STUDIES ON CANINE LOWER RESPIRATORY TRACT
WITH SPECIAL REFERENCE TO INHALED
CORTICOSTEROIDS

Marika Melamies

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Walter Room, EE-Building, on November 6th 2015, at 12 noon.

Helsinki 2015
Director of studies
Professor Thomas Spillmann
Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki, Finland

Supervised by
Docent Minna Rajamäki
Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki, Finland

Professor Outi Vainio
Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki, Finland

Professor emerita Anna-Kaisa Järvinen
Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki, Finland

Reviewed by
Associate professor Carol Norris Reinero
Department of Veterinary Medicine & Surgery
Veterinary Medical Teaching Hospital
University of Missouri, USA

Professor emerita Reeta Pösö
Department of Veterinary Biosciences
Faculty of Veterinary Medicine
University of Helsinki, Finland

Opponent
Professor Cecile Clercx
Department of Veterinary Clinical Sciences
Faculty of Veterinary Medicine
University of Liège, Belgium

Published in Dissertationes Scholae Doctoralis Ad Sanitatem
Investigandam Universitatis Helsinkiensis

No. 77/2015
http://ethesis.helsinki.fi

ISBN 978-951-51-1521-8 (PDF)

Hansaprint Oy, Vantaa 2015
ABSTRACT

Respiratory diseases, both chronic and acute, are relatively common in dogs. They often cause a dramatic reduction in quality of life, mainly due to repeated coughing, excessive secretion of mucus, respiratory distress and exercise intolerance, which also frequently results in substantial stress for the owner. If accurate diagnosis is not followed by appropriate treatment euthanasia of the dog is often considered. This means that there is a real need for reliable diagnostic methods and efficient, safe and easy to administer pharmacotherapy.

The aims of this thesis were to study a diagnostic method, bronchoalveolar lavage (BAL), and a treatment modality, corticosteroid inhalation, in healthy dogs. BAL is used to collect epithelial lining fluid (ELF) and the lavage fluid volumes used affect the amount of ELF recovered. Different techniques were compared to determine the best method of recovering a consistent volume of ELF. The canine pharmacokinetics of a commonly used inhaled corticosteroid (ICS), budesonide (BUD), were described. Systemic adverse effects of inhaled BUD were assessed and compared to those of a conventional therapy, oral prednisolone, and another readily available ICS, fluticasone propionate (FP).

We found that a BAL protocol adjusting for weight, i.e. adjusting the amount of fluid instilled according to the body weight of the dog, yielded more consistent ELF recovery than fixed-volume BAL. We therefore recommend use of a weight-dependent BAL protocol to ensure results are comparable and to quantify the constituents of bronchoalveolar lavage fluid (BALF).

Following BUD (1.0 mg) inhalation the mean maximum plasma concentration (C\text{max}) was 0.89 ng/ml, time to peak plasma concentration (T\text{max}) was 16 minutes and area under the concentration curve (AUC\text{0-6h}) was 1.1 ng h/ml. Inhaled BUD had moderately low systemic and pulmonary bioavailability in healthy dogs. Pulmonary bioavailability was evaluated by administering oral charcoal prior to inhalation in order to block gastrointestinal (GI) absorption of BUD.

A method to detect BUD in low-volume dog plasma samples was developed and validated. Instrumental analysis was carried out using high-pressure liquid chromatography tandem mass spectrometry (HPLC/MS/MS) in a positive ion electrospray mode, and overall the general method characteristics were excellent.

Our results also indicated that in healthy dogs a four-week course of clinically effective doses of inhaled FP, or oral prednisolone, but not inhaled BUD, produced dose-related adrenal suppression according to the adrenocorticotropic hormone (ACTH) stimulation test.

In summary, our findings contribute to knowledge of the BAL technique, and the pharmacokinetics and systemic adverse effects of inhaled BUD which
provides valuable information for treating canine patients with pulmonary symptoms.
CONTENTS

Abstract.......................................................................................................3

Contents......................................................................................................5

List of original publications ....................................................................8

Abbreviations ............................................................................................9

1 Introduction..........................................................................................10

2 Review of the literature ......................................................................11

  2.1 Canine respiratory tract...............................................................11

  2.2 Bronchoalveolar lavage..............................................................12

    2.2.1 Epithelial lining fluid .........................................................13

  2.3 Glucocorticoids ..........................................................................15

    2.3.1 Inhaled corticosteroids ......................................................15

    2.3.2 Budesonide.........................................................................16

      2.3.2.1 Reversible fatty acid conjugation ...............................16

      2.3.2.2 Quantitative analysis of budesonide.........................17

    2.3.3 Fluticasone propionate .......................................................18

    2.3.4 Bioavailability ....................................................................18

      2.3.4.1 Pulmonary bioavailability ..........................................18

    2.3.5 Drug delivery device ..........................................................19

      2.3.5.1 Valved holding chamber ............................................19

    2.3.6 Systemic adverse effects .....................................................20

3 Aims of the study .................................................................................21

4 Materials and methods ...........................................................................22

  4.1 Ethical approval of study protocols ............................................22

  4.2 Dogs ............................................................................................22
4.3 Bronchoalveolar lavage ......................................................... 23
4.3.1 Study design ................................................................. 23
4.3.2 Bronchoscopic examination and lavage ....................... 23
4.3.3 BALF analysis ............................................................... 24
4.4 Budesonide ..................................................................... 25
4.4.1 Pharmacokinetic study .................................................... 25
4.4.1.1 Study design ............................................................. 25
4.4.1.2 Treatments ............................................................... 25
4.4.1.3 Pharmacokinetic analysis ......................................... 26
4.4.2 Determination of budesonide in plasma ......................... 27
4.4.2.1 Reagents and chemicals .......................................... 27
4.4.2.2 Sample preparation.................................................. 27
4.4.2.3 LC-MS/MS procedure ............................................ 27
4.4.2.4 Method validation .................................................... 28
4.5 Endocrine effects ............................................................... 28
4.5.1 Study design ................................................................. 28
4.5.2 Treatments ................................................................... 29
4.5.3 ACTH stimulation test .................................................. 29
4.6 Statistical methods ............................................................ 29
5 Results ................................................................................ 31
5.1 Dogs ................................................................................ 31
5.2 Bronchoscopic findings and BALF analysis ...................... 32
5.3 Pharmacokinetic analysis ................................................. 33
5.4 Determination of budesonide in plasma ......................... 35
5.4.1 Method validation ........................................................ 35
5.4.2 LC-MS/MS analysis ..................................................... 36
5.5 Endocrine effects ............................................................. 36
5.5.1 ACTH stimulation test .........................................................36

6 Discussion........................................................................................39

6.1 Weight-dependent BAL technique provides more uniform ELF recovery ...........................................................................39

6.1.1 Evaluation of the amount of ELF using the urea method ...39

6.2 Budesonide......................................................................................... 41

6.2.1 Pharmacokinetic study in healthy dogs............................... 41

6.2.1.1 Drug delivery device..................................................42

6.2.1.2 Bioavailability...........................................................43

6.2.1.3 Study limitations.......................................................44

6.2.2 LC-MS/MS........................................................................45

6.2.3 Budesonide did not induce detectable adverse effects.......46

6.2.3.1 Results of serum biochemistry and urinanalysis .....46

6.2.3.2 Effect on the HPA axis .............................................46

7 Conclusions........................................................................................49

8 Acknowledgments..................................................................................50

References ................................................................................................52
This thesis is based on the following publications:


The publications are referred to by Roman numeral in the text. The texts of these publications are reprinted with the kind permission of the copyright holder.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>AUMC</td>
<td>area under the first moment curve</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BUD</td>
<td>budesonide</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum concentration</td>
</tr>
<tr>
<td>CL</td>
<td>total clearance</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DPI</td>
<td>dry powder inhaler</td>
</tr>
<tr>
<td>ELF</td>
<td>epithelial lining fluid</td>
</tr>
<tr>
<td>F</td>
<td>systemic bioavailability</td>
</tr>
<tr>
<td>FP</td>
<td>fluticasone propionate</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
</tr>
<tr>
<td>ICS</td>
<td>inhaled corticosteroid</td>
</tr>
<tr>
<td>iv</td>
<td>intravenous</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography – tandem mass spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>limit of quantification</td>
</tr>
<tr>
<td>MRT</td>
<td>mean residence time</td>
</tr>
<tr>
<td>pMDI</td>
<td>pressurised metered dose inhaler</td>
</tr>
<tr>
<td>PO</td>
<td>per os</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>elimination half-life</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>time to peak concentration</td>
</tr>
<tr>
<td>V&lt;sub&gt;d&lt;/sub&gt;</td>
<td>volume of distribution</td>
</tr>
<tr>
<td>VHC</td>
<td>valved holding chamber</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

Chronic respiratory diseases such as tracheal collapse, bronchomalacia, and inflammatory diseases, such as chronic bronchitis and eosinophilic bronchopneumopathy are relatively common in dogs (Johnson 2000, McKiernan 2000, Clercx and Peeters 2007). Along with thorough physical examination, thoracic radiographs, complete blood cell count and arterial blood sample, bronchoscopic examination is one of the most informative diagnostic techniques as it enables the airways to be visualised directly. Supplementing it with bronchoalveolar lavage (BAL) often enables identification of potential pathogens (bacteria, viruses, fungi, and parasites) as well as assessment of pathognomic changes in the cell distributions of the bronchoalveolar lavage fluid (BALF) and thus facilitates definitive diagnosis (Hawkins 2004). However, if BALF results are to be comparable the lavage protocol must be standardised.

Treatment of respiratory diseases has for decades relied on administration of oral glucocorticosteroids although they often have troublesome systemic adverse effects, e.g. iatrogenic hyperadrenocorticism. In humans and companion animals inhaled corticosteroids (ICSs) have become a common treatment for chronic respiratory disorders (Bexfield et al. 2006, Kirschvink et al. 2006, Cohn et al. 2008, Galler et al. 2013). Administration via inhalation allows the active drug to be delivered straight to the desired site of action, and thus systemic exposure is reduced relative to oral preparations. The first ICS, beclomethasone dipropionate, was introduced in early 1970s, and later structural modifications of the corticosteroid molecule led to the development of new ICSs such as budesonide (BUD) and fluticasone propionate (FP). The first published report of therapeutic use of ICSs in dogs is still comparatively recent (Bexfield et al. 2006). All corticosteroids, even ICSs, can induce side effects, and so it is important to understand the differences between types and doses of steroids in order to improve their therapeutic efficacy-safety profile.

The aim of the studies reported in this thesis was to collect information which would enable the BAL technique to be standardised, and to improve understanding of the pharmacokinetics and safety of ICS medication in order to provide a basis for future clinical studies within this field.
2 REVIEW OF THE LITERATURE

2.1 CANINE RESPIRATORY TRACT

The most important function of the respiratory tract is gas exchange; oxygen is delivered to the lungs via inhalation and distributed to the body via blood and carbon dioxide is excreted via exhalation.

The respiratory tract consists of the upper and lower airways and the lungs. The upper airways consist of the nasal cavity, nasopharynx and larynx and the lower airways of the trachea and main bronchi. The right lung is divided into four lobes (cranial, accessory, middle and caudal) and the left lung into cranial and caudal lobes. The main bronchi branch to give smaller bronchi and eventually respiratory bronchioles, which give rise to alveolar ducts, alveolar sacs and pulmonary alveoli (Fig. 1). All three of these respiratory compartments participate in the transfer of oxygen from the inspired air into the alveolar capillaries and the removal of carbon dioxide. (Harpster 2004)

![Figure 1](image)

A schematic drawing of bronchiole and alveoli.

The inner surface of the bronchi is coated with ciliated pseudostratified columnar epithelial cells, along with goblet, basal and Clara cells. The height of the cells diminishes distally to form a simple, cuboidal, ciliated layer of epithelium, which gradually develops into non-ciliated alveolar epithelium. In the alveoli the air and blood are separated only by a thin layer composed of the alveolar epithelial cells, basement membrane and the capillary endothelial cell (Fig. 2). The vast majority of the alveolar epithelium (95%) is made up of alveolar epithelial cells (type I pneumocytes), which are thin squamous epithelial cells. Type II pneumocytes are cuboidal epithelial cells which are responsible for the production and secretion of surfactant and for tissue
repair. The alveoli are surrounded by the lung interstitium. Macrophages are present in the airspaces of both the alveoli and interstitium.

Figure 2  A schematic drawing of an alveoli and pulmonary capillaries.

### 2.2 BRONCHOALVEOLAR LAVAGE

BAL is considered a minimally invasive, relatively safe method of obtaining samples from the lower respiratory tract. It involves infusing sterile isotonic saline into the bronchiolar and alveolar compartments of the lungs and re-aspirating the lavage fluid (Hawkins 2004). One author (Hawkins 2004) recommended using of two 25 ml boluses in each lung lobe in larger dogs and four or five 10 ml boluses per site in small dogs (< 8kg); another recommended 5-20 ml aliquots per site according to animal size (Zhu et al. 2015). Use of small-volume fluid washes with lavage volumes of 1-2 ml/5 kg have also been recommended for dogs (Cowell et al. 1999).

There is considerable variation in recommended lavage fluid volumes; in human research the infused volumes usually range from 100-300 ml per lung segment or sub-segment (Klech and Pohl 1989), but small-volume lavages with adjustment of fluid volume to weight (1 ml/kg, three aliquots) have been used in children (Ratjen and Bruch 1996).

BAL can be performed through the biopsy channel of a flexible bronchoscope, which permits visualisation of the airways and sampling from a specific area of lung; alternatively it can be performed blind by passing a soft tube (e.g. feeding tube) through the endotracheal tube until resistance is felt. Previous studies in dogs (Hawkins et al. 1995) and cats (Ybarra et al. 2012) have demonstrated that the extent and type of inflammation may differ between lung lobes, so bronchoscopic BAL is considered the more reliable diagnostic technique.
Aspiration - either gentle manual aspiration with handheld syringe or automated aspiration using a suction pump - of the instilled fluid should begin as soon as possible to decrease the time lavage fluid remains in lungs (the ‘dwell time’). Retrieval of BALF from dogs with pulmonary disease using both aspiration techniques has recently been studied by Woods et al. (2014b). They concluded that suction pump aspiration enhanced BALF retrieval but did not significantly influence the chances of achieving a definitive diagnosis. It has been suggested that the minimum representative sample is 40% of infused fluid in children (de Blic et al. 2000) whereas in dogs it has been suggested that an excellent quality specimen requires recovery of at least 50% of infused fluid with a foam layer rising to the top of the retrieved fluid (Hawkins 2004).

BAL is considered a safe procedure in appropriate patients. Respiratory distress (i.e. arterial oxygen pressure less than 60 mmHg (Lee and Drobatz 2004)) that does not resolve with supplementary oxygen is considered a contraindication for BAL (Hawkins 2004) but BAL has been performed successfully in dogs with lower arterial oxygen levels (Heikkilä et al. 2011). It has been reported that there is a significant decrease in arterial oxygen pressure after BAL in healthy dogs (Rajamäki et al. 2001) so oxygen supplementation during the procedure is recommended.

Minor complications of the BAL procedure in humans include coughing, a transient decrease in oxygen saturation, fever, haemorrhage, bronchospasm, vasovagal syncope and persistent hypoxaemia; more severe complications include pneumonia, pneumomediastinum, pneumothorax, arrhythmia, respiratory failure and cardiac arrest (Klech and Pohl 1989). The possible complications in canine patients are similar, but relatively rare. A mortality/euthanasia rate of 2% (2 out of 101 patients) for BAL at a veterinary referral centre was reported; it should be noted that both deaths occurred in patients which were overtly dyspnoeic prior to the lavage and showed severe systemic illness at autopsy (Hawkins et al. 1995). Cooper et al. (2005) reported severe complications in a dog with eosinophilic airway disease during BAL, probably caused by severe bronchoconstriction as a result of massive release of inflammatory mediators from disrupted eosinophils. At our hospital the mortality rate for BAL is less than 1% (Rajamäki pers. comm.).

2.2.1 EPITHELIAL LINING FLUID
ELF is a thin layer of fluid that coats the epithelial cell layer of the lower respiratory tract. During BAL ELF dissolves into the instilled saline and the resulting mixture of ELF and saline is known as BALF. The dilution of ELF affects the concentrations of constituents e.g. cells, proteins and bacteria, and so consistent recovery of ELF is necessary if the results are to be comparable and repeatable.

The amount of ELF can be calculated by determining the BALF concentration of an endogenous solute with the plasma concentration, or by administering an exogenous substance intravenously or via the lavage fluid
and then comparing the BALF concentration with the dose administered. The use of exogenous substances (e.g. methylene blue, inulin, radioactive tracers) requires additional non-routine procedures in both the lavage and analysis phases so it is simpler to use endogenous markers e.g. urea, albumin, protein, potassium (Rennard et al. 1998, Kirschvink et al. 2001). Collection of pure ELF using a bronchoscopic microsample probe has been reported in rats (Kamiyama et al. 2014) and human patients (Singh et al. 2015) but not dogs, and the technique is not commonly used.

Urea is regarded as the most reliable endogenous marker of dilution in healthy and diseased lungs (Rennard et al. 1998, Dargaville et al. 1999). Unlike albumin and protein, urea has the advantage of a low molecular mass and therefore diffuses rapidly from plasma into ELF, resulting in equal urea concentrations in both, so the volume of ELF in BALF can be calculated reliably. Urea has been used as a dilution indicator in humans, dogs, cats and horses. The reported mean volume of ELF in BALF for adults is 1.0 ml/100 ml of recovered lavage fluid (Rennard et al. 1986) and 2.0 ml/100 ml of BALF for children (Ratjen and Bruch 1996). In healthy dogs ELF makes up 1.0 - 1.1% of BALF (Hawkins et al. 1998, Mills and Litster 2005) and in healthy cats 4.6% (assessed after euthanasia) (Mills and Litster 2006); in healthy and heaves-affected horses the figure is 0.79% (Kirschvink et al. 2001).

It has however been shown that during BAL both fluids and urea diffuse from plasma into the alveolar space and so even the use of urea as an indicator of dilution is controversial (Marcy et al. 1987, Rennard et al. 1998). Free diffusion of urea into BALF may lead to overestimation of the volume of ELF recovered, especially with progressive administration of several aliquots of saline and longer dwell times, or where membrane permeability is altered as a result of disease processes (Marcy et al. 1987, Ward et al. 1992). In addition to urea the use of exogenous dilution indicator, intravenous (iv) technetium-99m diethylenetriaminepenta-acetic acid (99mTc-DTPA), has been described in dogs (Bayat et al. 1998).

The variation in the recovered ELF volume means that quantitative comparison of results is difficult (Marcy et al. 1987, Chinard 1992). Even in humans the BAL protocol has not yet been standardised to control for or adjust the factors which determine the volume of ELF recovered, including the lavage technique and the volume and dwell time of instilled saline. Fixed-volume lavage results in relatively large volumes in young children and lower volumes in older children; therefore Ratjen and Bruch (1996) investigated BAL technique in healthy children aged 3-15 years and were able to show that adjusting lavage fluid volume according to body weight yielded constant ELF fractions.
2.3 GLUCOCORTICOIDS

Glucocorticoids are vitally important steroid hormones that regulate multiple metabolic and homeostatic functions. The endogenous glucocorticoids, cortisol and corticosterone, are cholesterol-derived hormones secreted by the adrenal glands. Synthesis and release of glucocorticoids is regulated by the hypothalamic-pituitary-adrenal (HPA) axis in a circadian manner and levels increase in response to stress. Chronic imbalance in glucocorticoid levels can result in pathological conditions such as Cushing’s disease (chronic elevation) or Addison’s disease (chronic deficiency).

Synthetic glucocorticoids, such as prednisolone, BUD and FP, resemble endogenous glucocorticoids, but differ with respect to potency and metabolic clearance. Both endogenous and synthetic glucocorticoids act by binding to glucocorticoid receptors located in the cytoplasm of most cells. Synthetic glucocorticosteroids, particularly the ICSs, are the recommended treatment for chronic inflammatory respiratory diseases. They act by inhibiting the activation of proinflammatory description factors and thereby suppressing production of cytokines, chemokines and cell adhesion molecules by the airway epithelium. (Behrend and Kemppainen 1997, Kadmiel and Cidlowski 2013)

2.3.1 INHALED CORTICOSTEROIDS

ICSs have been the mainstay of treatment for human asthma for decades and in veterinary medicine they have been adopted for the treatment of chronic respiratory diseases in dogs (Bexfield et al. 2006, Cohn et al. 2008), cats (Kirschvink et al. 2006, Galler et al. 2013) and horses (Laan et al. 2004). The principle behind the administration of corticosteroids via inhalation is that the active drug will reach the desired site of action, the lower airways and lungs, before entering the circulatory system, and that this will minimise the incidence of the unpleasant adverse effects which often accompany use of oral preparations e.g. polydipsia and polyuria, polyphagia, weight gain, diabetes mellitus and iatrogenic hyperadrenocorticism (Cushing’s disease). The first-pass hepatic metabolism of ICSs is also very efficient and so any drug which is unintentionally swallowed is almost completely inactivated before it is distributed via systemic circulation. Compared with oral preparations, ICSs have a high topical anti-inflammatory potency and low systemic activity, and thus a better overall efficacy-safety profile (Stoloff and Kelly 2011).

