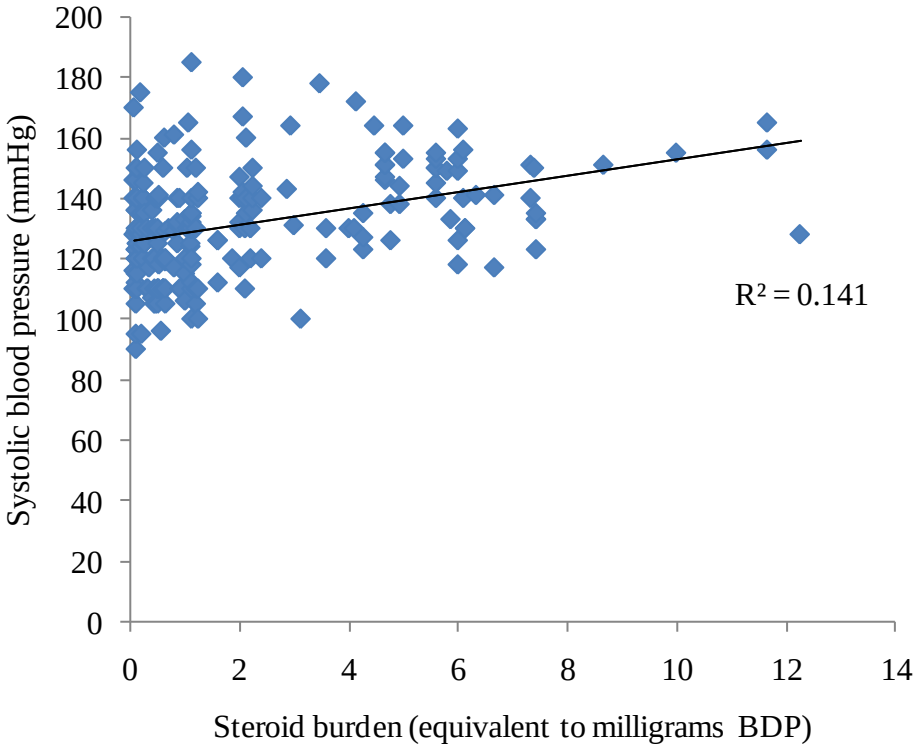


Figure 3.12 Correlation between (a) systolic blood pressure (SBP) and (b) diastolic blood pressure (DBP) with daily corticosteroid burden in all recruited patients (n = 259)

The analyses were repeated excluding the 14 patients prescribed antihypertensive medication thus leaving 99 patients with 229 blood pressure measurements (Figure 3.13). Regression analysis found a statistically significant positive correlation between SBP ($r = 0.376$; $p < 0.001$) and DBP ($r = 0.475$; $p < 0.001$) with corticosteroid burden. Further analysis with the Spearman rank correlation analysis showed a similar correlation coefficient as Pearson's correlation analysis for SBP ($r = 0.38$; $p < 0.0001$) and DBP ($r = 0.45$; $p < 0.0001$).

(a)



(b)

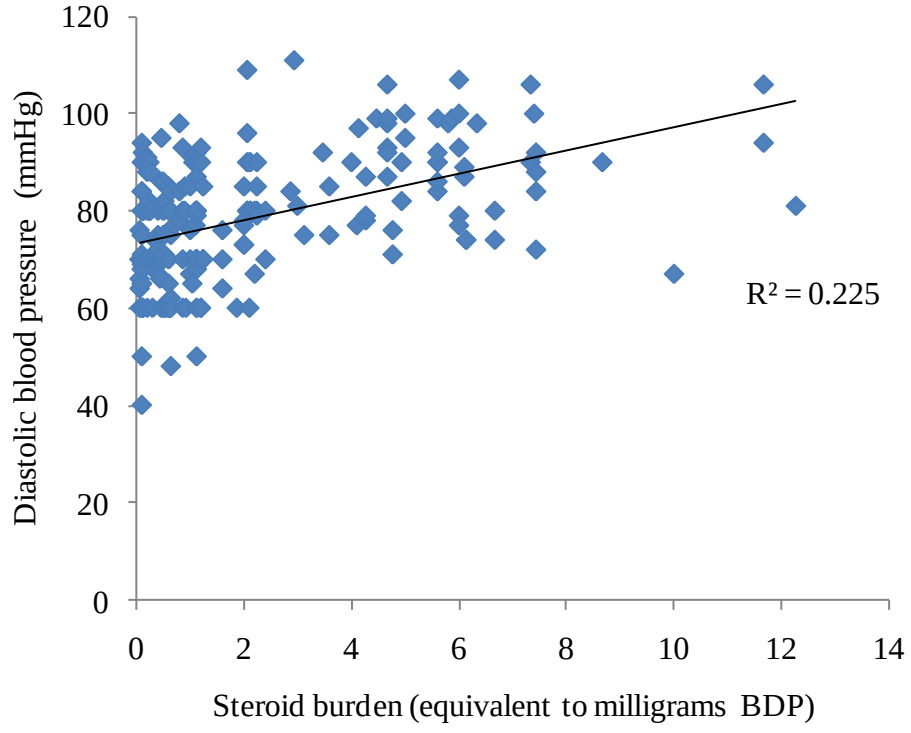
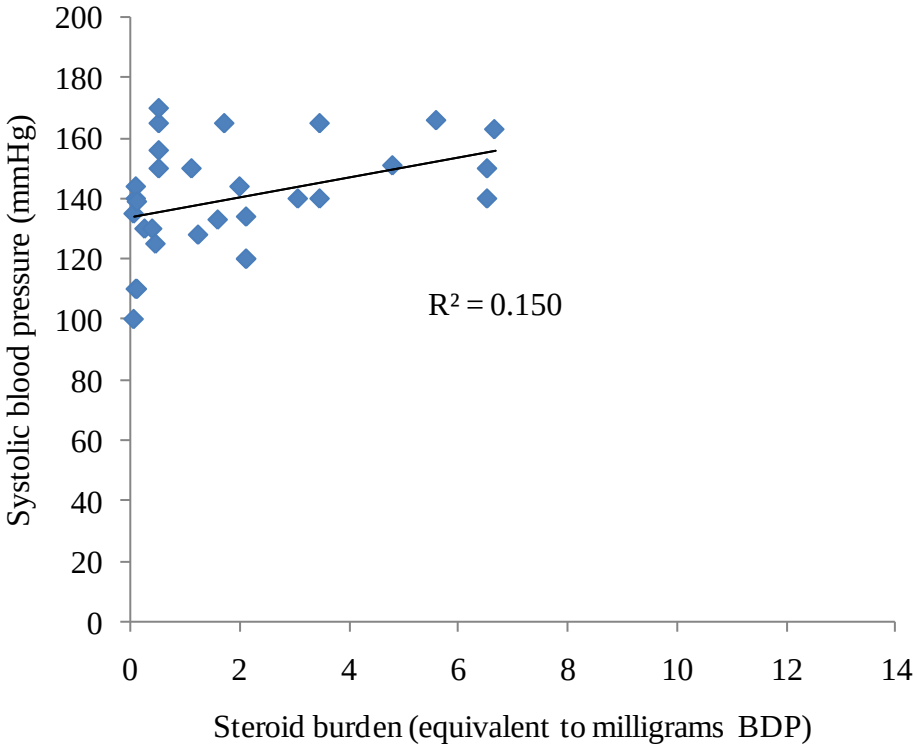


Figure 3.13 Correlation between (a) systolic blood pressure (SBP) and (b) diastolic blood pressure (DBP) with daily corticosteroid burden in patients not on antihypertensive medications (n = 229)

In the 14 patients who were treated with antihypertensive medications (n = 30), the analysis by Pearson's correlation showed a statistically significant relationship between the corticosteroid dose and the SBP ($r = 0.388$; $p = 0.034$) (Figure 3.14a). Further analysis with Spearman rank correlation analysis showed a similar correlation coefficient as the Pearson's correlation analysis for SBP ($r = 0.43$; $p = 0.018$). In contrast, no statistical correlation was found between DBP and the corticosteroid dose in patients prescribed antihypertensive medications ($r = 0.308$; $p = 0.098$) (Figure 3.14b). However, further analysis with the Spearman rank correlation analysis showed a statistically significant correlation between DBP and total corticosteroid dose ($r = 0.40$; $p = 0.027$). Therefore, it can be concluded that the correlation between the SBP and the corticosteroid dose are present and statistically significant ($p < 0.05$) whereas there is no correlation between DBP and corticosteroid burden.

(a)



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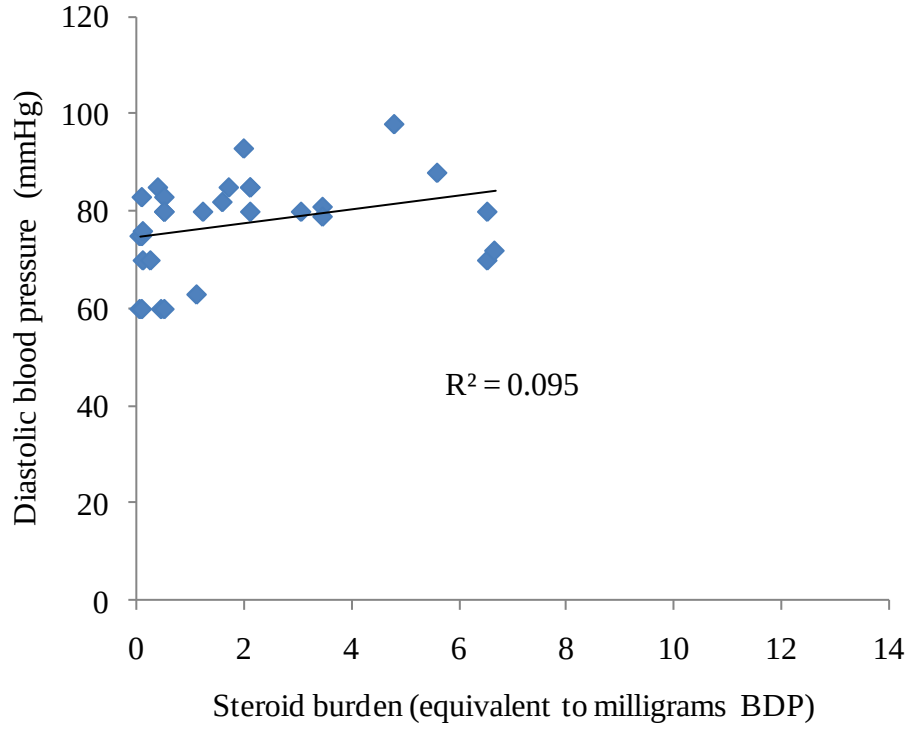


Figure 3.14 Correlation between (a) systolic blood pressure; (b) diastolic blood pressure (DBP) and daily corticosteroid burden in patients on antihypertensive medications (n = 30)

Patients with a corticosteroid burden equal to or greater than 1 milligram BDP equivalent daily were likely to suffer from corticosteroid induced hypertension compared to patients on a lower corticosteroid dose (less than 1 milligram BDP equivalent daily) (Table 3.9). This positive correlation and statistical significance indicates that the increase in blood pressure may be influenced by an increase in corticosteroid burden. However, this is partially true as there is significant correlation between SBP and corticosteroid burden in antihypertensive treated patients taking a corticosteroid dose less than 1 milligram BDP equivalent daily ($p < 0.01$).

