PHARMACOKINETIC AND PHARMACODYNAMIC EVALUATION OF
BECLOMETHASONE DIPROPIONATE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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This document is dedicated to my parents and all the friends who supported me.

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Beclomethasone dipropionate (BDP) was introduced as the first inhaled
glucocorticoid for the treatment of asthma in 1970's and is still a major candidate for
asthma treatment nowadays. In contrary to other glucocorticoids, BDP is an inactive
prodrug. Therefore, in order for BDP to be effective, BDP needs to be activated to its
active form, beclomethasone 17-monopropionate (17-BMP). Despite its long clinical
application, some crucial pharmacokinetic and pharmacodynamic data are still unknown
about BDP, such as the pulmonary targeting, pulmonary activation efficiency and plasma
protein binding of its active metabolite.

In this study, a sensitive HPLC/MS/MS assay was developed to facilitate the
pharmacokinetic study of BDP and 17-BMP in rats. Different routes of administration,
including intravenous and intratracheal doses, were studied to fully investigate the
pharmacokinetics of BDP and 17-BMP. Receptor occupancy was selected as a surrogate
marker for pharmacodynamic assessment. The receptor occupancy was monitored over

time in rats lung, liver, kidney and brain, using an *ex-vivo* receptor binding technique. Since the pharmacological desired and adverse effects of glucocorticoids are induced by the same receptors, pulmonary targeting has been defined in this study as the degree in which the occupancies of pulmonary (lung) and systemic (liver, kidney or brain) receptors differ. The plasma and tissue concentrations of BDP and 17-BMP were quantified to estimate the pulmonary activation efficiency of BDP into 17-BMP with non-compartmental analysis and compartmental modeling methods. The $EC_{50}$ values of 17-BMP in various rat tissues were also estimated based on pharmacodynamic modeling and plasma protein binding of 17-BMP.

The results showed that the prodrug property of BDP has a negative impact on the pulmonary targeting of BDP. This impact was quantified by the pulmonary activation portion of BDP into 17-BMP, which was about 56%. The EC50 values of 17-BMP in rat lung, liver and kidney were found to be consistent, confirming the homogeneity of glucocorticoid receptors.

The clinical study comparing two formulations of BDP, hydrofluoroalkane-134a(HFA)-BDP and chlorofluorocarbon-BDP, showed that a significant dose reduction is possible for HFA-BDP with the potential to reduce side effects while being equally effective to treat asthma as CFC-BDP at a higher dose.

CHAPTER 1
INTRODUCTION

Inhaled corticosteroids (ICS) have revolutionized the treatment of asthma in the past 30 years and now represent the first-line therapy for patients with chronic disease [1]. Numerous clinical studies have demonstrated the high anti-asthmatic efficacy (e.g. prevention and control of symptoms) and reduced systemic side effects of ICS therapy. Among the various ICS available in the market, beclomethasone dipropionate (BDP) (Figure 1) was the first one, introduced in 1972 in a pressurized metered-dose inhaler (MDI) and later in a dry powder inhaler and an aqueous nasal spray [2]. In this introduction section, the current literature on asthma pathogenesis and therapy, the mechanism of action of ICS in asthma therapy, the pharmacokinetic and pharmacodynamic concerns of BDP in asthma management will be discussed.

**Asthma**

Asthma is a chronic disorder of the airways characterized by airway inflammation, reversible airflow obstruction and airway hyperresponsiveness. It is believed that airway inflammation is an integral element in the pathogenesis of asthma and a driving force in airway hyperresponsiveness and a contributing factor for variable levels of airflow obstruction [3].

Asthma inflammation involves many cells, with mast cells, eosinophils, basophils, neutrophils, macrophages, epithelial cells, and lymphocytes all being active participants (see Figure 1.1). The interactions of these cells result in the release of different mediators leading to bronchoconstriction, microvascular leakage, mucus hypersecretion, epithelial

damage, and stimulation of neural reflex [4].  For example, activation of the mast cell by specific antigen through cell-bound IgE releases histamine and causes synthesis of leukotrienes, which can precipitate an acute episode of airway obstruction [5].  Mast cells also release inflammatory enzymes or proteases, which can contribute to airway inflammation and airway hyperresponsiveness. Finally, mast cells express and release a wide variety of proinflammatory cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, 4 and 5 [6].  These cytokines change the immunologic environment of the airway and are likely instrumental in initiating the inflammatory response in asthma through changes in adhesion proteins, cell recruitment, and inflammatory cell survival.  Eosinophils are recruited to the lung in asthma after antigen inhalation and are the main effector cells in the resulting inflammatory process. Eosinophils contribute to inflammation by release of mediators (i.e., leukotrienes and toxic oxygen products), by degranulation and release of granular proteins, and by generation of cytokines [5, 7]. Granular proteins, such as major basic protein, directly damage airway tissue, promote airway responsiveness, and injure airway epithelium. In general, the degree of airway eosinophilia is usually proportional to the severity of asthma [8].  Activated lymphocytes are another important element of airway inflammation. One of the T helper ($T_H$) lymphocytes, $T_{H2}$, shows a cytokine profile that contains IL-4 and IL-5, two cytokines that are essential for IgE synthesis (IL-4) and eosinophil production (IL-5).  Since lymphocytes are long lived, they are believed to be crucial to the persistence of the inflammatory process in asthma. Epithelial cells are another source of proinflammatory mediators in asthma including eicosanoids, cytokines, chemokines, and nitric oxide [9].
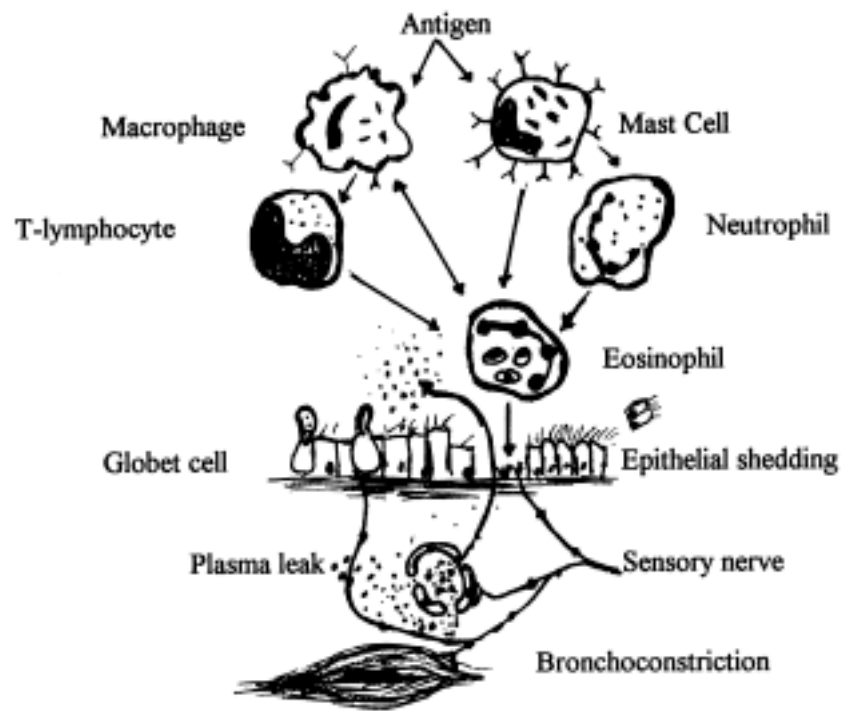
Figure 1-1. Cellular interactions after antigen inhalation in asthma [4].

The current concept of asthma therapy is based on a stepwise approach, depending on disease severity, and the aim is to reduce the symptoms that result from airway obstruction and inflammation, to prevent exacerbations and to maintain normal lung function [10]. Controlling the allergic response to the local environment, due to smoking and allergens found at home or in the workplace, is the first priority in minimizing asthma hyperresponsiveness. Traditionally, there are five classes of medications for asthma therapy: corticosteroids (e.g., beclomethasone dipropionate), short-acting (e.g., albuterol) and long-acting (e.g., salmeterol) beta-agonists, theophylline, cromolyn sodium and nedocromil sodium, and anticholinergics (e.g., ipratropium bromide) [11]. Corticosteroids, cromolyn sodium and nedocromil sodium can be classified as anti-inflammatory medications. Beta-agonists and theophylline are both brochodilators.

Anticholinergics work by blocking the contraction of the underlying smooth muscle of the bronchi. Among these medications, inhaled corticosteroids are the most effective for the treatment of asthma. Their efficacy is related to many factors including a diminution in inflammatory cell function and activation, stabilization of vascular leakage, a decrease in mucus production, and an increase in beta-adrenergic response. The strong scientific rationale behind the combination therapy of long-acting beta-aganists and corticosteroids has led to the development of two mixed combination inhalers: salmeterol/fluticasone and formoterol/budesonide [12]. They are increasingly used as a convenient controller in patients with persistent asthma. The advances in the knowledge of the pathophysiology of asthma in the past decade have opened the way to the development of novel therapeutic strategies that target more directly on the pathophysiology of asthma than those currently in use [13]. Various compounds able to interfere with the complex network of proinflammatory mediators, cytokines, chemokines, and adhesion molecules involved in the pathogenesis of asthma have been identified, such as leukotriene modifiers, tachykinin antagonists, endothelin antagonists, adenosine receptor inhibitors, tryptase inhibitors, cytokine and chemokine inhibitors, direct inhibitors of T-cell function and adhesion molecule blockers. New forms of immunotherapy, such as anti-IgE therapies, gene vaccination with plasmid DNA and mycobacterial preparations, are also emerging to aim at blocking the unbalanced $T_{H2}$ response that characterizes the pathophysiology of asthma. Despite the significant expansion of the experimentally available treatment options, only the leukotriene modifiers are on the market, among which leukotriene antagonists are now most frequently used in asthma treatment [14, 15]. The leukotriene antagonists have been shown to inhibit exercise-provoked bronchospasm,

to modify the airway reponse to inhaled antigen, and to improve airway function in patients with chronic asthma. But in head-to-head trials with inhaled corticosteroids, the leukotriene antagonists are less effective in terms of improvement in lung function and reduction in exacerbations [3]. Thus far, inhaled corticosteroids still represent the cornerstone of treatment for asthma.

<div align="center">**Corticosteroids in Asthma Therapy**</div>

Corticosteroids are able to affect many of the inflammatory pathways involved in the pathogenesis of asthma, including the complex cell to cell communications mediated by the so-called "cytokine network" [16]. Corticosteroids start their action when the lipophilic corticosteroid molecule crosses the cell membrane and binds to a specific, intracellular glucocorticoid receptor (GR) (Figure 1-2). The GR is located in the cytoplasma and belongs to the steroid thyroid/retinoic acid receptor superfamily [17]. Prior to binding corticosteroids, GR forms a large heteromeric complex with several other proteins, from which it dissociates upon ligand binding. A central role in this complex is played by 90 kDa heat shock protein (hsp90) [18], which binds as a dimer to the C-terminal ligand binding domain (Figure 1-2). The association with hsp90 has been shown to be required to maintain the C-terminal domain of GR in a favorable conformation for ligand binding [19], but GR is transcriptionally inactive at this stage. Binding of corticosteroids to GR dissociates hsp90 and other heat shock proteins, resulting in a conformational change which allows the GR complex to translocate to the nucleus or interact with cytoplasmic transcription factors. Once in the nucleus, the GR complex binds as a dimer to specific DNA sites through its central domain, which is structurally characterized by a DNA binding motif consisting of two "zinc fingers", each containing a zinc ion tetraedrically coordinated to four cysteine residues (Figure 1-3)

[20]. The GR interacts with DNA by targeting specific nucleotide palindromic sequences termed "glucocorticoid response elements" (GRE) (Figure 1-2). In particular, the dimer GR places its two DNA-binding fragments into adjacent major grooves of the DNA double helix in correspondence of appropriately spaced GRE half palindromes. Then the transcription of specific genes can be increased (transactivation) or decreased (transrepression) depending on whether the GRE is positive or negative. In particular, corticosteroids increase the expression of anti-inflammatory proteins such as lipocortin-1, interleukin-1 receptor antagonist (IL-1ra), interleukin-10 (IL-10), secretory leukocyte inhibitory protein, neutral endopetidase and the inhibitory protein (IκB) of nuclear factor-κB (NF-κB) [1]. It is known that lipocortin-1 inactivates the enzyme phospholipase $A_2$ thus inhibiting the production of lipid mediators (i.e., platelet-activating factor, leukotrienes, and prostaglandins) [21]and NF-κB positively regulated inflammatory and immune responses [22]. Corticosteroids are also able to enhance the transcription of the gene encoding the $\beta_2$-adrenergic receptor, thus reversing and/or preventing the down-regualtion possibly induced by long-term treatments with $\beta_2$-agonist bronchodilators [23]. However, the genomic mechanism of transactivation is believed to be mainly responsible for the unwanted side effects of corticosteroids, rather than for their anti-inflammatory and immunoregulatory actions. The very effective control of airway inflammation exerted by corticosteroids in asthma is largely mediated by inhibition of the transcriptional activity of several different genes encoding pro-inflammatory proteins such as cytokines (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13, TNF-α, GM-CSF), chemokines (IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxin), adhesion molecules (ICAM-1, VCAM-1, E-selectin), and mediator-synthesizing enzymes (i-NOS,

COX-2, cytoplasmic $PLA_2$) [16]. which are mostly due to GR-dependent repression of pro-inflammatory genes. Corticosteroids exert their repressive action by multiple routes, such as genomic mechanisms (Figure 1-2), direct interaction with transcription factors and post-transcriptional regulation (Figure 1-4) [16].

**Pharmacokinetic and Pharmacodynamic Concerns of Beclomethasone Dipropionate in Asthma Management**

Inhaled corticosteroids (ICS) exert their anti-inflammatory effects by binding to the glucocorticoid receptors which are localized in the cytoplasm of the target pulmanory cells.  Unfortunately, glucocorticoid receptors are expressed in almost every type of cell although the density of glucocorticoid receptors may differ from cell to cell.  In addition, there is no evidence that shows the glucocorticoid receptor bond by corticosteroids has subtype of differing affinity in different tissues.  Therefore, avoiding systemic side effects and increasing the targeting effect of ICS are always the goals of developing a good corticosteroid for the treatment of asthma.  To achieve this, an ideal ICS should have high pulmonary deposition, prolonged residence time in the lung, low oral bioavailability and high systemic clearance [24].  Among the various ICS in the market, beclomethasone dipropionate  (9-chloro-11β-hydroxy-16β-methyl-pregna-1, 4-diene-3,20-dione) (Figure 1-5) is unique in that it is the first ICS and a prodrug.  In order to be effective, BDP needs to be converted to its active metabolite, beclomethasone-17-monopropionate (17-BMP), via hydrolysis by esterases in vivo while BDP itself and another metabolite, beclomethasone, also show very weak anti-inflammatory activity according to their relative receptor affinity (Table 1-1).  Therefore, the pharmacokinetic and pharmacodynamic properties of BDP deserved special concerns.
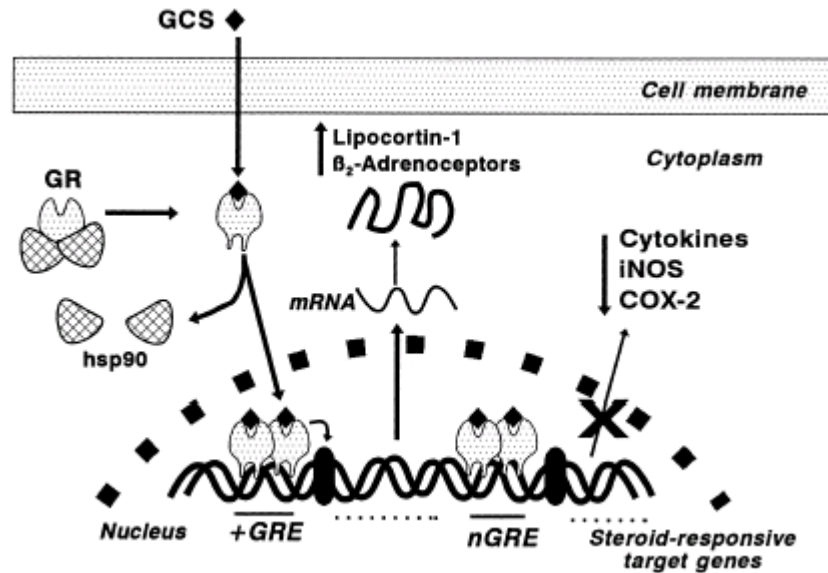
Figure 1-2. Classical model of glucocorticoid action. The glucocorticoid enters the cell and binds to a cytoplasmic glucocorticoid receptor (GR) that is complexed with two molecules of a 90kDa heat shock protein (hsp 90). GR translocates to the nucleus where, as a dimer, it binds to a glucocorticoid recognition sequence (GRE) on the 5-upstream promoter sequence of steroid-responsive genes. GREs may increase transcription and nGREs may decrease transcription, resulting in increased or decreased messenger RNA (mRNA) and protein synthesis (Adapted from Barnes [25]).
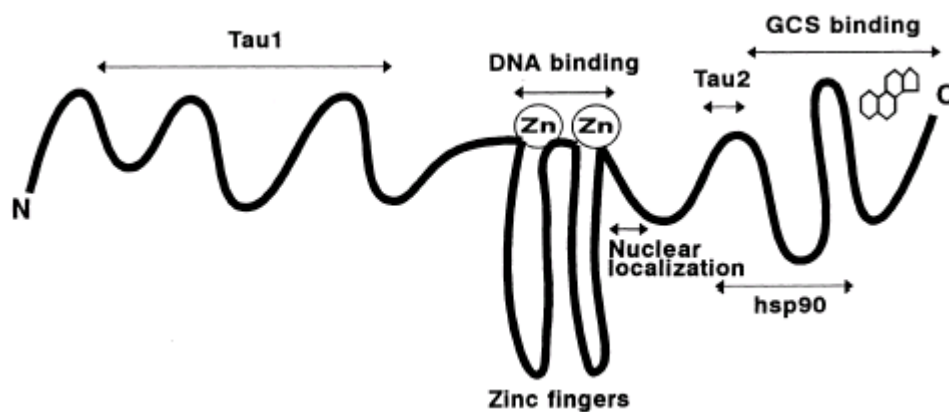