At present there are several ICSs available, including beclomethasone dipropionate, BUD, FP, mometasone furoate, flunisolide and ciclesonide. The pharmacological properties and formulations available for use in animals are variable. For this thesis we chose to study the pharmacokinetic profile of one specific ICS, BUD, and to compare the endocrine effects of BUD and FP, which are both readily available in suitable formulations.
2.3.2 BUDESONIDE

BUDESONIDE is a non-halogenated corticosteroid with potent glucocorticoid activity and weak mineralocorticoid activity. BUDESONIDE is a 1:1 racemic mixture of 2 epimers, (22R)- and (22S)-, that do not interconvert. BUDESONIDE has a high relative affinity for the glucocorticoid receptor. The anti-inflammatory effects of BUDESONIDE are mediated by numerous anti-inflammatory cells (e.g. eosinophils, neutrophils, lymphocytes, mast cells, macrophages) and humoral inflammatory mediators (e.g. histamine, leukotrienes, eicosanoids and cytokines). (Donnelly and Seale 2001)

BUDESONIDE has low oral bioavailability; approximately 90% of the swallowed drug is inactivated by first-pass hepatic metabolism (Donnelly and Seale 2001). Inhaled BUDESONIDE has been used for the treatment of naturally occurring asthma and chronic bronchitis in cats (Galler et al. 2013); it has also been administered via oral capsules to treat canine inflammatory bowel disease (Dye et al. 2013, Pietra et al. 2013).

![Image of BUDESONIDE chemical structure]

2.3.2.1 Reversible fatty acid conjugation

An in vivo experiment in rats showed that topically administered BUDESONIDE forms lipophilic intracellular fatty acid esters in the cells of the airway and lung tissue (Miller-Larsson et al. 1998). The same study also demonstrated that within 20 minutes of BUDESONIDE administration approximately 70–80% of BUDESONIDE retained in the trachea and main bronchi had been conjugated. Reversible fatty acid conjugation of BUDESONIDE has also been reported in the cells of the intestinal wall in dogs (Pietra et al. 2013) and in in vitro human lung microsomes (Tunek et al. 1997). Measurement of BUDESONIDE concentrations in lung tissue and plasma 90 minutes after single inhalation in humans undergoing resection of a lung lobe or whole lung indicated that concentrations in lung tissue were 8 times higher than in plasma (Van den Bosch et al. 1993).

The esterification of BUDESONIDE is reversible and creates a depot of latent, slowly regenerating drug; this may enhance the airway selectivity of BUDESONIDE, prolong local anti-inflammatory effect and improve airway retention (Miller-Larsson et al. 1998). It may also explain why daily doses of BUDESONIDE are an effective
treatment for mild to moderate asthma in humans (Campbell et al. 1998, Herjavecz et al. 1999, Donnelly and Seale 2001).

2.3.2.2 Quantitative analysis of budesonide

When BUD is administered via inhalation the dose delivered to the lungs is low, and therefore plasma concentrations of the drug are very low. This means that a robust, sensitive and selective method is required for quantitative analysis of plasma BUD following inhalation (Li et al. 2001). A multi-drug high throughput screening method has been used to assay BUD in the urine of racehorses (Leung et al. 2005), and BUD has been detected in human plasma and urine (Kronkvist et al. 1998, Deventer et al. 2006). To date dog plasma samples have not been analysed using a method which takes full advantage of a deuterated internal standard in high-pressure liquid chromatography tandem mass spectrometry (HPLC-MS/MS).

Several different techniques have been used for quantitative analysis of BUD in specimens from humans and other mammals. The simplest procedure is probably acidification to precipitate plasma proteins followed by evaporation and reconstitution as a chromatographic solvent; this was used on human plasma samples (Wang et al. 2003). Extraction with methyl tert-butyl ether followed by evaporation and reconstitution into 30% methanol has been used with racehorse plasma samples (Luo et al. 2005). Traditional diethyl ether extraction has recently been applied to human plasma samples (Borges et al. 2011). Ethyl acetate extraction of BUD from alkalised urine samples obtained after inhalation exposure has also been used (Deventer et al. 2006). Reversed phase solid-phase extraction has been used to screen horse urine samples for corticosteroids (Leung et al. 2005).

The chromatographic analysis of BUD from various matrices relies mainly on liquid chromatography, regardless of whether the goal is to quantify separate epimers of BUD or total BUD (Deventer et al. 2006, Gupta and Bhargava 2006, Streel et al. 2009). Although choice of column and mobile-phase composition varies considerably, reversed-phase columns with C8 or C18 stationary phases are invariably used (Leung et al. 2005, Deventer et al. 2006, Gupta and Bhargava 2006, Streel et al. 2009). Isocratic or linear gradient systems with mobile phases consisting of low molarity (typically millimolar range) buffer at acidic pH and methanol or acetonitrile as organic modifiers have been used with C8 phases. Isocratic elution modes with mobile phases consisting of low molarity buffer adjusted to acidic pH and acetonitrile as an organic modifier have been used with C18 phases.

In HPLC detection has typically been with ultraviolet light or MS. Atmospheric pressure chemical ionisation, electrospray ionisation (ESI) and recently atmospheric pressure photoionisation have been used with MS (Deventer et al. 2006, Fält et al. 2007, Borges et al. 2011). Multiple reaction monitoring (MRM) of the product ions has been used in tandem with MS and full scan mode with ion trap instrumentation (Deventer et al. 2006, Fält et al. 2007).
2007). The ionisation characteristics of BUD in LC-MS/ESI conditions have been investigated to aid in the identification of the breakdown products (Hou et al. 2005).

2.3.3 FLUTICASONE PROPIONATE

FP is a potent ICS with a high affinity for the glucocorticoid receptor. The oral bioavailability of FP is also very low; approximately 99% of the orally absorbed dose is inactivated by first-pass hepatic metabolism (Derendorf 1997, Derendorf et al. 1998). FP is highly lipophilic and therefore diffuses very slowly from the lungs into the systemic circulation (Kelly 1998). This lipophilic nature of FP is thought to enhance its relative respiratory efficacy, however it also means that it has the longest elimination half-life of the currently available ICSs; in humans it is over 10 hours following inhalation (Derendorf et al. 1998).

2.3.4 BIOAVAILABILITY

The bioavailability of a drug relates to the proportion of the drug - or its active metabolite - which reaches systemic circulation. ICSs enter the systemic circulation by via the respiratory tract and lung or the gastrointestinal (GI) tract. Absorption of an ICS will depend on the relative lipid solubility of the drug and once the drug has been absorbed into the systemic circulatory system the elimination rate will determine its clearance from the circulation and hence its half life.

The systemic (also referred to as absolute) bioavailability compares the bioavailability of the active drug in systemic circulation following inhalation with the bioavailability of the same drug following iv administration. The systemic bioavailability of a drug is obtained by measuring the plasma concentration over time following both inhaled and iv administration. The systemic bioavailability (F) is the dose-corrected area under curve (AUC) after inhalation divided by AUC after iv administration, as follows:

\[
F = 100 \times \left( \frac{AUC_{\text{inhaled}}}{AUC_{\text{iv}}} \right) \times \left( \frac{\text{Dose}_{\text{iv}}}{\text{Dose}_{\text{inhaled}}} \right)
\]

2.3.4.1 Pulmonary bioavailability

The pulmonary bioavailability of ICS relates to the proportion of drug found in systemic circulation after GI absorption of the drug has been blocked. In human studies it has been shown that concomitant oral charcoal does not affect the elimination and distribution of BUD; it is therefore assumed that when absorption of BUD from the GI tract is blocked the inhaled drug only reaches systemic circulation via absorption from the lungs (Thorsson and Edsbacker 1998, Lähelma et al. 2005, Grekas et al. 2014).
Figure 4  A schematic drawing of the pharmacokinetic route of inhaled budesonide (1 mg) in healthy dog. The proportional amounts of budesonide are derived from our pharmacokinetic study (Study II).

2.3.5 DRUG DELIVERY DEVICE
ICSs can be delivered to the patient as an aerosol via a pressurised metered dose inhaler (pMDI), as a dry powder via a dry powder inhaler (DPI) or as a nebulised solution with sterile saline via a nebuliser.

A pMDI is constructed so that the drug is dissolved or suspended in a propellant under pressure; when the device is fired an aliquot of drug and propellant is released through a valve system. The propellant is needed to propel and disaggregate the drug particles. Activation of DPIs depends on inspiratory flow to disperse the drug into small particles which are then delivered into the lower airways. Children under 6 years and elderly or mentally disabled people, and also animals, are not able to generate sufficient inspiratory pressure to operate a DPI. Nebulisation requires specific equipment and very careful cleaning of the device to avoid growth of bacteria within the device and is therefore usually reserved for hospital use.

2.3.5.1 Valved holding chamber
Spacers and valved holding chambers (VHCs) were originally designed to overcome the problems encountered by patients using pMDIs. VHCs work by slowing down the speed of the aerosol cloud, and the drug is released from the chamber only when the patient is inhaling. VHCs have been shown to reduce oropharyngeal deposition of the drug and increase pulmonary bioavailability
(Dempsey et al. 1999a, Dempsey et al. 1999b). In animals VHCs are essential as animals lack the ability to inhale forcefully when the pMDI is fired.

2.3.6 SYSTEMIC ADVERSE EFFECTS

Sometimes ICSs may have systemic adverse effects, especially when used in high doses or for long periods of time. In humans the safety of the various ICSs has been comprehensively evaluated. The most commonly reported systemic complications are related to the hypothalamic-pituitary-adrenal (HPA) axis, skin, bones, eyes, growth and immunity; local complications include oral candidiasis and hoarseness (Pandya et al. 2014). The reported prevalence of adverse events depends on several factors, including the pharmacological properties and dosage of the drug as well as the delivery device and method of administration. At low doses (0.4 mg/day) ICSs do not appear to produce adrenal suppression regardless of the device or drug used (Lipworth 1999). The systemic side effect profile seems to vary among ICSs. In children with mild to moderate asthma chronic BUD treatment (400 μg/day over a three-year period) produced no effects on HPA axis function (Bacharier et al. 2004). At higher doses (>0.4 mg/day) FP has been shown to cause adrenal suppression in asthmatic children, although BUD at the same dose did not (Clark et al. 1996). Investigation of the endocrine effects of pMDI-administered BUD or FP in asthmatic adults found that urinary cortisol levels were low in 8.3% of patients using BUD and 58% of patients using FP (Clark and Lipworth 1997). Thus FP seems to be more potent adrenal suppressor than BUD (Pandya et al. 2014).

In dogs adverse effects associated with corticosteroid therapy e.g. polydipsia and polyuria, polyphagia, weight gain, hair loss and suppression of the HPA axis, leading to a reduction in endogenous glucocorticoid production and potentially adrenal atrophy, are mainly associated with the use of oral glucocorticoids, although they may also occur with use of ICSs (Cohn et al. 2008, Canonne-Guibert et al. 2013). The adrenocorticotropic hormone (ACTH) stimulation test is the most reliable method of diagnosing adrenal dysfunction in dogs (Herrtage 2005, Lathan et al. 2008, Behrend et al. 2013).

The systemic adverse effects of FP and oral prednisone have been studied in healthy dogs; it is reported that FP produces less suppression of HPA axis function than prednisone (Cohn et al. 2008). We are not aware of any investigation into the systemic adverse effects of inhaled BUD, but there has been a report of significant suppression of the HPA axis in dogs with inflammatory bowel disease following oral BUD at a dose of 3 mg/m² for 30 days (Tumulty et al. 2004).
3 AIMS OF THE STUDY

The main objective of this thesis was to study the pharmacokinetic properties of an ICS, BUD, and compare its systemic adverse effects in healthy dogs to those of other corticosteroids. An additional aim was to create a more standardised protocol for BALF collection in dogs. Detailed aims were as follows:

1. To assess whether a BAL technique adjusted for the body weight would yield more consistent ELF recovery than fixed-volume BAL.

2. To investigate the pharmacokinetic properties of BUD administered iv, via inhalation or via inhalation with concomitant oral administration of charcoal, and to verify effectiveness of the charcoal in preventing GI absorption of BUD.

3. To develop and validate a sensitive, selective method for quantitative analysis of BUD in dog plasma samples.

4. To compare the endocrine effects of inhaled BUD, inhaled FP and oral prednisolone at clinically relevant doses.
4 MATERIALS AND METHODS

4.1 ETHICAL APPROVAL OF STUDY PROTOCOLS

The protocol used in Study I was approved by the Committee of Experimental Animals, University of Helsinki (HY 132-05; date of approval 19 January 2005). The protocols for Studies II, III and IV were approved by the Committee of Experimental Animals of Western Finland (ESLH-2007-02827/Ym-23; date of approval 31 May 2007).

4.2 DOGS

All dogs participating in these studies were purpose bread healthy laboratory beagles and they were housed and taken care according to the guidelines of the Ethics Committee for Animal Experimentation at the University of Helsinki.

**Study I**: thirteen dogs (5 females and 8 males) participated in the first study. The dogs were aged from 5 to 11 years (median, 8 years) and their body weight ranged from 9 to 20 kg (median, 15 kg). Dogs were given a weight index based on a five-point body condition scoring system (1: underweight; 2: lean; 3: optimum; 4: overweight; 5: obese). Twelve dogs had a weight index of 3 (optimum) and 1 had a weight index of 4 (overweight).

**Studies II and III**: eight dogs were enrolled (4 males and 4 spayed females). The age of the dogs ranged from 8 to 12 years (median, 10 years) and their weight from 10 to 22 kg (median, 14 kg). All dogs participated in the inhalation studies and 7 dogs in the iv and per os (PO) studies.

**Study IV**: the study group consisted of 6 neutered female dogs. At the beginning of the study the dogs were 2.5 years old and their weight ranged from 11 to 19 kg (median, 14 kg).

One week before the experiments the health status of the dogs was assessed with a physical examination, serum biochemistry and haematology panels, arterial blood sample (ABL 800 FLEX, Radiometer A/S), faecal flotation and sedimentation tests and ventro-dorsal and latero-lateral thoracic radiographs. Radiographs were viewed by two independent examiners.

In studies II and III bronchoscopic examination and bronchoalveolar lavage were also performed, under general anaesthesia, as described in detail in Section 4.3. In Study IV urine samples were also analysed (bacterial culture, protein-to-creatinine ratio, reagent strip (Multistix 10 SG, Siemens Healthcare Diagnostics), sediment, and specific gravity).
4.3 BRONCHOALVEOLAR LAVAGE

4.3.1 STUDY DESIGN
In Study I dogs were randomly assigned to one of two groups. Group A underwent BAL of the right caudal lung lobe using a fluid volume based on the dog’s weight (2ml/kg, divided into 2 aliquots) and BAL of the left caudal lung lobe with a fixed volume of fluid (50 ml per dog, divided into 2 aliquots). Group B underwent BAL of the right caudal lung lobe with a fixed volume of fluid and BAL of the left caudal lung lobe with a weight-dependent volume of fluid. In dog with a suboptimal weight index the lavage fluid volume was based on the estimated optimal weight.

4.3.2 BRONCHOSCOPIC EXAMINATION AND LAVAGE
Dogs were sedated with medetomidine (Domitor, Orion Oyj) 20 μg/kg combined with butorphanol (Torbugesic, Scan Vet Animal Health A/S) 0.1 mg/kg administered intramuscularly. General anaesthesia was induced with 1 mg/kg iv propofol (Rapinovet, Schering-Plough A/S), and the same dose of propofol was repeated if needed. During bronchoscopy dogs were positioned in sternal recumbency and additional oxygen was applied with a small catheter placed in the trachea.

Bronchoscopic examination was performed by slowly advancing a video bronchoscope (Olympus GIP type N30, Olympus Optical Co Ltd.) through the trachea, through the bifurcation and into the openings of the main bronchi (right cranial, middle, accessory and caudal lobes; left cranial and caudal lobes). The structure of the respiratory tract and the colour and smoothness of the epithelium and any abnormal secretions were recorded.

The lavage (BAL) was performed by first wedging the tip of the bronchoscope into the right caudal lung lobe until a tight seal was established between the bronroscope and the wall of the bronchus. Then sterile warmed (37°C) 0.9% saline solution was infused through the biopsy channel, followed by infusion of 5 ml of air to empty the channel. Gentle manual suction was applied immediately using a 20 ml syringe attached to the biopsy channel with a plastic adapter. As much BALF as possible was retrieved and stored in a glass container on ice. The same procedure was repeated immediately with the second fluid aliquot. The left caudal lung lobe was lavaged in a similar manner except that a different fluid volume was used.

The inclusion criterion for this study was recovery of ≥40% of the instilled fluid volume. The total duration of BAL, i.e. time from the beginning of the saline instillation until the end of the last suction and the time between fluid instillation and the first aspiration (dwell time) were measured.
4.3.3 BALF ANALYSIS

BALF specimens from the right and left caudal lung lobes were analysed within 30 min of collection. Before filtration a 10-μl aliquot of BALF was used to inoculate on a blood agar plate for quantitative bacterial culture. The plate was incubated at 37°C for 48 h and the indicator of bacterial infection was bacterial growth of more than $1.7 \times 10^3$ colony-forming units/ml (Peeters et al. 2000).

BALF samples were filtered through a single-layer cotton gauze. The volume of fluid recovered was measured and expressed as a percentage of the total volume of fluid infused. The BALF cell count was determined with a haemocytometer using trypan blue stain (1:1). An aliquot containing 40,000 cells was then cytcentrifuged (202 g for 10 min) on a slide and stained. Numbers of macrophages, lymphocytes, neutrophils, eosinophils, plasma cells, mast cells and epithelial cells were assessed by counting 300 nucleated cells. The remaining portion of each sample was centrifuged (100 g for 10 min), and then the supernatant was removed and used for urea concentration analysis.

Urea concentrations both in serum and BALF were determined using a kinetic enzymatic method (Gutmann and Bergmeyer 1974) involving use of a clinical chemistry analyser (Kone Spesific, ThermoFisher Scientific) and a commercial reagent (UREA UV 250, BioMerieux SA). The intra- and inter-assay coefficients of variation (CVs) for serum urea concentration were 2.5% (mean, 48.3 mg/dl; $n=10$), and 2.8% (mean, 55.9 mg/dl; $n=30$) respectively. The intra-assay variability of 8 BALF urea assays at concentrations of 0.62 and 2.54 mg/dl was 4.5% and 0.9%, respectively. The observed concentrations of BALF urea were 97.6% to 101.5% of the expected concentrations after addition of known amounts of urea ranging from 0.31 to 2.50 mg/dl. The detection limit of the BALF urea assay was 0.08 mg/dl.

The amount of ELF in BALF was estimated using the ratio of urea in BALF ($\text{Urea}_{\text{BALF}}$) to urea in serum ($\text{Urea}_{\text{serum}}$), assuming that the urea concentration in the ELF equals that of serum (Rennard et al. 1986).

\[
\text{ELF (%) = } \left( \frac{\text{Urea}_{\text{BALF}}}{\text{Urea}_{\text{serum}}} \right) \times 100
\]
4.4 BUDESONIDE

4.4.1 PHARMACOKINETIC STUDY

4.4.1.1 Study design

This was a prospective open-label study. All dogs participated in four experiments separated by three-week wash-out periods. Dogs received no preliminary training in breathing through a mask. Before each experiment food was withdrawn for 12 h and water was available *ad libitum*. BUD was administered intravenously (TREAT_IV) in the first study, via inhalation (TREAT_INH) in the second study, via inhalation in conjunction with activated charcoal PO (TREAT_INHC) in the third study and orally in conjunction with activated charcoal (TREAT_PO) in the fourth study.

4.4.1.2 Treatments

In all studies, 5 ml venous blood samples for assessment of BUD concentration were drawn from the cephalic vein via an iv catheter (Optiva 20G, Smiths Medical) into K₂EDTA tubes. The catheter was used only for sampling at 10, 20, 30, 45, 60 and 90 min and 2, 4 and 6 h after BUD administration (time zero). After sampling, 5 ml of physiological saline was administered iv to replace the blood loss. Samples were centrifuged within 30 min and refrigerated at -70°C until analysis.

In TREAT_IV, BUD powder (Budesonide micronised, Astra Zeneca PLC) was dissolved in ethanol and physiological saline (0.9%) to give a final BUD concentration of 100 μg/ml and ethanol 30% (w/w). 20 μg/kg BUD (total volume 2.0–4.4 ml per dog) was injected through a second plastic iv catheter (Optiva 20G, Smiths Medical) placed in the cephalic vein not used for sampling. Medication was injected over 10 s, after which the catheter was immediately flushed with 5 ml of physiological saline and removed.

In TREAT_INH BUD (Pulmicort HFA, AstraZeneca) was administered at 1.0 mg (5 x 200 μg) per dog through a VHC (AeroDawg, Trudell Medical International) attached to an anaesthetic mask (face mask with rubber diaphragm, Kruuse) (Fig. 5). The drug canister was first shaken carefully and the mask was then fitted to cover the nose and mouth of the dog so that breathing through the mouth was not possible. Medication was fired into the chamber, and the dog was allowed to take eight breaths. The mask was then removed, and the dog was allowed to breathe standard room air for 5 s before the manoeuvre was repeated; the inhalation procedure was carried out 5 times in total.