Table 3.9 Correlation between SBP and DBP and corticosteroid dose

Parameter	Corticosteroid dose	
	< 1 milligram daily$^{\infty}$	≥ 1 milligram daily$^{\infty}$
Systolic blood pressure (SBP) (mmHg) (Median (IQR))		
All patients	125 (110 – 140)	138 (128 – 150)*
Patients prescribed antihypertensive agents	137 (121 – 152)*	142 (133 – 160)
Patients not prescribed anti-hypertensive medication	125 (110 – 140)	135 (126 – 150)*
Diastolic blood pressure (DBP) (mmHg) (Median (IQR))		
All patients	72 (65 – 80)	81 (74 – 90)*
Patients prescribed antihypertensive agents	75 (60 – 81)	81 (79 – 85)
Patients not prescribed antihypertensive medication	71 (66 – 80)	81 (74 – 90)*

$^{\infty}$BDP equivalent daily *significant at $P < 0.001$

3.5.2.5 Hyperglycemia

Of the 113 patients, 2 patients (1.8 %) reported that they were prescribed antidiabetic medications in the clinical study questionnaire. One patient was found to have a random blood glucose (RBG) greater than 11 mmol L^{-1} which indicated hyperglycemia. This patient was previously diagnosed with diabetes mellitus type 2. Of the 113 patients, 255 random blood glucose (RBG) readings were available. The random blood glucose (RBG) was compared to the corticosteroid burden (Figures 3.15 and 3.16). There was no correlation observed between the random blood glucose and the corticosteroid burden using Pearson's correlation analysis in all patients recruited to this study ($n = 255$; $r = 0.061$; $p = 0.335$) (Figure 3.15). Further analysis with the Spearman rank correlation analysis showed a similar correlation coefficient to the Pearson's correlation analysis for RBG ($r = 0.43$; $p = 0.485$). Therefore, based on both correlation analyses, no correlation was found between the total corticosteroid dose and plasma glucose in all recruited patients.

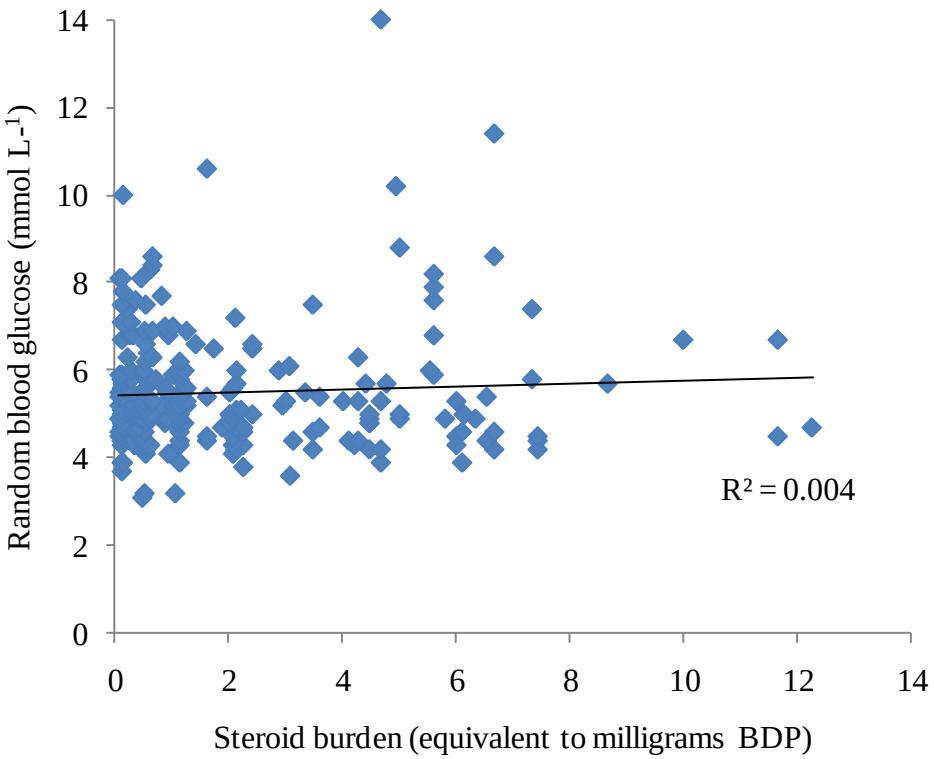


Figure 3.15 Correlation between random blood glucose (RBG) and daily corticosteroid burden in all recruited patients (n = 255).

Only four plasma glucose measurements (n = 4; 1.6 %) were collected from the two patients prescribed antidiabetic medications. Therefore, it was not possible to draw any conclusions in relation to potential correlation between random blood glucose and the corticosteroid burden.

Further statistical analysis of the effect of corticosteroid burden on the random blood glucose excluding patients prescribed antidiabetic medications was calculated. A Pearson's correlation analysis found no linear correlation between the RBG and the corticosteroid burden in 111 patients not prescribed antidiabetic medication (n = 251; r = -0.022; p = 0.725) (Figure 3.16). Further analysis with Spearman rank correlation analysis showed similar correlation coefficient to the Pearson's correlation analysis

for RBG (r = -0.08; p = 0.211). Therefore, based on both correlation analyses, no correlation was found between the total corticosteroid burden and the plasma glucose in patients not prescribed antidiabetic medication.

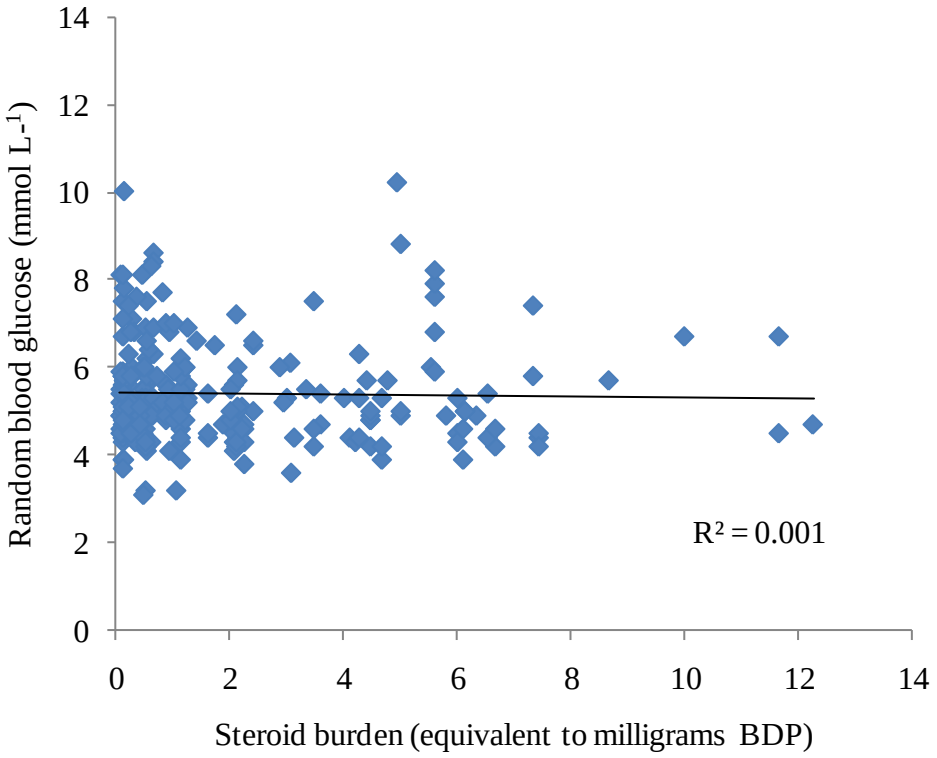


Figure 3.16 Correlation between random blood glucose (RBG) and daily corticosteroid burden in patients not prescribed antidiabetic medications (n = 251)

3.5.2.6 The symptom scores

3.5.2.6.1 Sino Nasal Outcome Test (SNOT-22) score

The SNOT-22 questionnaire was used to measure the symptom control in patients attending the ENT clinic at GRI. The scores were calculated by summating the symptom score (Gillett *et al*., 2009). The calculated SNOT-22 score is not divided into five domains as in previous studies (Pynnonen *et al*., 2009; Browne *et al*., 2007).

Only 129 samples from 62 patients have a matched salivary cortisol and SNOT-22 (Figure 3.17). The Pearson's correlation analysis showed a weak statistical correlation between the SNOT-22 scores and the salivary cortisol which is not statistically significant (n = 129; r = 0.079; p = 0.376). The severity of inflammation did not account for the reduction in salivary cortisol nor was it related to adrenal suppression.

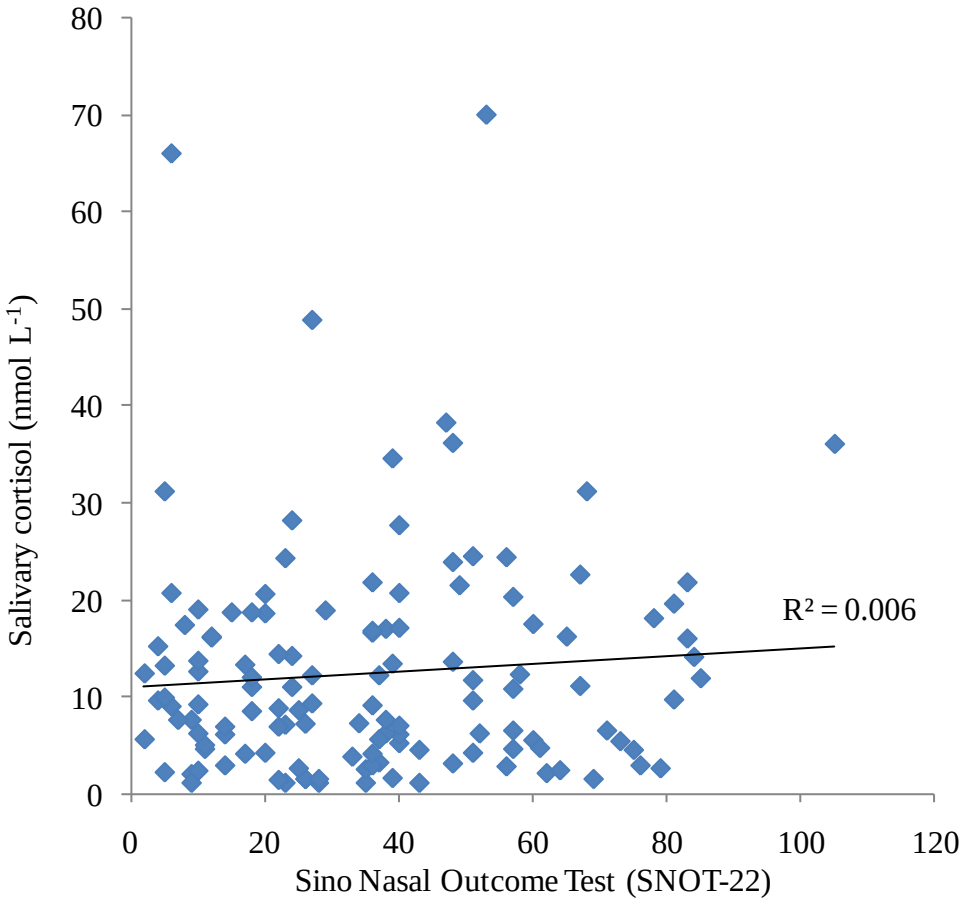


Figure 3.17 Correlation between salivary cortisol against sino nasal outcome test (SNOT-22) score (n = 129)

3.5.2.6.2 Asthma Control Questionnaire (ACQ) score

The symptom control of patients attending the Problem Asthma clinic at SH was assessed by the Asthma Control Questionnaire (ACQ). Good asthma control is defined as an ACQ score of equal to or less than 0.75 (Juniper *et al*., 2006). Patients with ACQ score of equal or greater than 1.5 are considered to have poor control of their asthma. Only two samples were collected from patients considered as having well control asthma (ACQ $\leq$0.75). Only 18 samples from 15 patients had a matched salivary cortisol and ACQ score. A Pearson's correlation analysis found no correlation between salivary cortisol and the ACQ score ($r = 0.089$; $p = 0.723$) (Figure 3.18). The lack of symptom control in patients with asthma did not account for reduction in salivary cortisol nor related to adrenal suppression.