Figure 1-3. Domains of glucocorticoid receptor (GCS: glucocorticoids. Adapted from Barnes [25])

A

Cytokine

Cell Membrane

Fos    Jun

AP-1

Cytoplasm

AP-1

Nucleus

AP-1

AP-1   GRE    Target Gene

Ap-1   GRE
DNA                    Transcription   mRNA   Translation   Protein

B

Translation

Cytoplasm                              Jun

mRNA

GR

Nucleus

GR
nGRE              Transcription

AP-1

GR
Ap-1   GRE                         GR
DNA                               AP-1

GR                   Complex does
GRE                  not bind DNA

Transcription +

mRNA   Translation              mRNA   A   A   AAAAA

Ribonuclease

C

Cytoplasm                          GR
                                   GR

NFκB
        DNA
Nucleus

NFκB        −
          IκBα

GR                           NFκB
GR
NFκB                         GR
                             GR
Complex does not bind DNA    NFκB

                             Complex does
                             not bind DNA

GR
GR
GRE
DNA   IκBα    Transcription   mRNA   AAAA   Translation   IκBα
                          +                              (NFκB
                                                         inhibitor)

Figure 1-4. Effects of corticosteroids (GC) on transcription factor action. A: Normal
pathway of activation for transcription factor activator protein-1 (AP-1).  An
extracellular signal, such as the binding of a cytokine to its receptor, is
transduced to the cytoplasm where it initiates a sequence of events leading to
increased or decreased levels of specific gene products.  For example,
stimulation of the cell by tumor necrosis factor-$\alpha$ (TNF-$\alpha$) results in the
binding of Fos protein with Jun protein and causes the formation of the
transcription factor complex, AP-1. AP-1 then binds to specific binding sites
wihin the 5' promoter region of target genes and upregulates the transcription
of mRNA. B: GR interactions with transcription factor, AP-1 and post-
transcriptional regulation.  The homodimer GR binds through a protein–
protein interaction with the transcription factor AP-1. This interaction can
occur in the cytoplasm or nucleus and prevents the binding of the transcription
factor to the promoter region of target gene(s).  The GR may also inhibit the
transcription of Jun, a component of AP-1, or stearically inhibit the binding of
AP-1 to the AP-1 binding site.  Post-transcriptional regulation by the GR
involves the upregulation of ribonucleases, which increase the instability of
various mRNA molecules.  Stability of the mRNA is normally enhanced by
the presence of poly-A tail of the mRNA, which is cleaved by the
ribonuclease. C: GR interactions with NF-$\kappa$B. The GR also may inhibit the
transcription factor NF-$\kappa$B by binding to the factor and preventing NF-$\kappa$B-
modulated transcription. As with AP-1, this interaction can occur in the
cytoplasm or nucleus. In addition, the activated GR may indirectly inhibit the
function of NF-$\kappa$B by upregulating the transcription of its inhibitor, I$\kappa$B$\alpha$
[16].

| | R | R' |
|---|---|---|
| **BDP** | $COC_2H_5$ | $COC_2H_5$ |
| **17-BMP** | H | $COC_2H_5$ |
| **21-BMP** | $COC_2H_5$ | H |
| **BOH** | H | H |
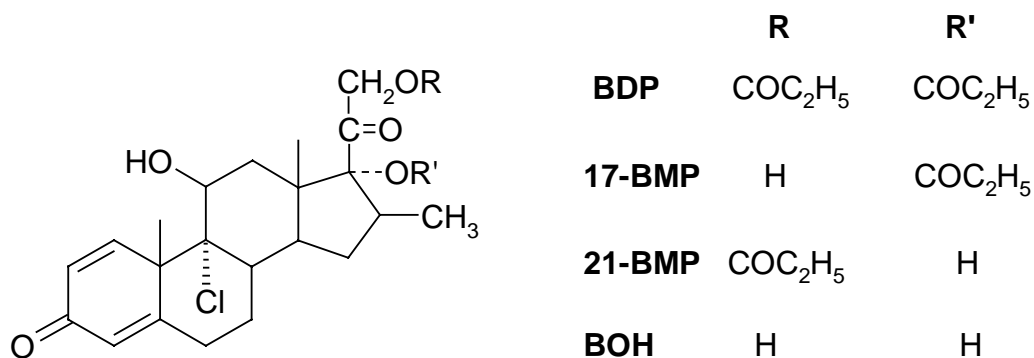
Figure 1-5. Molecular Structures of BDP, 17-BMP, 21-BMP and BOH

**Pharmacokinetics**

**Absorption.** After being inhaled by man, BDP will enter the respiratory track and

also deposit in the mouth and throat and then be swallowed. Both of these two fractions

will contribute to the systemic exposure.  For the traditional chlorofluorocarbon (CFC)-

BDP MDIs, a recent study [26] reported that only 3-8% of the inhaled dose deposited in

the lung and 94% was swallowed. But for the newly developed hydrofluoroalkane-134a

(HFA)-BDP, the corresponding numbers are 56% and 32%, with lung deposition being

improved dramatically because of the high percentage of fine particle mass in the new

Table 1-1. Relative binding affinity (RBA) of some glucocorticoids for the human
glucocorticoid receptor (RBA for dexamethasone = 100)

| Glucocorticoid | RBA |
|---|---|
| Fluticasone propionate | 1800[a] |
| Beclomethasone-17-monopropionate | 1345[b] |
| Budesonide | 935[c] |
| Beclomethasone (BOH) | 76[b] |
| Beclomethasone dipropionate | 43[b] |
| Beclomethasone-21-monopropionate | 0.9[b] |

a [27], b [28], c [29]

formulation. It is agreed that all pulmonary available BDP will be absorbed systemically.

As a result, pulmonary bioavailability is assumed to be 100% and used as a reference to

calculate the oral bioavailability. However, there exists a discrepancy about the oral

bioavailability of BDP. The early study by Martin et al. reported that 90% of BDP in the

microfine suspension was absorbed from the gastrointestinal track compared with 61% to

70% in the capsule formulation based on a radiochemical assay. In this study, BDP was

not differentiated from its metabolites. But according to the later metabolism studies and

pharmacokinetic studies, most BDP that are swallowed will be rapidly hydrolyzed to 17-

BMP in intestinal and there will be no unchanged BDP left after the first-pass metabolism

[30] Therefore, the oral bioavailability of intact BDP is thought to be zero, which

indicates that the detectable BDP after inhalation is absorbed from pulmonary deposited

BDP. But little was known about the bioavailability of 17-BMP, the active metabolite.

The appearance of HFA-BDP and the availability of a sensitive assay for 17-BMP drove

investigators to put more efforts into the pharmacokinetic study of 17-BMP. Based on

the lung deposition results from Leach et al. [26] (56% of inhaled HFA-BDP deposited in lung and 34% swallowed), Soria et al. [31] calculated the 17-BMP relative bioavailability of oral versus inhaled BDP to be 21%, which was comparable to the 26% estimated from BOH concentration, a surrogate for 17-BMP before the analytical assay was available for 17-BMP. But if the same method is applied to the data from another study [32] comparing the pharmacokinetics of CFC-BDP and HFA-BDP, the 17-BMP oral bioavailability (F) is calculated to be 47% according to equation (1) with the assumption that the systemic availability of drug fraction deposited in the lungs is complete and F is independent of the BDP formulation.

$$\frac{D_{HL} + D_{HS} \times F}{D_{CL} + D_{CS} \times F} = \frac{AUC_H}{AUC_C} \quad (1)$$

$D_{HL}$: lung deposition fraction after HFA-BDP inhalation (56%)

$D_{HS}$: swallowed fraction after HFA-BDP inhalation (34%)

$AUC_H$: area under the curve for 17-BMP after HFA-BDP inhalation (8603 pg.h/ml)

$D_{CL}$: lung deposition fraction after CFC-BDP inhalation (4%)

$D_{CS}$: swallowed fraction after CFC-BDP inhalation (94%)

$AUC_C$: area under the curve for 17-BMP after CFC-BDP inhalation (5755 pg.h/ml)

F: 17-BMP oral bioavailability

In a recent study [30], charcoal was administered to block the absorption of BDP that was swallowed after inhalation of HFA-BDP and the AUCs of 17-BMP with and without charcoal administration were compared to obtain the percentages of pulmonarily absorbed 17-BMP and orally absorbed 17-BMP in the total systemic available 17-BMP. Then based on the same lung deposition data for HFA-BDP, a 16% oral bioavailability of 17-BMP was obtained according to equation (2).

$$\frac{D_L}{D_L + D_S \times F} = \frac{AUC_{W/}}{AUC_{W/O}} \quad (2)$$

$D_L$: lung deposition fraction after HFA-BDP inhalation (56%)

$D_S$: swallowed fraction after HFA-BDP inhalation (34%)

$AUC_{w/}$: area under the curve for 17-BMP after HFA-BDP inhalation with charcoal administration

$AUC_{w/o}$: area under the curve for 17-BMP after HFA-BDP inhalation without charcoal administration

F: 17-BMP oral bioavailability

Instead of using these lung deposition data from the scintigraphic method, Daley-Yetes et al. [30] performed intravenous study along with intranosal, inhalation and oral studies to get 17-BMP related pharmacokinetic parameters, assuming that all injected BDP would be biotransformed to 17-BMP in vivo. Charcoal block method was also used in this study and the efficiency of this method was estimated to be 96% by the oral study with and without charcoal administration. All the bioavailability calculations were corrected for this charcoal efficiency. Traditional CFC-BDP was investigated and the deposition result was found to be 36% deposited in the lung and 64% swallowed, significantly different from the result obtained with the scintigraphic method. The oral bioavailability was calculated to be 41% relative to intravenous study. In another study [33] involving asthmatic children, the oral bioavailability of 17-BMP from HFA-BDP was reported to be 90% relative to intravenous study. It is obvious that the variability in 17-BMP oral bioavailability reports could be due to many factors, such as the formulation of BDP, selection of subjects, inhaling skills of different subjects, inhaler device and

methodology. A further well-designed study is needed to provide a relatively consistent result.

While the bioavailability reflects how much of the drug is absorbed into the systemic circulation, the time to reach maximum plasma concentration ($T_{max}$) is a measurement of how fast the drug is systemically absorbed. After inhalation of CFC-BDP from metered dose inhaler (MDI), $T_{max}$ of BDP was observed to be 3 - 5 minutes [34, 35], but a recent study reported it to be 18 minutes [30]. In a study comparing CFC-BDP MDI and CFC-BDP dry power inhaler (DPI), a longer Tmax of 13 minutes for BDP was observed in the CFC-BDP MDI group than the $T_{max}$ of 3 minutes in CFC-BDP DPI group. The slower dissolution of dry power could be the reason for this delay. For 17-BMP, $T_{max}$ observed ranged from 1 to 3 hours in different studies [30, 32, 34, 35]. Total BOH was used as a surrogate for beclomethasone esters and its $T_{max}$ was found to be 2 hours, which indicated that 17-BMP is the dominating ester [36]. For HFA-BDP, Tmax of 17-BMP was found to be 0.3 hour in a study involving mild to moderate asthmatics and 0.9 hour in another study involving healthy adults, generally shorter than Tmax of CFC-BDP. If total BOH is used as the marker, $T_{max}$ was found to be 0.8 hour, consistent with the result for 17-BMP. It is believed that the solution characteristic of HFA-BDP compared with the suspension characteristic of CFC-BDP results in a higher percentage of fine particle mass of BDP that deposits in the lung, which contributes to the faster absorption of BDP and 17-BMP.

**Distribution.** It is well known that the volume of distribution correlates strongly with lipophilicity of drugs. But as a very lipophilic drug, BDP only showed a volume of distribution of 167L [37]during elimination phase, even smaller than that of BUD [38], a

much less lipophilic drug. A recent study [30] even reported a 20L volume of distribution at steady state for BDP after intravenous administration of 1mg of BDP. The unusual low volume of distribution of BDP could be a result of its very rapid metabolism in vivo, which prevented extensive tissue distribution. 17-BMP is less lipophilic than BDP, but its volume of distribution was found to be 424L after intravenous administration of 1mg BDP, which indicated the relatively high tissue concentration of 17-BMP. But in another study with children [33], the mean steady state volume of distribution of 17-BMP was reported to be 84L. The significant difference between these two studies could be due to the difference in subjects, with one choosing healthy adults and the other choosing mild asthmatic children.

**Metabolism and elimination.** The metabolism of BDP has been studied extensively in a variety of media, including human plasma [28, 39], human lung [28, 39, 40] and liver homogenate [40], simulated gastric fluid, simulated intestinal fluid, TRIS-buffer and phosphate buffer [28]. A new scheme about the degradation pathway of BDP in human plasma was proposed after the structures of two more unknown metabolites, D2 and D3, were identified (Figure 1-6) [41].

When incubated with 9000g human liver supernatant [40], BDP was immediately biotransformed to 17-BMP, which then was metabolized to unknown metabolites. The half-life of 17-BMP in human liver homogenate is calculated to be 39 minutes, assuming a pseudo-first order degradation. No 21-BMP and BOH were detected in the liver homogenate according to the author. The metabolism of BDP in human lung homogenate is slower than in the human liver homogenate, but still much faster than in human plasma. Three different groups [28, 40, 41] investigated the metabolism of BDP

in human lung homogenate and reported significant different results about the half-life of

BDP. Initial concentrations of 10-40 ug/ml BDP and 4 mg/ml lung protein concentration

in lung homogenate were used in Foe et al.'s study [41].
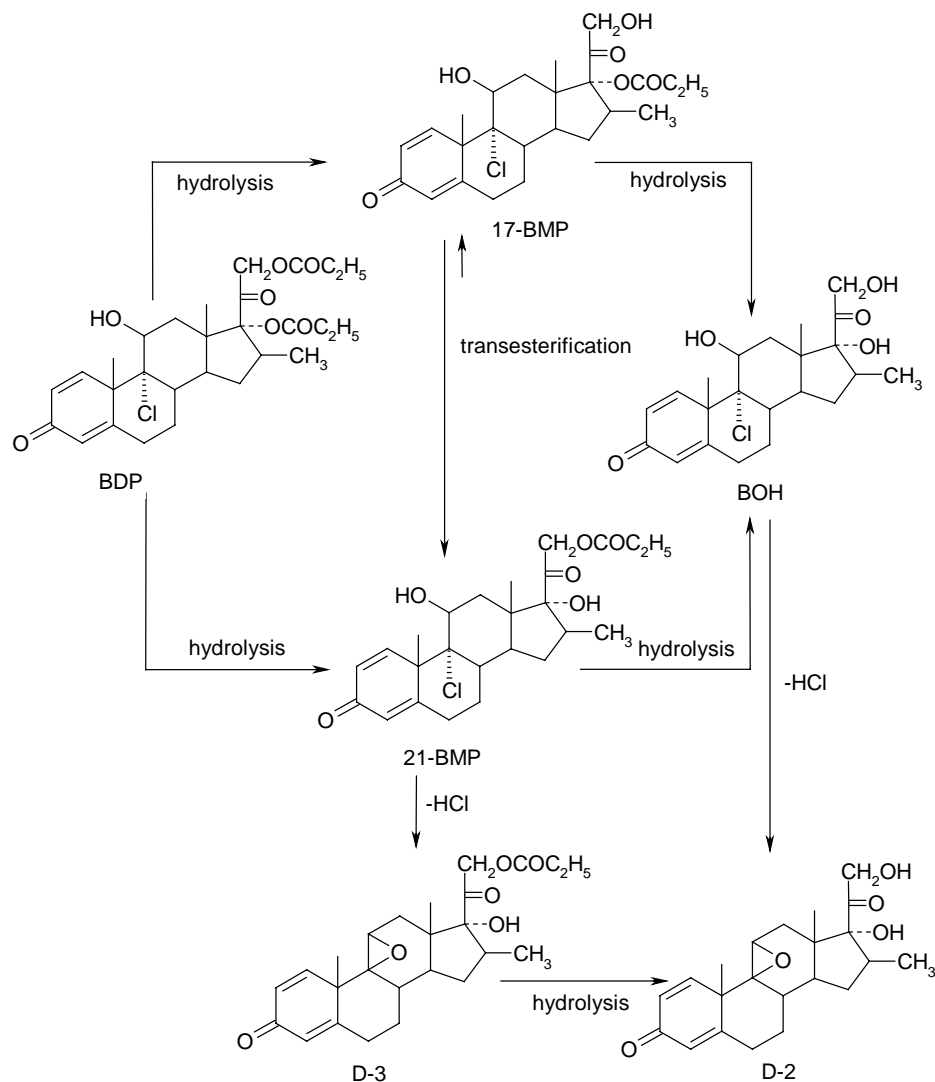


Figure 1-6. Decomposition pathway of BDP in human plasma [41]

The initial concentration of BDP seemed too high for the esterases in human lung

homogenate and an enzyme saturation was observed, which resulted in a nonlinearity on

the kinetics of BDP and 17-BMP. As a result, a much longer half-life of BDP

($t_{1/2}$=35min) was found in this study. In Wurthwein and Rohdewald's study [28], the

initial concentration of BDP was 50 ug/ml and the protein concentration in human lung homogenate was 10 mg/ml, indicating higher esterase activity. There seemed to be still some enzyme saturation happening according to the relatively long half-life of 10.5 minutes compared with a half-life of 5 minutes from another study [40], in which 0.05 ug/ml initial BDP concentration and 4 mg/ml protein concentration were used. In addition, nonlinearity was also noticed for the metabolism of BDP in both human lung homogenate and intestinal fluid, which could be due to product inhibition. Despite the difference in half-life, all the three groups found that BDP was selectively hydrolyzed to 17-BMP in human lung homogenate, without producing any detectable 21-BMP. And 17-BMP is relatively stable in human lung homogenate with a half-life of 3.5±1.2 hours [41]. It seems that human lung homogenate has a much higher percent of esterases that can only hydrolyze 21-ester bond in BDP than human plasma since BDP can be biotransformed to 17-BMP and 21-BMP in human plasma to a similar extent [41, Wurthwein, 1990 #56]. Both 17-BMP and 21-BMP will be further metabolized to BOH, which undergoes non-catalytic degradation to a recently identified metabolite, D-2. The non-catalytic transesterification reaction between 17-BMP and 21-BMP preferentially happens from 17-BMP to 21-BMP with a half-life of 5.4 hours in phosphate buffer, which provided another way of inactivation of 17-BMP [28]. Foe et al. explained that the lack of any detectable 21-BMP in human lung homogenate could be the result of non-catalytic transesterification being eliminated by the high esterase activity in human lung homogenate. It is, however, possible that 21-BMP is still produced from 17-BMP through transesterification, but is rapidly hydrolyzed to BOH because of the much higher percent of 21-ester specific esterase in human lung homogenate. The esterase that highly

catalyzes BDP to 17-BMP should have similar, or even higher, catalytic activity for 21-BMP to BOH.