TREAT_INHC used the same procedures as TREAT_INH, but 5 min before inhalation a suspension liquid charcoal (Carbo-mix, Leiras) was
Materials and methods

administered at 2.5 g/kg PO. Charcoal powder (50 g per bottle) was mixed with 250 ml of tap water according to the manufacturer’s instructions such that the total amount of suspension per dog varied from 155 to 340 ml. The charcoal suspension was administered slowly via bottle feeding over 5 min, and the dogs swallowed at least half of the dose.

**TREAT_PO** consisted of administration of activated charcoal as described in TREAT_INHC followed by PO administration of a BUD capsule (BUD powder weighed into gelatine capsules) at 10 mg per dog.

![Inhalation device](image)

**Figure 5** Inhalation device; a pressurised metered-dose inhaler (pMDI), a valved holding chamber (VHC) and an anaesthetic mask.

**4.4.1.3 Pharmacokinetic analysis**

Pharmacokinetic parameters, AUC$_{0-\infty}$, elimination half-life (T$_{1/2}$), total clearance (CL) and volume of distribution (V$_d$) were calculated using a non-compartment model and Kinetica™ software (Thermo Electron Corp., Waltham).

AUC was calculated using the trapezoidal method. T$_{1/2}$ was calculated by dividing ln2 by the rate constant of the elimination phase (β). The V$_d$ was calculated by the area method as V$_d$ = Dose/AUC x (β), and the total plasma clearance as CL = Dose/AUC. Mean residence time (MRT) was calculated as MRT = AUMC$_{0-\infty}$/ AUC$_{0-\infty}$, where AUMC is the area under the first moment curve. Maximum concentration (C$_{max}$) and time to peak concentration (T$_{max}$) were determined directly from individual plasma curves.

Systemic bioavailability (F) of BUD after inhalation was calculated from as F = (AUC$_{0-\infty}$ inhaled / AUC$_{0-\infty}$ iv) x (Dose$_{iv}$/ Dose$_{inhaled}$). When the extrapolated AUC exceeded 20%, AUC$_{0-6 h}$ was used.
4.4.2 DETERMINATION OF BUDESONIDE IN PLASMA

4.4.2.1 Reagents and chemicals
BUD (≥ 99% pure) was purchased from Sigma and the deuterated internal standard d8-budesonide (isotopic purity >99.6% determined with MS spectra peak ratio) was a gift from Orion Corporation (Orion Pharma). Stock standard solution (1 mg/ml), working standard solutions (0.1 and 0.01 μg/ml), stock internal standard solution (1 mg/ml) and working internal standard solution (0.1 μg/ml), were all prepared in methanol. Typically, six calibration standards were prepared in the range of 0.75–50 ng/ml in the mobile phase. The concentration of the internal standard in calibration standards was 25 ng/ml. Methanol, acetonitrile and formic acid were HPLC grade, and ammonium formate was analytical grade (J.T. Baker). Water was Milli-Q water purified with a Millipore Milli-Q Plus System (Millipore). Solid-phase extraction columns (C18, 500 mg, 6 ml) were from J.T. Baker.

4.4.2.2 Sample preparation
The method was an in-house modification of published BUD methods (Hou et al. 2005, Gupta and Bhargava 2006). Internal standard (BUD-d8, 0.1 μg/ml, 50 μl) and formic acid (4% in water, 100 μl) was added to plasma samples. Samples were carefully mixed with a shaker, and then ethanol (99%, 1ml) was added. Samples were incubated for 15 min at 4 °C and subsequently centrifuged (3500 rpm, 15 min). The supernatant was then separated, diluted with water (4 ml) and subjected to solid phase extraction.

Prior to use cartridges were conditioned with methanol (5 ml) followed by water (5 ml). Cartridges were not allowed to dry between conditioning and sample introduction. A sample solution was introduced to the column and the column was washed with methanol (20% in water, 3 ml). Finally, the analytes (BUD epimers) were eluted from the column with methanol (80% in water, 3ml). The effluent was collected and evaporated to dryness using nitrogen flow and gentle heating (50°C). The extract was suspended in 200 μl of the mobile phase (ammonium formate, 2 mM, pH 3.4 and methanol in a ratio of 40:60) used in LC-MS/MS.

4.4.2.3 LC-MS/MS procedure
The LC-MS/MS system consisted of a Waters Alliance 2695 Separation Module (Waters) and a Micromass Quattro Micro tandem mass spectrometer (Micromass UK Ltd.). The instrument was operated in the positive ion electrospray mode with the following parameters: capillary voltage 3.50 kV, source temperature 120 °C, desolvation temperature 300 °C, N2 cone gas flow 15 L/h, N2 solvation gas flow 700 L/h. Argon was used as the collision gas. Ion
transitions were investigated by MRM. Transitions and collision energies for BUD were 431>323 and 14 eV and 431>413 and 12 eV, respectively. Transition 439>421 and collision energy 12 eV were used for the internal standard. The LC separation of BUD was carried out on a Symmetry C18 column (3.5 μm, 2.1x150 mm; Waters) with mobile phase of ammonium formate (2 mM, pH 3.4) and methanol in a 40:60 ratio. The flow rate of the mobile phase was 0.2 ml/min and the injection volume was 10 μl. Quantification was based on the internal standard method. The area ratios of the product ion of BUD (m/z 323) to the product ion of the internal standard (m/z 421) were plotted against concentration. Six calibration standards were prepared in the concentration range of 0.75–50 ng/ml in the mobile phase.

4.4.2.4 Method validation
The following validation parameters were determined: selectivity, specificity, linearity, recovery, repeatability, reproducibility and limits of detection (LOD) and quantification (LOQ). Selectivity was assessed by comparing (two-sided t-test, 95% confidence) the slopes of the calibration curves being recorded with and without matrix at six different concentrations in the range of 0.75–50 ng/ml. Calibration lines were constructed using weighted (1/x) linear regression.

Specificity was evaluated by analysing ten blank plasma samples to assess matrix interference. The linearity of each point of the calibration curves was examined using the method described by van Trijp and Roos (1991). The recovery, repeatability and within-laboratory reproducibility of the method were determined by analysing blank plasma samples fortified at three concentration levels (0.2, 0.3 and 5.0 ng/ml). Fortified plasma samples were used for validation instead of incurred plasma samples because of the limited plasma volume.

4.5 ENDOCRINE EFFECTS

4.5.1 STUDY DESIGN
A prospective, randomised, placebo-controlled cross-over study was designed to compare the systemic effects of inhaled and oral corticosteroids. All six dogs received the following 4 treatment protocols in a random order: 1) BUD inhalation (Pulmicort HFA, AstraZeneca), 200 μg 12-hourly; 2) FP inhalation (Flixotide Evohaler, GlaxoSmithKline), 250 μg 12-hourly; 3) prednisolone orally (Prednisolon, Leiras), 1 mg/kg 24-hourly; 4) placebo inhalation, 12-hourly for 28 consecutive days.
4.5.2 TREATMENTS

Inhaled medications were administered with a similar inhalation device to that used in the pharmacokinetic study (Fig. 5). The pMDI was shaken prior to each treatment and attached tightly to the chamber. Medication was then administered as described for study II, except in that the dogs in the placebo group breathed room air through the chamber and mask. All dogs were assigned their own mask, VHC and pMDI for the four-week duration of the study and the inhalation devices were washed carefully after each treatment period. All blood samples (serum and K$_2$EDTA samples, both 6 ml) were always drawn by the same two persons, at the same time of the day (between 9 and 11 a.m.), in the environment where the dogs were housed. Treatments were administered 24 h before sampling.

The duration of all treatment and washout periods was 4 weeks. On days 0, 14, 28 and 35 a thorough physical examination, haematology and serum biochemistry and urinanalysis were performed. On days 0, 28 and 35 the ACTH stimulation test was also carried out. Blood samples were drawn from the cephalic vein and urine samples obtained by cystocentesis under ultrasonographic guidance. All samples were stored at room temperature and analysed within 2 h.

4.5.3 ACTH STIMULATION TEST

The function of the HPA axis was assessed using a low-dose ACTH stimulation test in which a synthetic analogue of ACTH (Synachten, Alliance) was administered at 5 $\mu$g/kg iv through a plastic catheter (Optiva 20G, Smiths Medical) (Kerl et al. 1999, Frank et al. 2004). First the zero blood sample was obtained, then the ACTH analogue was administered and the catheter was flushed immediately with 5 ml of physiological saline. The second blood sample was taken 60 min later. Serum samples were then frozen at -80°C until analysis.

Another technician performed cortisol assays in coded samples. Samples were analysed in duplicate using a commercial radioimmunoassay (RIA) kit validated for canine use (Coat-A-Count Cortisol, Siemens Healthcare Diagnostics Inc.). The intra-assay CV for serum cortisol determination was 5.6% at the mean concentration (42.6 nmol/l) and 6.4% at the 280.2 nmol/l concentration; both CVs were calculated from 10 duplicate determinations. The samples were analysed in one series and no inter-assay CV% was determined. The LOD of the serum cortisol assay was 6.9 nmol/l.

4.6 STATISTICAL METHODS

Statistical analyses were performed by the author in collaboration with a statistician using GraphPad Prism version 4.01 (GraphPad Software Inc), Statistix 9.0 for Windows (Analytical Software) and SAS System for Windows.
(SAS Institute Inc.) version 9.1 (Study I) and 9.2 (Study IV). Parametric analyses were used for normally distributed variables. The criterion for statistical significance was \( p < 0.05 \).

**Study I**: BALF recovery percentages for fixed-volume and weight-dependent BAL were compared using paired-samples \( t \)-tests. Wilcoxon matched-pairs sign rank tests were used to compare lavage times and BALF total cell counts for the two techniques. The association between lavage time and BALF urea concentration was analysed using Spearman’s correlation coefficient.

The SDs of the ELF amounts in samples from fixed-volume and weight-dependent BAL were compared using repeated-measures mixed ANOVA. Unstructured covariance structure (which enables differing SDs) was tested against compound symmetry structure (which requires equal SDs) using a general linear models procedure.

**Study II**: Non-parametric variables (\( C_{\text{max}} \) and \( T_{\text{max}} \)) were compared using the Wilcoxon signed-rank test and parametric variables were compared using a paired-samples \( t \)-test.

**Study IV**: The effect of treatments on the continuous response variables was assessed using a linear mixed effects model, where treatment, period, day of period, and interaction terms between treatment and period, and treatment and day of period were used as fixed effects and dog as a random effect. With urine protein-to-creatinine ratio and urine specific gravity, the dog-related variance component was very close to zero. To get the model to converge, linear analysis was performed, excluding the very small random effect of individual dogs.

Carry-over effect was excluded from all models, as the day 0 ACTH-stimulated cortisol results indicated that the four-week wash-out period was long enough to normalise HPA axis function before the next experimental period.
5 RESULTS

5.1 DOGS

All dogs had normal physical examination results. No significant changes were detected in serum biochemistry and haematology panels, (except in Study IV (Table 1)), arterial blood samples or thoracic radiographs and all tests for faecal parasites were negative.

Study IV: Results of haematological analyses on days 0, 14, 28 and 35, including differential cell counts, were within the reference range in all dogs, with minor exceptions. In the prednisolone group significant elevations in serum alkaline phosphatase (ALP), protein values and the urine protein-to-creatinine ratio were detected on days 14 and 28 in the serum biochemistry panels and urinalysis (Table 1).

Table 1  
Results of serum biochemistry and urinalysis (mean (SD)) on days 0, 14, 28 and 35 for healthy dogs after four weeks of treatment with placebo inhalation, oral prednisolone, inhaled fluticasone propionate (FP) or inhaled budesonide (BUD) (n = 6 per group).

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/l)</td>
<td>110 (43)</td>
<td>107 (48)</td>
<td>114 (46)</td>
<td>109 (46)</td>
<td></td>
</tr>
<tr>
<td>Prot (g/l)</td>
<td>57 (3)</td>
<td>55 (4.7)</td>
<td>57 (5.7)</td>
<td>55 (1.8)</td>
<td></td>
</tr>
<tr>
<td>U pr/cr</td>
<td>0.08 (0.02)</td>
<td>0.11 (0.02)</td>
<td>0.11 (0.02)</td>
<td>0.10 (0.02)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Prednisolone</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/l)</td>
<td>105 (45)</td>
<td>261 (91) *</td>
<td>246 (101) *</td>
<td>119 (43)</td>
<td></td>
</tr>
<tr>
<td>Prot (g/l)</td>
<td>58 (2.1)</td>
<td>65 (3.4) *</td>
<td>65 (2.3) *</td>
<td>57 (3.0)</td>
<td></td>
</tr>
<tr>
<td>U pr/cr</td>
<td>0.08 (0.02)</td>
<td>0.15 (0.04)</td>
<td>0.47 (0.33) *</td>
<td>0.15 (0.03)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>FP</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/l)</td>
<td>115 (47)</td>
<td>93 (19)</td>
<td>99 (22)</td>
<td>103 (34)</td>
<td></td>
</tr>
<tr>
<td>Prot (g/l)</td>
<td>58 (2.3)</td>
<td>58 (2.3)</td>
<td>58 (2.7)</td>
<td>55 (3.1)</td>
<td></td>
</tr>
<tr>
<td>U pr/cr</td>
<td>0.10 (0.04)</td>
<td>0.10 (0.03)</td>
<td>0.16 (0.12)</td>
<td>0.10 (0.02)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>BUD</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/l)</td>
<td>100 (25)</td>
<td>107 (28)</td>
<td>117 (38)</td>
<td>107 (42)</td>
<td></td>
</tr>
<tr>
<td>Prot (g/l)</td>
<td>56 (3.5)</td>
<td>57 (2.8)</td>
<td>58 (1.6)</td>
<td>55 (2.9)</td>
<td></td>
</tr>
<tr>
<td>U pr/cr</td>
<td>0.09 (0.03)</td>
<td>0.12 (0.03)</td>
<td>0.11 (0.02)</td>
<td>0.10 (0.02)</td>
<td></td>
</tr>
</tbody>
</table>

FP, fluticasone propionate; BUD, budesonide; ALP, alkaline phosphatase (reference range, 33-215 U/l); Prot, protein (reference range, 58-77 g/l); U pr/cr, urine protein-to-creatinine ratio (ref, <0.5).

* p <0.001, comparison with placebo.
5.2 BRONCHOSCOPIC FINDINGS AND BALF ANALYSIS

Bronchoscopic examination revealed no abnormal changes in the stucture of airways or on the airway epithelium.

**Study I:** Mean percentage (SD) recovery of infused lavage fluid was 58% (13) and 57% (11) for fixed-volume and weight-dependent BAL respectively; there was no difference between the techniques. Total cell count and cell counts for the various cell types were also similar (Table 2). No intracellular bacteria were seen and bacterial cultures yielded negative results.

There was no difference in lavage time for a single lung lobe lavage ($p = 0.15$, fixed-volume BAL: median, 11 min (range, 9.4 to 18 min), weight-dependent BAL: median, 10 min (range, 9.1 to 15 min). BALF urea concentration was not correlated with lavage time in either protocol (fixed-volume: $r = 0.12$, $p = 0.69$; weight-dependent: $r = 0.43$, $p = 0.19$). Dwell time was less than 30 s in all dogs.

Serum urea concentrations ranged from 17 to 29 mg/dl (median, 19 mg/dl), and BALF urea concentrations from 0.27 to 1.1 mg/dl (median, 0.53 mg/dl). Mean (SD) ELF amount determined using the urea method was 2.9% (0.89) for fixed-volume BAL and 2.3% (0.39) for weight-dependent BAL. The SDs for the two techniques were different ($p = 0.041$) (Fig. 6).

**Studies II and III:** BALF analysis yielded normal results, aside from evidence of mild lymphocytosis in four dogs (median, 17%; range, 6-23%; (95% confidence interval 10-16% (Rajamäki et al. 2002)).

<table>
<thead>
<tr>
<th></th>
<th>Fixed-volume</th>
<th>Weight-dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell count (cells/μl)</td>
<td>270 (120-730)</td>
<td>250 (190-820)</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>2.7 (0.7-5.4)</td>
<td>2.0 (1.4-7.0)</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0 (0-3.4)</td>
<td>0.4 (0-1.0)</td>
</tr>
<tr>
<td>Mast cells (%)</td>
<td>1.7 (1.0-7.0)</td>
<td>2.0 (0.4-5.7)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>19 (11-34)</td>
<td>15 (9.0-31)</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>74 (62-87)</td>
<td>78 (61-87)</td>
</tr>
<tr>
<td>Plasma cells (%)</td>
<td>0 (0-1.0)</td>
<td>0 (0-3.7)</td>
</tr>
<tr>
<td>Epithelial cells (%)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
</tr>
</tbody>
</table>
Figure 6  Proportion of epithelial lining fluid (ELF) recovered using fixed-volume and weight-dependent BAL techniques in 13 dogs. Each dot represents results for 1 dog and horizontal line indicates mean values. Standard deviations (SDs) for the two techniques were different ($p = 0.041$).

5.3 PHARMACOKINETIC ANALYSIS

The values for various pharmacokinetic parameters after iv administration of BUD (20μg/kg) (TREAT_IV) are presented in Table 3, along with the results of a previous study (Ryrfeldt et al. 1979).

<table>
<thead>
<tr>
<th></th>
<th>20 μg/kg</th>
<th>10 μg/kg</th>
<th>100 μg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Melamies et al.</td>
<td>Ryrfeldt et al. (1978)</td>
<td>Ryrfeldt et al. (1978)</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (ng h/ml)</td>
<td>14 (2.1)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>T½ (h)</td>
<td>2.8 (0.8)</td>
<td>2.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Cl (l h/kg)</td>
<td>1.5 (0.25)</td>
<td>2.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Vd (l/kg)</td>
<td>6.2 (2.8)</td>
<td>6.5</td>
<td>4.7</td>
</tr>
</tbody>
</table>

AUC, area under the curve; T½, elimination half-life; Cl, total clearance; Vd, volume of distribution.
**Results**

Table 4  
*Pharmacokinetic data (mean (SD)) calculated from plasma concentration time curves for budesonide (BUD) in healthy dogs (n=8) after inhalation of BUD (1 mg) (TREAT_INH) and after inhalation of BUD (1 mg) with concomitant oral charcoal (2.5 g/kg) (TREAT_INHC).*

<table>
<thead>
<tr>
<th></th>
<th>TREAT_INH</th>
<th>TREAT_INHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC₀–₆ h (ng h/ml)</td>
<td>1.1 (0.59)</td>
<td>0.55 (0.23)</td>
</tr>
<tr>
<td>Cₘₐₓ (ng/ml)</td>
<td>0.89 (0.59)</td>
<td>0.60 (0.19)</td>
</tr>
<tr>
<td>Tₘₐₓ (h)</td>
<td>0.27 (0.12)</td>
<td>0.22 (0.08)</td>
</tr>
<tr>
<td>F (%)</td>
<td>2.1 (0.98)</td>
<td>1.2 (0.59)</td>
</tr>
</tbody>
</table>

AUC, area under the time-drug concentration curve; Cₘₐₓ, maximum plasma drug concentration; Tₘₐₓ, time when Cₘₐₓ is measured; F, fraction of drug absorbed.

Figure 7  
Plasma concentrations of budesonide (BUD) in healthy beagles (n = 8) over a 90 min-period following inhalation of 1.0 mg BUD (open boxes) or inhalation of 1.0 mg BUD plus concomitant oral charcoal 2.5 g/kg (striped boxes). Lines within the boxes indicate medians; the lower and upper boundaries of the boxes indicate the values of the 25th and 75th percentiles, respectively. Whiskers represent the smallest and largest values.
Comparison of BUD inhalation (TREAT_INH) and BUD inhalation with concomitant charcoal (TREAT_INHC) revealed that Cmax and Tmax were similar following both treatments, but there was a difference in AUC0–6h values (p = 0.049). Table 4 gives pharmacokinetic data for both inhalation groups. Median plasma BUD concentrations for both inhalation groups are illustrated in Fig. 7.

In TREAT_PO BUD was not detected in plasma samples of three dogs, in the remaining four dogs the mean (SD) Cmax of BUD was 0.15 (0.15) ng/ml. The mean (SD) systemic bioavailability for inhaled BUD was 2.1% (0.98); the pulmonary bioavailability of BUD when administered with oral charcoal was 1.2% (0.59).

5.4 DETERMINATION OF BUDESONIDE IN PLASMA

5.4.1 METHOD VALIDATION
The selectivity of the analytical method was tested by comparing (two-sided t-test, 95% confidence) the slopes of the calibration curves for standards with and without matrix. Since statistical evaluation revealed no matrix effect, standards without matrix were used for calibration. Each point of the calibration line was tested for linearity; tolerance of 100 ± 10 was accepted. The method was considered as being linear over the concentration range investigated.