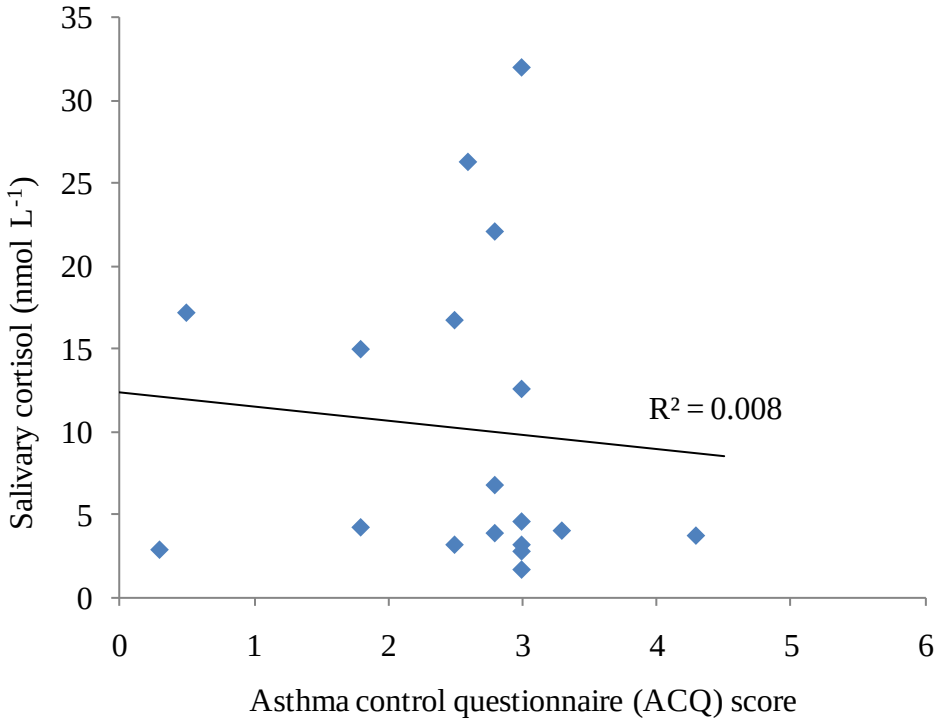


Figure 3.18 Correlation between salivary cortisol and the asthma control questionnaire (ACQ) score (n = 18)

3.5.2.7 Steroid treatment card

The Medicines and Healthcare products Regulatory Agency (MHRA) (1998) have advised that a steroid treatment card is issued to patients on prolonged corticosteroid treatment defined as more than 3 weeks treatment and a high dose of inhaled corticosteroid, defined as greater than or equal to 800 micrograms BDP equivalent daily. The steroid treatment card contains advice and information for the healthcare practitioner and the patient regarding corticosteroid therapy for the patient. Of 113 patients, 44 (38.9 %) confirmed that they received a steroid treatment card but only 24 (54.5 %) patients carried the card with them. Patients were not asked for the reasons for not carrying their steroid treatment card. In 44 patients who had a steroid

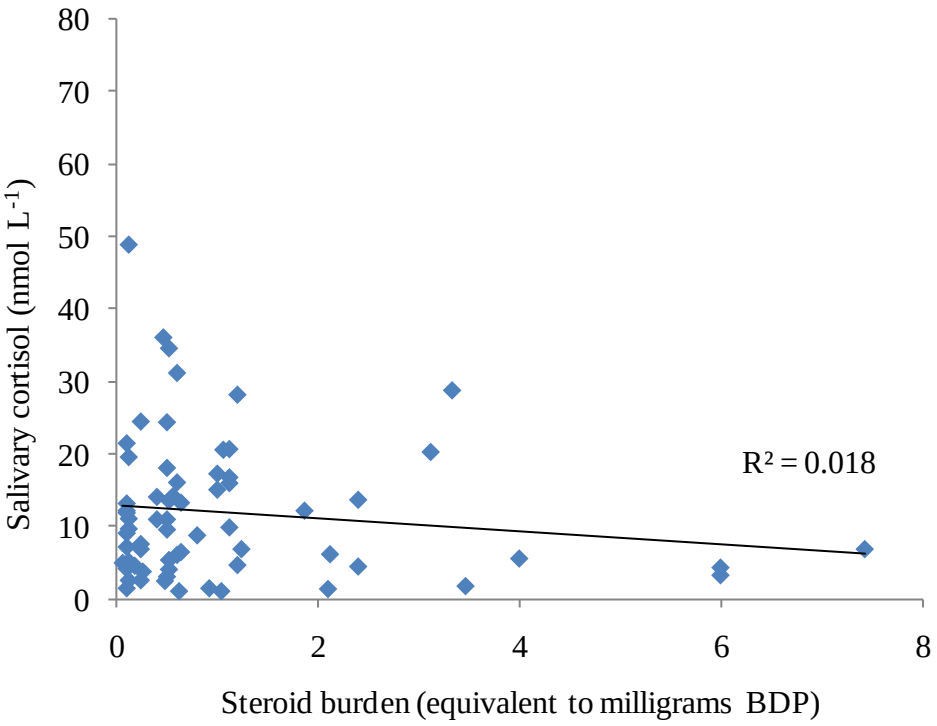
treatment card, 9 (20.5 %) patients were on a corticosteroid dose less than 800 micrograms BDP equivalent daily and 13 (29.5 %) needed a replacement card. Based on the MHRA (1998) recommendations, 57 (50.4 %) patients from this clinical study should have been issued a steroid treatment card but only 35 (31 %) confirmed they had received one.

3.5.2.8 First visit against all visits

The collected data were examined by inputting values for the first visit for all patients and the data available for all visits to see if multiple data points from a single patient influenced the outcome of the analysis. Correlation analysis was performed using the Fisher transformation analysis.

Fisher transformation analysis found that the difference between the data on salivary cortisol and the corticosteroid burden (Figure 3.19) collected at the first visit was not statistically significantly different from the data collected at all visits ($p = 0.448$). Thus, the analysis which was carried out with all the data relating to salivary cortisol and the corticosteroid burden collected at all visits was not influenced by multiple visits by patients.

(a)



(b)

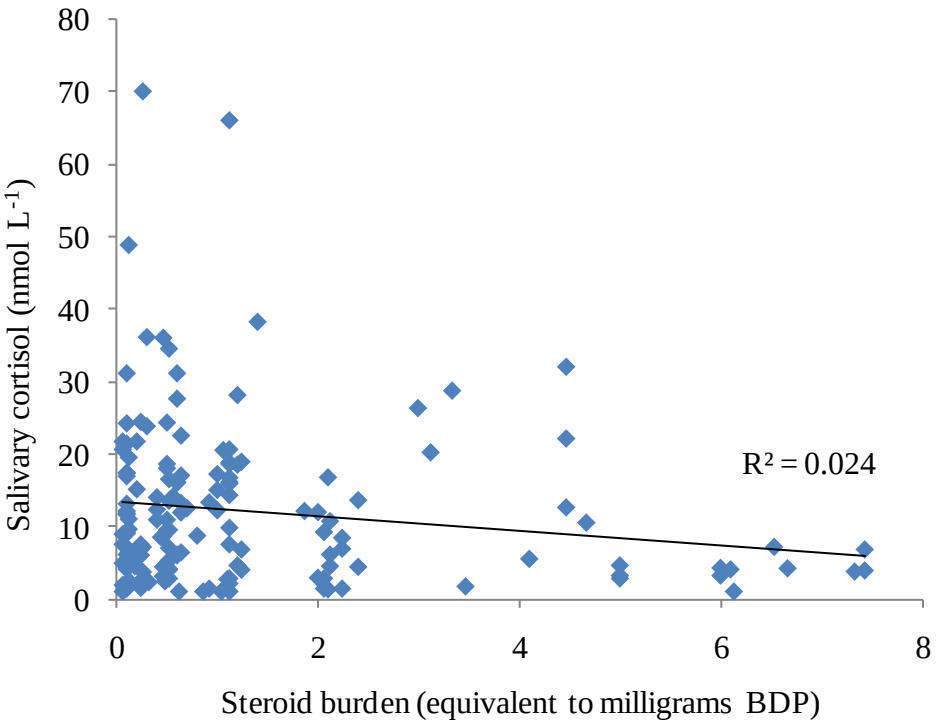
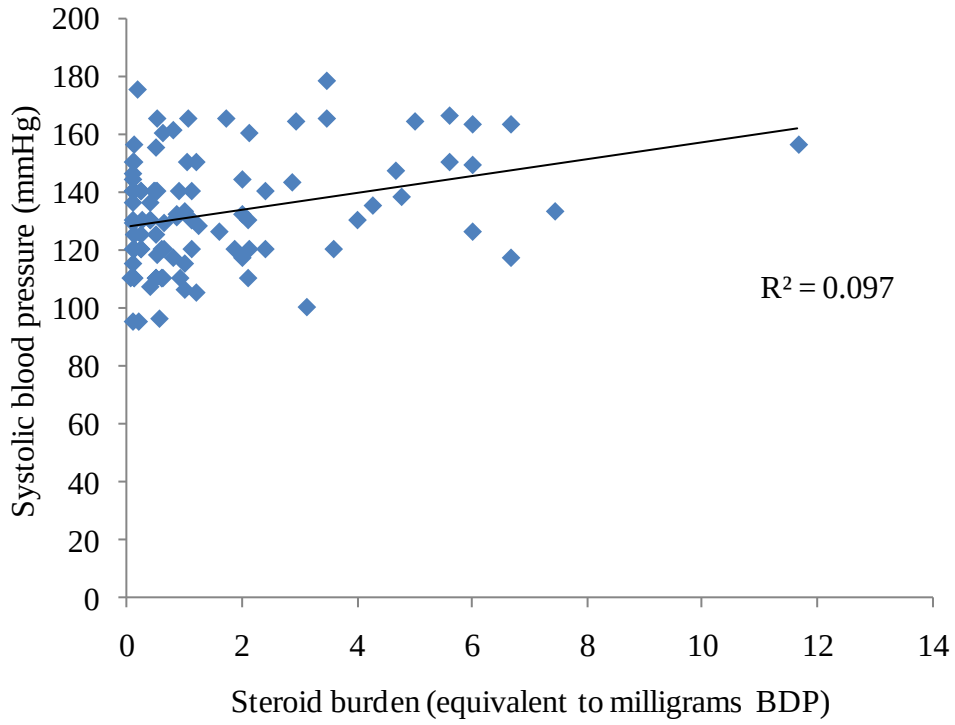


Figure 3.19. Salivary cortisol against daily corticosteroid burden at (a) first visit (n = 66) and (b) all visit (n = 155) (Fisher transformation r-to-z; p = 0.448)

Fisher transformation analysis found that the difference between data on systolic blood pressure and the corticosteroid burden collected at the first visit (Figure 3.20) was not statistically significantly different from the data collected at all visits ($p = 0.352$). Thus, the analysis which was carried out with all the data relating to systolic blood pressure and the corticosteroid burden collected at all visits was not influenced by multiple visits by patients.

(a)



(b)