The half-lives of BDP measured in vitro are much longer than that obtained in vivo study, which indicates the dominating effect of hepatic metabolism for in vivo BDP. The half-lives of BDP and 17-BMP were reported to be 30 and 37 minutes respectively after intravenous administration of 1 mg radiolabeled BDP [37]. But the result about 17-BMP is not reliable because of the dramatic cross-reaction of 21-BMP (1600%) with the 17-BMP antiserum. Another study [33]with asthmatic children reported 6.6 minutes and 1.7 hours for the half-lives of BDP and 17-BMP after intravenous administration of 60 μg BDP. After inhalation of CFC-BDP or HFA-BDP, the half-life of 17-BMP was calculated to be 2.7 hours. A recent study [30] involving healthy adults reported that after intravenous administration of 1 mg BDP, the half-life of BDP and 17-BMP are 30 minutes and 2.7 hours. After inhalation of CFC-BDP, the half life of 17-BMP was found to be 2.7 hours without charcoal administration or 2.3 hours with charcoal administration, not significantly different from the result after intravenous administration of BDP, which suggested that the disposition of 17-BMP was elimination rate-limited and the slow dissolution of BDP in the lung would not influence the terminal slope of 17-BMP concentration. The same conclusion was drawn in Hill and Vaughan's study [34], in which they found that the half-lives of 17-BMP after inhalation of CFC-BDP MDI and CFC-BDP DPI were both 3.3 hours despite that the half-life of BDP for DPI (50-60min) was much longer than that for MDI (less than 10 min). Additional evidence came from the study of HFA-BDP. The half-lives of 17-BMP from this new formulation of BDP was found to be 2.7 [33] and 2.1[42] hours, not significantly different from the result for

CFC-BDP even though the dissolution of HFA-BDP is agreed not to be a rate-limiting

factor because of the very fine particle size. A relatively early study [43] concluded that

the longer elimination half-life of 17-BMP (93min) was due to the retard effect of slowly

dissolving BDP. According to the recent studies, however, the 37 minutes half-life of 17-

BMP after intravenous injection of BDP used in this study is obviously not a proper

reference. The half-life of 6.5 hours reported in another study [35] is much longer than

the others. In contrast to BDP, the in vivo elimination of 17-BMP is consistent with its in

vitro elimination in human plasma, which indicates that hepatic metabolism is not the

dominating factor in 17-BMP in vivo metabolism. There are few studies about the

clearance of BDP and 17-BMP. A clearance of 230L/hr was reported for BDP and high

extra-hepatic clearance of BDP was concluded [37]. A recent study [30] also reported

high clearance for BDP (150L/hr). Despite the difference in numbers, an extra-hepatic

clearance is still obvious. But only 15% of the injected dose was excreted into the urine,

and most of the dose was finally disposed to faceas via biliary secretion. High esterase

activity in certain tissues could account for this non-hepatic metabolism. The clearance

of 17-BMP was reported to be 54L/hr in a study involving 14 mild asthmatic children.

But another study involving 12 healthy adults reported it to be 120L/hr. The high

clearance of 17-BMP resulted in a very low accumulation ratio of 1.1-1.4 at steady state

after multiple doses of three HFA-BDP dose levels [44], having no clinical consequence,

which is in contrast to fluticasone propionate with a significant drug accumulation after

multiple doses [45].

**Pharmacodynamics**

   **Relative receptor affinity.** Wurthwein *et al.* determined the receptor affinities of

inhaled glucocorticoids by performing *in vitro* competition assays using human lung

cytosol and reported them as relative receptor affinities with respect to the receptor affinity of dexamethasone.  Compared with currently available inhaled corticosteroids, the active form of BDP, 17-BMP, has the second highest relative receptor affinity (Table 1-1).  While higher receptor affinity does not necessarily mean higher activity because receptor antagonists can also have high receptor affinity, there appears a direct correlation between the corticosteroids receptor affinity and the activity at the site of action [29, 46].  Recent PK/PD investigations on systemic effects, such as cortisol suppression, degree of lymphocytopenia and increase in granulocytes of currently available corticosteroids, showed a positive correlation between the receptor affinity and the activity [47, 48].  We should keep in mind, however, that corticosteroids with high affinity for lung glucocorticoid receptors will also have high receptor affinity for all other glucocorticoid receptors in other tissues and cells, which contributes to the systemic side effects.  Therefore, a high receptor affinity does not lead to pulmonary selectivity, but only to high local activity or low dose.  This fact implies that only pharmacokinetics can change the therapeutic index (pulmonary activity/systemic activity) of different corticosteroids.

**Topical and systemic glucocorticoid potency.** With topical application, the anti-inflammatory potency of corticosteroids is typically assessed with the skin blanching or vasoconstriction assay [49, 50].  The corticosteroids to be tested are applied to the skin in an alcoholic solution or ointment and covered with a plastic film to prevent evaporating for several hours.  Then the consequent blanching will be visually assessed by the investigators.  The blanching activity of corticosteroids is related to their vasoconstriction effect.  However, it has been realized that this assay may be unreliable for predicting

clinical efficacy of different corticosteroids for the treatment of asthma in that BDP was reported to have higher topical potency than 17-BMP based on this assay and budesonide has 2-3 times better vasoconstriction properties than BDP [51] while later studies showed that 17-BMP is the active form of BDP and budesonide and BDP have equivalent efficacy when administered at the same dose via the same type of delivery device [52]. The more specific tests of pharmacological activity associated with anti-inflammation in asthma treatment such as regulation of gene transcription [53] or inhibition of T-cell proliferation [54] are under development and may provide more reliable information about the clinical efficacy of corticosteroids than skin blanching in the future.

The concern about the systemic effects of corticosteroids always exists, even after the inhaled corticosteroids substituted the oral corticosteroids and became the mainstay of asthma treatment for their substantially improved pulmonary/systemic activity. With the trend towards long-term use of high dose of corticosteroids, systemic effects draw more attention when corticosteroids are compared. The major systemic effects include growth suppression in children, bone demineralization, hypothalamic-pituitary-adrenal (HPA) axis suppression, ocular effect and thinning and bruising of skin [55]. Even though there are markers for each of these systemic HPA axis suppression is the most common one that is used to assess the systemic effect of corticosteroids due to its sensitivity and easily measured markers. As a result, HPA axis suppression effect is the most extensively evaluated systemic effect of corticosteroids therapy and has been thoroughly reviewed by Barnes [1] and Dluhy [56]. Several markers have been used to measure the HPA axis suppression effect, which are generally classified as two types of test of adrenal functions, assessing the basal secretary functions (single morning plasma cortisol

measurement, 24 hour serum corisol profile, 24 hour urinary free cortisol excretion and

urinary cortisol-creatinine ratio) and dynamic stimulation tests ( corticotropin ,

cosyntropin or metyrapone or insulin-induced hypoglycemia).  In one study with BDP

[57], healthy adults were given BDP for 10 days at the dose of 800 ug/day or 2000

ug/day.  Changes in morning plasma cortisol were found to be the least sensitive marker

and were not statistically significant for either dose.  At the same time, urinary

cortisol/creatinine ratio in timed urine samples was shown to be as sensitive as 24 hour

urinary free cortisol secretion in detecting the HPA axis suppression effect of BDP with

the advantage of avoiding the need for accurate measurement of urine volume and for

complete 24 hour collections.  Another study gave the same order of indices sensitivities

for different tests of HPA axis suppression effect [58].  Although basal adrenal function

can be acutely perturbed and sensitively measured, clinically relevant suppression of

HPA axis with subsequent adrenal cortical atrophy requires weeks or months of

exogenous corticosteroids exposure.  The dynamic stimulation test can be used to

evaluate the impairment of adrenal reserve and insufficient corticol response to the stress.

In a study with asthmatic children [59], the responsiveness of the adrenal glands to

corticotropin remained unchanged after 3 months of 400 ug/day BDP treatment in all

patients despite the substantial suppression in corticol secretion according to the 24 hour

serum cortisol profile and urinary free cortisol.  The author suggested that the suppression

happened at the hypothalamus-pituitary level.  Brown et al. [60] studied 78 patients with

asthma who had been treated with high doses of BDP or BUD (1500-2000 ug/day) for 6

months.  HPA axis suppression was found in 15 of 69 patients who had taken BDP.

Fourteen of the 15 patients had subnormal cortisol values after short cosyntropin

stimulation. On the other hand, the results of this study showed that 80% of the patients did not exhibit HPA axis suppression and 10 of 15 patients with suppressed adrenal function had received prior long-term systemic steroid therapy. Another study [61] investigated the corticol response of 13 patients, who had been treated with high dose BDP or BUD for 2 to 6 years, to the insulin-induced hypoglycemia stress test. Six patients failed to achieve plasma cortisol values above 500 nmol/l, but there was no dose response relationship in these patients and they were less stressed as indicated by high arterial oxygen content and peak expiratory flow. These studies suggested that even patients receiving high dose BDP treatment do not appear to have a suppressed adrenal function with clinical relevance.

Since the appearance of the BUD and FP, there have been quite a number of studies that compared the systemic effects of BDP with these two drugs. Most of them have been thoroughly reviewed by Lipworth [55] and Barnes et al. [1]. Despite the amount of available information, inconsistencies still exist in the findings because of the fact that many factors can influence the systemic effects of corticosteroids, such as dose and frequency of administration, timing and duration of treatment, difference in inhalor and propellant, systemic corticosteroids use history, subject selection (normal vs patients, children vs adults), interindividual varibility and markers used. But in general, it is agreed that long-term therapy with dose up to 800 ug/day of BDP is not associated with clinically relevant systemic effects and none of these three drugs has obvious advantage over the other two.

**Effects on airway inflammation.** Eosinophils, mast cells and T-lymphacytes are believed to be he major types of inflammatory cells for the bronchial asthma [62]. The

number of these inflammatory cells has therefore been a major focus of endobronchial

biopsy assessment in the studies of inhaled corticosteroids. One early study [63] found

that BDP significantly reduced the number of eosinophils in the epithelium and lamina

propria of biopsy samples from asthmatic patients. In one study involving 10 atopic

asthmatic patients, inhaled BDP (2 mg/day for 2 week followed by 1 mg/day for 4 weeks)

caused a significant reduction in epithelial and mucosal mast cells and eosinophils and

submucosal T-lymphocytes [64]. A placebo controlled study of mildly asthmatic patients

reported that 4 months of treatment with inhaled BDP 1000 ug/day resulted in significant

decrease in the number of both mast cells and eosinophils [65]. Another uncontrolled

study showed that inhaled BDP 2000 µg/day not only reduced eosinophils and mast cell

counts, but also caused a significant reduction in the epithelial cell number, which is

believed to play an important role in the regulation of airway funtion [66]. A recent

study [67] compared the effects of ketotifen, an anti-allergic compound, disodium

cromoglycate and BDP on bronchial mucosa and asthma symptoms in atopic asthmatic

patients. The findings confirmed the results from earlier studies. By reducing airway

inflammation, BDP also consistently reduced airway hyperresponsiveness in adults and

children with asthma [68].

**Effects on the clinical indices.** The clinical indices most commonly used to

evaluate the status of asthmatic patients are symptom score, peak expiratory flow (PEF)

and forced expiratory volume in 1 second (FEV1), improvements of which can be used to

assess the efficacy of inhaled corticosteroids. Numerous studies have shown that inhaled

BDP can lead to significant increase in PEF and PEV1 and improvement in symptom

score. After 2 weeks of therapy with BDP at 2000 ug/day, asthmatic patients showed a

significant increase in morning PEF from a median of 424L/min to 471L/min (p=0.01) and FEV1 from a mean of 99.8% to 107.5% (p=0.02).  After a further 4 weeks of BDP therapy at 1000 ug/day, there was a significant reduction in total asthmatic symptom score (p=0.008).  The improvements in the mean morning PEF and FEV1 were maintained over that time [64].  Most recent data came from the comparative studies between BDP with BUD or FP.  A review [52] about 18 comparative studies of BDP vs BUD showed that BDP and BUD were clinically equivalent in both efficacy and tolerability on an equal dose base.  Due to the fact that many of the studies were short-term trials in small number of patients, the conclusion is not very convincing.  Since then, there have been more studies comparing the efficacy of BDP and BUD.  A study by Tjwa [69] suggested that if administered by Turbuhaler, BUD is more effective than BDP Rotahaler at an equal dose of 400 ug/day in improving PEF, FEV1 and airway responsiveness.  In another two studies [70, 71], BUD Turbuhaler was found to be as effective as or even more effective than BDP pMDI even at twice dose, i.e. BUD:BDP=1:2.  It appeared that the delivery device improved the lung deposit percent of BUD and as a result increased the efficacy of BUD.  Two recent studies [72, 73] comparing hydrofluoroalkane-134a (HFA)-BDP Autohaler, a new formulation of BDP, and BUD Turbohaler found that HFA-BDP Autohaler at half the dose of BUD Turbohaler produced equivalent improvement in morning PEF with additional benefits on reduction of symptom score.  It is therefore not possible to draw any conclusion about the comparative efficacy of BDP and BUD before an equivalent delivery system is available for both of them.

In a recent review, Barnes et al. [74] performed a meta-analysis of seven studies comparing BDP and FP and concluded that BDP is as effective as FP in improving PEF if given clinically equivalent dose, i.e. BDP 400-2000 ug/day and FP 200-1000 ug/day. Another review by Kelly [75] compared other results besides PEF from 6 previously reviewed studies and another one not covered by Barnes, all of which were considered to be well-designed and sufficiently powered clinical comparison between BDP and FP. Five of 6 studies showed that BDP has the same efficacy as FP in improving FEV1 at equipotent dose. The reason why the other study showed different result is that patients were treated with same doses of BDP and FP, i.e. both at 750 ug twice daily. In terms of improvement in symptom score, 5 of 6 studies showed no difference for BDP and FP with the other one showing that FP achieved more improvement in symptom score during the nighttime, but BDP was more effective during the daytime. In general, these results supported the conclusion from Barnes et al.. There have been some new comparative studies in recent years. Raphael et al. [76] reported that at both low-dose (88 ug twice daily) and medium-dose (220 ug twice daily) FP was more effective than BDP in improving FEV1 even though BDP was given higher doses (168 ug twice daily and 336 ug twice daily). Another study [77] also showed that FP 1000 ug/day was more effective than BDP 2000 ug/day in improving lung function in terms of change of FEV1. But Bootsma et al. [78] reported that BDP 1500 ug/day was as effective as FP 750 ug/day according to their effects on parameters of inflammation in peripheral blood of patients with asthma and PC20 histamine tolerance and FEV1 change. In a study [79] involving 30 children with persistent asthma, it was shown that BDP 1500 ug/day is as effective as FP 750 ug/day in improving PEF and symptom score. Another study [80] also showed

that BDP 1500 ug/day and FP 750 ug/day improved FEV1, PEF, asthma symptoms, and

bronchial hyperresponsiveness to the same extent.  The recently developed new

formulation of BDP, hydrofluoroalkane-134a (HFA)-BDP, has been reported to be 2-2.5

times more effective than the conventional chlorofluorocarbon (CFC)-BDP [81].  As a

result, HFA-BDP and FP was reported to be equally effective at the same dose of 400

ug/day.  In addition, at higher doses, HFA-BDP 800 ug/day appeared to be as effective as

FP 1000 ug/day in improving lung function (PEF, FEV1) and asthma control (symptom

score) [82].  Further studies are needed to assess the relative efficacy of HFA-BDP.

## Objectives

Despite the fact that BDP has been in the market for more than 30 years, the lung

targeting of BDP has never been studied and no well-defined pharmacokinetic and

pharmacodynamic models have been developed for BDP while these models were

developed for some newer glucocorticoids, such as triamcinolone acetonide, budesonide

and fluticasone propionate, to provide helpful guidance for better asthma treatment and

systemic side effect control [83]. It is yet to be determined how the prodrug property of

BDP affects the pulmonary targeting. The fast metabolism of BDP in human body and

the lack of a sensitive analytical assay for BDP and 17-BMP made it difficult to develop

accurate pharmacokinetic model for BDP and 17-BMP. The total concentration of

beclomethasone (BOH), the metabolite of 17-BMP, was used to approximate the

concentration of 17-BMP to study the pharmacokinetics of 17-BMP because it was

believed that the majority of total BOH was from 17-BMP [36, 44, 84]. This approach,

however, appeared to underestimate the bioavailability of the oral route based on a recent

study by Daley-Yates et al. [30]. The availability of sensitive assays for BDP and 17-

BMP made it possible to investigate the detailed pharmacokinetics of BDP and 17-BMP

after intravenous study along with intranasal, inhalation and oral studies, assuming that all injected BDP would be biotransformed to 17-BMP in vivo [30].

In the present study, with intratracheal and intravenous administration of BDP or 17-BMP, the lung targeting of BDP will be evaluated and the detailed pharmacokinetics of BDP and 17-BMP will be studied to determine the extent of BDP activation in the lung. The receptor occupancy in tissues will be used as the pharmacodynamic marker to build a pharmacokinetic and pharmacodynamic model for BDP and 17-BMP.

**Specific Aim 1: Assay Development And Validation**

To develop and validate a sensitive bioanalytical assay to quantify the concentration of beclomethasone dipropionate and its major metabolite, 17-beclomethasone monopropionate, in rat plasma and various tissues, such as lung, liver and kidney.

**Specific Aim 2: Animal Study**

To evaluate the pulmonary targeting of beclomethasone dipropionate after inhalation of micronized dry powder by an ex-vivo receptor binding method in rats.

**Specific Aim 3: Pharmacokinetic And Pharmacodynamic Modeling**

To develop a pharmacokinetic and pharmacodynamic model for beclomethasone dipropionate and 17-beclomethasone monopropionate using the receptor occupancy in the tissues as the pharmacodynamic marker.