The specificity of the method was tested by analysing blank dog plasma samples. No interference signals were detected in the region of the retention times for the analyte. The average recovery of BUD varied between 86% and 99%, and the relative CV% for repeatability and reproducibility (SDwlr) were 6–15% and 11–17%, respectively. The absolute recovery was determined by analysing blank plasma samples fortified at three concentrations (0.2, 0.3 and 5.0 ng/ml, n=7). The average absolute recovery of BUD varied from 64 to 89% (CV% from 7% to 15%). The LOD of BUD was estimated based on the analyte and background response level of the lowest spike concentration. Using this information the LOD concentration was estimated based on signal-to-noise calculation of 3:1. The LOQ was defined to be 2 x LOD. LOD was defined as 0.05 ng/ml and the LOQ was 0.1 ng/ml.

We did not systematically investigate the stability of BUD or d8-budesonide in stock solutions and spiked dog plasma samples. However signal intensity data provided no evidence of degradation or other chemical instability. This is in line with recently published investigations of the stability of BUD in human plasma samples (Streel et al. 2009, Borges et al. 2011).
5.4.2 LC-MS/MS ANALYSIS

The protonated molecule \([\text{M+H}]^+\) of BUD (m/z 431) was fragmented in the collision cell to the product ions m/z 413 (M-18) and m/z 323 (M-108). The product ion m/z 413 originated from the protonated molecular ion as a result of cleavage of water. The product ion 323 was used for the quantification. Product ion m/z 421 of the internal standard was used for quantification. The fragmentation of BUD in the applied instrumental settings is in line with published data (Hou et al. 2005, Deventer et al. 2006). Control dog plasma samples and blank solvent samples were analysed between the consecutive sequences (about six samples in a sequence) and revealed no carry over or other interfering signals in chromatograms. The ion suppression/matrix effect was not specifically studied because of the use of the deuterated analogue of the analyte.

5.5 ENDOCRINE EFFECTS

5.5.1 ACTH STIMULATION TEST

These results are presented in Table 5.

<table>
<thead>
<tr>
<th></th>
<th>Cortisol (nmol/L)</th>
<th>Day 0</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>basal</td>
<td>36 ± 10 (20-51)</td>
<td>36 ± 10 (26-51)</td>
<td>37 ± 14 (24-64)</td>
<td></td>
</tr>
<tr>
<td>peak</td>
<td>288 ± 40 (237-335)</td>
<td>298 ± 25 (266-333)</td>
<td>297 ± 38 (251-346)</td>
<td></td>
</tr>
<tr>
<td><strong>Prednisolone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>basal</td>
<td>39 ± 18 (23-71)</td>
<td>14 ± 4 (8-19)</td>
<td>51 ± 25 (31-93)</td>
<td></td>
</tr>
<tr>
<td>peak</td>
<td>333 ±22 (305-372)</td>
<td>111 ± 15 (91-126) *</td>
<td>266 ± 20 (249-301)</td>
<td></td>
</tr>
<tr>
<td><strong>FP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>basal</td>
<td>42 ± 11 (26-60)</td>
<td>20 ± 10 (11-35)</td>
<td>55 ± 7 (45-65)</td>
<td></td>
</tr>
<tr>
<td>peak</td>
<td>314 ± 59 (246-417)</td>
<td>156 ± 67 (72-254) *</td>
<td>267 ± 18 (242-294)</td>
<td></td>
</tr>
<tr>
<td><strong>BUD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>basal</td>
<td>46 ± 17 (22-70)</td>
<td>35 ± 14 (16-50)</td>
<td>41 ± 15 (19-59)</td>
<td></td>
</tr>
<tr>
<td>peak</td>
<td>288 ±38 (253-361)</td>
<td>274 ± 19 (249-292)</td>
<td>292 ± 35 (243-332)</td>
<td></td>
</tr>
</tbody>
</table>

FP, fluticasone propionate; BUD, budesonide.

*Comparison with placebo or BUD, \( p < 0.001 \).
**Day 0.** There was no difference in either baseline or ACTH-stimulated peak cortisol levels when treatment groups were compared with the placebo group.

**Day 28.** Neither the placebo group nor the BUD groups showed any changes in basal or peak cortisol levels after 4 weeks of treatment relative to day 0 (all $p > 0.05$). However, on day 28 basal cortisol levels were suppressed in the prednisolone group relative to the placebo ($p = 0.016$) and BUD ($p = 0.029$) groups (Fig. 8). Peak cortisol levels after the ACTH stimulation test were significantly lower in the prednisolone and FP groups than in the placebo and BUD groups (Fig. 9; all $p < 0.001$).

**Day 35.** In the FP group basal cortisol levels were higher than on day 28 ($p < 0.001$) and higher than in the placebo group (Fig. 8; $p = 0.035$), but they were not higher than in the FP group on day 0 ($p = 0.099$). There were no group differences in ACTH-stimulated peak cortisol concentrations (Fig. 9).

![Figure 8](image.png)

**Figure 8** Basal cortisol concentrations (nmol/l) for the 4 treatment groups ($n = 6$ in each) on days 0, 28 and 35. Normal values range from 10 to 100 nmol/l (indicated as light grey bar beside the vertical axis). Outliers are marked as open circles (*). Differences at the $p < 0.05$ level are designated with an asterisk (*). The smallest and largest non-outlying values are shown by whiskers. On day 28 the cortisol levels in the prednisolone group were significantly suppressed relative to the placebo ($p = 0.016$) and budesonide ($p = 0.029$) groups. On day 35 the cortisol level in the fluticasone propionate group was elevated relative to the placebo group ($p = 0.035$).
Results

Figure 9  ACTH stimulated peak cortisol concentrations (nmol/l) in the 4 treatment groups \((n = 6\) in each) on days 0, 28 and 35. Normal results range from 200 to 600 nmol/l (indicated as a light grey bar beside the vertical axis). Outliers are marked as open circles (°). Differences at the \(p < 0.05\) level are designated with an asterisk (°). The smallest and largest non-outlying values are shown with whiskers. On day 28 the peak cortisol level in the prednisolone and fluticasone propionate groups was lower than in the placebo and budesonide group (all \(p < 0.001\)).
6 DISCUSSION

6.1 WEIGHT-DEPENDENT BAL TECHNIQUE PROVIDES MORE UNIFORM ELF RECOVERY

BALF is collected to diagnose and study alveolar and small airway diseases (Hawkins et al. 1995). The proportion of ELF recovered in BALF does not affect relative cell counts providing sufficient fluid is infused to ensure samples do not come primarily from the large airways (Rennard et al. 1990), but when BALF is used for quantitative assessment of constituents in the recovered fluid fluctuations in ELF recovery can have a marked influence on results. It is therefore vital that the volume of ELF recovered in consecutive lavages is consistent.

We compared different lavage methods in order to investigate the problems associated with variability in the volume of ELF retrieved. Fixed-volume BAL has been the conventional technique in dogs; reported lavage fluid volumes range from 5 to 25 ml and number of aliquots from one to five per site (Hawkins 2004, Zhu et al. 2015). In humans total lavage fluid volume usually varies between 100 and 300 ml/lobe (Klech and Pohl 1989); however, evidence from studies of children has shown that adjusting lavage fluid volumes according to body weight ensures that the proportion of ELF remains relatively constant (Ratjen and Bruch 1996). Like Ratjen et al. (1996) we found that in healthy dogs adjusting lavage fluid volume on the basis of body weight reduced the variability in the volume of ELF recovered (indexed by SD) relative to use of fixed-volume lavage.

In our study the mean percentage of ELF in recovered fluid was 2.3% for the weight-dependent technique and 2.9% for the fixed-volume technique, slightly higher than previously reported values for dogs (1.0 to 1.1%; Hawkins et al. 1998, Mills and Litster 2005). This difference is probably due mainly to methodological differences between the studies, including variations in aspiration technique, aspiration pressure, volume of lavage fluid, number of aliquots, dwell time, BAL duration and the preparation of BALF sample (Klech and Pohl 1989).

6.1.1 EVALUATION OF THE AMOUNT OF ELF USING THE UREA METHOD

We used the urea method described by Rennard et al. (1986) to assess the dilution of ELF in BALF. Urea is commonly used as an indicator of dilution in human studies and has also been used in dogs (Mills and Litster 2005) and cats (Mills and Litster 2006). The decision to use urea was based on several previous studies, e.g. Dargaville et al (1999), comparing data on various indicators of dilution in infants with and without lung disease; the conclusion
from this work was that urea was a more reliable indicator of dilution than protein, albumin, sphingomyelin, or IgA secretory component.

Urea is a good indicator of dilution; it is a physiological molecule which is not metabolised in lung cells, it is present in similar concentrations in several body fluids and it is easy to quantify. Our study was conducted in healthy dogs and therefore the previously mentioned influence of disease on urea influx into BALF was not relevant. Van de Graaf et al. (1991) reported that influx of urea into BALF may be higher in the presence of lung disease as a result of alterations in respiratory membrane permeability and suggested the use of additional endogenous or extrinsic indicators of dilution to verify the accuracy of the urea method. The influx of urea into BALF in the presence of disease remains controversial, however; Dargaville et al. (1999) found no evidence in children that lung disease was associated with influx of urea into the lavage fluid.

Long dwell times can cause problems with the urea method, as they allow urea to diffuse into BALF and thus result in overestimation of ELF recovery (Marcy et al. 1987, Ward et al. 1992). This problem notwithstanding the urea method is considered reasonably reliable at short dwell times i.e. when the instilled saline is aspirated without delay (de Blic et al. 2000, Pocino et al. 2015). In this study dwell times were short (<30 s) and so diffusion of urea was not considered likely to pose a problem.

Although total lavage times in this study were >7.0 min, because we instilled lavage fluid in two aliquots in an effort to maximise recovered fluid volumes, we did not find evidence that the increase in duration of the procedure caused an increase in the concentration of urea in BALF. This finding is consistent with a previous study in healthy humans which found no relationship between influx of urea and duration of BAL with lavage times varying from 2.7 to 7.0 minutes (van de Graaf et al. 1991). It has been reported that use of an automated suction pump enhances recovery of fluid relative to manual aspiration, in both healthy dogs and dogs with respiratory disease (Woods et al. 2014a, Woods et al. 2014b); however using a suction pump does not reduce the duration of the lavage procedure.

The use of dogs with a broader range of body weights (i.e. from small-breed dogs to giant-breed dogs) would have provided more evidence on the effects of adjusting lavage fluid volumes on the basis of body weight. The variation in body weight in our study sample was only moderate; however our finding that it reduced variability in ELF recovery is consistent with the findings of an earlier study in children between 3 and 15 years of age with a much wider range of body weights (Ratjen and Bruch 1996). The growth of airways is linked to overall growth (Hislop et al. 1972); this provides some justification for adjustment of lavage fluid volumes on the basis of body weight. Our results allow us to recommend use of a weight-dependent BAL protocol, at least in dogs with broadly similar weights.
6.2 BUDESONIDE

6.2.1 PHARMACOKINETIC STUDY IN HEALTHY DOGS

We assessed the pharmacokinetic characteristics of inhaled BUD in dogs. The pharmacokinetic and therapeutic properties of inhaled BUD, a commonly used anti-asthmatic drug in adults and children, have been comprehensively studied in humans. A series of case reports on the therapeutic use of FP and beclomethasone dipropionate in chronic inflammatory airway disease in dogs has been published (Bexfield et al. 2006); however the reported dosage protocols and the dosages we used in our research were based on an adaptation of human protocols as there were no data available on the pharmacokinetics of inhaled BUD or other ICSs, in dogs.

The relatively high dose of BUD (1.0 mg) used in our inhalation studies was chosen to ensure measurable plasma concentrations of the drug, and because similar (Godfrey et al. 2002) or larger doses (1.6 mg twice daily) (Minto et al. 2000) have been used in human studies. The results of our study on use of inhaled BUD without charcoal (TREAT_INH) are in line with an earlier study of the pharmacokinetics of nebulised BUD in children which used a 1mg dose of the drug (Agertoft et al. 1999). In our study the mean T\textsubscript{max} was 0.27 h; in children T\textsubscript{max} varied from 0.17 to 0.5 h. Mean C\textsubscript{max} was slightly lower in our dogs than in children (0.89 ng/ml and 1.12 ng/ml respectively); mean AUC was 1.1 ng h/ml in dogs and 2.0 ng h/ml in children. Systemic exposure to the drug (indexed by AUC) was lower for dogs than children; this suggests that in dogs the systemic activity of BUD treatment may be minor.

A study conducted in healthy humans showed that administration of an oral charcoal suspension before and after PO administration of a BUD capsule decreased GI absorption of BUD by about 96% (Lähelma et al. 2005). We showed that charcoal almost totally abolished GI absorption of an orally administered high dose (10mg; a tenfold increase on the inhaled dose) of BUD. In three dogs BUD was not detectable in plasma and in the remaining four dogs the mean C\textsubscript{max} was low (i.e. 0.15 ng/ml). In addition, when BUD inhalation was administered concomitantly with oral charcoal there was a 50% reduction in AUC, indicating a marked reduction in absorption of swallowed drug from the GI tract. There was no difference between the inhalation treatments (BUD with and without oral charcoal) in C\textsubscript{max} and T\textsubscript{max} values. Interestingly, the dog with the highest maximum plasma BUD concentration was the same for both inhalation treatments; it is unclear whether this was due to an efficient breathing technique or individual differences in drug metabolism.

None of our dogs were trained to breathe through a mask before the experiments, unlike human studies, where patients are usually given the chance to get accustomed to the inhalation device. The delivery of ICSs is influenced by several patient-level factors, some also applicable to our dogs, including hand-lung co-ordination (the ability to inhale deeply as the pMDI is
Discussion

fired), tidal volume and breathing pattern. A period of familiarisation with the inhalation device might have reduced the inter-individual variation in the plasma BUD concentrations in our dogs. It should be noted, however, that Minto et al. (2000) reported considerable inter-individual variability in lung absorption in healthy human volunteers after administration of inhaled BUD (1600 \( \mu g \) twice daily). In humans differences in the anatomy of the upper respiratory tract between children and adults have been shown to influence delivery of ICSs; similar factors, e.g. variation in muzzle and skull structures, probably contribute to variance in delivery in dogs.

The pharmacokinetic characteristics of iv administered radio-labelled BUD in dogs has been described by Ryrfeldt et al. (1979), and their results including elimination half-life, total clearance, and volume of distribution, are in accordance with human study (Ryrfeldt et al. 1984). Our findings of the iv pharmacokinetics of BUD are comparable with and verify the previous results (Ryrfeldt et al. 1979).

6.2.1.1 Drug delivery device

ICS delivery is affected by numerous technical and patient related factors which have been extensively investigated in humans. Important technical factors include the volume, shape and electrostatic charge of the chamber, the configuration of the inspiratory and expiratory valves, dead space volume, and fit of the mask. We used a commercially available VHC that was designed for dogs, and it probably enhances delivery of inhaled medication to lung. It has been suggested that in humans use of a VHC may as much as double the amount of medication reaching the lower airways (Dempsey et al. 1999b) and the use of such devices is recommended in small children and animals that are not able to control their breathing while using a pMDI, which is the only suitable method of administering ICSs in dogs. In our studies the VHC was not primed or washed with detergent to reduce electrostatic charge; it is therefore very likely that there was electrostatic attachment of drug molecules to the chamber walls (Barry and O'Callaghan 1995). We used an anaesthetic mask because it fitted tightly around the muzzle of the dog and prevented opening of the mouth; however this choice also meant that there was a large dead space volume and may have resulted in retention of BUD within the mask.

The dogs inhaled BUD via the nasal route, since oral inhalation is not feasible with currently available devices. Schulman et al. (2004) demonstrated the viability of the nasal administration route in healthy cats, showing that nebulised radio-labelled aerosol reached the lower airways and was distributed throughout the lung fields in all cats. We also noticed that the BUD inhalation procedure was well-tolerated by the dogs.
6.2.1.2 Bioavailability

The anti-inflammatory effect of inhaled BUD is thought to be due to local pulmonary effects rather than systemic activity (Toogood et al. 1990, Donnelly and Seale 2001). BUD found in the systemic circulation after inhalation is derived from absorption through the respiratory epithelium or from the GI tract following inadvertent ingestion of the drug and to a lesser extent from absorption through the buccal mucosa (Derendorf et al. 1998, Donnelly and Seale 2001). Although most of the nominal dose from a pMDI is deposited in the oropharynx (approximately 80% in humans) it is assumed that buccal absorption has little effect on the systemic bioavailability of the drug, because the oropharynx presents only a small absorptive surface area and the mucosal exposure time is relatively short. Approximately 90% of BUD absorbed from the GI tract is inactivated during first-pass hepatic metabolism, so the systemic bioavailability of BUD is mainly determined by deposition and absorption from the lung (Lipworth 1995, Donnelly and Seale 2001).

In our study the mean systemic bioavailability of inhaled BUD was 2.1%, and the mean pulmonary bioavailability, calculated on the basis that oral charcoal blocked GI absorption, was 1.2%. A study of nebulised BUD (1 mg dose) in children aged 3-6 years estimated the systemic bioavailability of BUD at 6.1% of the nominal dose (Agertoft et al. 1999). The total BUD clearance rate of 1.5 l h/kg for observed in our iv study is very similar compared to the clearance value reported for children, 1.8 l h/kg (Agertoft et al. 1999). The combination of lower systemic bioavailability of BUD and a similar clearance rate in dogs relative to children suggests that similar or larger doses of inhaled BUD can be used safely in dogs.

One factor which could account for the moderately low systemic bioavailability of inhaled BUD in our studies is that dogs cannot be instructed to take a deep breath when inhaled medication is given; this lack of co-ordination as well as an unfavourable breathing pattern, i.e. low tidal volume, reduces delivery of ICSs to the lower airways. The VHC and mask used in our study might have led to retention of BUD in the administration devices, as discussed in Section 6.2.1.1.

Our dogs inhaled the drug via the nasal route, which in humans results in lower mean lung deposition than the oral inhalation route; in a study of healthy adults deposition of radio-labelled aerosol was reduced from 75% to 38% (Everard et al. 1993). Chua et al. (1994) have also demonstrated in children with cystic fibrosis that nasal inhalation resulted in low total lung deposition of radio-labelled nebulised saline aerosol using planar and single-photon emission computed tomography (SPECT) scans; values ranged from 0.3% to 1.6% in infants (who were asleep) and from 1.6% to 4.4% in older children. There has been no comparable study in dogs.

BUD can form esters with long-chain fatty acids in cells; depots of the inactive drug can be de-esterified and released in concentration-dependent manner (Donnelly and Seale 2001). It has been established that BUD-ester conjugates are formed in the lung cells of humans (Tunek et al. 1997, Nave et
al. 2007, van den Brink et al. 2008) and rats (Miller-Larsson et al. 1998), but ester conjugation has not yet been verified in the respiratory tract cells of dogs. An earlier study (Pietra et al. 2013) showed that when dogs with inflammatory bowel disease were given oral BUD capsules there was a gradual accumulation of the drug during the first 8 days of treatment. It was speculated that this was due to biotransformation of BUD into inactive lipophilic intracellular fatty acid esters in the intestinal cells and that this provided a store of the active drug. If a similar esterification process occurs in the lung cells of dogs, the depot of inactive drug could in part explain the high pulmonary efficacy of inhaled BUD.

The range of body weights in our study sample was fairly narrow (10-22 kg); however, previous research suggests that body weight should not have affected the pharmacological parameters. Barry and O’Callaghan (1999) showed that in vitro there is a linear relationship between tidal volume (which increases with body weight) and the output of BUD from a VHC comparable to that used in our studies; the drug dose should therefore be kept constant to ensure that all patients receive the same dose per kilogram.

6.2.1.3 Study limitations

Our results were achieved in a small sample of healthy beagles. The breed may have influence on the values of the pharmacological parameters investigated. In humans BUD is metabolised mainly by cytochrome p-450 (CYP) enzymes (Jönsson et al. 1995) and there are known to be genetic differences between the CYP enzymes of beagles and other dog breeds (Trepanier 2006, Court 2013) so these results may not generalise completely to other dog breeds. Health is another factor which may have affected pharmacokinetic parameters; our dogs were healthy and in humans it has been shown that respiratory disease affects the proportion of inhaled FP which reaches the lower airways (Brutsche et al. 2000) although Dalby et al. (1998) showed the systemic bioavailability of BUD was similar in healthy humans and people with chronic obstructive pulmonary disease. Mortimer et al. (2007) studied the effects of induced bronchoconstriction on plasma concentrations of FP and BUD following inhalation and concluded that bronchoconstriction produced a greater reduction in plasma concentrations of FP.

These studies were not designed to determine the therapeutic dose of inhaled BUD, which is affected by the severity of the inflammatory process (Gulliver et al. 2007). Further clinical trials in dogs with respiratory disease will be needed to determine the clinically effective dose. Comparative analysis of the various inhalation devices should also be carried out.
6.2.2 LC-MS/MS

The method of liquid-liquid extraction combined with a subsequent solid-phase extraction was developed, validated and tested with authentic dog plasma samples. The product, an interference-free extract of dog plasma, was compliant for instrumental analysis. The leftover extracts were suitable to be archived in a freezer for possible future use. Although sample processing consisted of two separate tasks, namely liquid–liquid extraction and solid-phase extraction, the method was not considered too laborious.