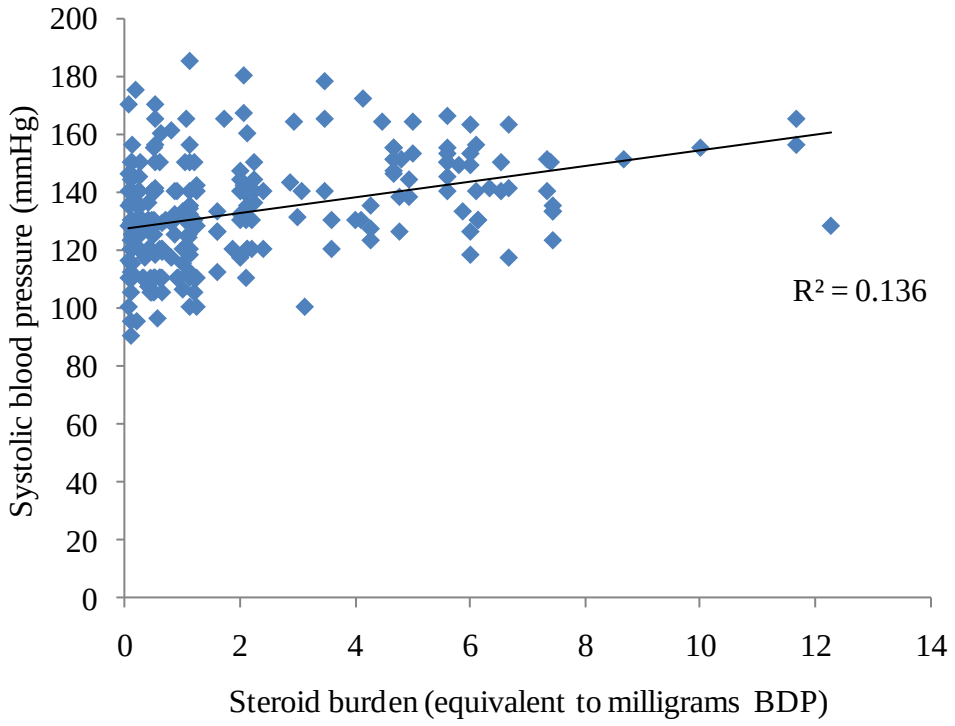
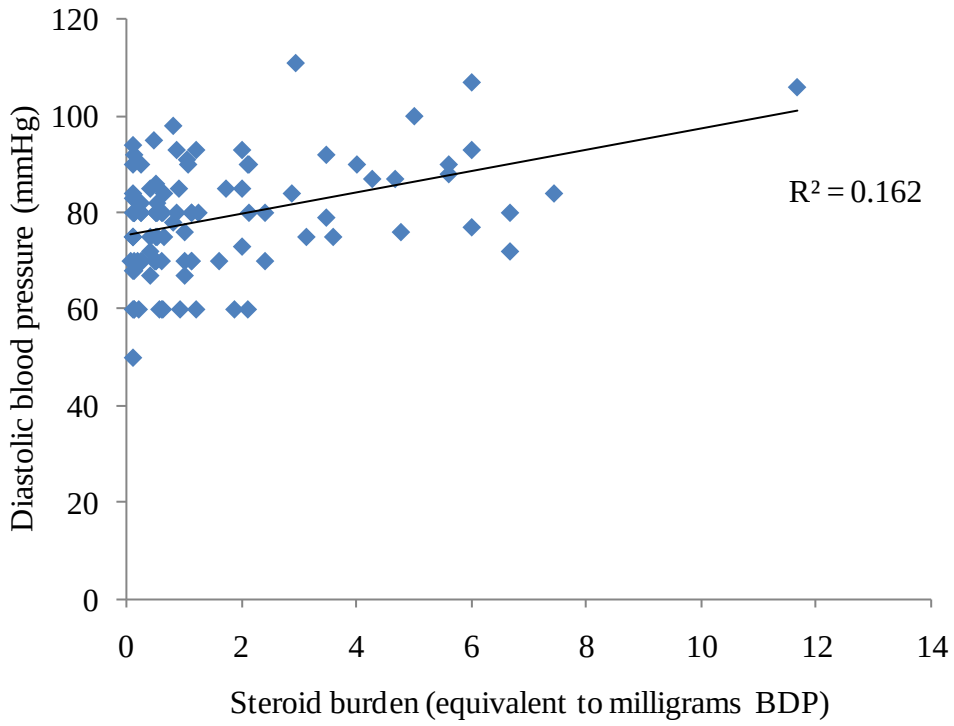


Figure 3.20 Systolic blood pressure against daily corticosteroid burden at (a) first visit (n = 104) and (b) all visits (n = 259) (Fisher transformation r-to-z; p = 0.352)

Fisher transformation analysis found that the difference between the data on diastolic blood pressure and the corticosteroid burden collected at the first visit (Figure 3.21) was not statistically significantly different from the data collected at all visits ($p = 0.337$). Thus, the analysis which was carried out with all the data relating to diastolic blood pressure and the corticosteroid burden collected at all visits was not influenced by multiple visits by patients.

(a)



(b)

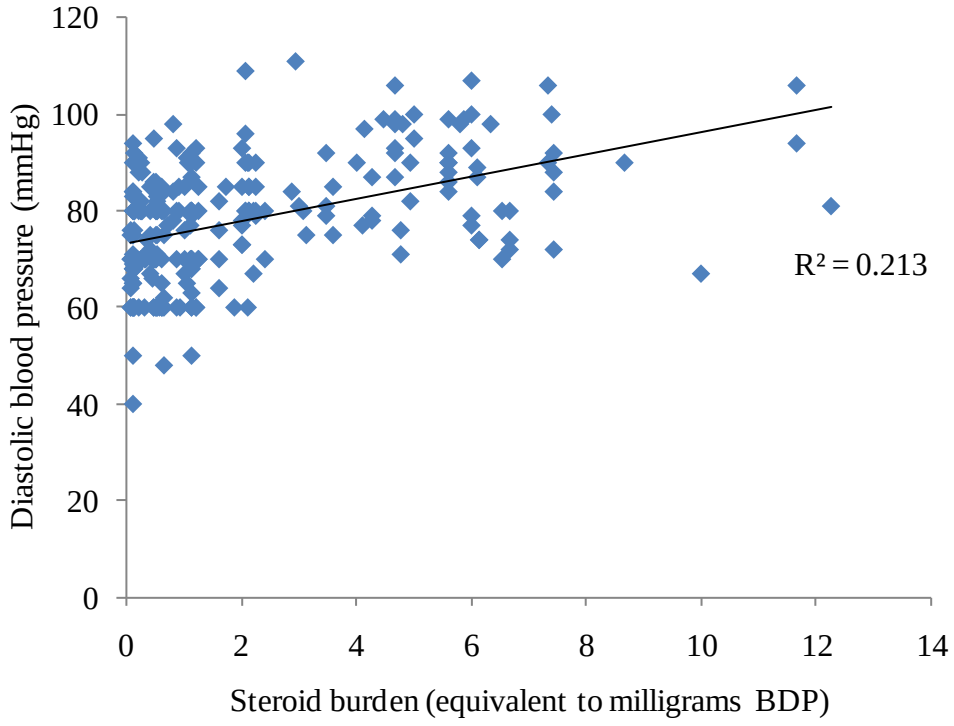
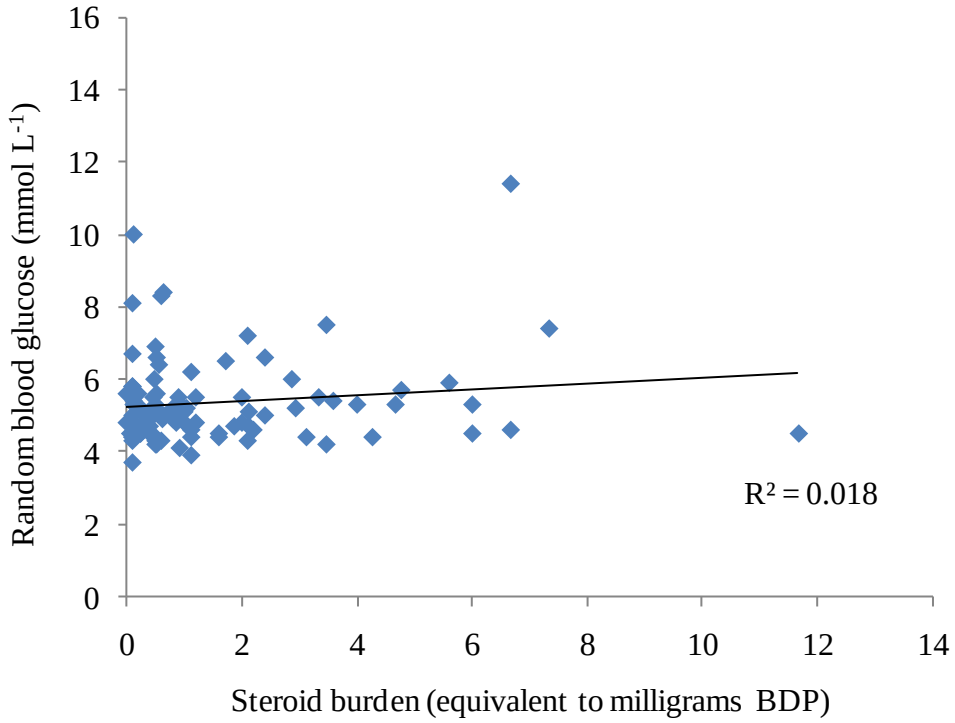


Figure 3.21. Diastolic blood pressure against daily corticosteroid burden at (a) first visit (n = 104) and (b) all visits (n = 259) (Fisher transformation r-to-z; p = 0.337)

Fisher transformation analysis found that the difference between the data on random blood glucose and the corticosteroid burden collected at the first visit (Figure 3.22) was not statistically significantly different from the data collected at all visits ($p = 0.264$). Thus, the analysis which was carried out with all the data relating to random blood glucose and the corticosteroid burden collected at all visits was not influenced by multiple visits by patients.

(a)



(b)

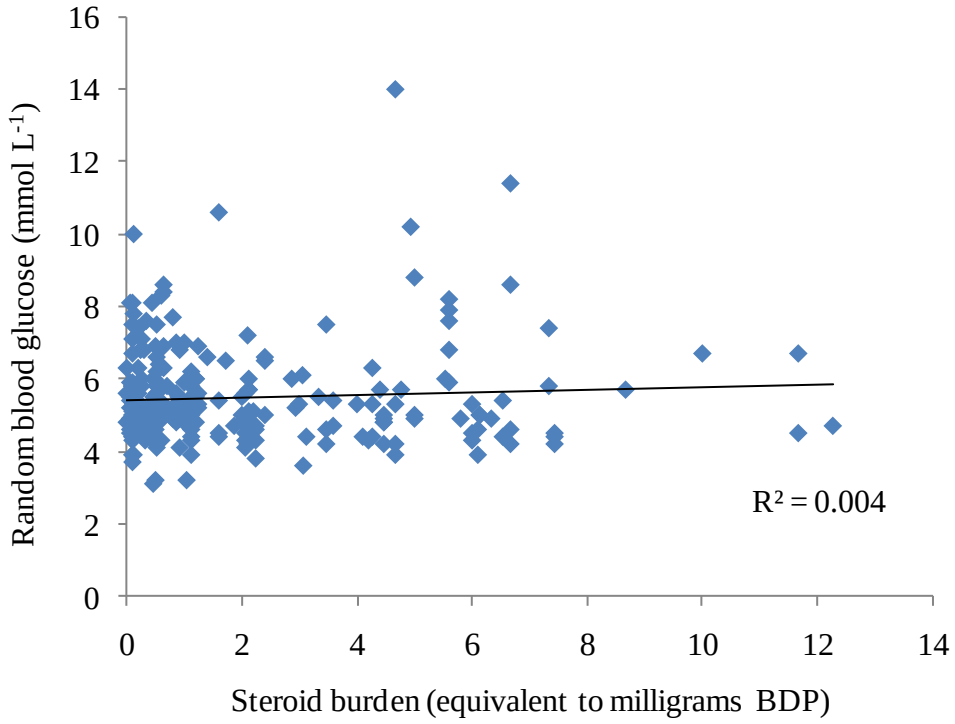
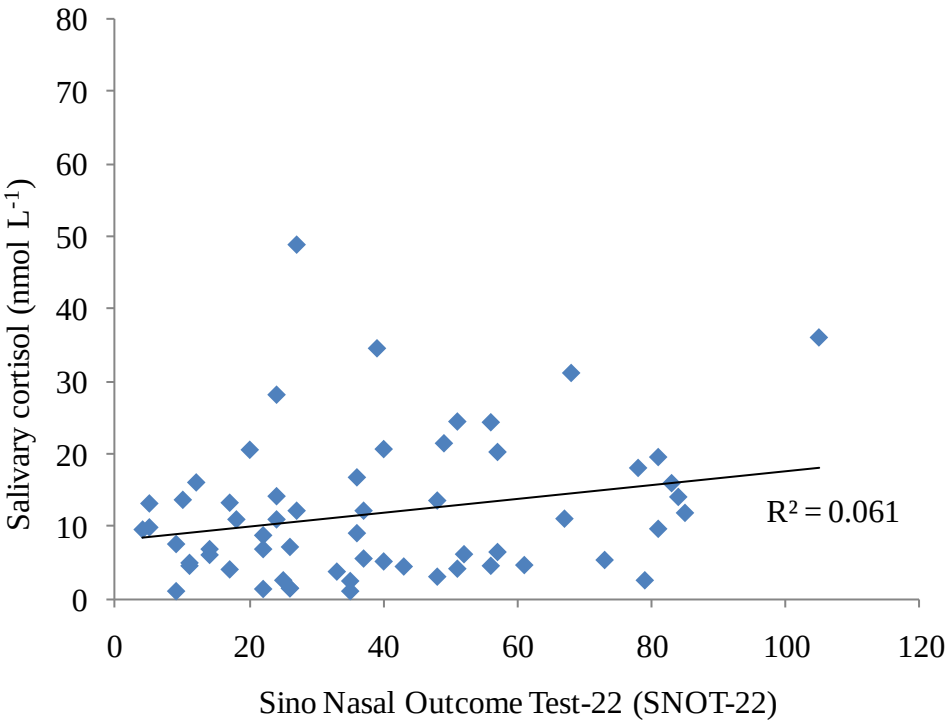


Figure 3.22 Random blood glucose against daily corticosteroid burden at (a) first visit (n = 98) and (b) all visits (n = 256) (Fisher transformation r-to-z; p = 0.264)

Fisher transformation analysis found that the difference between data on salivary cortisol and the SNOT-22 score collected at the first visit (Figure 3.23) was not statistically significantly different from the data collected at all visits ($p = 0.125$). Thus, the analysis which was carried out with all the data relating to salivary cortisol and the SNOT-22 collected at all visits was not influenced by multiple visits by patients.