**Specific Aim 4: Formulation Comparison**

To compare pharmacokinetics and pharmacodynamics of chlorofuorocarbon (CFC) and hydrofluoroalkane (HFA) metered-dose inhalers of beclomethasone dipropionate in clinical studies.

CHAPTER 2
DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS

**Introduction**

The lack of sensitive analytical assays for beclomethasone dipropionate (BDP) and

its active metabolite, beclomethasone 17-monopropionate (17-BMP), was one of the

main reasons for the delayed clarification of pharmacokinetics of BDP. A very sensitive

HPLC/MS assay was used in one study [34], but no details were given about the assay.

In another study, a very sensitive assay was developed for the metabolite of 17-BMP,

beclomethason (BOH), and the total BOH concentration was measured to approximate

the concentration of 17-BMP to study the pharmacokinetics of 17-BMP because it was

believed that the majority of total BOH was from 17-BMP [36, 44, 84]. This approach,

however, appeared to underestimate the bioavailability of the oral route based on a recent

study by Daley-Yates et al. [30]. A sensitive assay for BDP and 17-BMP is, therefore,

necessary for accurate evaluation of the pharmacokinetics of BDP and 17-BMP. In this

chapter, a sensitive analytical assay will be developed and validated to quantify the

concentrations of BDP and 17-BMP in rat plasma and various rat tissues, such as lung,

liver and kidney.

**Materials and Methods**

**Chemicals**

Micronized beclomethasone dipropionate (Figure 2.1) was kindly provided by 3M.

17-beclomethasone monopropionate (Figure 2.1) was purchased from European

Directorate for the Quality of Medicine (EDQM). The internal standard, fluticasone

propionate, was kindly provided by Glaxo Group Research (Herts, UK). Double distilled

deionized water was prepared in our lab (Corning AG-3 still, Corning, NY) and filtered

through 0.2μm filter. Methanol, ethanol, formic acid, ammonium acetate, ethyl acetate

and heptane were of HPLC grade and purchased from Fisher Scientific (Fisher,

Springfield, NJ). Rat and human blank plasma were obtained from the Civitan regional

blood system (Gainesville, FL). The solid phase $LC_{18}$ (3 ml) cartridges for sample

extraction were obtained from Supelco (Bellefonte, PA).

**HPLC/MS/MS Conditions**

The high performance liquid chromatography was performed isocratically at

ambient temperature using a Waters $C_{18}$ 3.5um column (Symmetry, 2.1×50mm i.d.,

Milford, MA) preceded by a Whatman 3.5-μm ODS $C_{18}$ guard column cartridge (Clifton,

NJ). The mobile phase consisted of 33mM ammonium acetate/0.33% formic acid buffer

(pH 3.44) -methanol (30:70, v/v), delivered by a flow-rate of 0.3 ml $min^{-1}$ by a LDC

Analytical constaMetric®3500 solvent delivery system (LDC/Milton Roy, Riviera

Beach, FL). The mobile phase was degassed using helium for approximately 10 min

before use. The mass spectrometer was a Micromass Quattro-LC-Z (Beverly, MA) triple

quadrupole mass spectrometer equipped with an electrospary ionization (ESI) ion source.

Positive ESI was chosen after tuning with three analytes. The source temperature was set

to 120° C and the desolvation temperature was set to 450° C. Capillary and cone

voltages were set to 3.0 kV and 30 V respectively. Argon was used as the collision gas.

The mass spectrometer was linked to a Perkin Elmer ISS 200 autosampler via contact

closure and the operation was controlled by computer software, Masslynx 3.1. Data

analysis was performed using Masslynx software. The calibration curves were plotted as

the peak area ratio (analyte/I.S.) versus analyte concentration with a weighting factor of the reciprocal of the analyte nominal concentration.

## Preparation of Calibration Standards And Quality Control Samples

### Plasma samples

Primary stock solutions were prepared by dissolving the compounds or internal standard in methanol.  Appropriate dilutions of the stock solutions with drug-free rat plasma were made subsequently in order to prepare the working solutions for BDP and 17-BMP.  Two different series of stock solutions were prepared from different weightings for calibration standards (CCs) and quality control samples (QCs).  The CCs and QCs ranged from 0.05-5 ng ml$^{-1}$ for both analytes.  Aliquots of 1 ml sample were transferred into thick wall glass tubes with enzyme inhibitor (EDTANa$_2$+NaF, 17mg: 17.5mg), capped and stored at approximately -69 °C.  The internal standard (I.S.) stock solution was diluted with methanol to produce a working solution of 5 ng ml$^{-1}$.

### Tissue samples

The primary stock solution for CCs was diluted with methanol to produce the working solutions of 1, 2, 4, 10, 20, 40 and 100 ng/ml.  Fifty microliters of each working solution was added to 1 ml blank rat tissue homogenate (liver, lung or kidney taken from the placebo rat as described under Receptor Binding Assays in Chapter 3) to generate 0.05, 0.1, 0.2, 0.5, 1, 2 and 5 ng/ml CCs.  For QCs, the primary stock solution for QCs was diluted with methanol to produce the working solutions of 1, 2, 10 and 40 ng/ml which then were diluted in 1 ml blank rat tissue homogenate (liver, lung or kidney) to generate 0.05, 0.1, 0.5, and 2 ng/ml QCs.  Fifty microliters of I.S. working solution was added into each CC or QC sample.  After vortexing, four milliliters of ethyl acetate was

added to inhibit the metabolism of BDP. The samples were capped and stored at approximately -69 °C.

**Sample Preparation**

**Plasma samples**

BDP and 17-BMP were extracted from rat or human plasma by solid phase extraction. Plasma samples (1ml) were thawed at room temperature. After addition of 50 µl of I.S. working solution to 1ml plasma, they were then vortexed and 1ml of 30% ethanol (v/v) was added into the plasma sample. After 15 minutes of incubation at 4°C, the mixture was centrifuged at 4000rpm for 15 minutes to remove the protein precipitate. The supernatant was then transferred onto the SPE extraction column preconditioned with ethanol and water. The column was drained under necessary vacuum to ensure that the sample was aspirated at a drop wise flow rate. The column was then washed with a) one column volume of 25% ethanol solution, b) one column volume of water solution and c) 2 ml of 2:98 (v/v) ethyl acetate/n-heptane mixture. Finally, the sample was eluted with 3 ml of 35:65 (v/v) ethyl acetate/n-heptane mixture at a drop wise flow rate, evaporated in a vacuum centrifuge, and reconstituted in 50 µl of mobile phase. A volume of 30 µl was injected into the HPLC/MS/MS system.

**Tissue samples**

Rat tissues (liver, lung and kidney) were homogenized in appropriate amount of buffer (1g in 10 ml of buffer for liver and 1g in 4 ml of buffer for lung and kidney, see 2.2.3.4 for details about the buffer system). One milliliter of the homogenate was transferred to a thick wall glass tube to which 50 µl of I.S. working solution was added. After vortexing, 4 ml of ethyl acetate was added. The mixture was stored at approximately -69 °C after mixing. At the time of analysis, the frozen samples were

thawed at room temperature and then were shaken on a shaker for 15 minutes. After

centrifugation (5 min, 3070 g), the upper organic layer was transferred into a new tube,

evaporated in a vacuum centrifuge, and reconstituted in 50 µl of mobile phase. A volume

of 30 µl was injected into the HPLC/MS/MS system.

**Validation**

**Selectivity**

Plasma and tissue samples from six different rats were extracted and analyzed to

assess the potential endogenous interferences from human plasma. Any apparent

response at the retention times of BDP, 17-BMP and I.S. was compared to the response at

the lower limit of quantification for BDP, 17-BMP and to the response at the working

concentration for I.S..

**Recovery**

The recovery of BDP and 17-BMP was evaluated in triplicate at three

concentrations (0.1, 0.5 and 2 ng/ml) by comparing the peak areas of BDP and 17-BMP

to the peak areas of corresponding compounds in samples prepared by spiking extracted

drug-free plasma or tissue samples with the same amount of compounds at the step

immediately prior to injection. The same method was used to assess the recovery of I.S.

at the working concentration.

**Calibration and sample quantification**

Duplicate seven point standard curves ranging from 0.05 to 5 ng/ml of BDP and

17-BMP were run on three separate days. Calibration curves (y=ax+b), represented by

the plots of the peak-area ratios (y) of the analyte to I.S. versus the nominal concentration

(x) of the calibration standards, were generated using linear least square regression.

Actual, quality control and stability samples were calculated from the resulting area ratio of the analyte to I.S. and the regression equation of the calibration curve.

**Accuracy and precision**

Within-day accuracy and precision were evaluated by analysis of quality controls at concentrations of 0.05, 0.1, 0.5 and 2 ng/ml (n=6 at each concentration) on the same day. The same experiment was repeated on three different days to assess the between-day accuracy and precision. Accuracy was calculated as the percentage ratio of measured concentration to nominal concentration. Precision was expressed as the coefficient of variation. The lower limit of quantification (LLOQ) of the assay was also determined during this process.

**Stability**

Freeze and thaw stability was determined by analyzing triplicate quality control samples at the concentrations of 0.1, 0.5 and 2 ng/ml following one cycle of freezing at -69 °C and thawing. Short-term temperature stability was evaluated at the same concentrations after the samples (plasma or tissue samples) were thawed and kept at room temperature for 6 hours. The bench top stability at the same concentrations after extraction and reconstitution was evaluated over 5 hours. Stability was expressed as the percentage ratio of measured concentration to the nominal concentration.

<div align="center">

**Results and Discussions**

</div>

**Mass spectrometry/Chromatography**

Both atmosphere pressure chemical ionization (APCI) and electrospray ionization (ESI) modes were tuned for all compounds. Positive ESI (ESI+) was chosen for the better sensitivity. All these parent ions generated specific strong daughter ions under multiple reaction monitoring (MRM) mode. The parent mass spectra and daughter ion

mass spectra of two analytes and of the I.S. are shown in Figure. 2-1.  The transitions

selected for monitoring all compounds are listed in table 2.1.  The instrument was tuned

to give maximum abundance of the daughter ion for each compound.  Figures. 2-2, 2-3,

2-4 and 2-5 showed the typical chromatograms for blank plasma and tissue matrices and

their corresponding samples spiked with 0.05 ng/ml BDP, and 17-BMP and 0.25 ng/ml

I.S..  The analysis time was 7.0 min with 17-BMP eluted at 2.38 min, I.S. at 2.94 min and

BDP at 5.37 min.  The resulting calibration curves were linear with correlation

coefficients >0.99.  The metabolite of 17-BMP, beclomethasone (BOH) was also tuned

for assay development, but the preliminary data showed that almost no detectable BOH

was found in rat plasma or tissue samples. No extra efforts were tried.

Table 2-1.  The transition channels for two analytes and the internal standard

| Compound | Parent ion | Daughter ion |
| --- | --- | --- |
| Beclomethasone dipropionate (BDP) | 521 | 319 |
| beclomethasone 17-monopropionate (17-BMP) | 465 | 279 |
| Beclomethasone (BOH) | 409 | 313 |
| Fluticasone propionate (I.S.) | 501 | 313 |

**Internal Standard Selection**

Isotopically-labeled compounds were not available.  Therefore, structural analogs

were screened to find suitable compounds for internal standard.  Fluticasone propionate

(FP) was finally chosen as the internal standard for its better sensitivity and simultaneous

good correction for both BDP and 17-BMP as shown by the data.

**Sample Preparation**

Due to the relatively small number of samples but many different matrices (plasma

and three tissues) it was not the goal to perform extensive method optimization but to

provide sample analysis with sufficient accuracy, precision and robustness of the assays.

It was preferred to apply the same sample preparation and analysis technique for all

compounds and matrices with minor variations to save method development time and to allow a rapid switching from one assay to another. Therefore, a simple liquid-liquid extraction method modified from a previous study [85] was tried for all the matrices. Unfortunately, this method did not work well for plasma samples in terms of the sample cleanness even though it worked for the tissues. A solid phase extraction method from [86] was adopted for plasma samples. Further optimization was tried, but the original method seemed to provide the best recovery balance between BDP and 17-BMP.
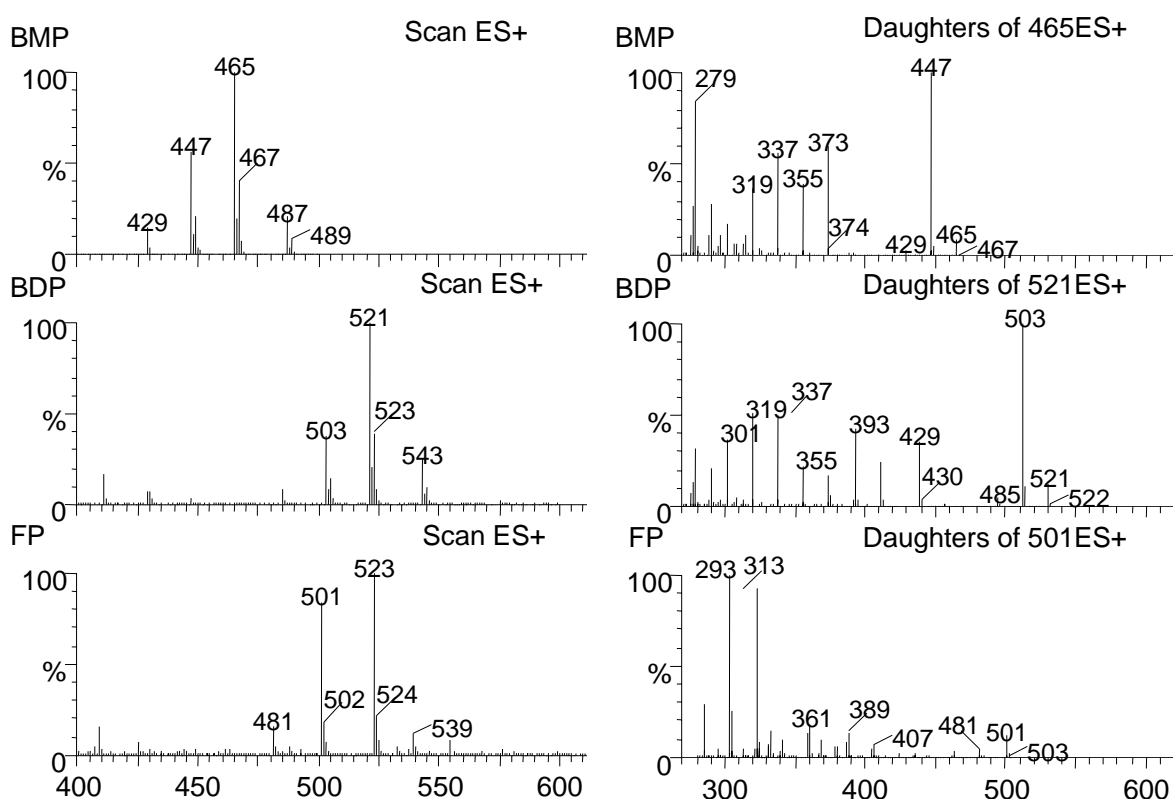


Figure 2-1. Full scan (left) and daughter scan (right) spectra of 17-BMP, BDP and I.S.

**Mobile Phase And Buffer System Selection**

In order to achieve the maximum signal response under ESI (+) condition and the shortest analysis time, the percentage of organic phase in the mobile phase was
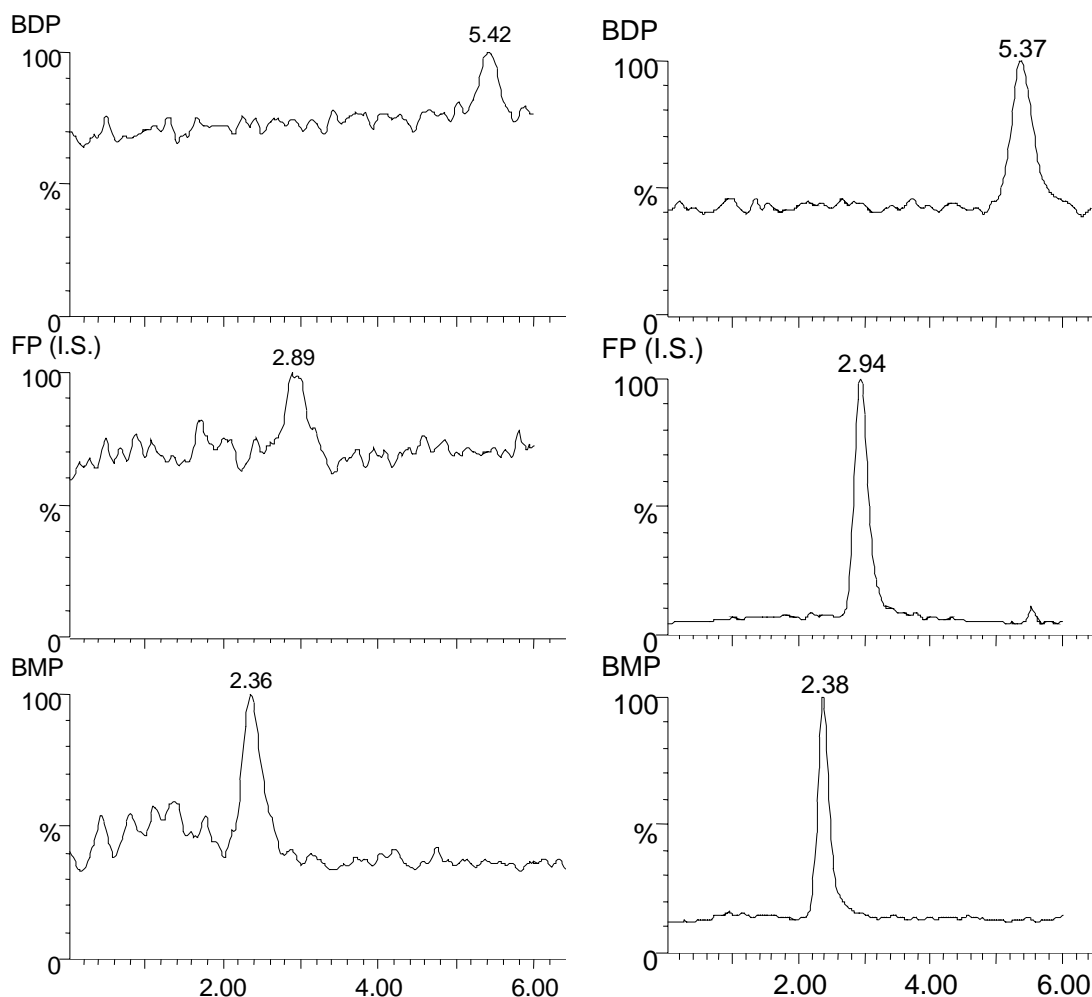
Figure 2-2. Chromatograms of blank plasma (left) and plasma spiked with 0.05 ng/ml analytes and 0.25ng/ml I.S (right).

maintained as high as possible while still avoiding the early front peak which contained

most of the hydrophilic response-suppressing endogenous interferences (Figure 2-6).