Recoveries from human plasma samples at low levels of BUD, i.e. < 1 ng/ml plasma, have had a tendency to show better recoveries. A study using a multianalyte method reported recovery rates varying between 74% and 85% (Wang et al. 2003) and using single analyte methods recovery varies from 77% to 98% (Li et al. 2001, Wang et al. 2003, Borges et al. 2011). It could be argued that extensive sample handling is a source of inaccuracy and/or poor recovery. Our recovery rates - between 86% and 97% - are well within the reported range although we carried out more extensive sample purification before analysis.

This is the first method of assessing BUD in dog plasma to take full advantage of the use of deuterated BUD as an internal standard. A recently developed method, based on photoionisation in MS/MS, for analysing human plasma samples (Borges et al. 2011) achieved a slightly lower LOQ than our method. But even the level of sensitivity with our method was far below than necessary to detect BUD in dogs in clinical settings. Use of ultra-high-performance liquid chromatography (UPLC) combined with more modern and sensitive MS/MS instrumentation might improve sensitivity. It is reasonable to suggest that increased sensitivity could be achieved using more modern equipment because it is evident that a low background noise/matrix effect can be achieved with good sample handling. The chromatographic analysis time in our studies was short, less than five minutes, although we used conventional HPLC with a traditional column diameter (2.1 mm). Use of UPLC might further reduce analysis times.

The method was fully validated. The sample workout demonstrated excellent recoveries, even at low BUD levels. At the three BUD levels tested the method had good repeatability and reproducibility. We did not test the stability of BUD or d8-BUD, but the indirect evidence, i.e. the constant signal intensity of stock standards and spiked dog plasma samples, indicates that BUD is stable in dog plasma if samples are stored in a freezer and handled at standard room temperatures. This conclusion is consistent with the observation that BUD is stable in human plasma samples (Streel et al. 2009, Borges et al. 2011).

The performance of the method was excellent with respect to sample handling and instrumental analysis of dog plasma samples for BUD. We have successfully used the method in pharmacokinetic studies of inhaled BUD in dogs. To demonstrate that the method possessed the requisite sensitivity we analysed samples from randomly selected dogs treated with BUD aerosols. In general, BUD in dog plasma was detected close to the LOQ within 2 h and the
Discussion

MRM chromatogram (transitions, m/z 431>413 and 431> 323) was free of interfering fragments. This suggests that the method could be used for quantification of BUD in dog plasma samples.

6.2.3 BUDESONIDE DID NOT INDUCE DETECTABLE ADVERSE EFFECTS

6.2.3.1 Results of serum biochemistry and urinanalysis

After 2 and 4 weeks of prednisolone treatment we observed an increase in serum ALP and protein concentrations; after 4 weeks there was also an increase in the urine protein to creatinine ratio relative to the placebo group. Oral corticosteroid treatment is commonly associated with elevation of ALP levels; this effect is mainly due to formation of the corticosteroid-induced ALP isoenzyme (Solter et al. 1994). Slight elevations in the urine protein to creatinine ratio in dogs have been reported following 42 days of treatment with immunosuppressive doses of corticosteroids; these effects were attributed to mesangial cell proliferation and glomerular changes in the kidneys (Waters et al. 1997). The elevations in urine protein to creatinine ratio in our study might have been due to similar changes, although the dose of prednisolone used was lower.

Elevated serum protein concentrations in dogs after a five-week course of anti-inflammatory glucocorticoid therapy using a dose equivalent to that in our study, have been reported (Moore et al. 1992). In our dogs serum protein concentrations tended to increase but as they remained within the reference range this finding is clinically unremarkable. That same study also found reductions in the numbers of eosinophils and lymphocytes (Moore et al. 1992), however we did not find any changes in differential cell counts, perhaps because of the shorter duration of treatment.

6.2.3.2 Effect on the HPA axis

We compared the systemic effects of inhaled and oral corticosteroids. Inhalation delivers a relatively small dose of drug straight to the lungs, in our study about 1.2% of the nominal dose reached the lungs, and swallowed drug is almost completely inactivated by first-pass hepatic metabolism (90% for BUD; 99% for FP) (Ryrfeldt et al. 1982, Harding 1990, Lipworth 1995). BUD and FP are both potent corticosteroids and are used in inhalation formulations to treat chronic respiratory diseases; however there was no research on the endocrine effects of inhaled BUD in dogs. We compared the effects of inhaled BUD also with those of a conventional drug treatment, oral prednisolone. Comparison of the results of Study II with research on iv BUD in children
(Agertoft et al. 1999) indicated that the pharmacokinetic characteristics of BUD, specifically the elimination half-life, total clearance and volume of distribution, do not markedly differ between dogs and children. We therefore based the estimates of clinically effective doses of BUD and FP used in our study on recommendations for the treatment of asthmatic children (Campbell et al. 1998, Herjavec et al. 1999, Levy et al. 2009, Schramm and Carroll 2009, Stoloff and Kelly 2011).

Suppression of the HPA axis is the most common systemic adverse effect of prolonged corticosteroid administration in dogs. We observed marked suppression after 4 weeks of treatment with FP or prednisolone, which is consistent with previous research on use of FP and prednisone in dogs (Cohn et al. 2008) and humans (Clark et al. 1996, Clark and Lipworth 1997, Kaliner 2006). It should be noted, however, that most of the studies conducted in humans and all the studies in dogs were of healthy individuals. Harrison et al. (2001) showed that FP given via a DPI at a dose of 1500 μg/day has greater effect on the HPA axis in healthy subjects than in asthmatics, whereas BUD at a dose of 1600 μg/day had similar effect on both groups.

Unlike Cohn et al. (2008) we found that both FP and oral prednisolone suppressed the HPA axis relative to a placebo; interestingly BUD did not cause adrenal suppression. Whether a similar pattern of results would be found in dogs with respiratory disease is uncertain, given the possibility that FP inhalation has a smaller effect on the HPA axis in diseased dogs (Harrison et al. 2001).

We detected normal basal and ACTH stimulated peak cortisol levels in all treatment groups on days 0 and 35. In the FP group there was an increase in basal cortisol level on day 35 relative to the placebo group, but levels were still within the reference range and so the clinical importance of the finding is unclear; it is probably a reflection of recovery of the adrenal gland after suppression. This finding suggests that there is complete recovery in HPA axis function one week after discontinuing a four-week period of treatment with prednisolone at an anti-inflammatory dose. Moore and Hoenig (1992) investigated recovery following a five-week course of a daily dose of prednisolone (equivalent to the dose used in our study); they concluded that HPA axis recovery was complete 2 weeks after the end of drug treatment. In this study we also standardised the environment and sampling procedures as much as possible in order to minimise the influence of circadian rhythm of cortisol excretion (Derendorf 1997), and stress-induced elevation of cortisol levels.

The pharmacokinetic and pharmacodynamic properties of ICSs are very complex (Clark and Lipworth 1997, Lipworth and Jackson 1999, Adams et al. 2007, Stoloff and Kelly 2011). It has been suggested that FP is a more effective ICS than BUD both because it is retained better in the lung tissue owing to its higher lipophilicity, and because its higher glucocorticoid receptor affinity extends the duration of local anti-inflammatory action (Hogger and Rohdewald 1994, Johnson 1996). However the lower lipophilicity of BUD
means that it dissolves better in the bronchial fluid (Hogger et al. 1993) and, unlike FP, is able to form fatty acid esters in the cells of the respiratory tract (Tunek et al. 1997, Nave et al. 2007, van den Brink et al. 2008). The lower systemic bioactivity of BUD compared with FP in our study might be due to differences in pharmacological properties. With all corticosteroids the systemic effects can be reduced by tapering the dose, but equivalent doses for different drugs – in terms of endocrine effects or clinical response – are not known as there have been no clinical trials comparing the efficacy of FP, BUD and prednisolone in dogs.

Our findings suggest that at current doses inhaled BUD would represent a safer anti-inflammatory therapy for chronic respiratory diseases in dogs that are sensitive to the systemic adverse effects of corticosteroid treatment.
7 CONCLUSIONS

1. A method which improves the consistency of ELF recovery in BALF in healthy dogs was developed. Use of weight-dependent lavage yielded more consistent results than use of fixed-volume lavage.

2. Pharmacokinetic variables relating to administration of BUD iv (T<sub>1/2</sub>, Cl, V<sub>d</sub>, and AUC<sub>0-∞</sub>), and inhaled BUD (C<sub>max</sub>, T<sub>max</sub>, and AUC<sub>0-6h</sub>) in healthy dogs were assessed. We found that inhaled BUD has moderately low systemic and pulmonary bioavailability.

3. A liquid chromatography-tandem mass spectrometry method which allows low-volume dog plasma samples to be handled using liquid–liquid extraction combined with reversed-phase solid-phase extraction was developed.

4. We demonstrated that a four-week period of treatment with clinically effective doses of FP or prednisolone, but not BUD, strongly suppressed HPA axis function in healthy dogs.
Studies included in this thesis were carried out at the Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki. Financial support provided by the Faculty of Veterinary Medicine, the Finnish Veterinary Foundation, the Helvi Knuuttila Foundation, and the Orion Pharmos Research Foundation, is gratefully acknowledged.

After laying the foundations for small animal respiratory research in Finland, Docent Minna Rajamäki introduced me to the fascinating world of respiratory research, with the assistance of Professor emerita Anna-Kaisa Järvinen. I express my deepest gratitude to my supervisor Minna Rajamäki, for her enthusiasm for the subject, for her ability to listen, discuss and speculate, but above all for her ever friendly and compassionate attitude to life in general. I also owe my gratitude to my supervisor Professor Outi Vainio for her vast scientific expertise and support, as well as her patience with this long project. I want to thank Professor Thomas Spillmann for providing the facilities that made these studies possible.

This research would not have been possible without my co-workers, Anu Lappalainen, DVM, PhD, Kaisa-Liina Juhajoki, DVM and Noora Salin, DVM. For technical support, my sincere thanks to Professor Satu Sankari, Merja Ranta, Lilja Jääskeläinen, and Kirsi Laukkanen, for handling the samples, and to Matti Järvinen and Laura Parikka for their contribution in editing pictures and graphs, and help in general whenever needed.

I owe my gratitude to Seija Berg and Kimmo Peltonen for their scientific and technical expertise with budesonide analysis, and Marikki Peltoniemi for her assistance with the pharmacokinetic data analysis. Warm thanks go to Hannu Rita and Jouni Junnila for helping me with the statistical analyses.

I am deeply grateful to Associate Professor Carol Norris Reinero and Professor emerita Reeta Pösö for reviewing the manuscript of this thesis, and for their valuable comments, and to Professor Cecile Clercx for accepting the position of opponent at the public examination.

In addition, my thanks to all my colleagues and staff at the Faculty of Veterinary Medicine, especially to Karoliina Autio, Merja Laitinen, and Henna Laurila, for their invaluable support during the writing process.

My deepest gratitude and thanks go to my husband, Jaakko. Without his excellent sense of humour, loving care, constructive criticism and support, this thesis would never have been accomplished. Our children, Akseli, Meeri, and Toivo, mean everything to us and bring joy to our life. My parents, Ritva and Pentti Immonen, thank you for the support, help and love during all these years, as well as for the curiosity and sceptical attitude that I have inherited from you. My parents-in-law, Arja and Lauri Melamies, I warmly thank you for your love and support. I owe a debt of gratitude to my sister, Sari Paukku, for always being there for me to share the joys and miseries of life. My dearest
friends, Sanna Vartiainen and Niina Metsä-Simola, I would never have made it without your comfort and support.
REFERENCES


Barry, P. W. & O’Callaghan, C. 1999. The output of budesonide from spacer devices assessed under simulated breathing conditions. The Journal of Allergy and Clinical Immunology 104, 1205-1210.


References


References

Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 28 (5): 1398-1404.


The central issue in BAL is the recovery of a uniform amount of ELF for analysis of cellular and noncellular constituents. The ELF is a thin layer of fluid that covers the epithelium of the alveoli and small airways. During BAL, ELF dissolves in saline (0.9% NaCl) solution to yield BALF. The determination of total amounts of constituents (eg, cells, proteins, and bacteria) in BALF is affected by variations in ELF dilution. The volume of ELF can be calculated by determining the amount of an endogenous solute that exists naturally in BALF or an exogenous molecule that is added to the lavage fluid and comparing that amount with the plasma concentration or the initial concentration of the exogenous molecule, respectively. However, feasibility for the use of extrinsic molecules (eg, methylene blue, insulin, or radioactive tracers) requires additional measures for both the lavage and analysis phases; therefore, it is easier to use endogenous markers (eg, urea, albumin, protein, or potassium). Urea has been considered to be the most reliable endogenous marker of dilution in healthy and diseased lungs. Compared with the use of albumin and protein, urea has the advantage of a low molecular mass that allows rapid diffusion from plasma into ELF, which results in equal urea concentrations in both the plasma and ELF. Use of urea as a dilutional marker to calculate ELF recovery has been reported in horses, humans, dogs, and cats. Marked variations in volume of ELF recovered have been described, which makes it difficult to compare results among studies. Factors (including the lavage technique, volume of lavage, and amount of time that

Comparison of results for weight-adjusted and fixed-amount bronchoalveolar lavage techniques in healthy Beagles

Marika A. Melamies, DVM; Anna-Kaisa Järvinen, DVM, PhD; Kati M. Seppälä, DVM; Hannu J. Rita, PhD; Minna M. Rajamäki, DVM, PhD

Objective—To compare recovery of epithelial lining fluid (ELF) in bronchoalveolar lavage fluid (BALF) by use of weight-adjusted or fixed-amount volumes of lavage fluid in dogs.

Animals—13 healthy Beagles.

Procedures—Dogs were allocated to 2 groups. In 1 group, the right caudal lung lobe was lavaged on the basis of each dog’s weight (2 mL/kg, divided into 2 aliquots) and the left caudal lung lobe was lavaged with a fixed amount of fluid (50 mL/dog, divided into 2 aliquots). In the second group, the right and left caudal lung lobes were lavaged by use of the fixed-amount and weight-adjusted techniques, respectively. The BALF was collected by use of bronchoscopy. A recovery percentage ≥ 40% was required. The proportion of ELF was calculated by use of the following equation: (concentration of urea in BALF/concentration of urea in serum) X 100.

Results—Mean ± SD proportion of ELF in BALF was 2.28 ± 0.39% for the weight-adjusted technique and 2.89 ± 0.89% for the fixed-amount technique. The SDs between these 2 techniques differed significantly (calculated by comparing 2 covariance structures [unstructured and compound symmetry] in a repeated-measures mixed ANOVA).

Conclusions and Clinical Relevance—The findings strongly suggested that use of a weight-adjusted bronchoalveolar lavage technique provided a more uniform ELF recovery, compared with that for a fixed-amount bronchoalveolar lavage technique, when urea was used as a marker of dilution. A constant ELF fraction can facilitate more accurate comparisons of cellular and noncellular constituents in BALF among patients of various sizes. (Am J Vet Res 2011;72:694–698)
A recovery percentage ≥ 40% of the instilled fluid volume was required. Total duration of BAL for each lung lobe (ie, lavage time elapsed from the beginning of the instillation of the first aliquot until the end of the last aspiration attempt after infusion of the second aliquot) was measured for both groups. Dwell time (time that elapsed between fluid instillation and the first attempted aspiration) was < 30 seconds in all dogs.

**Examination of BALF**—The BALF specimens from the right and left caudal lung lobes were examined immediately after collection. Quantitative bacterial culture was performed by inoculating a 10-μL sample of unfiltered BALF on a blood-agar plate. The plate was incubated at 37°C for 48 hours, and bacterial growth of > 1.7 × 10^3 CFUs/mL was used as an indicator of bacterial infection.

Urea concentrations in serum and BALF were determined with a kinetic enzymatic method by use of a clinical chemistry analyzer and a commercial reagent. The intra-assay and interassay coefficients of variation for the serum urea determination were 2.5% (mean, 48.3 mg/dL; n = 10 observations) and 2.8% (mean, 55.9 mg/dL; 30 observations), respectively. The intra-assay variability of 8 BALF urea assays was 4.9% and 0.9% for concentrations of 0.62 and 2.54 mg/dL, respectively. The observed concentrations of BALF urea relative to expected concentrations after addition of known amounts of urea that ranged from 0.31 to 2.50 mg/dL were 97.6% to 101.5%. The detection limit of the BALF urea assay was 0.08 mg/dL. The proportion amount of ELF was calculated by use of the following equation: (concentration of urea in BALF/concentration of urea in serum) × 100%.

**Statistical analysis**—Data were expressed as mean ± SD or median and range. Parametric analyses were used when normal distribution of data was verified. Statistical software programs were used for the statistical analyses. For all analyses, values of P < 0.05 were considered significant.

Comparison of BALF recovery percentages between fixed-amount and weight-adjusted techniques was performed with paired t tests. Comparisons of lavage times and BALF total cell counts between techniques were analyzed with Wilcoxon matched-pairs sign rank tests. The association between lavage time and BALF urea concentration was analyzed via the Spearman correlation coefficient.
The SDs of the proportions of ELF for the weight-adjusted and fixed-amount techniques were compared by use of a repeated-measures mixed ANOVA. Unstructured covariance structure (which enables differing SDs) was tested against compound symmetry structure (which requires equal SDs) by use of a general linear models procedure.5

**Results**

**Dogs**—Results of physical examination were unremarkable in all dogs. Weight index was 3 (optimum) in 12 dogs and 4 (overweight) in 1 dog. Results of hematologic and serum biochemical analyses as well as mean ± SD PaO₂ (97.6 ± 7.4 mm Hg) and PaO₂-PaO₂ (9.9 ± 6.6 mm Hg) were within reference ranges in all dogs, with minor exceptions. All fecal analyses for parasites yielded negative results. Thoracic radiography revealed only mild age-related findings.

**BAL**—Mean ± SD recovery percentage of infused lavage fluid was 58 ± 13% for the fixed-amount BAL technique and 57 ± 11% for the weight-adjusted BAL technique; no significant difference (P = 0.81) was detected between the techniques. Total cell counts did not differ significantly (P = 0.31) between the fixed-amount (median, 270 cells/μL; range, 120 to 730 cells/μL) and weight-adjusted (median, 250.0 cells/μL; range, 190 to 820 cells/μL) BAL techniques. Median differential cell counts for the fixed-amount and weight-adjusted BAL techniques were 74.4% (range, 62.4% to 87.4%) and 78.4% (range, 61.0% to 87.0%), respectively, for macrophages; 19.4% (range, 11.0% to 33.7%) and 15.4% (range, 9.0% to 31.4%), respectively, for lymphocytes; 2.7% (range, 0.7% to 5.4%) and 2.0% (range, 1.4% to 7.0%), respectively, for neutrophils; 1.7% (range, 1.0% to 7.0%) and 2.0% (range, 0.4% to 5.7%), respectively, for mast cells; 0% (range, 0% to 3.4%) and 0.4% (range, 0% to 1.0%), respectively, for eosinophils; 0% (range, 0% to 1.0%) and 0% (range, 0% to 3.7%), respectively, for plasma cells; and 0% (range, 0% to 0%) and 0% (range, 0% to 0%), respectively, for epithelial cells. Bacterial cultures yielded negative results, and no intracellular bacteria were detected. Serum urea concentrations ranged from 16.5 to 28.6 mg/dL (median, 19.3 mg/dL), and BALF urea concentrations ranged from 0.27 to 1.1 mg/dL (median, 0.53 mg/dL).

Lavage time for 1 lung lobe did not differ significantly (P = 0.15) between the fixed-amount (median, 11.3 minutes; range, 9.4 to 18.2 minutes) and weight-adjusted (median, 10.4 minutes; range, 9.1 to 15.1 minutes) BAL techniques. No association between urea concentration in BALF and lavage time was detected for either BAL technique (fixed-amount technique, r = 0.12 [P = 0.69]; weight-adjusted technique, r = 0.43 [P = 0.19]).

Mean ± SD proportion of ELF calculated by use of the urea method was 2.89 ± 0.89% for the fixed-amount technique and 2.28 ± 0.39% for the weight-adjusted technique (Figure 1). The SDs differed significantly (P = 0.041) between the 2 BAL techniques.

**Discussion**

Examination of BALF is a method that is useful in the diagnosis and study of alveolar and small airway diseases in dogs. The proportion of ELF recovered in BALF does not affect relative cell counts provided sufficient fluid is infused to avoid collecting samples primarily from the large airways.14 However, when BALF is used for quantitative assessment of constituents in recovered fluid, fluctuations in ELF recovery may cause marked variation in results; thus, it is vital to collect a uniform amount of ELF in consecutive lavages. Few studies15-17 have been conducted to solve this problem via development of methods to collect pure ELF, and such techniques are not yet appropriate for routine use. In the present study, we found that adjustment of the volume of lavage fluid on the basis of body weight provides a more uniform recovery of ELF in dogs than does use of a fixed-amount volume of lavage fluid.

In the study reported here, dilution of ELF was determined by use of the urea method, as described elsewhere.2 Urea is a good marker of dilution; it is a physiologic molecule with no metabolism in lung cells, has comparable concentrations in various body fluids, and is easy to measure.2 The major problem with this method is the possible overestimation of the recovered ELF volume caused by diffusion of urea into ELF during lavage, especially in cases of prolonged dwell time or concomitant lung disease with altered membrane permeability.9,18 Despite these factors, the urea method is considered sufficiently reliable provided the aspiration of instilled saline solution is initiated without delay and the dwell time for lavage fluid remains short.9 In the present study, diffusion of urea was not expected because dwell times were short (ie, <30 seconds).