(a)



(b)

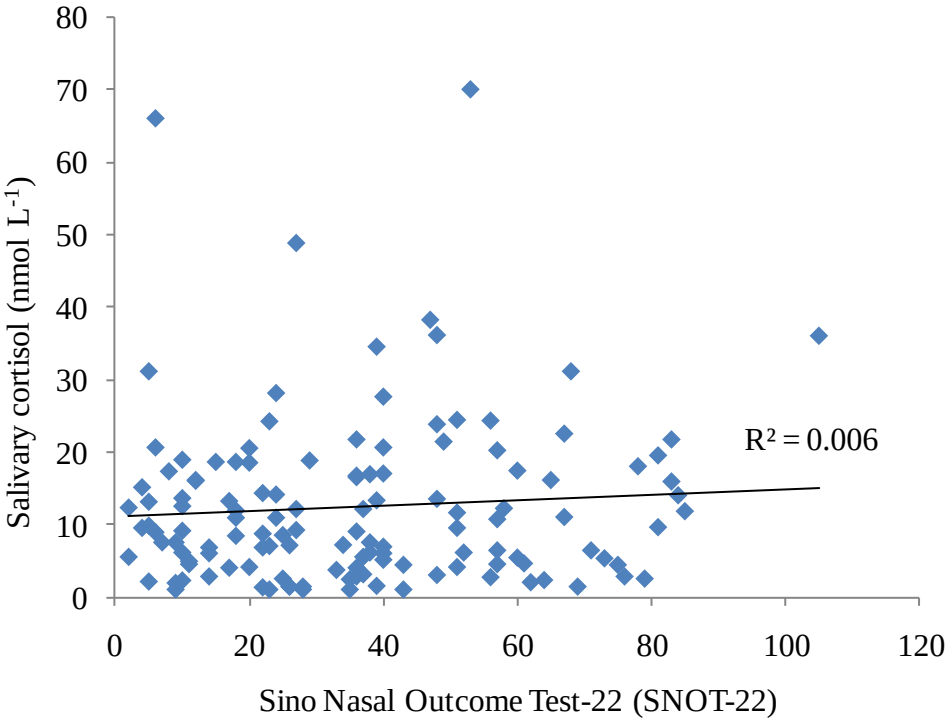
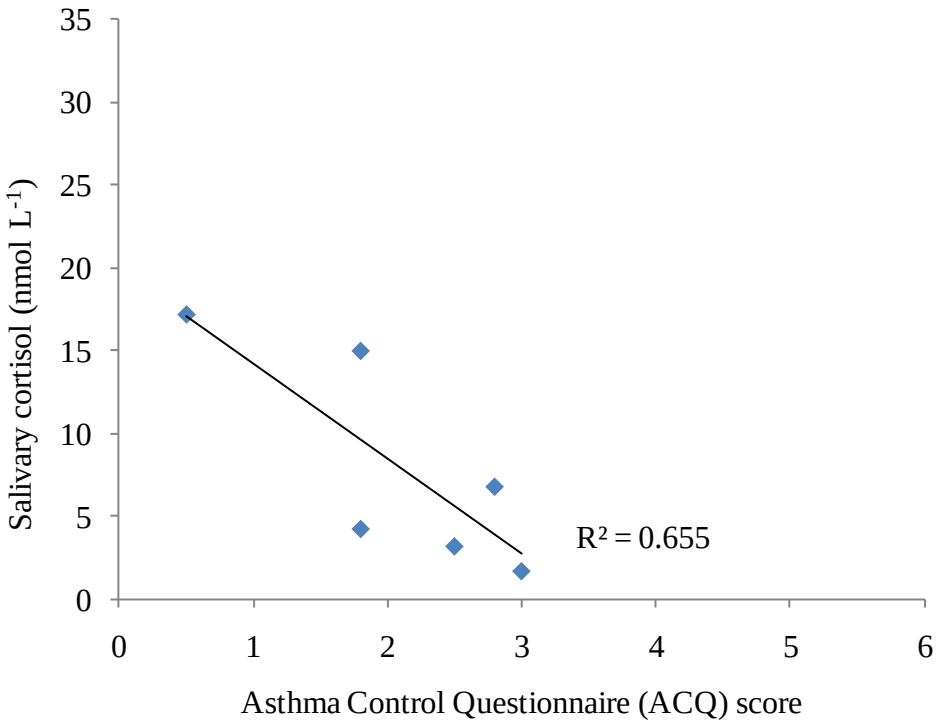


Figure 3.23 Salivary cortisol against SNOT-22 at (a) first visit (n = 59) and (b) all visits (n = 129) (Fisher transformation r-to-z; p = 0.125)

Fisher transformation analysis found that the difference between the data on salivary cortisol and the asthma control questionnaire (ACQ) score collected at the first visit (Figure 3.24) was not statistically significantly different from the data collected at all visits ($p = 0.452$). Thus, the analysis which was carried out with all the data relating to salivary cortisol and the asthma control questionnaire (ACQ) collected at all visits was not influenced by multiple visits by patients.

(a)



(b)

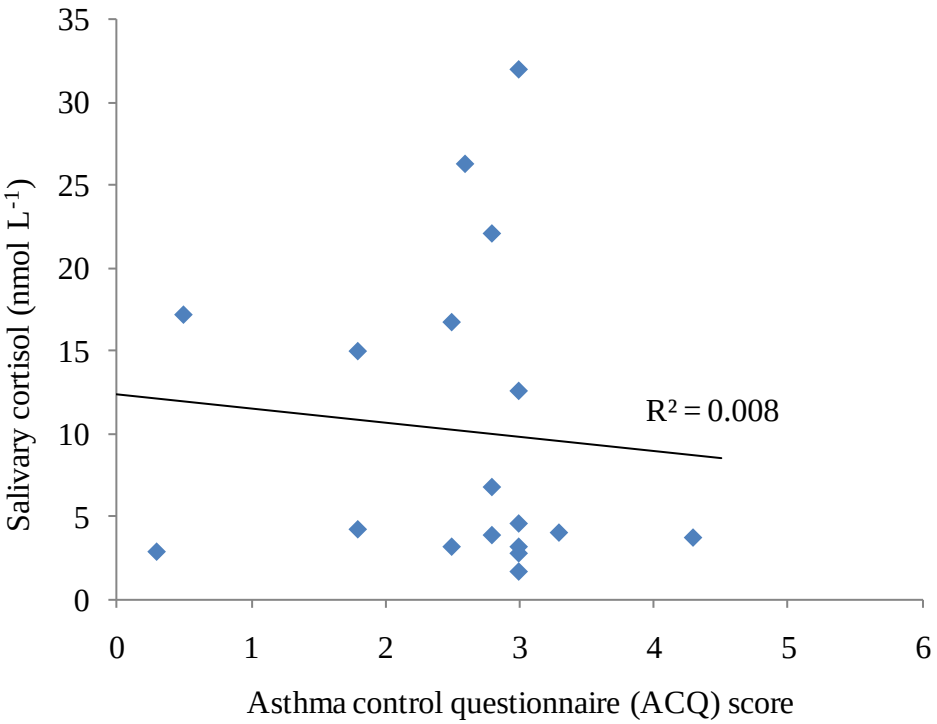


Figure 3.24 Salivary cortisol against ACQ score at (a) first visit (n = 6) and (b) all visits (n = 18) (Fisher transformation r-to-z; p = 0.452)

In summary, there was no statistically significant difference between the data for salivary cortisol (Figure 3.19), corticosteroid burden, systolic blood pressure (Figure 3.20), diastolic blood pressure (Figure 3.21), random blood glucose (RBG) (Figure 3.22) or the symptom score (SNOT-22, ACQ) (Figure 3.23 and 3.24), whether they were analysed at the first visit or included multiple visits for each patient. Thus the inclusion of multiple data points for individual patients did not affect any correlations reported.

3.6 Discussion

This study aimed to investigate the effects of corticosteroids in patients given corticosteroid therapy by a single or combination of routes of administration. The effects of corticosteroids such as hypothalamic pituitary axis (HPA) suppression, corticosteroid induced osteoporosis, development of diabetes mellitus and hypertension have been associated with the long term use of corticosteroids and high corticosteroid doses (Tayab *et al.*, 2007; Derom *et al*., 2005; Fardon *et al*., 2004; Patel *et al*., 2004). In this study, the corticosteroid burden, which is the total corticosteroids dose taken by the patients either by inhaled, intranasal or oral, was calculated and its impact on the physiological function in each individual was assessed.

3.6.1 Principal findings

3.6.1.1 Adrenal suppression

The main finding of this study was the absence of an association between the daily corticosteroid burden and the salivary cortisol. The analysis showed a weak correlation between the corticosteroid burden and adrenal suppression which was not statistically significant. The distribution of salivary cortisol measurements, with a median of 9.1 (4.1 – 16.9) nmol L^{-1} and a maximum of 70.0 nmol L^{-1} and minimum of 1.0 nmol L^{-1} showed variability in the patient's response to the corticosteroid therapy. The analysis showed that an increase in corticosteroid dose did not reduce the salivary cortisol. Several studies have shown that there is no adrenal suppression related to inhaled corticosteroids (Szelfer *et al*., 2005; Eichenhorn *et al*., 2003;

Martin *et al*., 2002) or intranasal corticosteroids (Sachanandani *et al*., 2009; Algorta *et al*., 2008; Dales-Yates *et al*., 2004).

Interestingly, patients with a corticosteroid dose of less than 1 milligram BDP equivalent daily were shown to be at the same risk of suppression of salivary cortisol as patients prescribed more than 1 milligram BDP equivalent daily. These findings were supported by Patel *et al*., (2004) who suggested that intranasal corticosteroids at a dose as low as 200 micrograms BDP equivalent daily are linked to adrenal suppression. However, most of the previous studies described the risk of developing adrenal suppression as greater in patients prescribed a high dose, greater than 800 micrograms BDP equivalent daily, than those prescribed a low to moderate corticosteroid dose (Tayab *et al.*, 2007; Whelan *et al*., 2005; Derom *et al*., 2005; Fardon *et al*., 2004). Studies have reported that a corticosteroid dose of less than 1 milligram BDP equivalent daily are generally safe (Sachanandani *et al*., 2009; Algorta *et al*., 2008; Szelfer *et al*., 2005; Fowler *et al*., 2002; Keith *et al*., 2000). The Medicines and Healthcare products Regulatory Authority (MHRA) (2006) suggested that the risk of adrenal suppression starts to increase at a corticosteroid dose of 1 milligram BDP equivalent daily and these patients should be provided with a steroid warning card. Patients prescribed high doses of corticosteroids should not have their corticosteroids withdrawn suddenly and corticosteroid replacement is to be considered in cases of intercurrent illness.

Based on the results of this study, the risk of suppression of salivary cortisol is not related to the administration route of corticosteroids. Surprisingly, the patients

taking a combination of inhaled and intranasal corticosteroids showed a similar median and wider range of salivary cortisol measurements compared to the patients prescribed intranasal corticosteroids only. It is possible that some of the patients prescribed inhaled corticosteroids did not comply with the instructions for saliva collection. The collection of saliva for salivary cortisol measurement involves a strict procedure including not eating, drinking or taking medication before saliva collection and adhering to an exact time of collection (Liening *et al*., 2009; Dorn *et al*., 2007; Jacobs *et al*., 2005). It is assumed that the noncompliance of patients with the strict procedure of salivary cortisol collection may affect the result (Broderick *et al*., 2004). In this study, clear written instructions detailing how the saliva should be collected (Appendix 3.6) was given at every visit along with the sample bottle. Several patients recruited to this study mentioned that they took their inhaled corticosteroid before saliva collection which might lead to an elevated salivary cortisol measurement. This demands a follow up assessment of the saliva collection method. In this study, no correction was made to any of the salivary cortisol measurements if the patients had taken their inhaled corticosteroids or not before sample collection.