Methanol and acetonitrile were screened for the organic phase. Methanol was selected

due to the better sensitivity. Ammonium acetate buffer system was optimized in terms of

the concentration and the composition of the buffer system. Acetate acid and formic acid

were screened for the acid component. Formic acid was selected due to its better

sensitivity and higher buffering capacity while achieving that sensitivity. Finally, the

mobile phase consisted of 10mM ammonium acetate/0.1% formic acid buffer (pH 3.44) -

methanol (30:70, v/v).
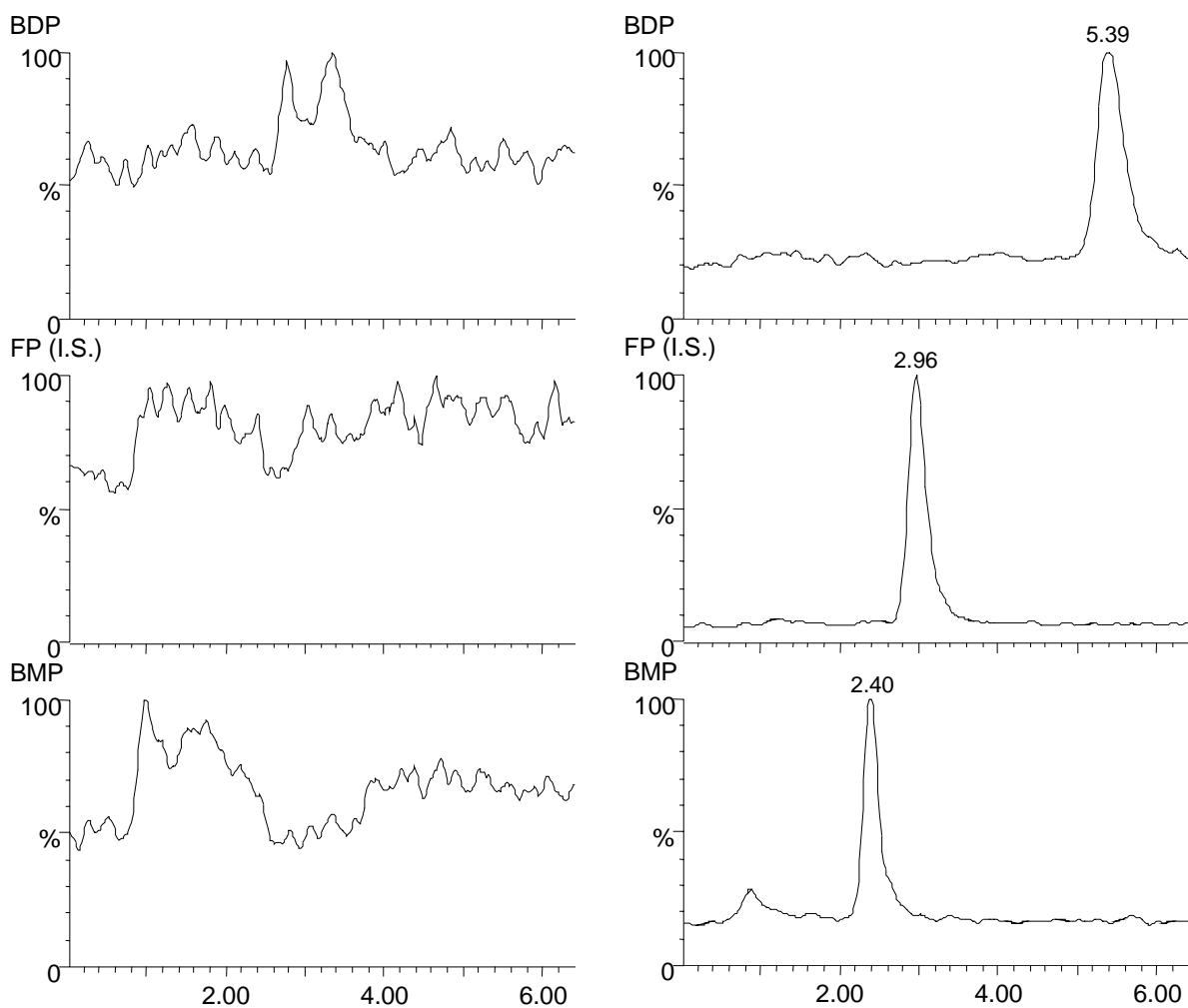


Figure 2-3. Chromatograms of blank liver (left) and liver spiked with 0.05 ng/ml analytes
            and 0.25ng/ml I.S (right).

**Matrix Effect**

The application of ESI and the relative fast chromatographic separation led to the

questions about the influence of matrix effects on the analyte determination.  To show the

potential matrix effects caused by matrix components, a post-column infusion experiment

[87] was carried out.  Continuous infusion of all compounds was performed via an
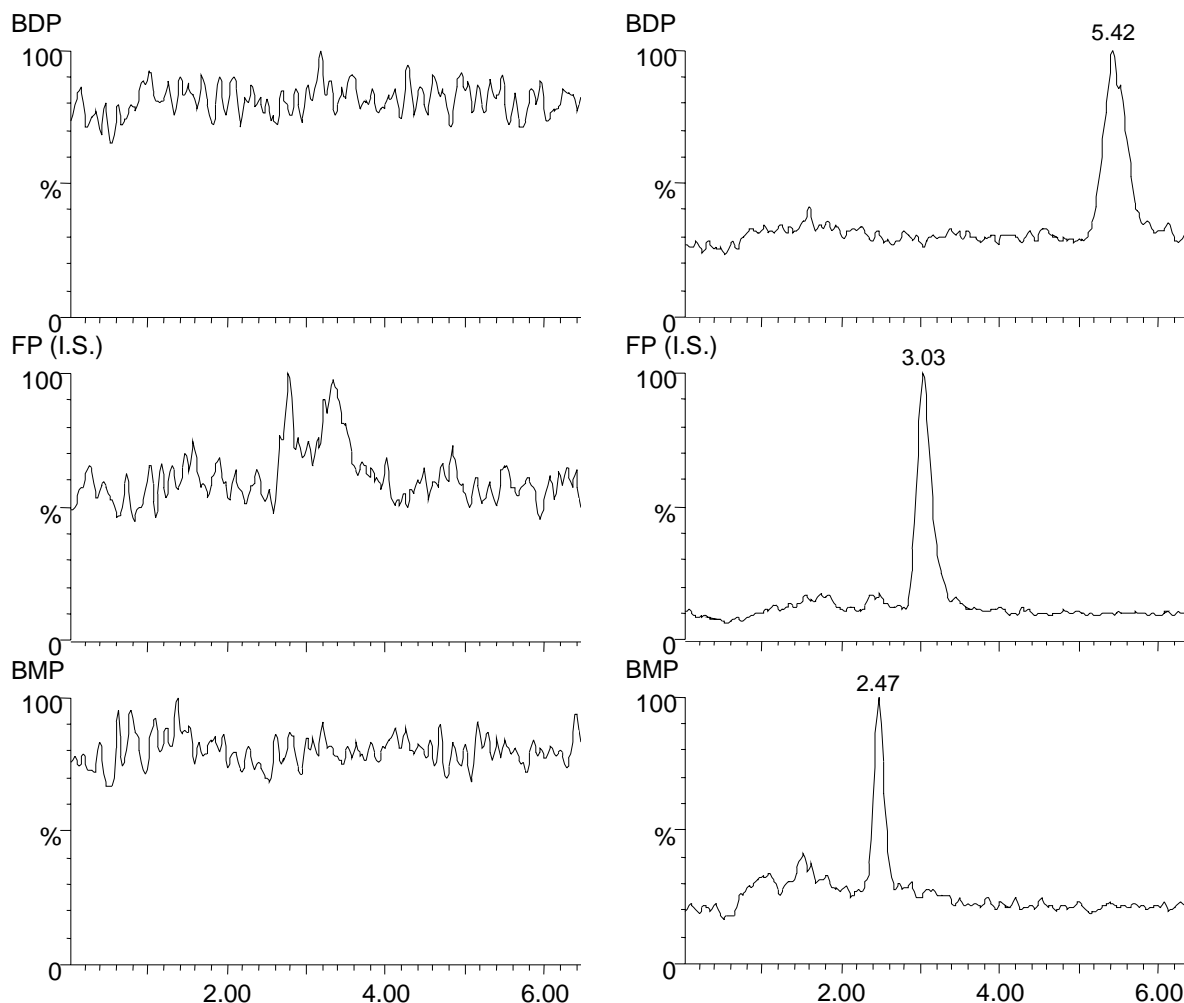
Figure 2-4. Chromatograms of blank lung (left) and liver spiked with 0.05 ng/ml analytes and 0.25ng/ml I.S (right).

infusion pump and T-piece after the analytical column, and blank matrix extract was

injected into the HPLC system.  Suppressive matrix effect was observed at the retention

times of 0.7 and 2.6 minutes after injection of all matrices compared to the injection of

mobile phase (Figure 2-6).  These suppressive effects were not compound-specific since

they were observed in the MRM channels for both the analytes and I.S..  However, the

determination of the analytes was not affected because BDP, 17-BMP and I.S. were

eluted at 5.37, 2.38 and 2.94 minutes.  Even though the infusion covered only 20 minutes

and there could be some late eluting matrix components after 20 minutes affecting the

subsequent runs, the satisfactory validation data (Table 2-3) indicated that this possibility was minor.

BDP

BDP
5.40

FP (I.S.)

FP (I.S.)
2.94

BMP

BMP
2.34

2.00    4.00    6.00

2.00    4.00    6.00
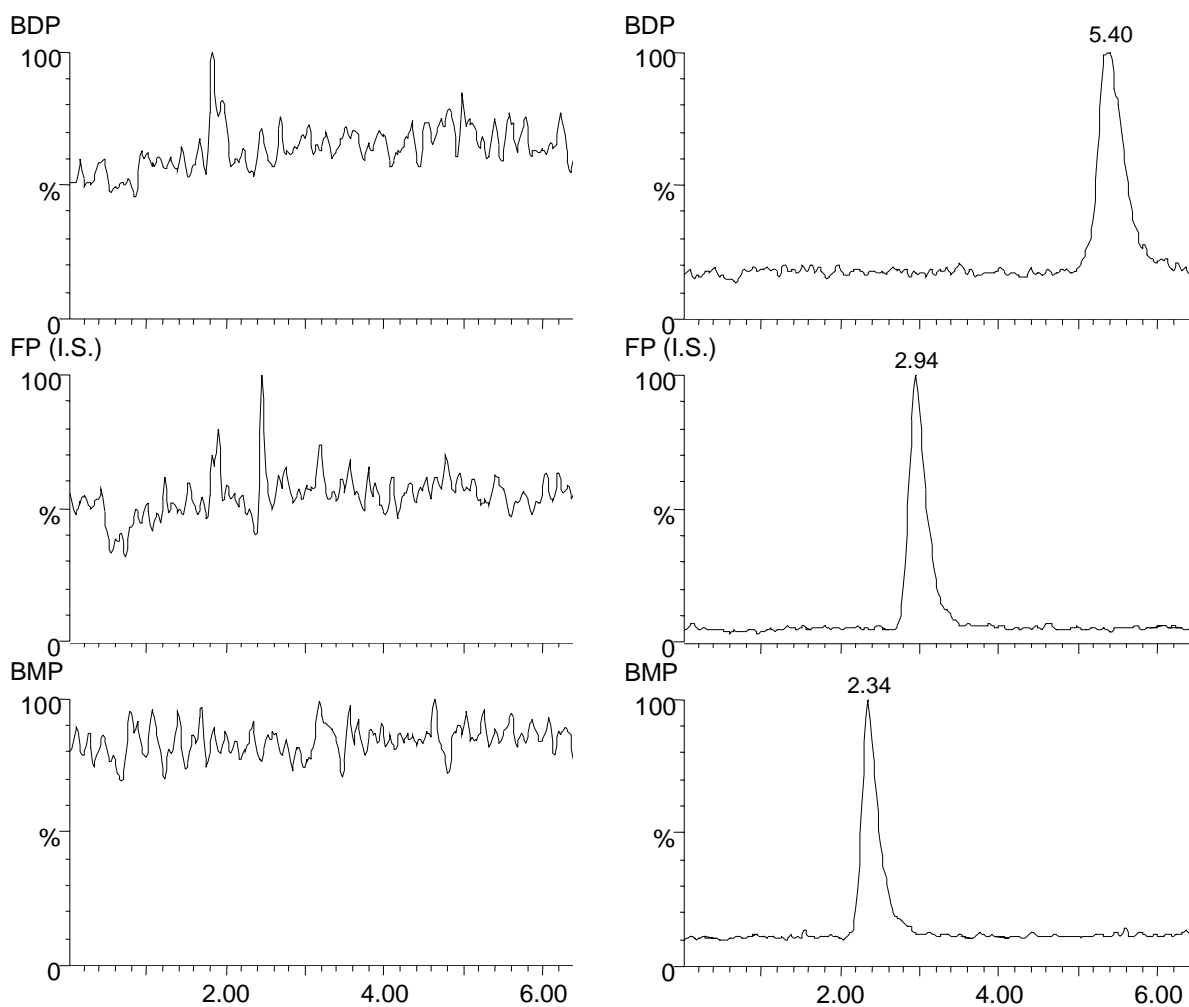
Figure 2-5. Chromatograms of blank kidney (left) and liver spiked with 0.05 ng/ml analytes and 0.25ng/ml I.S (right).

**Assay Performance**

**Selectivity**. Drug-free plasma and tissue samples from six rats were screened during method validation and Figures. 2-2, 2-3, 2-4 and 2-5 compared the chromatograms for extracted drug-free samples and samples spiked with 0.05 ng/ml BDP and 17-BMP and 0.25 ng/ml I.S..  Even though minor peaks were observed at the retention times of

BDP, 17-BMP and I.S. in plasma samples (Figure 2-2), the accuracy and precision of the

assay were within the acceptable range (Table 2-3). No significant interferences were

observed at the retention times of BDP, 17-BMP and I.S. in the tissue samples (Figures 2-

3, 2-4 and 2-5).
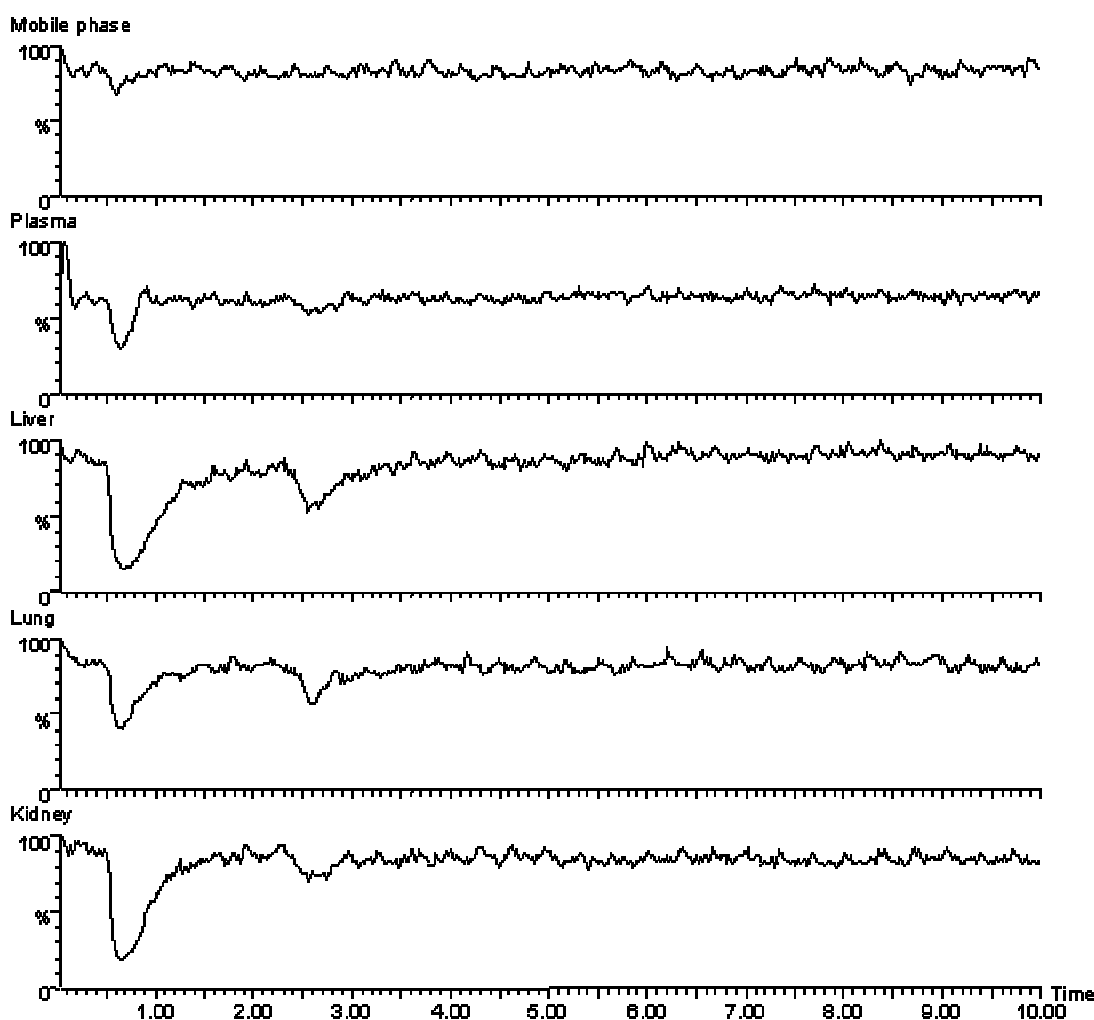


Figure 2-6: Influence of different matrices on analytes and I.S. signals with continuous
infusion of 5 μg/ml all compounds at 10 μl/min after column and injection of
blank matrix extracts into HPLC system.