Investigators in other studies9,19 have suggested that in addition to dwell time, lavage time (ie, duration of BAL) has an effect on urea diffusion. In 1 study,6 investigators performed BAL in healthy human volunteers with lavage fluid volumes of 100 and 300 mL and found that the diffusion of urea increased significantly beginning with the third 20-mL or with the 50-mL aliquot when BAL lasted 2.0 and 4.1 minutes, respectively. However, investigators in another study20 in healthy humans found no relationship between influx of urea and duration of BAL when lavage time varied from 2.7

![Figure 1—Proportions of ELF recovered by use of fixed-amount and weight-adjusted BAL techniques in 13 dogs. Each symbol represents results for 1 dog, and the horizontal line indicates the mean value for each technique. The SDs differed significantly (P = 0.041) between the 2 techniques.](image-url)
to 7.0 minutes. Although lavage times in the present study were > 7.0 minutes because of efforts to maximize the amount of recovered fluid and to enable us to evaluate the time effect on urea concentration in BALF we did not find that an increase in BAL duration caused an increase in urea concentration in BALF.

Mean ELF recovery of 2.3% for the weight-adjusted technique and 2.9% for the fixed-amount technique are slightly higher than the recovery percentages (range, 1.0% to 2.1%) reported for dogs in other studies.\(^3\),\(^6\),\(^7\)

This can be explained by differences in methods among studies, including variations in aspiration technique, aspiration pressure, volume of lavage fluid, number of aliquots, dwell time, BAL duration, and preparation of BALF sample. However, the key issue in the study reported here is that the variability in the proportion of recovered ELF described by the SDs was smaller for the weight-adjusted technique than for the fixed-amount technique. Therefore, we believe that the accuracy for analyses of constituent concentrations in BALF is better for the weight-adjusted technique and that the estimate of absolute amounts of constituents in ELF is more exact.

Healthy dogs were used in the study reported here. It has been speculated that lung disease can change the permeability of the alveolar-capillary membrane and allow additional influx of urea into BALF thus complicating the use of urea as a marker of dilution.\(^2\)\(^0\) In contrast, investigators in another study\(^3\) compared various markers of dilution in infants with and without lung disease and concluded that urea is a more reliable marker of dilution than is protein, albumin, sputum albumin, or IgA secretory component. In addition, that study\(^3\) revealed no evidence of additional influx of urea into the lavage fluid in association with epithelial disruption in diseased lungs. Significant variations in albumin and protein concentrations in ELF have been detected among diseased, recovering, and healthy lungs.\(^2\),\(^1\),\(^2\)\(^1\),\(^2\)\(^2\) Additionally, elevated albumin concentrations in ELF have been associated with increased age in humans.\(^2\)\(^3\) The use of urea and inulin as dilutional markers has been evaluated in healthy horses and horses with chronic obstructive pulmonary disease (ie, heaves).\(^1\) In that study,\(^1\) investigators found that ELF recovery was significantly higher when calculated via the inulin method and there was no correlation between the ELF percentages calculated with inulin or urea. However, they concluded that combined use of both markers may yield an advantage by providing upper and lower limits of ELF recovery. If the study reported here were to be repeated in dogs with lung disease, diffusion of urea might be altered and use of additional endogenous or extrinsic markers of dilution would be needed to verify accuracy of the urea method.

The use of dogs with a broader range of body weights (ie, from small-breed dogs to giant-breed dogs) would have further elucidated the effect of adjustment of the volume of lavage fluid on the basis of body weight. Although the study population had only moderate variation for size, the result of a more uniform ELF recovery is consistent with the findings in a study\(^6\) in children between 3 and 15 years of age with broad differences in weights. Because airways grow in parallel with overall body size, adjustment of the volume of lavage fluid on the basis of body weight appears to be justified.\(^2\)\(^4\)

We concluded that when the aim of BALF analysis is to measure exact amounts of constituents (eg, bacteria and proteins) in ELF for comparison of results, recovery of a uniform ELF volume is essential. Analysis of our results revealed that in healthy Beagles, the use of a volume of lavage fluid adjusted on the basis of body weight is 1 method for a more uniform ELF recovery.

References


Pharmacokinetics of budesonide following inhalation and intravenous administration in Beagle dogs

Marika Melamiesa,*, Outi Vainioa, Marikki Peltoniemib, Mia Sivenb, Kimmo Peltonenc, Seija Bergc, Kaisa-Liina Juhajokiia, Minna M. Rajamäkia

a Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, Viikintie 49, 00014 University of Helsinki, Finland
b Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, Viikinkaari 5, 00014 University of Helsinki, Finland
c Finnish Food Safety Authority EVIRA, Chemistry and Toxicology Research Unit, Mustialankatu 3, 00790 Helsinki, Finland

* Corresponding author. Tel.: +358 50 357 1878
E-mail address: marika.melamies@helsinki.fi (M. Melamies)
Abstract
Treatment of chronic respiratory diseases with inhaled corticosteroids (ICSs) has become common also in companion animals. Because the pharmacokinetics of ICSs in dogs have not been studied, a prospective open-label study was conducted where the pharmacokinetic properties of budesonide (BUD) administered intravenously (IV), via inhalation or via inhalation with concomitant charcoal were evaluated in eight healthy Beagles. Charcoal block was used to prevent gastrointestinal absorption of unintentionally swallowed drug. Blood samples were taken at predetermined times up to six hours after medication. Plasma concentrations of unchanged BUD were determined with a validated high-performance liquid-chromatography tandem mass spectrometry method.

The results of the inhalation studies (expressed as mean (standard deviation)) were as follows: $C_{\text{max}}$, $t_{\text{max}}$ and $\text{AUC}_{0-6}$ values after inhalation were 0.89 (0.59) ng/mL, 16 (7.4) min and 1.1 (0.59) ng h/mL and with concomitant charcoal 0.60 (0.19) ng/mL, 13 (4.6) min and 0.55 (0.23) ng h/mL, respectively. Inhalation studies revealed a marked inter-individual variation in the amount of inhaled drug reaching systemic circulation through respiratory epithelium and a low systemic and pulmonary bioavailability of BUD.

Keywords: Budesonide; Charcoal; Dog; Inhalation; Pharmacokinetics
Introduction

During the last decades treatment of canine chronic respiratory diseases, such as chronic bronchitis and eosinophilic bronchopneumopathy, has relied mainly on oral corticosteroids (McKiernan, 2000). Inhaled corticosteroids (ICSs) are currently the first-line treatment for human asthma and are generally regarded as effective also in the treatment of chronic respiratory diseases in dogs (Bexfield et al., 2006; Cohn et al., 2010), cats (Kirschvink et al., 2006; Galler et al., 2013) and horses (Laan et al., 2004). ICSs directly target the respiratory epithelium and have a low systemic bioavailability and a favourable efficacy-safety profile. Thus, they seldom cause the unpleasant adverse effects related to long-term oral corticosteroid therapy, e.g. polyphagia, polydipsia and polyuria, weight gain, panting, diabetes mellitus and especially iatrogenic hyperadrenocorticism, as we previously reported in our study comparing ICSs with prednisolone (Melamies et al., 2012).

The pharmacokinetic and therapeutic properties of budesonide (BUD), a potent anti-inflammatory corticosteroid, have been extensively investigated in man. The anti-asthmatic effect of inhaled BUD has been proposed to be due to local pulmonary action of the drug, not systemic activity (Toogood et al., 1990). BUD has a low oral bioavailability; approximately 90% of the swallowed drug is inactivated by first-pass hepatic metabolism (Donnelly and Seale, 2001). Unlike other currently available ICSs, BUD forms reversible intracellular fatty acid esters in the cells of the respiratory tract, which prolongs lung retention and explains the good efficacy of a single daily dose (Miller-Larsson et al., 1998; Selroos et al., 2004).

ICSs enter the systemic circulation by absorption from the respiratory epithelium or from the gastrointestinal (GI) tract after unintentional ingestion of the drug (Derendorf et al., 1998; Donnelly and Seale, 2001). Because we evaluated the systemic and pulmonary bioavailability of inhaled BUD, absorption of the drug via the GI tract was blocked with concomitant oral charcoal. Human studies have shown that oral charcoal does not have an effect on the elimination and distribution of BUD, and thus, when absorption of BUD from the GI tract has been prevented the drug found in systemic circulation is assumed to reflect solely absorption of the inhaled drug from the lungs (Thorsson and Edsbacker, 1998; Lähelma et al., 2005; Grekas et al., 2014).

To the authors’ knowledge, only one previous study has described the pharmacokinetic properties of intravenously (IV) administered BUD in dogs (Ryrfeldt et al., 1979). Since no published data about the pharmacokinetics of ICSs in dogs exist, the treatment dosages have been adapted from human studies, although drug absorption and metabolism may differ. The objective of our study was to investigate the pharmacokinetic properties of BUD administered IV, via inhalation or via inhalation with concomitant oral administration of charcoal, and to verify effectiveness of charcoal in preventing GI absorption of BUD in healthy Beagles.

Materials and methods

Dogs and verification of health

The study group consisted of eight clinically healthy laboratory Beagles (age 8-12 years; weight 10-22 kg; 4 males and 4 spayed females). Eight dogs were enrolled in the inhalation studies and 7 dogs in the IV and PO studies. One week before initiation of the trial, each dog’s health status was assessed with physical examination, serum biochemistry and haematology panels, arterial blood sample (ABL 800 FLEX, Radiometer A/S), faecal flotation and sedimentation tests and ventro-dorsal and latero-lateral thoracic radiographs. Additionally, bronchoscopy and bronchoalveolar
lavage were performed under general anaesthesia, as described in detail earlier (Melamies et al., 2011). Thoracic radiographs were viewed by two independent examiners.

Clinical examination was normal in all dogs. Serum biochemistry, haematology and arterial blood sample results were within reference ranges with minor exceptions. Faecal tests for parasites were negative in all dogs. Some mild age-related findings were detected in thoracic radiographs. Bronchoscopy revealed no abnormal changes, and results of laboratory analysis of bronchoalveolar lavage fluid were normal, except for mild lymphocytosis detected in four dogs (median 16.9% (range 6.0-22.9%); reference, 95% confidence interval 10.3-16.1%) (Rajamäki et al., 2002)

Study design
This was a prospective open-label study. All dogs participated in four experiments separated by three-week wash-out periods. Dogs received no preliminary training in breathing through a mask. Before each experiment food was withdrawn for 12 h and water was available *ad libitum.* BUD was administered intravenously (TREAT_IV) in the first study, via inhalation (TREAT_INH) in the second study, via inhalation in conjunction with activated charcoal PO (TREAT_INHC) in the third study and orally in conjunction with activated charcoal (TREAT_PO) in the fourth study. The study protocol was approved by the Committee of Experimental Animals of Western Finland (ESLH-2007-02827/Ym-23; date of approval 31 May 2007).

Treatments
In all studies, 5 mL venous blood samples for assessment of BUD concentration were drawn from the cephalic vein via an IV catheter (Optiva 20G, Smiths Medical) into K$_2$EDTA tubes. The catheter was used only for sampling at 10, 20, 30, 45, 60 and 90 min and 2, 4 and 6 h after BUD administration (time zero). After sampling, 5 mL of physiological saline was administered IV to replace the blood loss. Samples were centrifuged within 30 min and refrigerated at -70 °C until analysis.

In TREAT_IV, BUD powder (Budesonide micronised, AstraZeneca PLC) was dissolved in ethanol and physiological saline (0.9%) to give a final BUD concentration of 100 μg/mL and ethanol 30% (w/w). 20 μg/kg BUD (total volume 2.0–4.4 mL per dog) was injected through a second plastic IV catheter (Optiva 20G, Smiths Medical) placed in the cephalic vein not used for sampling. Medication was injected over 10 s, after which the catheter was immediately flushed with 5 mL of physiological saline and removed.

In TREAT_INH BUD (Pulmicort HFA, AstraZeneca) was administered at 1.0 mg (5 × 200 μg) per dog through a valved holding chamber (VHC) (AeroDawg, Trudell Medical International) attached to an anaesthetic mask (face mask with rubber diaphragm, Kruuse) (Fig. 1). The drug canister was first shaken carefully and the mask was then fitted to cover the nose and mouth of the dog so that breathing through the mouth was not possible. Medication was fired into the chamber, and the dog was allowed to take eight breaths. The mask was then removed, and the dog was allowed to breathe standard room air for 5 s before the manoeuvre was repeated five times in total.

TREAT_INHC used the same procedures as TREAT_INH, but 5 min before inhalation a suspension liquid charcoal (Carbo-mix, Leiras) was administered at 2.5 g/kg PO. Charcoal powder (50 g per bottle) was mixed with 250 mL of tap water according to the manufacturer’s instructions such that the total amount of suspension
per dog varied from 155 to 340 mL. The charcoal suspension was administered slowly via bottle feeding over 5 min.

**TREAT_PO** consisted of administration of activated charcoal as described in TREAT_INHC followed by PO administration of a BUD capsule (BUD powder weighed into gelatine capsules) at 10 mg per dog.

**HPLC tandem mass spectrometry**

Concentrations of unchanged BUD were determined from plasma samples using a method described in detail elsewhere (Berg et al., 2012). Briefly, after liquid-liquid extraction and solid-phase extraction the analytes were reconstructed in the mobile phase and liquid chromatography-tandem mass spectrometry with electrospray ionization was performed. Limit of detection (LOD) was defined as 0.05 ng/mL and limit of quantitation (LOQ) as 0.1 ng/mL.

**Pharmacokinetic analysis**

Pharmacokinetic parameters, area under the curve (AUC$_{0-\infty}$), elimination half-life ($T_{1/2}$), total clearance (CL) and volume of distribution ($V_d$) were calculated using a non-compartment model and Kinetica™ software (Thermo Electron Corp., Waltham). AUC was calculated using the trapezoidal method. $T_{1/2}$ was calculated by dividing $ln2$ by the rate constant of the elimination phase ($\beta$). The volume of distribution was calculated by the area method as $V_d = \text{Dose} / \text{AUC} \times (\beta)$, and the total plasma clearance as $\text{CL} = \text{Dose} / \text{AUC}$. Mean residence time (MRT) was calculated from the equation $\text{MRT} = \text{AUMC}_{0-\infty} / \text{AUC}_{0-\infty}$, where AUMC is the area under the first moment curve. Maximum concentration ($C_{\text{max}}$) and time to peak concentration ($T_{\text{max}}$) were determined directly from individual plasma curves.

Systemic bioavailability (F) of BUD after inhalation was calculated from: $F = (\text{AUC}_{0-\infty, \text{inhaled}} / \text{AUC}_{0-\infty, \text{iv}}) \times (\text{Dose \ iv} / \text{Dose \ inhaled})$. When the extrapolated AUC exceeded 20%, AUC$_{0-6 \text{h}}$ was used.

**Statistical analysis**

Statistical analyses were performed using commercially available software (GraphPad Prism for Windows, version 4, GraphPad Software). Normal distribution of the data was assessed using Kolmogrov-Smirnov’s test. Non-parametric variables ($C_{\text{max}}$ and $T_{\text{max}}$) were compared using the Wilcoxon signed-rank test and parametric variables were compared using a paired-samples $t$-test. The criterion for statistical significance was $P < 0.05$.

**Results**

Results are given as mean (SD). Calculated pharmacokinetic parameters for TREAT_IV were as follows: AUC$_{0-\infty}$ 14 (2.1) ng h/mL , $T_{1/2}$ 2.8 (0.8) h, CL 1.5 (0.25) L h/kg and $V_d$ 6.2 (2.8) L/kg.

Comparison of BUD inhalation (TREAT_INH) and BUD inhalation with concomitant charcoal (TREAT_INHC) revealed that $C_{\text{max}}$ and $T_{\text{max}}$ were similar following both treatments, but there was a difference in AUC$_{0-6 \text{h}}$ values ($P = 0.049$). Table 1 gives pharmacokinetic data for both inhalation groups. Median plasma BUD concentrations for both inhalation groups are illustrated in Fig. 2. In TREAT_PO BUD was not detected in plasma samples of three dogs, in the remaining four dogs the $C_{\text{max}}$ of BUD was 0.15 (0.15) ng/mL.
The systemic bioavailability for inhaled BUD was 2.1% (0.98); the pulmonary bioavailability of BUD when administered with oral charcoal was 1.2% (0.59).

### Discussion

We describe, for the first time, the pharmacokinetic characteristics of inhaled BUD in dogs. ICSs offer several therapeutic benefits over oral preparations; via inhalation the active drug reaches the bronchial and alveolar level of the lungs and exerts the desired therapeutic effect before entering systemic circulation. However, human studies have shown that a large proportion of ICS will not reach the lower respiratory tract, but will be deposited in the oropharynx and absorbed into the systemic circulation partly from the buccal mucosa and partly from the GI tract after being swallowed. Buccal absorption is assumed to have a minimal effect on the systemic availability of an ICS since the absorptive surface area available in the oropharynx is small and the mucosal exposure time is relatively short. Approximately 90% of BUD absorbed from the GI tract is inactivated during first-pass hepatic metabolism, so the systemic bioavailability of BUD is mainly determined by deposition and absorption from the lung (Lipworth, 1995; Donnelly and Seale, 2001).

The relatively high dose of BUD (1.0 mg) used in our inhalation studies was chosen to ensure measurable plasma concentrations of the drug, and because similar (Godfrey et al., 2002) or larger doses (1.6 mg twice daily) (Minto et al., 2000) have been used in human studies. The results of our study on use of inhaled BUD without charcoal (TREAT_INH) are in line with an earlier study of the pharmacokinetics of nebulised BUD in children which used a 1 mg dose of the drug (Agertoft et al., 1999). In our study the mean $T_{\text{max}}$ was 0.27 h; in children $T_{\text{max}}$ varied from 0.17 to 0.5 h. Mean $C_{\text{max}}$ was slightly lower in our dogs than in children (0.89 ng/mL and 1.12 ng/mL respectively); mean AUC was 1.1 ng h/mL in dogs and 2.0 ng h/mL in children. Systemic exposure to the drug (indexed by AUC) was lower for dogs than children; this suggests that in dogs the systemic activity of BUD treatment may be minor.

A study conducted in healthy humans showed that administration of an oral charcoal suspension before and after PO administration of a BUD capsule decreased GI absorption of BUD by about 96% (Lähelma et al., 2005). We showed that charcoal almost totally abolished GI absorption of an orally administered high dose (10mg; a tenfold increase on the inhaled dose) of BUD. In three dogs BUD was not detectable in plasma and in the remaining four dogs the mean $C_{\text{max}}$ was low (i.e. 0.15 ng/mL). In addition, when BUD inhalation was administered concomitantly with oral charcoal there was a 50% reduction in AUC, indicating a marked reduction in absorption of swallowed drug from the GI tract. There was no difference between the inhalation treatments (with and without oral charcoal) in $C_{\text{max}}$ and $T_{\text{max}}$ values. Interestingly, the dog with the highest maximum plasma BUD concentration was the same for both inhalation treatments; it is unclear whether this was due to an efficient breathing technique or individual differences in drug metabolism.

In our study the mean systemic bioavailability of inhaled BUD was 2.1%, and the mean pulmonary bioavailability, calculated on the basis that oral charcoal blocked GI absorption, was 1.2%. A study of nebulised BUD (1 mg dose) in children aged 3-6 years estimated the systemic bioavailability of BUD at 6.1% of the nominal dose (Agertoft et al., 1999). Our findings of the IV pharmacokinetics of BUD are comparable with and verify the previous results of Ryrfeldt et al. (1979). The total BUD clearance rate of 1.5 L h/kg observed in our IV study is very similar compared to the clearance value reported for children, 1.8 L h/kg (Agertoft et al., 1999).
combination of lower systemic bioavailability of BUD and a similar clearance rate in dogs relative to children suggests that similar or larger doses of inhaled BUD can be used safely in dogs. It has been established that BUD-ester conjugates are formed in the lung cells of humans (Tunek et al., 1997; van den Brink et al., 2008) and rats (Miller-Larsson et al., 1998), but not dogs. If a similar esterification process occurs in the lung cells of dogs, the depot of inactive drug could in part explain the moderately low pulmonary bioavailability of inhaled BUD detected in our study.

None of our dogs were trained to breathe through a mask before the experiments, unlike human studies, where patients are usually given the chance to get accustomed to the inhalation device. The delivery of ICSs is influenced by several patient-level factors, some also applicable to our dogs. Dogs cannot be instructed to take a deep breath when inhaled medication is given; this lack of co-ordination as well as an unfavourable breathing pattern, i.e. low tidal volume, reduces delivery of ICSs to the lower airways. A period of familiarisation with the inhalation device might have reduced the inter-individual variation in the plasma BUD concentrations in our dogs. It should be noted, however, that Minto et al. (2000) reported considerable inter-individual variability in lung absorption in healthy human volunteers after administration of inhaled BUD (1600 μg twice daily).