Several published studies have shown that intranasal betamethasone (BETA) exhibits higher risk of adrenal suppression when compared to intranasal mometasone (MF) or intranasal beclometasone (BDP) (Rosenblut *et al*., 2007; Fowler *et al*., 2002; Patel *et al*., 2004). A similar trend was seen in our study where patients prescribed intranasal BETA alone ($n = 22$) showed greater suppression of salivary cortisol than patients prescribed intranasal MF ($n = 19$) ($p < 0.05$) but there was no statistically significant

difference when compared to the patients prescribed intranasal BDP. This analysis suggests that the prescription of intranasal BETA may be associated with a higher risk of adrenal suppression than other intranasal corticosteroids.

The plasma concentrations of prednisolone were measured using the analytical method developed in this study. The absence of a relationship between salivary cortisol and plasma concentration of prednisolone was not unexpected as the patients cortisol production is affected by the corticosteroid burden as a total and not oral prednisolone dose alone. Due to the small number of plasma samples quantified by the study (n = 23), the findings observed are not sufficient to represent the whole study population.

A larger study population is needed and more sensitive analytical method that can quantify the low concentration of corticosteroids which may help to answer these questions. A larger study population may reveal more significant results.

3.6.1.2 Osteoporosis

In this study, the results suggested a poor correlation between an increase in the Z-score and T-score with the increase in corticosteroid dose but it was not statistically significant. In this study, the BMD was expressed as both the Z-score and the T-score. The T-score of a Dual Energy X-ray Absorptiometry (DEXA) scan has been suggested by the World Health Organisation (WHO) as the tool to be used to explain the BMD measurements rather than the actual BMD value (WHO, 1994). The Z-score may be more applicable than the T-score in this study as the age distribution of

the patients recruited is 54 (43 – 62) years old and thus the patients may already have an osteoporosis risk due to their age. However, since the Z-score is not recommended for risk assessment of osteoporosis, the risk of assessment was done by using T-score of the BMD measurements. Based on the lumbar spine T-score, two patients were considered to be osteoporotic and five patients were considered to be osteopenic based on the World Health Organisation (WHO) classification. Of the 16 patients, 13 (81.3 %) were on long term oral corticosteroid therapy (more than 6 months) and three patients were on high dose corticosteroids (greater than 1 milligram BDP equivalent). Of the 13 patients who received oral corticosteroids, only one patient was classified as osteoporotic and five patients were considered to be osteopenic. In the patients who were prescribed therapeutic bone protection (n = 4), no post treatment analysis was carried out since no follow up DEXA scan was recorded in the medical records.

Some studies have presented conflicting results on the correlation between use of corticosteroids and their impact on bone mineral density in corticosteroid treated patients. The decrease in bone mineral density (BMD) which leads to osteoporosis has been reported to be associated with long term corticosteroid use (Salem *et al*., 2010; Langhammer *et al*., 2007; Hiwatashi & Westesson, 2007; Vestergaard *et al*., 2007; de Vries *et al*., 2005; Dubois *et al*., 2002; Gudbjornsson *et al*., 2002; Israel *et al*., 2001). On the other hand, several studies have found higher BMD values with an increase in corticosteroid dose (Hubbard *et al*., 2002; Toogood *et al*., 1995) and some studies have found no correlation (de Vries *et al*., 2007; Angeli *et al*., 2006; Suissa *et al*., 2004).

Of the 16 patients with DEXA scan results, only four patients were postmenopausal women and three of them receive therapeutic bone protection. Two were classified as having osteoporosis and one was osteopenic based on their lumbar spine T-score. Evidence from past studies mentioned loss of BMD in postmenopausal women treated with oral corticosteroids (Israel *et al*., 2001; Ton *et al*., 2005) but conflicting results were observed elsewhere (Angeli *et al*., 2006; Kaji *et al*., 2008). Postmenopausal women are subject to benefit from the therapeutic bone protection compared to men of a similar age (Boonen *et al*., 2009; Campbell *et al*., 2004).

In summary, no significant correlation was found between the loss of bone density with an increase in corticosteroid dose. Additionally, the results of the DEXA scans in this population of corticosteroid treated patients (n = 16) is not large enough to represent the study population.

3.6.1.3 Hypertension

In our study, there was a significant positive correlation between the systolic blood pressure (SBP), diastolic blood pressure (DBP) and the corticosteroid burden. This association was seen in all patients whether the patient was treated with antihypertensive medication or not. The elevated blood pressure due to an increase in corticosteroid burden was similar to the cortisol induced hypertension in Cushing's syndrome (Arnaldi *et al*., 2003; Walker *et al*., 2000). A similar observation of the correlation between SBP and DBP with the corticosteroid burden has also been demonstrated in other corticosteroid studies (Fardet *et al*., 2007,

Whitworth *et al*., 2005; Severino *et al*., 2002). In one theory, Walker *et al*., (2005). Roy *et al*., (2009) believed that the mineralocorticoid properties of the corticosteroids cause sodium and water retention that leads to corticosteroid induced hypertension but Fardet *et al*., (2007) and Severino *et al*., (2002) found no correlation between sodium and water retention in elevated blood pressure in their normotensive patients. On the other hand, Walker *et al*., (2000) found a significant increase in DBP but not in SBP in normotensive patients and the effect only occurred in men in the study.

The relationship between hypertension and corticosteroid burden was seen in patients treated with high doses of corticosteroid (greater than or equal to 1 milligram BDP equivalent daily) ($p < 0.05$). It is believed that other risk factors might be contributing to this association in this group; 35.6 % of the patients recruited were considered to be obese, the mean age of the population was 51 years old and 37.8 % of the population were men.

Several studies describe the association between obesity and corticosteroid induced hypertension (Kidambi *et al*., 2007; Walker *et al*., 2000; Fraser *et al*., 1999). Normal and overweight patients on corticosteroid therapy might develop obesity and reduced insulin sensitivity in adipocytes due to the effect of corticosteroids on fat redistribution (Morton *et al*., 2010). The administration of corticosteroids in obese patients (body mass index (BMI) greater than or equal to 30 kg m^{-2}) might worsen the elevated blood pressure (Kidambi *et al*., 2007; Walker *et al*., 2000). Obesity is one of the factors that can contribute to hypertension (National Institute for Health

and Clinical Excellence, NICE, 2006) but in this study, obesity only accounted for 36 % of the factors behind hypertension related to administration of corticosteroids.

In a population of patients treated with oral corticosteroids, the increase in blood pressure was seen only in patients treated with oral corticosteroids at a dose equal to or greater than 7.5 milligrams but not those patients treated with lower doses (less than 7.5 milligrams of prednisolone daily), prednisolone equivalent respectively (Panoulas *et al*., 2008; Wei *et al*., 2004). The risk of hypertension in patients treated with low dose oral corticosteroids at a dose less than 7.5 milligrams oral prednisolone equivalent is similar to patients with no corticosteroid exposure.

Despite these reports, in our study, corticosteroids did cause an increase in blood pressure, the importance of these findings should be examined further since the weak correlation between corticosteroid dose and blood pressure explained only 10 – 21 % of the variance in blood pressure with the increase in corticosteroid dose.

3.6.1.4 Diabetes mellitus

In our study, the correlation between the patient's plasma glucose and their corticosteroid burden can be considered as nonexistent as it is not statistically significant. This observation is consistent with results found in non diabetic patients (Roberts & Monteiro, 2009) and diabetic patients (Faul *et al*., 2009). One of the patients who reported taking antidiabetic medication showed a RBG measurement of 14 mmol L^{-1} which is greater than level of recommended maximum glucose concentration level (less than 11 mmol L^{-1}). A theory was suggested by Roberts and

Monteiro (2009) and Severino *et al*., (2002) that diabetic patients on corticosteroids have reduced insulin sensitivity which may worsen the glucose control.

However, a few studies have shown a significant increase in plasma glucose in patients treated with corticosteroids (Suissa *et al*., 2010; Slatore *et al*., 2009; Gulliford *et al*., 2006; Iwamoto *et al*., 2004; Blackburn *et al.,* 2002). The impairment in glucose tolerance and increase in insulin resistance following the administration of corticosteroids can cause hyperglycemia in patients with type 2 diabetes mellitus (Jatwa *et al*., 2007; Severino *et al*., 2002) and patients with no history of diabetes (Iwamoto *et al*., 2004; Blackburn *et al.,* 2002). A larger increase in plasma glucose was observed in patients prescribed oral corticosteroids compared to the group prescribed inhaled corticosteroids (Gulliford *et al*., 2006; Blackburn *et al.,* 2002). The theory of the underlying mechanism of corticosteroid induced diabetes was the administration of corticosteroids can cause fat redistribution and down regulation of the glucose transporter 4 (GLUT-4) in muscle which leads to a decrease in insulin sensitivity and an increase in insulin resistance (Kidambi *et al*., 2007; Almon *et al*., 2005; Andrews & Walker, 1999).

In our study, corticosteroid therapy did not cause an increase in the patients plasma glucose, it is suggested that the patient's blood glucose should be monitored carefully especially in patients on long term oral corticosteroids.

3.6.1.5 Steroid treatment card

This study also highlights the need for the patient to carry a steroid treatment card for the benefit of the patients and the health practitioner. The steroid treatment card includes warnings and advice to the patients during the corticosteroid therapy and after withdrawal of the treatment (Appendix 3.7). Only 50.4 % patients in our study required a steroid treatment card and only 31 % patients had received a card in the past. Only half (54.5 %) of the population who had received a steroid treatment card actually carried the card all the time. Patients should be advised of importance of the steroid treatment card and be advised to carry it with them at all times (MHRA, 1998). The steroid treatment card should be supplied to the 19.4 % patients who have never possessed one.

3.6.1.6 Symptoms score

Our study also investigated whether the symptom scores which measure the symptom related to either asthma or rhinitis correlated with cortisol suppression. In our study, the symptom scores, Sino Nasal Outcome Test (SNOT-22) and Asthma Control Questionnaire (ACQ), were carried out on the patients to measure the symptom control based on patient's response to treatment (Baumann, 2010; Gillett *et al*., 2009; Juniper *et al*., 2006).

However, no relationship was observed between the salivary cortisol and either of the symptom scores. It is very unlikely that the absorption of corticosteroids at the target sites increases with a worsening of the disease. The SNOT-22 is a proven tool which is effective at assessing sino nasal symptoms but which may be affected by

similar symptoms related to other diseases (Hopkins *et al*., 2009; Gillett *et al*., 2009). The SNOT-22 scores measured did not correlate with the nasal polyp grade, thus, the relationship observed between cortisol suppression and nasal polyp grade could not be extrapolated.

Patients with an ACQ score above 1.5 can be considered as having poor control of asthma (Juniper *et al*., 2006) based on 88 % positive predictive value with a 66 % negative predictive value. Patients with an ACQ lower than 1.5 have a 66 % chance of having well controlled asthma. Based on this scale (Juniper *et al.*, 2006), the median (IQR) ACQ was 2.8 (1.8 – 3) with only two patients considered to have full asthma control and the rest were considered to have poorly controlled asthma. The median (IQR) of salivary cortisol in these patients was 6.8 (3.8 – 16.8) nmol L^{-1}. Visually, even when the patients have a similar ACQ score, the salivary cortisol measurement varied, thus, no relationship was observed between the ACQ and adrenal suppression. A moderate yet significant correlation ($p < 0.001$) between cortisol suppression and the ACQ was found in patients with asthma on oral and inhaled corticosteroids (AbuRuz *et al*., 2007).

3.6.1.7 Fraction of exhaled nitric oxide (FE_{NO})

The data on the patient's fraction of exhaled nitric oxide (FE_{NO}) was available for some patients who attended the Problem Asthma clinic at SH (n = 18). Seven of the FE_{NO} measurements were greater than 50 parts per billion (ppb) which indicates eosinophilic inflammation but is not specific for asthma (SIGN/BTS, 2008). Only a limited number of salivary cortisol values were available to match with the patient's

FE_{NO} (n = 3). Therefore, it was not possible to draw any conclusions in relation to potential correlation between cortisol suppression and the FE_{NO} result.