**Recovery**. The recoveries (Table 2-2) from plasma samples for BDP and 17-BMP

were $83.8 \pm 4.5\%$ and $48.3 \pm 1.2\%$, respectively, at 0.1 ng/ml (n=3), $83.0 \pm 4.1\%$ and

$59.5 \pm 1.8\%$ at 0.5 ng/ml (n=3) and $82.6 \pm 1.8$ and $60.0 \pm 4.9$ at 2 ng/ml (n=3). I.S.

recovery was also determined to be 70% at the working concentration of 5 ng/ml.  The

recoveries from tissue samples for 17-BMP and I.S. were listed in table 2-2.  Despite the

relatively low values for 17-BMP in plasma and some tissue (liver and kidney) samples,

the recoveries were fairly reproducible as shown by the data.

Table 2-2: The recoveries of BDP, 17-BMP and I.S. from plasma and tissue samples*

| Concentration (ng/mL) | BDP | | | 17-BMP | | | I.S. |
|---|---|---|---|---|---|---|---|
| | 0.1 | 0.5 | 2 | 0.1 | 0.5 | 2 | 0.25 |
| Plasma | 83.8 | 83.0 | 82.6 | 48.3 | 59.5 | 60.0 | 70 |
| | (4.5) | (4.1) | (1.8) | (1.2) | (1.8) | (4.9) | (6.5) |
| Liver | 83.5 | 83.2 | 81.1 | 49.9 | 49.5 | 51.1 | 65.3 |
| | (11.6) | (5.8) | (5.3) | (5.1) | (2.8) | (1.4) | (4.6) |
| Lung | 85.0 | 83.4 | 83.0 | 76.4 | 73.1 | 71.2 | 92.8 |
| | (14.9) | (11.2) | (12.7) | (1.1) | (13.8) | (1.5) | (9.8) |
| Kidney | 79.0 | 76.9 | 79.3 | 32.4 | 30.7 | 33.3 | 40.2 |
| | (13.3) | (7.9) | (3.5) | (5.9) | (4.1) | (5.6) | (5.6) |

*Mean (standard deviation) (n=6)

**Precision and accuracy**. Table 3 lists the within and between-day precision and

accuracy data for BDP and 17-BMP at four quality control levels in plasma and tissue

samples.  Accuracy was calculated as the percentage ratio of measured concentration to

nominal concentration.  Precision was expressed as the coefficient of variation.  The

results showed that this assay is consistent and reliable with good accuracy (86.3-106.4%

at LLOQ and 85.7-105.7% at other concentrations) and precision (<18% at LLOQ and <

15% at other concentrations).  LLOQs were set at 0.05ng/ml for both analytes with an

accuracy of 80-120% and a precision <=20% on a day-to-day basis [88].  The linear

range was 0.05 to 5 ng/ml of BDP and 17-BMP with correlation coefficients >0.99

(Figures 2-7 and 2-8).

**Stability**. BDP and 17-BMP are known to be the substrates for the esterase that is

widely distributed in plasma and various tissues.  $Na_2EDTA$ and NaF were added to the

blood collecting tube for each plasma sample as esterase inhibitor.  PMSF was added to

the tissue buffer as esterase inhibitor, but certain metabolism was still observed for BDP

during sample preparation. Therefore, 4 ml of ethyl acetate was added to the tissue

homogenate right after the homogenization to further inhibit the hydrolysis reaction.

Table 4 and 5 list the data for the freeze thaw stability, the short-term stability under

room temperature and the bench top stability at three concentrations for plasma and tissue

samples. Stability was expressed as the percentage ratio of measured concentration to the

nominal concentration. The results showed that both analytes were stable under

conditions investigated in this study since the measured concentrations were all within 85

-115% of the nominal concentrations.

Table 2-3 Within and between-day accuracy/precision for BDP and 17-BMP in rat
plasma and tissues (n=6)

| | Day | BDP | | | | 17-BMP | | | |
|---|---|---|---|---|---|---|---|---|---|
| Concentration (ng/ml) | | 0.05 | 0.1 | 0.5 | 2 | 0.05 | 0.1 | 0.5 | 2 |
| Plasma | 1 | 89.3/13.8 | 85.7/4.8 | 101.7/5.9 | 102.4/4.0 | 95.5/8.8 | 102.9/14.9 | 109.3/6.2 | 102.3/5.0 |
| | 2 | 97.9/18.0 | 92.2/11.4 | 103.1/5.0 | 101.2/10.0 | 97.1/10.2 | 94.3/9.1 | 89.4/7.9 | 98.6/9.2 |
| | 3 | 92.6/11.3 | 89.5/7.1 | 98.6/6.4 | 97.9/9.4 | 90.5/5.6 | 92.6/6.5 | 95.3/8.6 | 90.6/10.3 |
| Liver | 1 | 86.3/12.8 | 90.1/6.5 | 93.8/10.6 | 91.3/9.1 | 92.0/12.0 | 93.4/11.6 | 102.3/9.4 | 105.7/4.9 |
| | 2 | 90.6/10.3 | 92.4/7.8 | 98.9/8.3 | 95.4/7.6 | 96.7/9.9 | 91.8/9.6 | 102.6/6.2 | 97.0/4.1 |
| | 3 | 93.1/8.4 | 96.7/8.1 | 90.4/7.5 | 98.2/6.2 | 90.9/8.5 | 95.4/7.1 | 100.6/5.3 | 103.5/6.5 |
| Lung | 1 | 105.3/11.8 | 95.1/7.5 | 96.5/6.4 | 97.3/10.5 | 93.7/14.1 | 90.6/10.4 | 102.5/7.4 | 102.5/3.1 |
| | 2 | 106.4/10.6 | 90.4/8.3 | 91.8/7.9 | 94.8/7.3 | 101.3/7.6 | 107.6/11.8 | 103.2/5.5 | 99.8/3.1 |
| | 3 | 99.5/9.8 | 96.3/7.5 | 90.8/6.9 | 99.3/7.4 | 95.6/10.8 | 92.5/9.3 | 98.3/8.4 | 97.7/4.6 |
| Kidney | 1 | 90.6/12.5 | 98.3/7.9 | 94.2/8.9 | 89.3/10.1 | 97.3/10.8 | 85.4/11.3 | 98.9/6.1 | 102.7/2.1 |
| | 2 | 93.8/10.4 | 91.4/6.8 | 99.4/6.8 | 92.8/7.1 | 101.7/11.7 | 101.3/8.0 | 98.1/3.8 | 104.8/2.2 |
| | 3 | 89.3/9.4 | 90.6/9.2 | 93.6/9.6 | 98.2/8.8 | 95.3/9.4 | 90.3/7.9 | 93.9/7.1 | 98.4/5.3 |

Table 2-4. Stability of BDP and 17-BMP in rat plasma samples under various conditions*

| Analyte | BDP | | | 17-BMP | | |
|---|---|---|---|---|---|---|
| Nominal concentration (ng/ml) | 0.1 | 0.5 | 2 | 0.1 | 0.5 | 2 |
| Freeze thaw (1 cycle) | 93.9±2.0% | 95.1±8.6% | 106.6±5.6% | 99.1±12.5% | 103.7±5.4% | 107.8±5.5% |
| Short term (6 hours) | 90.1±10.1% | 92.4±13.1 | 101.9±9.5% | 92.8±9.4% | 93.87±3.6% | 104.0±4.1% |
| Bench top (7 hours) | 98.5±10.1% | 96.8±9.5% | 101.1±10.3% | 97.6±10.2 | 102.9±15.4% | 101.2±4.9% |

* Stability was expressed as the percentage ratio of measured concentration to the nominal concentration (n=3)
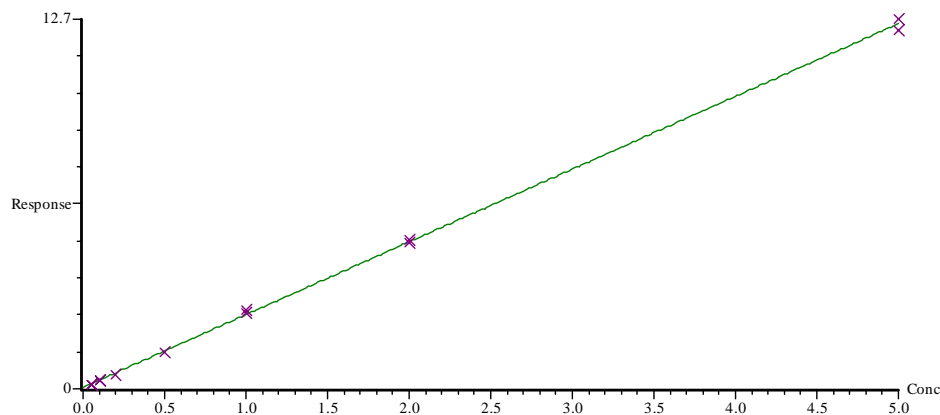
Figure 2-7. The calibration curve of BDP in rat plasma samples
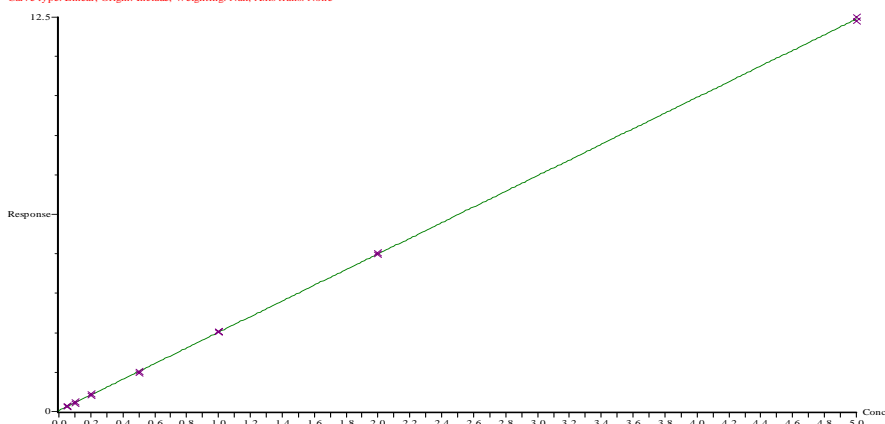


Figure 2-8. The calibration curve of 17-BMP in rat plasma samples

## Conclusion

A simple, sensitive and selective LC-(ESI+)-MS-MS method was developed using

a solid phase extraction procedure for simultaneously quantifying BDP and its major

metabolite, 17-BMP, in rat plasma and tissue samples.  Validation results have shown

that the method is robust and meets the requirements of the pharmacokinetic investigation

of inhaled BDP.  While this assay was being validated, another HPLC/MS/MS assay was

developed in a Perkin Elmer API-3000 mass spectrometer with gradient elution HPLC

system in human plasma [30]. The same LOQ of 50 pg/ml was achieved for BDP, 17-

BMP and BOH in the reported assay.

CHAPTER 3
ASSESSMENT OF PULMONARY TARGETING OF BECLOMETHASONE
DIPROPIONATE AFTER INHALATION OF MICRONIZED DRY POWDER USING
AN *EX VIVO* RECEPTOR BINDING ASSAY IN RATS

## Introduction

BDP is an inactive prodrug for the treatment of asthma. Due to the high
lipophilicity, BDP has a slow dissolution rate, which will retard the absorption of drug
from the airways and increase the mean residence time of BDP in the lung (change in the
pharmacokinetics of the drug) as well as the mean pulmonary effect (change in the
pharmacodynamics) and may improve the pulmonary targeting. At the same time,
however, the prodrug property of BDP requires pulmonarily deposited BDP to be
converted to its active form, beclomethasone 17-monopropionate (17-BMP), in the lung
to have targeting effect for asthma treatment. Since lung is a highly perfused organ and it
takes time to metabolize BDP into 17-BMP, a significant percentage of pulmonarily
deposited BDP might be directly absorbed into the circulation system before activation
happens in the lung. And it is well known that systemically absorbed glucocorticoids
will cause all kinds of side effects. As a result, intact BDP that is directly absorbed into
circulation system will be converted into 17-BMP in blood and other tissues and
contribute to the side effects without any targeting effect for asthma treatment.

## Hypothesis

The prodrug property will negatively affect the pulmonary targeting of BDP, which
can be evaluated by the difference in receptor occupancy in lung and a systemic organ.

## Rationale

Receptor occupancy was selected as a pharmacodynamic surrogate marker because early work in cell systems found a close correlation between the extent of receptor occupancy and the extent of the biological response.  In addition, a direct relation between the receptor affinity of a glucocorticoid and the activity at the site of action (e.g. the skin blanching activity) has been demonstrated.  Since the pharmacological desired and adverse effects of glucocorticoids are induced by the same receptors, lung targeting has been defined in this study as the degree in which the occupancies of pulmonary (lung) and systemic (liver, kidney or brain) receptors differ.

## Materials and Methods

### Chemicals and Devices

Micronized beclomethasone dipropionate was kindly provided by 3M.  17-beclomethasone monopropionate was purchased from European Directorate for the Quality of Medicine (EDQM).  Double distilled deionized water was prepared in our lab (Gainesville, FL).  Analytical grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO.).  $(1,2,4-^3H)$ triamcinolone acetonide (45 Ci/mmol) was purchased from New-England Nuclear (Boston, MA).  Extra-fine lactose monohydrate was purchased from EM$^®$ (Hawthorne, NY).

Insufflator (Model DP-3) for intratracheal administration of dry powder was purchased from Penn-Century (Philadelphia, PA). Microscope …

### Animals

The project was approved by the Animal Care Committee, University of Florida, an AAALAC approved facility.  Specific-pathogen-free, non-adrenalectomized male F-344 rats, weighing 200-250 grams, were obtained from Harlan Spraque Dawley Inc. (IN,

USA) and were housed 12 hrs. in a light/dark, constant temperature environment prior to the experiment.

**Particle size distribution of BDP and 17-BMP dry powder**

The particle size distributions of BDP and 17-BMP dry powder were measured with an electron microscope (EM) with Image Pro Optical Analysis (MediaCybernetics Inc., USA) at two magnifications (×35 and ×100).

**Administration of Drugs**

Each rat was weighed and placed in a rat holder for intraperitoneal administration of the anesthetics (fresh preparation of the combination of 1.5 ml of ketamine 10%, 1.5 ml of xylazine 2%, and 0.5 ml of acepromazine 1% at the dose 1 ml/kg). The depth of the anesthesia was checked by tail pinch or pedal withdrawal reflex. For the intratracheal administration (IT), after loss of reflexes, one inch of a special round-tipped canula attached to a delivery device for administration of dry powders (Penn-Century, Philadelphia, PA) was introduced into the trachea through the mouth. A mixture of lactose-drug mixture (100μg/kg of drug) or lactose alone was placed in the chamber of the device and instilled in the lungs with insufflation of 3 ml of air. For the intravenous administration (IV), either 100μl of IV solution (200μg/ml for BDP or 180 μg/ml for 17-BMP in a mixture of PEG 300 and saline 3:1 v/v) or 100μl of the solvent (for the placebo rat) was injected into the tail vein. Then the rats (one rat per time point) were decapitated with a guillotine at 0.5h, 1h, 2h, 4h, 7h, 12h after drug administration and 6 hours after lactose or saline administration. The 0.5h rat was kept anesthetized until decapitated. The rats being decapitated at 1, 2, 4, 6, 7 and 12h regained consciousness within about 1 hour and were kept in cages until they were decapitated. Before they were decapitated,

the rats were anesthetized with the same dose of mixture anesthetic mentioned above and decapitated just like the 0.5h rats. Blood was collected in tubes containing heparin and suitable enzyme inhibitors (EDTANa2 and NaF) and centrifuged at 3000 rpm for 10 minutes. The plasma was separated and stored at approximately -69 $^{o}$C until analyzed. After preparation of plasma, lungs, brains, kidneys and livers were immediately processed for receptor binding studies.

Experiments were performed on three different days for every form (IV or IT) of administration and for each type of preparation, e.g. BDP or 17-BMP. Each animal represented a single time point with grouped data for liver, lung, kidney and brain.

**Receptor Binding Assays**

Immediately after decapitation, the lung, without trachea, a lobe of the liver, the two kidney beans and the brain were resected and placed on ice.

The weighed tissue was added into appropriate amount (1 g in 10 ml of buffer for liver and 1g in 4 ml of buffer for lung, kidney and brain) of ice-cooled incubation buffer (10mM Tris/HCL, 10 mM sodium molybdate, 2mM 1,4-dithiothreitol and 2mM phenylmethylsulfonyl fluoride, PMSF) and homogenized in a Virtis 45 homogenizer at 40% of full speed, for three periods of 5 seconds each with a 30 sec cooling period between each step. Then 1 ml of the resultant homogenate was transferred into a thick wall tube and 50 µl of I.S. (part 3.1) was added. After vortexing, 4 ml of ethyl acetate was added and mixed. The tube was capped and stored at approximately - 69 °C for later analysis of BDP and 17-BMP in different tissues. Another 1 ml of the homogenate was transferred into a centrifuge tube to which 100 µl of 5% activated charcoal (prepared in incubation buffer) was added and mixed. After 5 minutes, the suspension was

centrifuged at 4 °C at 50,000g for 10 min. in a Beckman centrifuge equipped with a JA-21 rotor (Beckman instruments, Palo Alto, CA) to obtain a clear supernatant.

Aliquots of the supernatant (150μl) were transferred into microcentrifuge tubes. Then 50 μl of 40 nM $^3$H-TA in incubation buffer was added to determine total binding (TB) or 50 μl of mixture solution in incubation buffer with 40 nM $^3$H-TA and 40 μM unlabeled TA was added to determine the non-specific binding (NS). Aliquots of 150 μl of the resultant mixtures were used to determine the amount of total radioactivity (TR).