Dogs inhaled BUD via the nasal route, since oral inhalation is not feasible with currently available devices. Schulman et al. (2004) demonstrated the viability of the nasal administration route in healthy cats, showing that nebulised radio-labelled aerosol reached the lower airways and was distributed throughout the lung fields in all cats. However, when nasal breathing was compared with oral breathing in healthy adults the mean lung deposition of radiolabelled aerosol decreased from 75% to 38% (Everard et al., 1993).

In our study, BUD inhalation procedure was well-tolerated by the dogs. BUD was dosed in the only suitable rapid method for dogs, from a commercial pMDI, which does not require controlled forceful inspiration, unlike the other available formulation, a dry powder inhaler. We used a commercially available VHC that is designed for dogs and probably enhances delivery of ICSs to lung (Dempsey et al., 1999). We did not prime or wash the VHC with detergent to reduce electrostatic charge; it is therefore likely that there was attachment of drug molecules to the chamber walls (Barry and O'Callaghan, 1995). We used an anaesthetic mask because it fitted tightly around the muzzle of the dog and prevented opening of the mouth; however this choice also meant that there was a large dead space volume and may have resulted in retention of BUD within the mask.

Our results were achieved in a small sample of healthy Beagles. In humans BUD is metabolised mainly by cytochrome p-450 (CYP) enzymes and there are known to be genetic differences between the CYP enzymes of Beagles and other dog breeds (Trepanier, 2006; Court, 2013) so these results may not generalise completely to other dog breeds. In addition, the dogs participating in our study were healthy, and it is likely that respiratory disease alters the amount of inhaled drug reaching the lower airways. However, Dalby et al. (1998) have shown that the relative systemic availabilities of BUD between healthy humans and ones afflicted with chronic obstructive pulmonary disease (COPD) were similar. Our study population had a fairly narrow weight range (10-22 kg), which based on previous studies should not have any effect on the pharmacological parameters measured. Barry and O’Callaghan (1999) showed that in vitro there is a linear relationship between tidal volume (which increases with body weight) and the output of BUD from a VHC comparable to that
used in our studies; the drug dose should therefore be kept constant to ensure that all patients receive the same dose per kilogram.

The aim of the current study was not to determine the therapeutic dose of inhaled BUD, which is affected by the severity of the inflammatory process (Gulliver et al., 2007). Further clinical trials in dogs with respiratory disease will be needed to determine the clinically effective dose. Comparative analysis of the various inhalation devices should also be carried out.

**Conclusion**

We present pharmacokinetic variables of intravenous and inhaled BUD in healthy dogs. Our study demonstrates that a VHC and mask is a feasible way to administer inhaled BUD and that concomitant administration of liquid charcoal suspension is efficient in preventing GI absorption of BUD. We found that inhaled BUD has moderately low systemic and pulmonary bioavailability, and thus, further clinical trials evaluating the therapeutic use and effective doses of ICSs in dogs are warranted.

**Conflict of interest statement**

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

**Acknowledgements**

This work was conducted at the Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, and was supported by grants from the Finnish Veterinary Foundation, the Helvi Knuuttila Foundation and the Orion-Farmos Research Foundation.

Preliminary results of the current study were presented as a Poster at the 19th annual ECVIM-CA Congress, Porto, 10th - 12th September 2009.

**References**


Barry, P.W., O'Callaghan, C., 1999. The output of budesonide from spacer devices assessed under simulated breathing conditions. The Journal of Allergy and Clinical Immunology 104, 1205-1210.


Table 1
Pharmacokinetic data (mean (SD)) calculated from plasma concentration time curves for budesonide (BUD) in healthy dogs ($n = 8$) after inhalation of BUD (1 mg) (TREAT_INH) and after inhalation of BUD (1 mg) with concomitant oral charcoal (2.5 g/kg) (TREAT_INHC).

<table>
<thead>
<tr>
<th></th>
<th>TREAT_INH</th>
<th>TREAT_INHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{0-6h}$ (ng h/mL)</td>
<td>1.1 (0.59)</td>
<td>0.55 (0.23)</td>
</tr>
<tr>
<td>C$_{max}$ (ng/mL)</td>
<td>0.89 (0.59)</td>
<td>0.60 (0.19)</td>
</tr>
<tr>
<td>T$_{max}$ (h)</td>
<td>0.27 (0.12)</td>
<td>0.22 (0.08)</td>
</tr>
<tr>
<td>F (%)</td>
<td>2.1 (0.98)</td>
<td>1.2 (0.59)</td>
</tr>
</tbody>
</table>

AUC, area under the time-drug concentration curve; C$_{max}$, maximum plasma drug concentration; T$_{max}$, time when C$_{max}$ is measured; F, fraction of drug absorbed.
Figures

Fig.1. Inhalation device was constructed of a pressurized metered dose inhaler (pMDI), a valved holding chamber (AeroDawg, Trudell Medical International) and an anaesthetic mask (face mask with rubber diaphragm, Kruuse).

Fig.2. Plasma concentrations of budesonide (BUD) in healthy Beagles \( n = 8 \) over a 90 min-period following inhalation of 1.0 mg BUD (open boxes) or inhalation of 1.0 mg BUD plus concomitant oral charcoal 2.5 g/kg (striped boxes). Lines within the boxes indicate medians; the lower and upper boundaries of the boxes indicate the values of the 25th and 75th percentiles, respectively. Whiskers represent the smallest and largest values.
Liquid chromatography tandem mass spectrometry determination of total budesonide levels in dog plasma after inhalation exposure

Seija Berg · Marika Melamies · Minna Rajamäki · Outi Vainio · Kimmo Peltonen

Abstract A sensitive and selective method to quantify budesonide in dog plasma samples was developed and fully validated. Liquid–liquid extraction was followed by solid-phase extraction and liquid chromatography–tandem mass spectrometry with electrospray ionization. After reconstitution of the analytes in the mobile phase, samples were analysed by reversed-phase liquid chromatography with isocratic elution. d8-Budesonide was used as an internal standard, and characteristic transitions of d8-budesonide and budesonide were used for quantification. The method was validated with respect to selectivity, specificity, linearity, recovery, repeatability, reproducibility and limits of detection and quantification. The validated method was successfully applied to monitor the plasma levels of budesonide in dogs exposed to clinical doses of inhaled and intravenous drug.

Keywords Budesonide · Sample preparation · LC-MS/MS · Plasma · Inhalation

Introduction

Budesonide (16,17-butylidenebis(oxy)-11,21-dihydroxyprogna-1,4-diene-3,20-dione) (Fig. 1) is an anti-inflammatory synthetic glucocorticoid used in the treatment of human asthma by inhalation and in the therapy of inflammatory bowel disease with oral administration [1].

Budesonide is rapidly and almost completely absorbed following oral administration. However, the fraction of inhaled budesonide deposited in the lungs ranges from a mere 10% up to 30% in humans depending on the inhalation device and breathing technique. Since the dose delivered to the lungs is low, the amount of drug available for systemic absorption is small and plasma concentrations are very low. Thus, a robust, sensitive and selective method is required for the determination of budesonide in plasma after inhalation delivery [2]. Budesonide has been detected in human plasma and urine [3, 4] and it has been assayed in racehorse urine by a multi-drug high throughput screening method [5]. To the best of our knowledge, budesonide has not been determined in dog plasma samples with a method which takes a full advantage of deuterated internal standard in high-pressure liquid chromatography (HPLC)/MS/MS analysis.

Different approaches have been taken to isolate budesonide for analysis in specimens from humans and other mammalian species. In a very simple procedure, human plasma samples were only acidified to precipitate plasma proteins before evaporation and reconstituting into a chromatographic solvent [6]. For racehorse plasma samples, methyl tert-butyl ether was applied for extraction followed by evaporation and reconstituting into 30% methanol [7]. In addition, a traditional diethyl ether extraction was recently applied to human plasma samples [8]. In human urine samples, ethyl acetate extraction has been applied to alkalized urine samples to extract budesonide after inhalation exposure [4]. A reversed-phase solid-phase extraction was utilized for in a screen for corticosteroids in horse urine samples [5].
The chromatographic analysis of budesonide from various matrixes mainly relies on liquid chromatography, regardless of whether the separate epimers of budesonide or the total amount are the target of analysis [4, 9, 10]. Although column choice and mobile-phase composition vary considerably, the determinations have always been made with reversed-phase columns with C8 or C18 stationary phases [4, 5, 9, 10]. With C8 phases, isocratic or linear gradient systems have been used with mobile phases consisting of low molarity (typically millimolar range), buffer at acidic pH and methanol or acetonitrile as organic modifiers. With the C18 phases, isocratic elution modes have been used with mobile phases consisting of low molarity buffer adjusted to acidic pH and acetonitrile as an organic modifier.

In HPLC applications, the typical detection method has been ultraviolet detection or mass spectrometry. With mass spectrometry, atmospheric pressure chemical ionization, electrospray ionization (ESI) and recently atmospheric pressure photoionization have been employed [4, 8, 11]. Multiple reaction monitoring (MRM) of the product ions has been used in tandem mass spectrometry and full scan mode with ion trap instrumentation [4, 11]. The ionization characteristics of budesonide in LC-MS/ESI conditions have been investigated to aid in the identification of the breakdown products [12].

This communication is first to describe a method which includes the use of deuterated internal standard in the analysis of budesonide in dog plasma. The method combines liquid-liquid and solid-phase extraction in sample handling. A subsequent instrumental analysis of budesonide at low levels was demonstrated by using a HPLC/MS/MS. The method was fully validated and takes full advantage of the use of deuterated budesonide as an internal standard. The recoveries were typically slightly concentration dependent with a low coefficient of variation irrespective of the extensive purification. The limit of detection (LOD) of budesonide, determined at the signal-to-noise ratio of 3:1, was 0.05 ng/ml dog plasma. With the corresponding limit of quantification (LOQ) being 0.1 ng/ml.

Materials and methods

The Chemistry and Toxicology Research Unit is an accredited laboratory according to ISO 17025 and has an accreditation number T014. The laboratory has also Good Laboratory Practice (GLP) status and is included in the national GLP compliance program.

Reagents and chemicals

Budesonide (≥99%) was purchased from Sigma (St. Louis, MO, USA) and the deuterated internal standard d8-budesonide (the isotopic purity of >99.9% determined with MS spectra peak ratio) was a gift from Orion Corporation (Orion Pharma, Espoo, Finland). Stock standard solution (1 mg/ml), working standard solutions (0.1 and 0.01 μg/ml), stock internal standard solution (1 mg/ml) and working internal standard solution (0.1 μg/ml), were all prepared in methanol. Typically, six calibration standards were prepared in the range of 0.75–50 ng/ml in the mobile phase. The concentration of the internal standard in calibration standards was 25 ng/ml. Methanol, acetonitrile and formic acid were HPLC grade, and ammonium formate was analytical grade (J.T. Baker, Deventer, The Netherlands). Water was Milli-Q water purified with a Millipore Milli-Q Plus System (Millipore, Espoo, Finland). Solid-phase extraction columns (C18, 500 mg, 6 ml) were from J.T. Baker (Deventer, The Netherlands).

Blood samples

Blood samples for method development and validation were collected from adult healthy laboratory beagles during a study of pharmacokinetics of budesonide after inhalation exposure or intravenous administration. The study protocol was approved by the National Animal Experiment Board. Blood samples were collected from the cephalic vein into EDTA tubes (volume, 5 ml) through a plastic iv catheter 10, 20, 30, 45, 60, 90, 120, 240 and 360 min after medication was delivered. Plasma was separated within 30 min by centrifugation (3,500 rpm, 10 min; Multifuge, Heraeus), and samples were stored at −70 °C until analysis.

Sample preparation

Internal standard (50 μl, d8-budesonide, 0.1 μg/ml) and formic acid (4% in water, 100 μl) were added to plasma samples (1 ml) to acidify the plasma. After the samples had been carefully mixed with a shaker, ethanol (99%, 1 ml) was added. The samples were then incubated 15 min at 4 °C, and the mixture was centrifuged (3,500 rpm, 15 min) to remove precipitated protein. The supernatant was separated, diluted with water (4 ml) and subjected to solid-phase
extraction. Before use, the cartridges were conditioned with methanol (5 ml) and then water (5 ml). Cartridges were not allowed to dry between the conditioning and sample introduction. Sample solution was introduced into the column, and the column was flushed with methanol/water solution (20% methanol in water, 3 ml). Finally, the analytes (budesonide epimers) were eluted from the column with a methanol/water solution (80% methanol in water, 3 ml). The effluent was collected and evaporated to dryness using a nitrogen flow with gentle heating at 50 °C. The extract was suspended into 200 µl of the mobile phase (ammonium formate, 2 mM, pH 3.4 and methanol in ratio 40:60) used in liquid chromatography–tandem mass spectrometry (LC-MS/MS).

LC-MS/MS analysis

The LC-MS/MS system consisted of a Waters Alliance 2695 Separation Module (Waters, Milford, MA, USA) and a MicroMass Quattro Micro tandem mass spectrometer (Micromass UK Ltd., Altrincham, Cheshire, UK). The instrument was operated in the positive ion electrospray mode with the following parameters: capillary voltage 3.50 kV, source temperature 120 °C, desolvation temperature 300 °C, N₂ cone gas flow 15 L/h, N₂ solvation gas flow 700 L/h. Argon was used as the collision gas. Ion transitions were investigated by MRM. Transitions and collision energies for budesonide were 431>323 and 14 eV and 431>413 and 12 eV, respectively. Transition 439>421 and collision energy 12 eV were used for the internal standard. The LC separation of budesonide was carried out on a Symmetry C18 column (3.5 µm, 2.1×150 mm; Waters, Milford, MA, USA) with mobile phase of ammonium formate (2 mM, pH 3.4) and methanol in a 40:60 ratio. The flow rate of the mobile phase was 0.2 ml/min and the injection volume was 10 µl. Quantification was based on the internal standard method. The area ratios of the product ion of budesonide (m/z 323) to the product ion of the internal standard (m/z 421) were plotted against concentration. Six standards for calibration were prepared in the concentration range of 0.75–50 ng/ml in the mobile phase.

Method validation

The following validation parameters were determined: selectivity, specificity, linearity, recovery, repeatability, reproducibility and limits of detection and quantification. Selectivity of the analytical method was tested by comparing (two-sided t test, 95% confidence) the slopes of the calibration curves being recorded with and without matrix at six different concentrations in the range of 0.75–50 ng/ml. Calibration lines were constructed by weighted (1/x²) linear regression. Specificity was evaluated by analysing ten blank plasma samples for the presence of matrix interference. The linearity of each point of the calibration curves was examined with the method of Trijp and Roos [13]. The recovery, repeatability and within-laboratory reproducibility of the method were determined by analysing blank plasma samples fortified at three concentration levels (0.2, 0.3 and 5.0 ng/ml). Fortified plasma samples were used for validation instead of incurred plasma samples because of the limited plasma volume.

Dog plasma samples

Inhalation study

Budesonide (200 µg/dose, Pulmicort HFA; Astra-Zeneca AB, Södertälje, Sweden) was administered by inhalation at a dose of 1.0 g (5×200 µg) per dog through an aerosol chamber specially developed for dogs (AeroDawg, Trudell Medical International, Ontario, Canada). An anesthetic mask covered the nose and mouth of the dog so that breathing through the mouth was impossible. Before each puff, the drug canister was shaken carefully according to the manufacturer’s instructions. The medication was sprayed into the inhalation chamber and the dog was allowed to take eight breaths through the nose. The mask was then removed and the dog was allowed to breathe normal room air for 5 s before the next puff was administered.

Intravenous study

Budesonide powder (Budesonide micronized, Astra-Zeneca PLC, London, UK) was dissolved in ethanol and physiological saline (0.9%) so that the final concentration of budesonide was 100 µg/ml and ethanol 30% (w/w). Each dog was injected with 20 µg/kg of budesonide (2.0–4.4 ml) during 10 s through a plastic intravenous catheter placed in the cephalic vein, and the catheter was immediately flushed with 5 ml of physiological saline.

Results

Method validation

The selectivity of the analytical method was tested by comparing (two-sided t test, 95% confidence) the slopes of the calibration curves for standards with and without matrix. The parameters of the curves are summarized in Table 1. The curves were constructed by using the analyte peak areas of fortified plasma extracts and the analyte peak areas of fortified solvent. Since statistical evaluation revealed no matrix effect, standards without matrix were
used for calibration. Each point of the calibration line was tested for linearity. A tolerance of 100±10 was accepted for good linearity. The method was considered as being linear over the studied concentration range.

The specificity of the method was tested by analysing blank dog plasma samples. No interference signals were detected in the region of the retention times for the analyte. An MRM chromatogram of a blank dog plasma sample with ISTD is shown in Fig. 2a, a spiked dog plasma sample in Fig. 2b, and a dog plasma sample collected 10 min after the dog had been allowed to inhale budesonide (Pulmicort. 5×200 μg) Fig. 2c. The retention times of extracted budesonide and the internal standard were 3.66 and 3.62 min, respectively (Fig. 2 a–c).

The average recovery of budesonide varied between 86% and 99%, and the relative coefficients of variation (CV %) for repeatability and reproducibility (SD_{r,b}) were 6–15% and 11–17%, respectively. Recovery, repeatability, and within-laboratory reproducibility data are summarized in Table 2. The absolute recovery was determined by analysing blank plasma samples fortified at three concentrations (0.2, 0.3 and 5.0 ng/ml, n=7). The average absolute recovery of budesonide varied from 64 to 89% (CV% from 7% to 15%). The LOD of budesonide was estimated based on the analyte and background response level of the lowest spike concentration. Using this information the LOD concentration was estimated based on signal-to-noise calculation of 3:1. The LOQ was defined to be 2×LOD. LOD was defined as 0.05 ng/ml and the LOQ was 0.1 ng/ml (Fig. 3).

Stability of budesonide or d8-budesonide in stock solutions and spiked dog plasma samples were not systematically explored. However, no degradation or other chemical instability was observed as decreased signal intensity. This is in line with recently published reports in which stability of budesonide was studied in human plasma samples [8, 10].

**LC-MS/MS analysis**

The protonated molecule [M+H]^+ of budesonide (m/z 431) was fragmented in the collision cell to the product ions m/z 413 (M-18) and m/z 323 (M-108). The product ion m/z 413 originated from the protonated molecular ion as a result of cleavage of water. The product ion 323 was used for the quantification. Product ion m/z 421 of the internal standard was used for quantification. The fragmentation of budesonide in the applied instrumental settings is in line with the published literature [4, 12].

Control dog plasma samples and blank solvent samples were analysed between the consecutive sequences (about six samples in a sequence) demonstrating no carry over or other interfering signals in chromatograms (Fig. 2a). The ion suppression/matrix effect was not specifically studied because of the use of the deuterated analogue of the analyte.

**Discussion**

The liquid-liquid extraction combined with a subsequent solid-phase extraction was developed, validated and tested with authentic dog plasma samples. The product—an interference-free extract of dog plasma, was compliant for instrumental analysis. The leftover extracts were suitable to be archived in a freezer for possible future needs. Even though the sample handling includes two separate tasks, namely liquid–liquid extraction and solid-phase extraction, the method was not felt to be too laborious.

The recoveries from human plasma samples at low levels of budesonide, i.e. levels lower than 1 ng/ml plasma have had a tendency to show better recoveries at high concentrations. In a multianalyte method, the recovery varied from 74% to 85% [6] and in single analyte methods, the recoveries varied from 77% to 98% [6, 8, 14]. One could argue that extensive sample handling could be a source of inaccuracy and/or poor recovery. The recoveries we report, i.e. from 86% to 97% are well in line with the reported values even though more extensive sample purification was applied before the analysis.

The nonplanar 1,4-pregnadien-3-one corticosteroids have been successfully analysed/trapped to C18 reversed-phase column packing materials [3, 7, 8, 12]. In the case of budesonide, the chemical structure favours retention in the C18 phase. In particular, the cyclic acetal group of budesonide increases the retention in nonpolar-phase materials. This can be clearly seen if the retention times

**Fig. 2** MRM chromatograms of (a) typical control dog plasma sample spiked with budesonide-d8 (5 ng/ml plasma), (b) dog plasma sample spiked with 0.3 ng/ml plasma of budesonide and budesonide-d8 (5 ng/ml plasma) and (e) dog plasma sample taken after inhalation administration of budesonide (2 ng/ml plasma)
LC-MS/MS determination of total budesonide levels in dog plasma
in relation to chemical structure of prednisolone and budesonide are compared. Additionally, the RP-HPLC retention time for corticosteroids has been reported to be proportional to the length of the cyclic acetal side chain (Fig. 1) [15, 16].

This is the first method designed and developed to assess budesonide in dog plasma which takes full advantage of deuterated budesonide as an internal standard. A recently developed method for human plasma samples based on photoionization in MS/MS demonstrated a slightly lower LOQ than achieved in this report [8]. On the other hand, this level of sensitivity is far below than necessary to assess the detection of budesonide in dog in clinical settings. If needed, additional sensitivity may be gained by applying ultra-high-performance liquid chromatography (UPLC) combined with more modern and sensitive MS/MS instrumentation. Increased sensitivity with more modern instrumentation can be proposed because it is evident that a low background noise/matrix effect was achieved with good sample handling. The chromatographic analysis time was short, less than five minutes, even though a conventional HPLC with traditional column diameter (2.1 mm) was applied. Furthermore, the analysis time could be shortened even more if UPLC were to be applied.