3.7 Conclusion

It appears that the calculated corticosteroid burden may be useful in predicting an increase in blood pressure which may lead to an increased incidence of hypertension but the increase in corticosteroid burden was not correlated with an increase in the risk of adrenal suppression, osteoporosis or hyperglycemia. The presence of polyps alters corticosteroid absorption at the inflamed target sites and corticosteroid therapy is less effective in patients with total obstruction of the nasal turbinate compared to small nasal polyps in the middle meatus. The findings in this clinical study are relevant to patients who are taking corticosteroid therapy in single or multiple ways of administration. Salivary cortisol collection should be used as a clinical tool which is easy and reliable to assess the cortisol level. The patients should be assessed on saliva collection method to minimise sample contamination. The analytical method for quantification of corticosteroids developed in this study needs to be improved for lower concentrations of corticosteroids to be quantified.

Chapter 4

Conclusion

4.1 Strength and limitations of study

4.1.1 Strengths

This population based study was conducted from October 2008 until June 2010: 1 year and 8 months data collection. This clinical study assessed the effects of steroid burden for longer time than other published studies (Dahl *et al*., 2010; Dalby *et al*., 2009; Tayab *et al.*, 2007; Jerjes *et al*., 2006; Buhl *et al*., 2006; Aukema *et al*., 2005; Whelan *et al.*, 2005; Fardon *et al*., 2004). These studies of a shorter duration (two weeks up to six months) than our study suggested the possibility of suppressed cortisol concentration in corticosteroids treated patients whereas the results that we found failed to agree to their conclusion. There is also an absence of a link between corticosteroid burden and either bone density or diabetes. The effect of corticosteroids on blood pressure is believed to be similar to cortisol induced hypertension in Cushing's syndrome (Arnaldi *et al*., 2003; Walker *et al*., 2000). This study is relevant to the population at large since patients with asthma, rhinitis and/or nasal polyposis in this study were prescribed corticosteroids long term and not short term. This study concluded that increases in corticosteroid load is not associated directly with the corticosteroid side effects.

In the clinical study, the participants enrolled were maintained on corticosteroids either by inhaled, intranasal or oral in the primary care setting. The prescribing was uncontrolled and no active interventions were carried out on treatment or clinical procedures; it was an observational study. The findings are relevant to the population at large. This study had 39.8 % patients suffering from combination of rhinitis, and nasal polyposis and asthma and 23.9 % patients with rhinitis and nasal

polyposis, the inclusion of these patients reflected the reality of managing this group of patients in practice.

One of the strengths of the present study is that the analytical method developed allows the analyst to quantify and detected seven corticosteroids (prednisolone, betamethasone, budesonide, beclometasone-17-monopropionate, mometasone, fluticasone and beclometasone-17, 21-monopropionate) in a mixture. The gradient method uses methanol-water as its mobile phase which runs for 110 minutes and it is validated according to FDA (2001). The linearity, accuracy, precision, limit of quantification and limit of detection and recovery of corticosteroids from spiked plasma were determined for all corticosteroids except beclometasone-17-monopropionate as the amount of the compound was scarce. The analysis time was longer than most of the analytical studies involving only two to four of the analytes of interest (6 to 14 minutes) (Zou *et al*., 2008; Qu *et al.*, 2007; Deventer *et al.*, 2006; Taylor *et al.*, 2004). This analytical time was necessary as the analysis needs to obtain complete separation of all the compounds including budesonide with its diastereoisomeric profile and between mometasone and fluticasone with similar hydrophobic behaviour.

The solid-phase extraction method used in this study is simple and practical in extracting corticosteroids from a biological matrix. The SPE using Strata C_{18} obtained an extraction recovery between 50 – 95 % for all corticosteroids except for beclometasone – 17, 21 –dipropionate (BDP) (% recovery = 24 – 43 %). The method uses a combination of common organic solvents, methanol and water which

are easily accessible as the best solvents to elute the corticosteroids from the SPE cartridges as well as remove the interference matrix.

4.1.2 Limitations

This thesis has some limitations. This clinical study was conducted in an uncontrolled environment where the patients were considered as fully complant to their medication regimens. Patients were expected to have the correct inhaled and intranasal technique, follow the instruction for saliva sample collection carefully and answer the questionnaires truthfully. The patient's inhaled or intranasal technique was not assessed during the study. Poor technique of administration (inhalation or intranasal) has been proved to affect the distribution of the drug at the target sites and thus reduce their efficacy in treating the disease (Scally *et al*., 2010; Nair *et al*., 2010; Patel *et al*., 2004; Homer *et al*., 2002). Patients were expected to take their medication (antihypertensives, antidiabetic) as instructed. Blood glucose and blood pressure were measured during their visit and not as a specific time of the day.

The second limitation is that insufficient data points were available for salivary cortisol. The low number (68.5 %) of participants who returned saliva for analysis is believed to be due to lack of understanding on importance of salivary cortisol collection. In 68.5 % of participants who returned saliva samples, only 2.6 % were patients prescribed inhaled corticosteroids and 5.2 % were patients prescribed inhaled and oral corticosteroids. The majority of saliva samples (85.3 %) were from patients prescribed intranasal corticosteroids attending the ENT clinic at GRI. The high percentage of saliva collection from this clinic is due to the fact that saliva

collection is part of the routine care in the ENT clinic at GRI but not in Problem Asthma clinic at SH. Lack of patient's compliance with return of the saliva samples may affect the overall conclusions. Patients might be reluctant to post the salivary cortisol as the envelope provided is not stamped. In future, a stamped envelope should be provided to increase the patient's response. A better explanation of the importance of saliva collection and concerns about anonymity might help the patient's response to sample collection.

The sample size of patients with Dual Energy X-Ray Absorptiometry (DEXA) scan (n = 16) was too small to be meaningful. Hackshaw (2008) considered a sample size of less than 20 was too small. The data on the DEXA scan results were recorded in the patient's medical notes and no additional DEXA scans were recommended during the study due to cost and availability. A similar conclusion also can be made on salivary cortisol data of patients on oral prednisolone. Based on the analysis, the risk of salivary cortisol suppression is similar between patients prescribed oral prednisolone or not ($p = 0.149$) but the low number of salivary cortisol results from patients treated with oral corticosteroids (n = 19) may lead to an unreliable conclusion. The majority of the studies concluded that the oral corticosteroids have strong relationship with suppression of cortisol (van den Berge *et al*., 2009; Schuetz *et al*., 2008; Mortimer *et al*., 2006; Jerjes *et al*., 2006; Fardon *et al*., 2000) even though some researchers described the relationship as dose related (Mortimer *et al*., 2006; Hissaria *et al*., 2006). A larger number of salivary cortisol samples from patients treated with oral corticosteroids might provide a better correlation between adrenal suppression and oral corticosteroids.

One of the weaknesses of this study was the inability of the analytical method developed to detect and measure the corticosteroids, other than prednisolone, in the plasma of patients prescribed these medicines. Patients with respiratory diseases such as asthma, rhinitis and nasal polyps were prescribed with variety of corticosteroids such as betamethasone, budesonide, fluticasone, mometasone and beclometasone dipropionate. Since only prednisolone with concentration as low as 0.07 μg mL^{-1} could be quantified precisely, this may not represent the entire absorption of corticosteroids, making it is difficult to correlate with other measured parameters (salivary cortisol) or the corticosteroid dose. The limit of quantification (LOQ) of prednisolone reported in this study is higher than reported in other studies (LOQ = 1.3 – 10.7 ng mL^{-1}) (Ionita *et al*., 2009; Touber *et al*., 2007; Zhang *et al*., 2006; Frerichs & Tornatore, 2004).

The restricted use to only a single wavelength ultraviolet detector in this study contributed to the poor sensitivity of the present analytical method. In this analysis, the corticosteroids were measured at an ultraviolet (UV) wavelength of 239 nm, which is the maximum UV wavelength for fluticasone, beclometasone dipropionate, betamethasone and beclometasone-17-monopropionate but not for prednisolone, budesonide and mometasone. The use of diode array detector which monitors analytes simultaneously over a range of wavelengths is preferred over a variable wavelength UV detector. A few studies quoted that diode array detector showed lower limit of detection (LOD) for prednisolone (1 – 2 ng mL^{-1}) (Zhang *et al*., 2006; Reddy *et al*., 2009); betamethasone and budesonide (1 ng mL^{-1}) (Reddy *et al*., 2009) and fluticasone (0.15 ng mL^{-1}) (Chiang *et al*., 2010). Also, changing the ultraviolet

detector to a more sensitive detector, such as mass spectrometry might help since several studies using mass spectrometry as the detector reported a lower LOQ of the corticosteroids than present study such as betamethasone, 0.05 – 200 ng mL^{-1} (Touber *et al*., 2007; Fu *et al*., 2009; Pereira *et al.*, 2005); fluticasone, 10 – 20 pg mL^{-1} (Mascher *et al*., 2008; Li *et al.*, 2001; Krishnaswami *et al*., 2000); budesonide, 3.2 – 2300 pg mL^{-1} (Streel *et al*., 2009; Qu *et al.*, 2007; Touber *et al*., 2007; Deventer *et al.*, 2006); and mometasone, 15- 50 pg mL^{-1} (Affrime et al., 2000; Sahasranaman *et al.*, 2005).

The concept of an alternative internal standard was used in the present study. Since the internal standards chosen in this study, either betamethasone or mometasone are corticosteroids which may be taken by the participants, this resulted in the requirement for two different calibration standard solutions and the internal standard was spiked into plasma based on the corticosteroid prescribed to the patient. Some studies have explored the concept of alternative internal standards (Touber *et al*., 2007; Taylor *et al*., 2004), but the concept of one internal standard for all analysis is more practical. The need for one internal standard is crucial to minimise the preparation time, reducing the human error and improving the reliability of the analytical method.

4.2 General conclusion and future work

Through the studies described, the following findings were summarised:

The present research indicated that patients treated with corticosteroids showed no link between dose prescribed and the corticosteroid side effects except for increased blood pressure. However, the prescription of corticosteroids do appear to explain the 14 % variation in increase in systolic blood pressure and 23 % variation in diastolic blood pressure in patients treated with high corticosteroids (≥ 1 milligram BDP equivalent daily) but failed to show a link in patients prescribed a lower corticosteroid dose (< 1 milligram BDP equivalent daily). The mechanism of the corticosteroid effects on blood pressure were proposed to be similar to Cushing's syndrome. Patients on oral corticosteroids showed no significant difference in suppression of cortisol compared to patients prescribed no corticosteroid. This study suggests that there is no increased risk of adrenal suppression between patients treated with single (inhaled, intranasal or oral) or multiple forms of corticosteroids. Despite, the significant effects of intranasal betamethasone on cortisol, no firm conclusion can be made since the data obtained is relatively sparce (n = 22).

More patients need to be recruited to represent the whole population. Patients should be advised regularly on correct inhaler and/or intranasal technique, proper saliva sample collection and the importance of carrying a steroid warning card. A prospective study is preferred in the future to avoid the risk of recall bias as reported by several studies involving questionnaires (Suissa *et al*., 2007; Tashkin *et al*., 2004;

Walsh *et al*., 2001). Patients should be asked to bring their prescription to obtain the correct medication details.

To quantify corticosteroids in the solution and plasma, an analytical method was developed using solid-phase extraction coupled with HPLC-UV. The betamethasone and mometasone served as the internal standards in this study. In terms of method development, the analytical method succeeds in detecting and quantifying seven corticosteroids in one run of 110 minutes. The developed analytical method was validated to meet the precision, accuracy, linearity, specificity, selectivity, limit of detection and limit of quantification. The limit of detection (LOD) and limit of quantification (LOQ) of corticosteroids varies from 0.02 – 0.26 µg mL^{-1} and 0.07 – 0.87 µg mL^{-1}, respectively.

To optimise the analyte separation in the present analytical method, it is necessary to find an analytical technique which is more selectivity and sensitive and reducing the analysis time. It may be necessary to use photo diode array detector instead of single UV wavelength detector or using different type of detector which better sensitivity than ultraviolet detector (i.e mass spectrometry). This method could then be used to analyse the corticosteroids in other biological fluids (urine, tissue, hair). The additional step of evaporation of the sample after the sample extraction and then dissolving in a smaller volume of mobile phase might concentrate the final sample extract further; using a small volume could limit the re-analysis if needed.