After a 16-24 hour incubation period at 4 °C, the unbound glucocorticoid was removed by addition of a 5% suspension of activated charcoal in buffer (200 μl). The mixture was incubated for 5 min on ice and then centrifuged at 10,000 rpm for 5 min in a micro-centrifuge (Fisher model 235A). The radioactivity (dpm) in 300 μl of supernatant was determined using a liquid scintillation counter (Beckman model LS 5000 TD, Palo Alto, CA). All determinations were performed in triplicate.

Specific bindings for a certain tissue were directly used as a marker for the number of available binding sites since the concentration of $^3$H-TA (10nM) was chosen to be high enough to cover 100 % of the total binding sites and it was assumed that $^3$H-TA would not replace bound unlabeled corticosteroid to a significant level due to the very slow dissociation rate at 4°C. The specific binding for control rats was treated as the reference level or 100 % available binding sites. The ratio of the specific binding for drug-treated rats to that for control rats served as the estimate of percent of available binding sites. Then the percentage of receptor occupancy by the unlabeled corticosteroid could be easily calculated from this ratio.

For assessing differential receptor occupancy between lung and systemic organs (liver, kidney and brain), the cumulative change from baseline (AUC) was calculated for the 12-hour investigation period by the trapezoidal rule from percent occupied receptor ($E_x$)-time profiles. Pulmonary targeting (PT) was defined as

$$PT = AUC_{E\ Lung} - AUC_{E\ sys\ organ} \qquad (1)$$

where $AUC_{E\ sys\ organ}$ represents the $AUC_E$ of liver, kidney or brain. A positive PT would indicate preferential lung targeting. The area under the first moment curve ($AUMC_\infty$) was calculated by the trapezoidal rule from $E_x*t_x$ versus $t_x$-pairs. Effects after the last measurement points were extrapolated assuming a linear decline of the effect over time at late time points. These estimates were also used to derive $AUC_\infty$. The mean pulmonary effect times (MET) were calculated consequently from $AUC_{Lung\infty}$ and $AUMC_{Lung\infty}$ ($MET = AUMC_{Lung\infty}/AUC_{Lung\infty}$).

## Results and Discussions

### Particle Size Distribution

The particle size distributions of BDP and 17-BMP dry powder were listed in table 3-1. It is well known that particles with aerodynamic diameter 1 to 5 μm can achieve maximum pulmonary deposition while particles larger than 5 μm will impact in the back of the throat because of the large momentum and particles smaller than 1 μm will be breathed back out [89]. Since the insufflator was used for intratracheal administration, larger particles were also delivered into the trachea and subject to pulmonary absorption. The larger particle sizes, however, could lead to slow dissolution and as a result the overall pulmonary absorption process could be prolonged. The significant higher percentage of large particles (>5 μm) for 17-BMP (Tabel 3-1) suggested that the

absorption of 17-BMP could be significantly prolonged despite its better hydrophilicity compared to BDP, which was confirmed by the results in "IT Administration of 17-BMP".

Table 3-1: Particle size distributions (percent) of BDP and 17-BMP dry powder.

|        | <1 μm | 1-5 μm | 5-10 μm | >10 μm |
|--------|-------|--------|---------|--------|
| BDP    | 3.9   | 40.9   | 45.7    | 9.5    |
| 17-BMP | 1.5   | 17.3   | 30.0    | 51.2   |

## IV Administration of BDP

After IV administration of BDP, it was expected that all tissues have similar receptor occupancies (assuming no tissue sequestration) and no lung targeting should be observed.  The results, however, showed a certain degree of "liver targeting" if liver was used as the systemic organ (Figure 3-1) and lung targeting if brain was used as the systemic organ (Figure 3-2).  The trends were obvious even though the multiple comparisons were not statistically significant (Table 3-2) due to the small sample size and the large variability.  The higher liver receptor occupancy observed is understandable, if one considers the high intrinsic hepatic clearance of BDP, which leads to the formation of 17-BMP, the active form of glucocorticoid.  Because BDP is highly metabolized in the liver, (which is also indicated by its oral bioavailability of lower than 1%), at any given time point, both 17-BMP concentrations and liver receptor occupancies will be higher than those in the other organs.  An opposite result was observed for IV administered fluticasone propionate (FP) in a previous study [85], in which lower liver receptor occupancy than those in the other organs was observed due to the high intrinsic hepatic clearance of FP, a deactivation process for FP.  These findings suggest that the liver may not be an appropriate organ to assess the extent of systemic absorption of drugs with high intrinsic hepatic clearance such as BDP and FP.  The lower receptor occupancy

in brain was consistent with previous studies [85] (Figure 3-1), suggesting the

involvement of P-glycoprotein in the distribution of 17-BMP or BDP in the brain because

the blood brain barrier should not prevent significant amount of BDP and 17-BMP from

entering the brain given their lipophilicity.  The similarity between lung and kidney

receptor occupancies suggests that the kidney may be the appropriate organ to assess the

pulmonary selectivity of BDP (Figure 3-3).



Figure 3-1. The comparison of receptor occupancies in lung and liver after IV
administration of BDP (mean±SE, n=3).



Figure 3-2. The comparison of receptor occupancies in lung and brain after IV
administration of BDP  (mean±SE, n=3).

Figure 3-3. The comparison of receptor occupancies in lung and kidney after IV administration of BDP (mean±SE, n=3).

**IV Administration of 17-BMP**

After IV administration of 17-BMP, lung, liver and kidney showed similar receptor occupancies (Figure 3-4) with brain still showing much lower receptor occupancy. This result confirmed the potential local activation of BDP in liver since the direct administration of 17-BMP did not cause preferential receptor binding in the liver. At the same time, the low receptor occupancy in the brain suggested the involvement of P-glycoprotein in the distribution of 17-BMP in the brain. Obviously, brain is not the appropriate systemic organ to be used to assess the pulmonary targeting of 17-BMP or BDP. This result, however, provided important information about the potential adverse effect of this type of drug on very young patients' brain development since it is known that the p-glycoprotein system in these patients is not fully developed.

**IT Administration of BDP**

After IT administration of BDP, the receptor occupancy in the liver was higher than that in the lung for most of the investigated time even though the receptor occupancy was temporarily higher in the lung during the first two hours (Figure 3-5). As stated earlier, the local activation of BDP into 17-BMP in the liver could be the reason for this

phenomenon.  The short early pulmonary targeting was obviously due to the fact that the

lung was the absorption site.  Since kidney was found to be the appropriate systemic

organ to assess the pulmonary targeting of BDP, the comparison between the cumulative
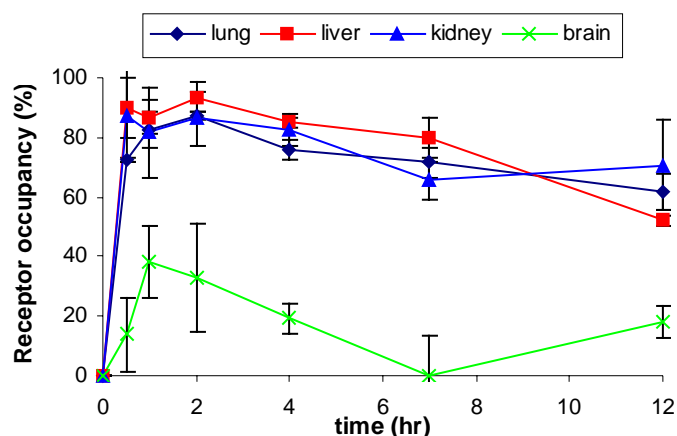


Figure 3-4. The receptor occupancies in lung, liver, kidney and brain after IV
administration of 17-BMP  (mean±SE, n=3).

receptor occupancies in lung ($AUC_{E\,lung}$=723 ± 116%*hr) and kidney ($AUC_{E\,kidney}$=647 ±

125 %*hr) indicated preferential lung targeting (p<0.05), which was mainly due to the

higher receptor occupancy in the lung during the early time (Figure 3-6).  The

significantly longer (p<0.05) MET (28.0±13.5 hr) after IT administration of BDP

compared with that (9.8±4.9 hr) after IV administration of BDP was an indication of the

long pulmonary residence time which could be the result of slow dissolution rate of BDP

dry powder.  Again, the receptor occupancy in the brain was significantly lower (p<0.05)

compared to that in the lung for the possible reason mentioned earlier (Figure 3-7).

**IT Administration of 17-BMP**

After IT administration of 17-BMP, lung showed consistent high receptor occupancy for

most of the investigated time (Figure 3-8), indicating a consistently high concentration of

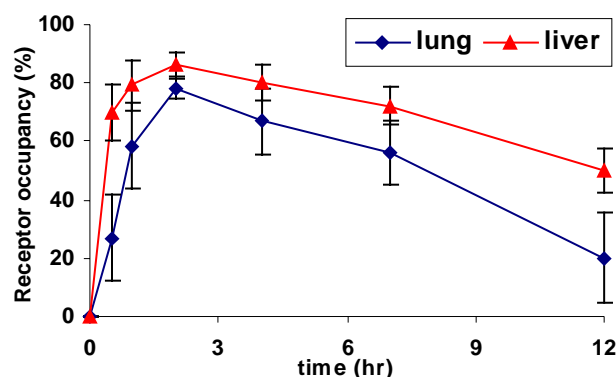17-BMP in the lung.  The receptor occupancy in the liver and kidney
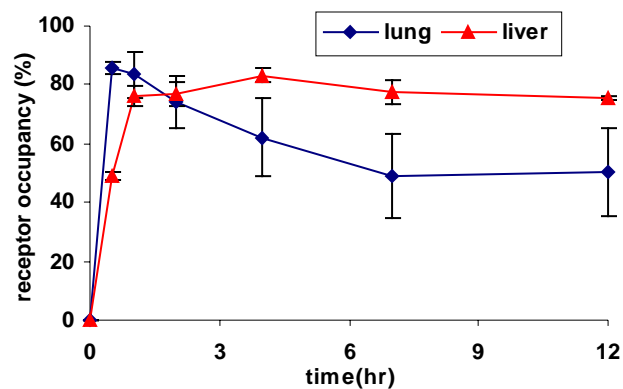
Figure 3-5. The comparison of receptor occupancies in lung and liver after IT administration of BDP (mean±SE, n=3).
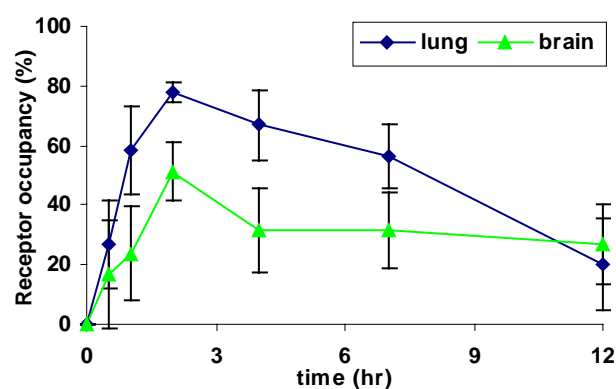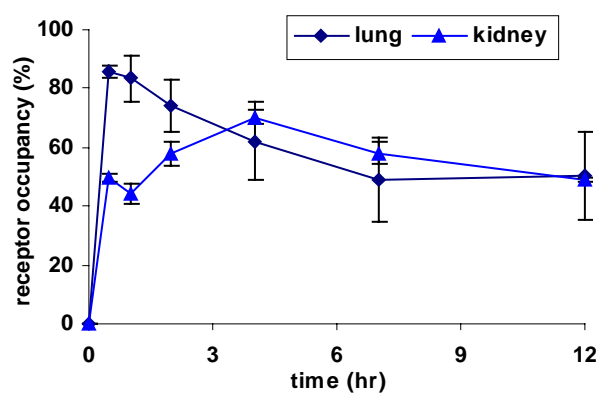


Figure 3-6. The comparison of receptor occupancies in lung and kidney after IT administration of BDP (mean±SE, n=3).



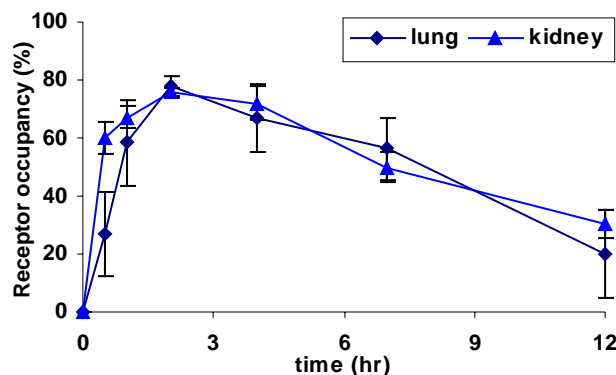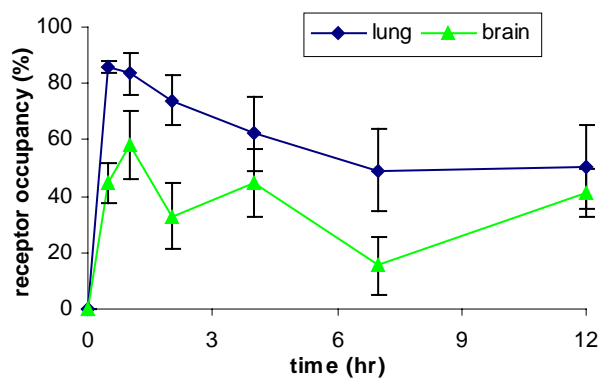Figure 3-7. The comparison of receptor occupancies in lung and brain after IT administration of BDP (mean±SE, n=3).

were relatively lower, which indicated a preferential lung targeting (p=0.09). As in other

routes of administration, brain showed the lowest receptor occupancy. Since no

micronized 17-BMP powder was available and the amount of raw 17-BMP powder was

not enough for micronization, the raw powder was directly mixed with micronized

lactose and given to the rats. The bigger particle size of 17-BMP may contribute to the

slow absorption of 17-BMP from the lung and the much longer MET (Table 3-2).
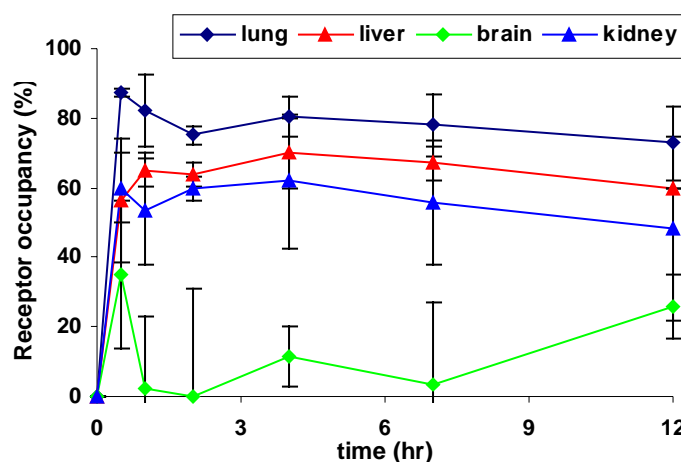


Figure 3-8. The receptor occupancies in lung, liver, kidney and brain after IT
administration of 17-BMP (mean±SE, n=3).

Table 3-2: Cumulative receptor occupancy ($AUC_E$), pulmonary targeting and mean
pulmonary effect time after IT and IV administration of beclomethasone
dipropionate (BDP, 100 µg/kg), IT and IV administration of beclomethasone
17-monopropionate (17-BMP, 90 µg/kg).

|  | BDP sol IV adm | BDP pw IT adm | 17-BMP sol IV adm | 17-BMP pw IT adm |
|---|---|---|---|---|
| $AUC_E$ (% * h) |  |  |  |  |
| Lung | 585±258 | 723±115 | 839±53 | 913±50 |
| Liver | 837±126 | 905±29 | 864±47 | 750±136 |
| Kidney | 648±59 | 647±125 | 836±41 | 678±167 |
| Brain | 377±257 | 392±74 | 149±79 | 158±91 |
| Pulmonary targeting %*hr |  |  |  |  |
| $AUC_{ELung}$ - $AUC_{ELiver}$ | -251 ± 143 | -182 ± 156 | -24 ± 40 | 150 ±130 |
| (p-value) | (0.046) | (0.09) | (0.20) | (0.15) |
| $AUC_{ELung}$ - $AUC_{EKidney}$ | -63 ± 219 | 76 ± 18 | 3 ± 38 | 235 ± 203 |
| (p-value) | (0.33) | (0.009) | (0.45) | (0.09) |
| $AUC_{ELung}$ - $AUC_{Ebrain}$ | 208 ± 227 | 331± 209 | 691 ± 125 | 754 ± 104 |
| (p-value) | (0.12) | (0.06) | (0.005) | (0.003) |
| Mean pulmonary effect time (h) | 9.8±4.9 | 28.0±13.5 | 25.4 ± 11.5 | 47.7 ± 19.7 |

**Conclusion**

Despite the high lipophilicity and the resulting long dissolution time, the fast

systemic absorption of BDP negatively affected the overall pulmonary targeting after IT

administration. The local activation of BDP in the liver into 17-BMP was also a negative

factor for pulmonary targeting. Even though direct 17-BMP IT administration resulted in

longer MET and favorable pulmonary targeting, the non-micronized powder is not

acceptable in clinical application since the bigger particles will not be able to get into the

patients' lung, but swallowed by the patients and absorbed from the gastro-intestinal

track, leading to the loss of pulmonary targeting. Further experiment should be

performed to study the pulmonary targeting of micronized 17-BMP powder to mimic the

clinical situation.

Several *in vitro* and *in vivo* studies have shown differences in the topical and

systemic potency [90], receptor affinity[91], and pharmacokinetic properties [92, 93] of

inhaled glucocorticoids. In addition, pharmacokinetic/pharmacodynamic (PK/PD)

models have been developed to assess the relationship between PK/PD and the extent of

glucocorticoid systemic effects such as lymphocyte and cortisol suppression. [48] These

studies, however, gave no indication of the extent of pulmonary selectivity, as it is

determined by both the extent of local, as well as systemic effects. Furthermore,

pulmonary targeting has been rather difficult to evaluate because good surrogate markers

for pulmonary effects are not available for human pharmacological studies. In this study

we used the *ex vivo* animal model to assess pulmonary selectivity of one commercially

available inhaled glucocorticoid and its active metabolite.