The method was fully validated. The sample workout demonstrated excellent recoveries, even at low budesonide levels. In addition, the repeatability and reproducibility tested at three budesonide levels displayed good results. We did not test the stability of budesonide or d8-budesonide but the indirect evidence seen as the constant signal intensity of stock standards and spiked dog plasma samples warrants the conclusion that budesonide is a stable compound in dog plasma if stored in a freezer and samples are handled out at normal room temperature. This conclusion is in line with the observation that budesonide is stable in human plasma samples [8, 10].

Budesonide has been analysed by using both positive and negative ion modes in electrospray from human and equine plasma samples [6–8, 12]. The majority of the reported methods have used the positive ion mode, however in the analysis of budesonide and its major metabolites, the negative ion mode was concluded as being advantageous [6]. The reason for the use of negative ion mode was that in positive ion mode, the most abundant signals were evenly split to M+H and sodium (M+Na) and potassium (M+K) adducts. Naturally, this decreased the sensitivity of the assay. The problem was solved by using the negative ion mode and assaying the fragments originating from the acetate adducts. The problem of extensive formation of sodium and potassium adducts is at odds with our experience in our assay. Furthermore, our data are in line with other reports in which budesonide has been analysed from human plasma samples [8, 12].

The method provides excellent performance in sample handling and instrumental analysis for dog plasma samples for budesonide. We have successfully applied the method in pharmacokinetic studies of budesonide in dogs after drug inhalation exposed to budesonide. A full presentation of the pharmacokinetics of budesonide in dog, as monitored with the use of the analytical method presented here, will be provided elsewhere. To demonstrate that the necessary sensitivity had been achieved, we analysed samples originating from randomly selected dogs treated with budesonide aerosols. In general, the budesonide in dog plasma was detected close to the LOQ within 2 h, the MRM chromatogram (transitions, m/z 431>413 and 431>323) was free of interfering fragments, and thus it could be successfully used for budesonide quantification from dog plasma samples.

### Conclusions

A method was developed which allows low-volume dog plasma samples to be handled by liquid–liquid extraction combined with reversed-phase solid-phase extraction. The method made full advantage of the deuterated d8-
budesonide internal standard. Instrumental analysis was carried out by using HPLC/MS/MS operated in a positive ion electrospray mode. Overall the general method characteristics were excellent. The LOD was 0.05 ng/ml, and the LOQ was 0.1 ng/ml. The applicability of the method for dog plasma samples was demonstrated by its ability to detect budesonide in randomly selected samples.

Acknowledgement  Ms. Merja Orpana is thanked for her excellent technical skills in MS analysis.

References

Endocrine effects of inhaled budesonide compared with inhaled fluticasone propionate and oral prednisolone in healthy Beagle dogs

Marika Melamies\textsuperscript{a,}\textsuperscript{⇑}, Outi Vainio\textsuperscript{a}, Thomas Spillmann\textsuperscript{a}, Jouni Junnila\textsuperscript{b}, Minna M. Rajamäki\textsuperscript{a}

\textsuperscript{a}Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, Viikintie 49, 00014 University of Helsinki, Finland
\textsuperscript{b}4Pharma, Metsänneidonkuja 6, 02130 Espoo, Finland

**A B S T R A C T**

Orally administered corticosteroids are commonly used to treat chronic respiratory disease, but adverse effects suggest that the inhalation route may be safer. To compare the systemic effects of inhaled and oral corticosteroids, a prospective, randomised, placebo-controlled cross-over study was conducted. Six healthy neutered female Beagle dogs were randomly allocated to four treatment groups: (1) budesonide inhalation (200 l g twice daily); (2) fluticasone inhalation (250 l g twice daily); (3) oral prednisolone (1 mg/kg once daily); and (4) placebo inhalation (room air twice daily). Each treatment and wash-out period lasted 4 weeks. The endocrine status of each dog was assessed on days 0, 28 and 35 using the adrenocorticotropic hormone (ACTH) stimulation test. The effects of treatments were assessed using a linear mixed effects model.

After the 4 week treatment period, a significant decrease was observed in the basal serum cortisol level of the prednisolone group ($P < 0.03$), and a decrease was also seen in the ACTH-stimulated peak cortisol levels of both the prednisolone and fluticasone groups ($P < 0.001$), compared with the budesonide group in which no suppression was detected. The results showed that cortisol production in dogs was strongly suppressed by oral prednisolone and by inhaled fluticasone.

**Introduction**

Chronic inflammatory airway diseases, most notably chronic bronchitis and eosinophilic bronchopneumopathy, are common causes of coughing in dogs. Cornerstone therapies have relied on the oral administration of corticosteroids, although this is frequently associated with undesirable adverse effects. Inhaled corticosteroids (ICSs) have replaced oral products as the first-line anti-inflammatory therapy for asthma in humans, and species-specific modification of inhalation chambers and masks has led to ICSs being more commonly used in companion animals. However, to our knowledge, there is only a single report describing the therapeutic use of fluticasone propionate (FP) and beclomethasone dipropionate in canine chronic inflammatory airway disease (Bexfield et al., 2006). No studies comparing the clinical efficacy of different ICSs in dogs have been published.

The most common systemic adverse effects related to oral corticosteroid treatment in dogs are polyuria, polyphagia, weight gain, hair loss and suppression of the hypothalamic–pituitary–adrenal (HPA) axis, leading to decreased endogenous cortisol production. ICSs are also capable of causing dose-related systemic adverse effects, but to a lesser extent than orally administered preparations (Ahmet et al., 2011). ICSs are delivered via inhaled air to the target organ and they reach the systemic circulation either by direct absorption through the respiratory epithelium or via the gastrointestinal tract after unintentional ingestion (Derendorf et al., 1998; Donnelly and Seale, 2001). The swallowed proportion of the drug is, however, almost completely inactivated by the effective first-pass hepatic metabolism (99% for FP and 90% for budesonide (BUD)) (Ryrfeldt et al., 1979; Harding, 1990; Lipworth and Jackson, 1999).

The systemic adverse effects of inhaled FP and oral prednisone have already been studied in healthy dogs, with less HPA axis suppression when using FP compared to prednisone (Cohn et al., 2008). There do not appear to be any reports describing the systemic adverse effects of inhaled BUD, whereas significant suppression of the HPA axis has been detected with oral BUD at a dose of 3 mg/m$^2$ for 30 days in dogs with inflammatory bowel disease (Tumulty et al., 2004). In humans, the long-term use of FP has been found to cause dose-related adrenal suppression more often than BUD at equal dosages (Clark et al., 1996; Clark and Lipworth, 1997; Lipworth, 1999; Kaliner, 2006). The objective of the current study was to compare the endocrine effects of inhaled BUD, inhaled FP and oral prednisolone in healthy Beagle dogs at clinically relevant doses.
Materials and methods

Dogs and verification of health

Six healthy neutered female laboratory Beagle dogs were enrolled in the study. At the start of the study, the dogs were 2.5 years old and their mean bodyweight (BW) was 17 kg (range: 11–19 kg). The health status of each dog was assessed by thorough physical examination, haematology and serum biochemistry panels (alanine aminotransferase, albumin, alkaline phosphatase (ALP), blood urea nitrogen, calcium, creatinine, glucose, potassium, protein, sodium and total bilirubin) as well as by urinalysis (bacterial culture, protein-to-creatinine ratio, reagent strip (Multistix 10 SG, Siemens Healthcare Diagnostics), sediment and specific gravity), latero-lateral and ventro-dorsal thoracic radiographs were taken. Additionally, faecal flotation and sedimentation tests were performed.

The study protocol was approved by the Committee of Experimental Animals of Western Finland.

Study design

This study was an open-label, prospective, randomised, placebo-controlled, cross-over design. All dogs received all of the following four treatment protocols in random order: (1) BUD inhalation (Pulmicort HFA, AstraZeneca) 200 µg twice daily; (2) FP inhalation (Fluticort Eviolator, GlaxoSmithKline) 250 µg twice daily; (3) prednisolone orally (Prednisolon, Leiras) 1 mg/kg once daily; (4) placebo inhalation twice daily. Inhaled medications were administered through an aerosol chamber (AeroDawg, Trudell Medical International) attached to an anaesthetic mask (face mask with rubber diaphragm, Kruse). Before each medication, the metered-dose inhaler (MDI) was shaken and attached tightly to the aerosol chamber. An anaesthetic mask was then fitted to cover the dog’s nose and mouth, the medication was sprayed into the chamber and eight breaths were counted. The placebo group breathed room air through the chamber and mask. All dogs had their own masks, aerosol chambers and MDIs during the 4-week study period, and the inhalation devices were carefully washed after each treatment period. The last medication was given 24 h before blood sampling. Blood samples (serum and K3 EDTA samples, 6 mL each) were taken at the same time of the day (between 09:00 and 11:00 h) in the same environment where the dogs were housed. To avoid the possible effect of circadian rhythm on cortisol excretion (Derendorf, 1997), as well as stress-induced elevation in cortisol levels, we standardised the environmental factors and sample-taking protocol as far as possible, by constant sampling time and place and the same two persons collecting the samples.

Each treatment and each washout period lasted 4 weeks. The health status was assessed on days 0, 14, 28 and 35 by physical examination, haematology, serum biochemistry and urinalysis, and additionally by ACTH stimulation test on days 0, 28 and 35. All blood samples were taken from the cephalic vein and urine samples by cystocentesis using ultrasonographic guidance. All haematological, serum biochemical and urinalysis samples were stored at room temperature and analysed within 2 h.

ACTH stimulation test

The adrenocorticotropic hormone (ACTH) stimulation test is the most reliable way to diagnose adrenal dysfunction in dogs (Herrtage, 2005; Lathan et al., 2008), and in our study the adrenocorticotropic status was assessed with the low-dose ACTH stimulation test (Kerli et al., 1999; Frank et al., 2004) using a synthetic analogue of ACTH (Synacthen, Alliance; 5 µg/kg IV) administered via a plastic catheter (Optiva 20 G, Smiths Medical). The catheter was immediately flushed with 5 mL of physiological saline and a second blood sample was taken 60 min later. Serum samples for cortisol assays were frozen at −80 °C until analysis.

Cortisol assays were performed by a separate technician who was blinded to the treatments. Samples were analysed in duplicate with a commercial radioimmunoassay (RIA) kit validated for canine use (Coat-A-Count Cortisol, Siemens Healthcare Diagnostics). The intra-assay coefficient of variation (CV) for serum cortisol determination was 5.6% at the mean concentration level of 42.6 nmol/L and 6.4% at the level of 280.2 nmol/L (both CVs were calculated from 10 duplicate determinations).

The samples were analysed in one series, and no inter-assay CVs were determined. The detection limit of the serum cortisol assay was 6.9 nmol/L.

Statistical analysis

The effect of treatments on the continuous response variables was assessed using a linear mixed effects model, where treatment, period, day of period, and interaction terms between treatment and period, and treatment and day of period were used as fixed effects and dog as a random effect. With urine protein-to-creatinine ratio and urine-specific gravity, the dog-related variance component was very close to zero. To get the model to converge, linear analysis was performed, excluding the very small random effect of individual dogs.

Results

Dogs and verification of health

All of the dogs showed normal physical examination results. No significant changes were detected in thoracic radiographs, and all faecal tests to detect parasites were negative. Results on days 0, 14, 28 and 35 of haematological analyses, including differential cell counts, were within the reference range in all dogs with minor exceptions. Serum biochemistry panels and urinalysis results were within the reference range, except changes detected in serum ALP and protein values and urine protein-to-creatinine ratio in the prednisolone group on days 14 and 28 (Table 1).

ACTH stimulation test

Test results are presented in Table 2. On day 0, no significant differences were seen in baseline or ACTH-stimulated peak cortisol levels when treatment groups were compared with the placebo group. Furthermore, no significant changes were noted in the placebo or BUD groups in basal or peak cortisol levels after 4 weeks of treatment relative to day 0 (P > 0.05 in each case). However, on day 28, prednisolone had significantly suppressed the basal cortisol level compared with the placebo (P = 0.016) or BUD (P = 0.029) group (Fig. 1). Additionally, the peak cortisol level after the ACTH stimulation test was significantly lower in the prednisolone and FP groups than in the placebo or BUD group (Fig. 2; P < 0.001 in each case).

One week after discontinuation of treatment (day 35) in the FP group, significant elevation of the basal cortisol level was found compared with day 28 (P < 0.001). The level was also higher than that in the placebo group (Fig. 1; P = 0.035), but no significant change was observed compared with the basal cortisol level in the FP group on day 0 (P = 0.099). On day 35, no significant differences between the treatment groups were detected in ACTH-stimulated peak cortisol concentrations (Fig. 2).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Serum biochemistry and urinalysis results (mean ± SD) in the four treatment groups (n = 6 per group).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Day 0</td>
</tr>
<tr>
<td>Placebo</td>
<td>ALP (U/L)</td>
</tr>
<tr>
<td>Prot (g/L)</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>U/pr</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>ALP (U/L)</td>
</tr>
<tr>
<td>Prot (g/L)</td>
<td>58 ± 2.1</td>
</tr>
<tr>
<td>U/pr</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>FP</td>
<td>ALP (U/L)</td>
</tr>
<tr>
<td>Prot (g/L)</td>
<td>58 ± 2.3</td>
</tr>
<tr>
<td>U/pr</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>BUD</td>
<td>ALP (U/L)</td>
</tr>
<tr>
<td>Prot (g/L)</td>
<td>56 ± 3.5</td>
</tr>
<tr>
<td>U/pr</td>
<td>0.09 ± 0.03</td>
</tr>
</tbody>
</table>

FP, fluticasone propionate; BUD, budesonide; ALP, alkaline phosphatase (reference range, 33–215 U/L); Prot, protein (reference range, 58–77 g/L); U/pr, urine protein-to-creatinine ratio (reference range, <0.5).

* Significant difference compared to placebo, P < 0.001.
Bexfield et al. (2006) described the use of FP and beclomethasone in small animals, particularly in the treatment of chronic respiratory diseases as inhaled formulas, although few studies have been undertaken to investigate efficacy and adverse effects of these compounds in small animals. Both BUD and FP are potent corticosteroids used for the treatment of chronic respiratory diseases in dogs and humans (Ryrfeldt et al., 1979; Donnelly and Seale, 1991). The dosages of BUD and also FP used in our study were therefore determined according to the existing treatment recommendations for asthmatic children and were estimated to be clinically relevant (Campbell et al., 1998; Herjavecz et al., 1999; Busse et al., 2001). The dosages of BUD and also FP used in our study were therefore determined according to the existing treatment recommendations for asthmatic children and were estimated to be clinically relevant (Campbell et al., 1998; Herjavecz et al., 1999; Busse et al., 2001; Levy et al., 2009; Schramm and Carroll, 2009).

### Discussion

Both BUD and FP are potent corticosteroids used for the treatment of chronic respiratory diseases as inhaled formulas, although few studies have been undertaken to investigate efficacy and adverse effects of these compounds in small animals, particularly dogs. Bexfield et al. (2006) described the use of FP and beclomethasone, compared with oral or parenteral corticosteroid, in canine chronic inflammatory airway disease. Treatment with ICSs resulted in reduction of symptoms and side-effects, although both of these responses were owner-assessed. In experimental canine asthma models, inhaled BUD has been shown to significantly reduce airway eosinophilia, airway hyperresponsiveness and production of allergen-induced bone marrow progenitors (Woolley et al., 1994a, 1994b; Inman et al., 1997).

Table 2

<table>
<thead>
<tr>
<th>Cortisol (nmol/L)</th>
<th>Day 0</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>36 ± 10 (20–51)</td>
<td>36 ± 10 (26–51)</td>
<td>37 ± 14 (24–64)</td>
</tr>
<tr>
<td>Peak</td>
<td>288 ± 40 (237–335)</td>
<td>298 ± 25 (266–333)</td>
<td>297 ± 38 (251–346)</td>
</tr>
<tr>
<td><strong>Prednisolone</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>39 ± 18 (23–71)</td>
<td>14 ± 4 (8–19)</td>
<td>51 ± 25 (31–93)</td>
</tr>
<tr>
<td>Peak</td>
<td>333 ± 22 (305–372)</td>
<td>111 ± 15 (91–126)</td>
<td>266 ± 20 (249–301)</td>
</tr>
<tr>
<td><strong>FP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>42 ± 11 (26–60)</td>
<td>20 ± 10 (11–35)</td>
<td>55 ± 7 (45–65)</td>
</tr>
<tr>
<td>Peak</td>
<td>314 ± 59 (246–417)</td>
<td>156 ± 67 (72–254)</td>
<td>267 ± 18 (242–294)</td>
</tr>
<tr>
<td><strong>BUD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>46 ± 17 (22–70)</td>
<td>35 ± 14 (16–50)</td>
<td>41 ± 15 (19–59)</td>
</tr>
</tbody>
</table>

**FP**, fluticasone propionate; **BUD**, budesonide.

- Significant difference compared to placebo or BUD, *P* < 0.001.
after 4 weeks also in the urine protein-to-creatinine ratio, compared with the placebo group. An elevated ALP value is commonly associated with oral corticosteroid treatment and is mainly due to the formation of the corticosteroid-induced ALP isoenzyme (Soler et al., 1994). Moore et al. (1992) previously reported elevated protein concentrations in dogs after 5 weeks’ treatment with nearly identical anti-inflammatory doses of prednisone. In our dogs, the serum protein concentrations tended to increase whilst remaining within the reference range, thus rendering the clinical importance of this finding as unremarkable. Mild elevations in the canine urine protein-to-creatinine ratio reported with immunosuppressive doses of corticosteroids for 42 days were caused by mesangial cell proliferation and glomerular changes in the kidneys (Waters et al., 1997). Although the dose of prednisolone was lower in our study, the detected elevations in the urine protein-to-creatinine ratio might be attributed to similar changes. Five weeks of anti-inflammatory glucocorticoid treatment in dogs has previously been associated with decreased numbers of eosinophils and lymphocytes (Moore et al., 1992). In the current study, however, changes in differential cell counts were not detected, which might be due to the shorter duration of medication.

The most common systemic adverse effect in dogs after prolonged corticosteroid administration is HPA axis suppression. Accordingly, marked suppression was observed after 4 weeks of FP or prednisolone therapy in our study. This finding was in agreement with earlier findings in FP- and prednisone-treated dogs, and also with human studies (Clark et al., 1996; Clark and Lipworth, 1997; Kaliner, 2006; Cohn et al., 2008). However, unlike a previously reported study (Cohn et al., 2008), our work also demonstrated that FP but not BUD significantly suppressed the HPA axis relative to placebo. One week after discontinuation of treatment, we observed a significant increase in the basal cortisol level in the FP group compared with the placebo group. However, the values did not exceed the reference range, and thus, the clinical importance of this finding is negligible.

There was a minor systemic bioactivity of BUD relative to FP at clinically relevant doses which was probably due to differences in the pharmacological properties of these drugs. Tapering the dosage of any corticosteroid reduces systemic effects, but the equivalent dosages for equal endocrine effects or clinical responses are unknown since clinical trials comparing the efficacies of FP, BUD and prednisolone are lacking. Numerous studies of ICs in humans have established flaws in the pharmacokinetics and pharmacodynamics of these drugs are very complex (Clark and Lipworth, 1997; Lipworth, 1999; Adams et al., 2007). It has been suggested that FP may be more efficient than BUD because of superior retention in the lung tissue due to its higher lipophilicity and its higher glucocorticoid receptor binding affinity resulting in a longer duration of local anti-inflammatory action (Hogger and Rohdewald, 1994; Johnson, 1996). However, BUD dissolves better in bronchial fluid due to its lower lipophilicity (Hogger et al., 1993) and is also able to form fatty acid esters in the cells of the respiratory tract, unlike FP. These ester conjugates provide a local depot of latent, gradually regenerating pharmacokinetics.

The HPA axis was strongly suppressed after 4 weeks treatment with FP or prednisolone, but not BUD, at clinically relevant doses in healthy dogs. These findings suggested that inhaled BUD may be a safer choice in the treatment of chronic respiratory diseases in dogs sensitive to the systemic adverse effects of corticosteroid treatment. However, further studies are required to assess the therapeutic doses of ICs in dogs and to compare the systemic adverse effects of dosages with equivalent clinical efficacy.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

This study was carried out at the Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, and supported by a grant from the Helvi Knuuttila Foundation. Anu Lappalainen, DVM, is thanked for assessing the radiographs. The contributions of Satu Sankari and technician Kai-sa Aaltonen to cortisol analyses are greatly appreciated.

Conclusions

The HPA axis was strongly suppressed after 4 weeks treatment with FP or prednisolone, but not BUD, at clinically relevant doses in healthy dogs. These findings suggested that inhaled BUD may be a safer choice in the treatment of chronic respiratory diseases in dogs sensitive to the systemic adverse effects of corticosteroid treatment. However, further studies are required to assess the therapeutic doses of ICs in dogs and to compare the systemic adverse effects of dosages with equivalent clinical efficacy.

References