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Appendices

Appendix 3.1

Sino Nasal Outcome Test (SNOT-22)

Sino-Nasal Outcome Test-22 Questionnaire

Name:_____Date:_____

Below you will find a list of symptoms and social/emotional consequences of your nasal disorder. We would like to know more about these problems and would appreciate you answering the following question to the best of your ability. There are no right or wrong answers, and only you can provide us with this information. Please rate your problems, as they have been over the past two weeks. Thank you for your participation.

Considering how severe the problem is when you experience it and how frequently it happens, please rate each item below on how 'bad' it is by circling the number that corresponds with how you feel using this scale

	No problem	Very mild problem	Mild or slight problem	Moderate problem	Severe problem	Problem as bad as it can be	
1. Need to blow nose	0	1	2	3	4	5	
2. Sneezing	0	1	2	3	4	5	
3. Runny nose	0	1	2	3	4	5	
4. Cough	0	1	2	3	4	5	
5. Post nasal discharge	0	1	2	3	4	5	
6. Thick nasal discharge	0	1	2	3	4	5	
7. Ear fullness	0	1	2	3	4	5	
8. Dizziness	0	1	2	3	4	5	
9. Ear pain/pressure	0	1	2	3	4	5	
10. Facial pain/pressure	0	1	2	3	4	5	
11. Difficulty falling asleep	0	1	2	3	4	5	
12. Waking up at night	0	1	2	3	4	5	
13. Lack of a good night's sleep	0	1	2	3	4	5	
14. Waking up tired	0	1	2	3	4	5	
15. Fatigue during the day	0	1	2	3	4	5	
16. Reduced productivity	0	1	2	3	4	5	
17. Reduced concentration	0	1	2	3	4	5	
18. Frustrated/restless/irritable	0	1	2	3	4	5	
19. Sad	0	1	2	3	4	5	
20. Embarrassed	0	1	2	3	4	5	
21. Sense of taste/smell	0	1	2	3	4	5	
22. Blockage/congestion of nose	0	1	2	3	4	5	

In the column to the far right, please mark the most important items affecting your health.

Please list any other items important to you, which you feel are not mentioned above

_____

Appendix 3.2

Asthma Control Questionnaire (ACQ)

Asthma Control Questionnaire

Name:_____ Date:_____

Please answer questions 1 - 6.

Circle the number of the response that best describes how you have been during the past week

1	On average, during the past week, how often were you woken by your asthma during the night?	0 Never 1 Hardly ever 2 A few minutes 3 Several times 4 Many times 5 A great many times 6 Unable to sleep because of asthma
2	On average, during the past week, how bad were your asthma symptoms when you woke up in the morning?	0 No symptoms 1 Very mild symptoms 2 Mild symptoms 3 Moderate symptoms 4 Quite severe symptoms 5 Severe symptoms 6 Very severe symptoms
3	In general, during the past week, how limited were you in your activities because of your asthma?	0 Not limited at all 1 Very slightly limited 2 Slightly limited 3 Moderately limited 4 Very limited 5 Extremely limited 6 Totally limited
4	In general, during the past week, how much shortness of breath did you experience because of you asthma?	0 None 1 A very little 2 A little 3 A moderate amount 4 Quite a lot 5 A great deal 6 A very great deal
5	In general, during the past week, how much of the time did you wheeze?	0 Not at all 1 Hardly any of the time 2 A little of the time 3 A moderate amount of the time 4 A lot of the time 5 Most of the time 6 All the time
6	On average, during the past week, how many puffs of short-acting bronchodilator (eg. Ventolin) have you used each day?	0 None 1 1 - 2 puffs most days 2 3 - 4 puffs most days 3 5 - 8 puffs most days 4 9 - 12 puffs most days 5 13 - 16 puffs most days 6 More than 16 puffs most days

Appendix 3.3

Patient information leaflet

Department of Respiratory Medicine

Glasgow Royal Infirmary

Glasgow G31 2ER

Patient Information Leaflet

Intranasal and Inhaled Corticosteroids and Side Effects

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Consumers for ethics in Research (CERES) publish a leaflet entitled "Medical Research and You". This leaflet gives more information about medical research and looks at some of the questions you may want to ask. We have a copy available for you to read or take away.

Thank you for reading this information leaflet.

What is the purpose of the study?
You are currently using steroids in your inhaler or nose spray or both to control your asthma or nose condition. Although these medicines are safe they can sometimes cause side effects which you may not notice. These are generally not troublesome but it may be possible to predict them. This study has been designed to see if this is a problem and if there is a simple way to identify these potential problems and prevent them.

Why have I been chosen?
You have been chosen to take part in this study because you use a steroid inhaler for your nose and airway.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw from the study at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I decide to take part?
If you decide to take part in the study you will be asked to complete a short questionnaire. You will continue to take your medicine as normal and this will be monitored in the usual way. We will ask you for a blood sample taken from your vein, a blood sample taken using a fingerprick test and some urine each time you come to the clinic. We will also take your blood pressure and ask you a few questions about the medicines you have taken since your last appointment. We will also ask you to collect a sample of saliva for us.

What do I have to do?
You will have to continue to take your normal treatments and come to your normal appointments. The samples we will ask you for are simple to collect. We will give you full instruction on how to get the saliva sample for us. This is easy to obtain.

What are the drugs being tested?
There are no new medicines being tested in this study – it is looking at how the medicine you already take affects your body.

What are the alternatives for treatment?
The alternative is for you to continue taking your normal medication without any samples being taken.

What are the side effects of taking part?
The blood samples we will take may cause slight pain but this is a routine test carried out in the hospital.

What are the possible benefits of taking part?
We hope to find out more about what happens in your body when you take these medicines. In the future we may be able to predict which patients are likely to have side effects from these medicines and prevent them from happening.

What if new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment/medicine that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study (but not from your treatment). They will explain the reasons and arrange for your care to continue.

What happens when the research study stops?
You will carry on taking your medicines as prescribed by your doctor.

What if something goes wrong?
If you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism will be available to you. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the study will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. If you agree to participate in the study, your G.P. will be informed about your participation.

What will happen to the results of the research study?
The results of the study will be published as a report in the future for other health care workers to read. You will not be identified in that report. If you wish, you will be informed of how to obtain a copy.

Who is organising and funding the study?
The study is being organised by the Departments of Respiratory Medicine and ENT at Glasgow Royal Infirmary and the Division of Pharmaceutical Sciences in the Strathclyde Institute of Pharmacy and Biomedical Sciences at the University of Strathclyde.

Who has reviewed the study?
The study has been reviewed by the Glasgow Royal Infirmary Research Ethics Committee and the University of Strathclyde, University Ethics Committee.

Contacts for further information.
If you require any further information please contact Dr CE Bucknall, Consultant Respiratory Physician on 0141 201 3717, Dr G McGarry, ENT surgeon on 0141 211 4423 or Dr AC Boyter, Lecturer and Honorary Principal Pharmacist on 0141 548 4594.

Please keep this information sheet. If you agree to take part in the study you will be asked to sign a consent form and will be given a copy to keep. Thank you for reading this information sheet.

Appendix 3.4

Consent form

Department of Respiratory Medicine

Glasgow Royal Infirmary

Glasgow G31 2ER

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **Intranasal and Inhaled Corticosteroids and Side Effects**

Name of Researchers: Dr AC Boyter, Dr CE Bucknall, Mr G McGarry

		Please initial box
1.	I confirm that I have read and understand the information sheet dated January 2008 (version 2) for the above study and have had the opportunity to ask questions.	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
3.	I understand that sections of any of my medical notes may be looked at by responsible individuals or from regulatory authorities where it is. relevant to my taking part in research. I give permission for these individuals to have access to my records.	☐
4.	I agree to provide samples of blood and urine for analysis in the study and to their disposal at the end of the study.	☐
5.	I agree to my GP being informed of my participation in the study	☐
4.	I agree to take part in the above study.	☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 3.5

Clinical study questionnaire

Study number

Date:
Date of Birth : _____
Sex : Female/Male

Please tick (√) the answer which best describes your treatment.
Check your steroid inhaler (BROWN, MAROON, RED OR PURPLE INHALER) if you have it with you.

1. Are you taking **steroid tablets (prednisolone)** every day?
   ☐ No, go to Question 2
   ☐ Yes,
   Usual dose_____ mg

2. Since the last visit dated _____,have you been given steroids tablets by your GP or by the hospital?
   ☐ No, go to question 3
   ☐ Yes, how many **courses in total**
   ☐ 1 course ☐ 2 courses ☐ 3 courses
   ☐ 4 courses ☐ 5 courses ☐ more than 5 courses
   ☐ can't remember

About your **steroid inhaler.**

3. Please circle the name of your inhaler from the list below.
   Aerobec, Aerobec Forte, Alvesco, Asmabec, Asmanex, Beclometasone, Becloforte, Beclazone Becotide, Becodisks, Budesonide, Ciclesonide, Clenil, Flixotide, Fluticasone, Fostair, Mometasone, Novolizer, Pulmicort, Qvar, Seretide, Symbicort,

4. Type of inhaler **(**puffer) ☐ **Accuhaler** ☐ **Metered dose inhaler** ☐ **Turbohaler** ☐ **Other _____**

5. Strength of your inhaler (micrograms)
   ☐ 50 ☐ 100 (or 100/6) ☐ 200 (or 200/6) ☐ 250 ☐ 400
   ☐ 500

6. How **many puffs** do you use at one time?
   ☐ 1 puff ☐ 2 puffs ☐ 3 puffs ☐ 4 puffs ☐ more than 4 puffs

   How **many times** a day as you use this inhaler?
   ☐ Once ☐ Twice ☐ 3 times ☐ 4 times
   ☐ more than 4 times ☐ when you need it

Please turn over.

7. Do you take any medicines for your nose?
   □ No, go to Question 9
   □ Yes – Please circle the name of the medicine from the list below:

   Beclometasone, Beconase, Betnesol, Budesonide, Flixonase, Flixonase Nasule, Nasacort, Nasonex, Nasofan, Rhinocort, Syntaris, Vista-Methasone

8. How **many times** a day do you use this medicine?
   □ Once □ Twice □ 3 times □ 4 times
   □ more than 4 times

9. Have you ever been given a blue steroid warning card?

   □ Yes □ No

   If yes, do you still carry this card?

   □ Yes □ No

10. <u>About yourself</u> (please tick the box if the answer is "yes" to any of the following statements

   □ Have you ever had a wrist, back or hip fracture?
   □ Do you take any medicine for your thyroid (thyroxine)

   Do you:
   □ smoke?
   □ have a history or family history of osteoporosis?
   For female patients only
   □ Have you passed the menopause (that is, have your periods stopped)?

11. How much alcohol would you drink in an average week?
   _____

12. Please write down all the medicines you take from your doctor or the hospital:

☺ Thank you for taking part in the study

Appendix 3.6

Salivary sample collection instruction

North Glasgow University Hospital Division

From: Dr Christine Bucknall

Department of Respiratory Medicine

Stobhill Hospital

Dear…….

I enclose tubes and instructions for spit collection. Your spit will be analysed to assess your current treatment, as explained at your clinic appointment.

1. Collect two samples over two days (one spit each morning) by spitting into one tube after getting out of bed in the morning.
2. Rinse your mouth with water, 10 minutes before spitting
3. Spit before brushing your teeth, taking nose drops or eating
4. Label each tube with your name and the time and date of spitting
5. Return both bottles in addressed envelope supplied

Thank you for your help with this project.

Appendix 3.7

Steroid treatment card

STEROID TREATMENT CARD

I am a patient on STEROID treatment which must not be stopped suddenly

- If you have been taking this medicine for more than three weeks, the dose should be reduced gradually when you stop taking steroids unless your doctor says otherwise.
- Read the patient information leaflet given with the medicine.
- Always carry this card with you and show it to anyone who treats you (for example a doctor, nurse, pharmacist or dentist). For one year after you stop the treatment, you must mention that you have taken steroids.
- If you become ill, or if you come into contact with anyone who has an infectious disease, consult your doctor promptly. If you have never had chickenpox, you should avoid close contact with people who have chickenpox or shingles. If you do come into contact with chickenpox, see your doctor urgently.
- Make sure that the information on the card is kept up to date.