CHAPTER 4
PHARMACOKINETIC AND PHARMACODYNAMIC MODELING OF
BECLOMETHASONE DIPROPIONATE AND BECLOMETHASONE 17-
MONOPROPIONATE IN RATS

## Introduction

Pharmacokinetic (PK)and pharmacodynamic (PD)modeling has gained increasing

attention during the last decade in the regulatory bodies, industry and the academic area

due to the fact that it could correlate the drug dose with the therapeutic or side effect of

the drug by making use of various mathematical equations.  Pharmacokinetics is what the

body does to the drug.  By describing the absorption, distribution, metabolism and

excretion of the drug, pharmacokinetics determines the blood or plasma concentrations

with certain pharmacokinetic parameters such as volume of distribution, clearance and

absorption rate constant etc.  A pharmacokinetic model allows us to simulate the

concentrations of the drug under different circumstances and optimize doses or select the

route of administration to achieve the desired blood or plasma concentrations.  Both

compartmental and noncompartmental methods can be used to describe the

pharmacokinetics of a drug. For compartmental methods, the body is divided into well-

stirred hypothetical areas for either drug or metabolite and the disposition of the drug or

the metabolite is described by proper mathematical equations.  For noncompartmental

method, statistical moment theory is used to estimate pharmacokinetic parameters such as

bioavailability, clearance, apparent volume of distribution, and the fraction of a dose of a

drug that is converted to a specific metabolite etc.  Compartmental analysis is essential

for PK/PD modeling and simulation even though noncompartmental analysis can also provide important information about certain pharmacokinetic parameters.

Pharmacodynamics is what the drug does to the body, which could be either desired or side effects. Pharmacodynamic modeling characterizes the change of a certain physiological parameter compared to its predose or baseline values, which is defined as the effect, through various mathematical equations. The sum of all beneficial effects is termed as efficacy. A proper physiological parameter needs to be able to quantify the drug effect and be easy to measure. The result should be reproducible, corrected for baseline and correlated to the therapeutic outcome.

**Hypothesis**

A compartmental model (Figure 4-1) is hypothesized to describe the pharmacokinetics of BDP after inhalation administration in the rat model. It is assumed that the pulmonary absorption is complete. The receptor occupancies in tissues will be used as the pharmacodynamic marker, which is hypothesized to be linked with the tissue concentration of 17-BMP via an E-max model.
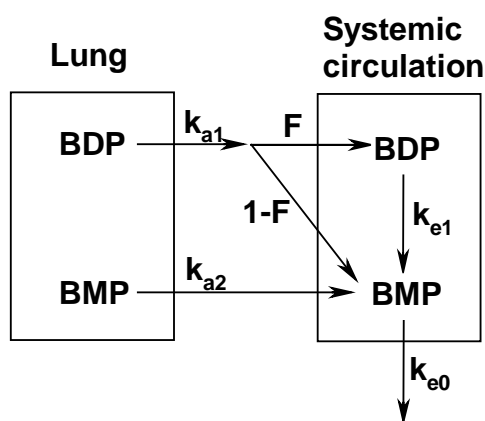


Figure 4-1. The hypothesized compartmental model for the pharmacokinetics of inhaled BDP ( $k_{a1}$ and $k_{a2}$: absorption rate constants for BDP and 17-BMP, $k_{e1}$: elimination rate constant for BDP, $k_{e0}$: the elimination rate constant for 17-BMP, F: fraction of intact BDP absorbed into systemic circulation)

**Rationale**

The pharmacokinetic parameters for 17-BMP can be obtained from plasma and tissue concentrations of 17-BMP in the intravenous and intratrecheal studies of 17-BMP. Then the pharmacokinetics of BDP after inhalation can be derived from the plasma and tissue concentrations of BDP and 17-BMP in the intravenous and intratrecheal studies of BDP together with the pharmacokinetic information for 17-BMP. In vitro metabolism studies have shown that BDP was rapidly metabolized in rat lung homogenate [41, 94], suggesting the activation of BDP in the lung. The degree of activation, however, is determined by the fraction parameter, F. Clarification of these parameters will provide valuable information about the degree of activation of BDP in the lung, allow us to predict plasma concentrations of BDP and 17-BMP under different circumstances, such as different dosages, multiple doses etc. Certain parameters can also be obtained from non-compartmental analysis. By comparing the area under the concentration curve vs time for 17-BMP after intravenous administration of BDP ($AUC_{BMP(BDPIV)}$) to that after intravenous administration of an equimolar dose of 17-BMP ($AUC_{BMP(BMPIV)}$) (Equation 4-1), the fraction (Fr) of BDP that is converted to 17-BMP can be estimated.

$$Fr = \frac{AUC_{BMP(BDPIV)}}{AUC_{BMP(BMPIV)}} \qquad 4\text{-}1$$

The percentage of intact BDP (F) that is absorbed into the systemic circulation after inhalation of BDP can also be estimated by equation 4-2

$$F = \frac{AUC_{BDP(BDPIT)}}{AUC_{BDP(BDPIV)}} \qquad 4\text{-}2$$

where $AUC_{BDP(BDPIT)}$ is the area under the concentration curve vs time for BDP

after inhalation of BDP and $AUC_{BDP(BDPIV)}$ is the area under the concentration curve vs

time for BDP after IV administration of BDP.

Since 17-BMP is the active corticosteroid for asthma control, the concentrations of

17-BMP in various tissues are believed to be directly linked to the receptor occupancies

in different tissues via the well-known Michaelis-Menten equation.  Therefore, an E-max

model (Equation 4-3) will be used to correlate the tissue concentrations of 17-BMP (C)

with the corresponding receptor occupancy where E is the observed receptor occupancy,

$E_{max}$ is the maximum receptor occupancy and $EC_{50}$ is the concentration of 17-BMP to

achieve 50% of $E_{max}$.  Since the total tissue concentration, instead of the free

concentration, of 17-BMP is used in the model, the estimated $EC_{50}$ ($EC_{50est}$) will not be

the actual $EC_{50}$, but $EC_{50}$/Fu where Fu is the fraction of free 17-BMP in the tissue.

Therefore, the outcome parameters will allow assessment of differences in tissue binding

in various tissues. If the tissue binding fractions can be measured, the actual $EC_{50}$ in

different tissues can be obtained based on Equation 4-4.

$$E = \frac{E_{max} \cdot C}{EC_{50} + C} \qquad\qquad 4\text{-}3$$

$$EC_{50} = EC_{50est} \cdot F_u \qquad\qquad 4\text{-}4$$

## Materials and Methods

### Chemicals

Micronized beclomethasone dipropionate was kindly provided by 3M.  17-

beclomethasone monopropionate was purchased from European Directorate for the

Quality of Medicine (EDQM).  The internal standard, fluticasone propionate, was kindly

provided by Glaxo Group Research (Herts, UK). Double distilled deionized water was prepared in our lab (Corning AG-3 still, Corning, NY) and filtered through 0.2μm filter. Methanol, ethanol, formic acid, ammonium acetate, ethyl acetate and heptane were of HPLC grade and purchased from Fisher Scientific (Fisher, Springfield, NJ). Rat and human blank plasma were obtained from the Civitan regional blood system (Gainesville, FL). The solid phase $LC_{18}$ (3 ml) cartridges for sample extraction were obtained from Supelco (Bellefonte, PA).

**HPLC/MS/MS Conditions**

The high performance liquid chromatography was performed isocratically at ambient temperature using a Waters $C_{18}$ 3.5um column (Symmetry, 2.1×50mm i.d., Milford, MA) preceded by a Whatman 3.5-μm ODS $C_{18}$ guard column cartridge (Clifton, NJ). The mobile phase consisted of 33mM ammonium acetate/0.33% formic acid buffer (pH 3.44) -methanol (30:70, v/v), delivered by a flow-rate of 0.3 ml min$^{-1}$ by a LDC Analytical constaMetric®3500 solvent delivery system (LDC/Milton Roy, Riviera Beach, FL). The mobile phase was degassed using helium for approximately 10 min before use. The mass spectrometer was a Micromass Quattro-LC-Z (Beverly, MA) triple quadrupole mass spectrometer equipped with an electrospary ionization (ESI) ion source. Positive ESI was chosen after tuning with three analytes. The source temperature was set to 120° C and the desolvation temperature was set to 450° C. Capillary and cone voltages were set to 3.0 kV and 30 V respectively. Argon was used as the collision gas. The mass spectrometer was linked to a Perkin Elmer ISS 200 autosampler via contact closure and the operation was controlled by computer software, Masslynx 3.1. Data analysis was performed using Masslynx software. The calibration curves were plotted as

the peak area ratio (analyte/I.S.) versus analyte concentration with a weighting factor of the reciprocal of the analyte nominal concentration.

**Preparation of Calibration Standards And Quality Control Samples**

**Plasma samples**

Primary stock solutions were prepared by dissolving the compounds or internal standard in methanol. Appropriate dilutions of the stock solutions with drug-free rat plasma were made subsequently in order to prepare the working solutions for BDP and 17-BMP. Two different series of stock solutions were prepared from different weightings for calibration standards (CCs) and quality control samples (QCs). The CCs and QCs ranged from 0.05-5 ng ml$^{-1}$ for both analytes. Aliquots of 1 ml sample were transferred into thick wall glass tubes with enzyme inhibitor (EDTANa$_2$+NaF, 17mg: 17.5mg), capped and stored at approximately -69 °C. The internal standard (I.S.) stock solution was diluted with methanol to produce a working solution of 5 ng ml$^{-1}$.

**Tissue samples**

The primary stock solution for CCs was diluted with methanol to produce the working solutions of 1, 2, 4, 10, 20, 40 and 100 ng/ml. Fifty microliters of each working solution was added to 1 ml blank rat tissue homogenate (liver, lung or kidney taken from the placebo rat as described under Receptor Binding Assays in Chapter 3) to generate 0.05, 0.1, 0.2, 0.5, 1, 2 and 5 ng/ml CCs. For QCs, the primary stock solution for QCs was diluted with methanol to produce the working solutions of 1, 2, 10 and 40 ng/ml which then were diluted in 1 ml blank rat tissue homogenate (liver, lung or kidney) to generate 0.1, 0.5, and 2 ng/ml QCs. Fifty microliters of I.S. working solution was added into each CC or QC sample. After vortexing, four milliliters of ethyl acetate was added

to inhibit the metabolism of BDP. The samples were capped and stored at approximately -69 °C.

**Sample Preparation**

**Plasma samples**

After the rats were decapitated at different time points following drug administration, blood was collected in tubes containing heparin and suitable enzyme inhibitors (EDTANa$_2$ and NaF) and centrifuged at 3000 rpm for 10 minutes. The plasma was separated and stored at approximately -69 $^o$C until analyzed.  BDP and 17-BMP were extracted from rat plasma by solid phase extraction.  Plasma samples (1ml) were thawed at room temperature.  After addition of 50 µl of I.S. working solution to 1ml plasma, they were then vortexed and 1ml of 30% ethanol (v/v) was added into the plasma sample.  After 15 minutes of incubation at 4°C, the mixture was centrifuged at 4000rpm for 15 minutes to remove the protein precipitate.  The supernatant was then transferred onto the SPE extraction column preconditioned with ethanol and water.  The column was drained under necessary vacuum to ensure that the sample was aspirated at a drop wise flow rate.  The column was then washed with a) one column volume of 25% ethanol solution, b) one column volume of water solution and c) 2 ml of 2:98 (v/v) ethyl acetate/n-heptane mixture.  Finally, the sample was eluted with 3 ml of 35:65 (v/v) ethyl acetate/n-heptane mixture at a drop wise flow rate, evaporated in a vacuum centrifuge, and reconstituted in 50 µl of mobile phase.  A volume of 30 µl was injected into the HPLC/MS/MS system.

**Tissue samples**

After the rats were decapitated at different time points following drug administration, various tissues (liver, lung and kidney) were homogenized in appropriate amount of buffer (1g in 10 ml of buffer for liver and 1g in 4 ml of buffer for lung and

kidney). The buffer system consisted of 10mM Tris/HCL, 10 mM sodium molybdate, 2mM 1,4-dithiothreitol and 2mM phenylmethylsulfonyl fluoride, PMSF. One milliliter of the homogenate was transferred to a thick wall glass tube to which 50 µl of I.S. working solution was added. After vortexing, 4 ml of ethyl acetate was added. The mixture was stored at approximately -69 °C after mixing. At the time of analysis, the frozen samples were thawed at room temperature and then were shaken on a shaker for 15 minutes. After centrifugation (5 min, 3070 g), the upper organic layer was transferred into a new tube, evaporated in a vacuum centrifuge, and reconstituted in 50 µl of mobile phase. A volume of 30 µl was injected into the HPLC/MS/MS system.

**Plasma Protein Binding of 17-BMP**

**Diluted plasma samples**

Drug-free rat plasma was diluted with phosphate buffered saline (PBS, pH 7.4) to 1%. The diluted plasma was used to prepare 1, 2, 5, 20 and 40 ng/ml 17-BMP. Aliquots of 2 ml sample were transferred into thick wall glass tubes with enzyme inhibitor (EDTANa$_2$+NaF, 7mg: 17.5mg).

**Diluted plasma quality control samples**

The diluted plasma (1% in PBS) was centrifuged through Centricon$^{TM}$ 30 microconcentrator (30KD cutoff value, Millipore Corporation) to obtain enough filtrate to prepare quality samples at 0.5, 1, 2, 10 and 20 ng/ml. One hundred microliters of resulting solution was added to 900 µl rat plasma in thick wall glass tubes with enzyme inhibitor (EDTANa$_2$+NaF, 17mg: 17.5mg).

**Sample process**

Three replicates of 2 ml sample at each concentration were incubated at 37 °C for 30 minutes. One milliliter of the incubated sample was centrifuged through Centricon$^{TM}$

30 microconcentrator (30KD cutoff value, Millipore Corporation) for 1 minute at 4000

rpm (1935g, Beckman Centrifuge model JA-20). Less than 20% of the total volume was

filtered to prevent disturbance of the equilibrium. One hundred microliters of the filtrate

was added to 900 μl rat plasma in thick wall glass tubes with enzyme inhibitor

(EDTANa$_2$+NaF, 17mg: 17.5mg). The other milliliter of the incubated sample was used

to assess total concentrations. One hundred microliters of this incubated sample was

added to 900 μl rat plasma in thick wall glass tubes with enzyme inhibitor

(EDTANa$_2$+NaF, 17mg: 17.5mg). Then the samples were extracted according to the solid

phase extraction method described above and quantified by the HPLC/MS/MS method.

**Protein binding calculation**

The measured concentration of 17-BMP in the filtrate times 10 gave the free

concentration of 17-BMP in the diluted plasma ($C_f$). Similary, the measured

concentration of 17-BMP in the un-filtered sample times 10 gave the total concentration

of 17-BMP in the diluted plasma ($C_t$). The free fraction in the diluted plasma ($F_{ud}$) was

then calculated based on Equation 4-5. The free faction in the full plasma (Fu) can be

calculated based on Equation 4-6 (Appendix A for derivation). Protein binding of 17-

BMP in full rat plasma is then 1-$F_u$.

$$F_{ud} = \frac{C_f}{C_t} \qquad\qquad 4\text{-}5$$

$$F_u = \frac{F_{ud}}{100 - 99F_{ud}} \qquad\qquad 4\text{-}6$$

**Pharmacokinetic Analysis**

Plasma and tissue BDP and 17-BMP concentrations over time will be subject to standard non-compartmental pharmacokinetic analysis with WinNonlin® (Pharsight Corporation, CA, USA) and compartmental pharmacokinetic analysis with NONMEM V (University of California, San Francisco, CA, USA). Non-compartmental analysis is mainly used to estimate the fraction (Fr) of BDP that is converted to 17-BMP according to Equation 4-1 and the percentage of intact BDP (F) that is absorbed into the systemic circulation after inhalation of BDP according to Equation 4-2. Compartmental pharmacokinetic models for BDP and 17-BMP after different routes of administration were built sequentially. The basic disposition pharmacokinetic models for BDP and 17-BMP were first built based on the plasma concentrations of BDP and 17-BMP after intravenous administration of BDP and 17-BMP, respectively. Then the absorption kinetics of BDP and 17-BMP after inhalation administration was modeled by fixing the disposition parameter. The models were fitted to the data from three days' studies simultaneously based on non-linear mixed-effect modeling. The parameter for a specific day (Pi) can be described by an exponential error model (Equation 4-7):

$$P_i = TVP \times \exp(\eta_i) \qquad \text{4-7}$$

where TVP is the mean parameter and $\eta_i$ is a random variable for inter-day variability that follows normal distribution with mean zero and variance $\omega^2$.( $\eta_i \sim N(0, \omega^2)$). The residual error is modeled by a proportional error model:

$$C_{obs} = F \times (1 + \varepsilon_1) \qquad \text{4-8}$$

where Cobs is the observed concentration, F is the predicted concentration and $\varepsilon_1$ is a random variable that follows normal distribution with mean zero and variances $\sigma_1^2$.

**Pharmacodynamic Analysis**

Glucocorticoid receptor occupancy was considered as a pharmacodynamic end point. 17-BMP tissue concentrations will be correlated to the pharmacodynamic effects (the percentage of occupied receptors in different tissues) via an $E_{max}$ model (Equation 4-3) with WinNonlin® (Pharsight Corporation, CA, USA). 17-BMP tissue concentrations and the receptor occupancies from different studies were pooled together for a specific tissue (lung, liver or kidney) to have a wider coverage of receptor occupancy so that $EC_{50}$ and $E_{max}$ could be estimated with better precision.

### Results and Discussions

**Plasma Protein Binding of 17-BMP**

The results of free fraction of 17-BMP in presence of different drug concentrations in 1% diluted rat plasma and 100% rat plasma were summarized in Table 4-1 and Table 4-2. The estimated values for free fraction is independent of the total concentration of 17-BMP, suggesting linear protein binding. The overall mean of free fraction of 17-BMP in full rat plasma was calculated to be 0.016 and therefore the plasma protein binding is 98.4%.

Table 4-1: Free fraction of 17-BMP in 1% diluted rat plasma

| Concentration (ng/ml) | 1 | 2 | 5 | 20 | 40 |
|---|---|---|---|---|---|
| 1 | 0.44 | 0.64 | 0.55 | 0.65 | 0.55 |
| 2 | 0.67 | 0.59 | 0.70 | 0.67 | 0.72 |
| 3 | 0.61 | 0.52 | 0.67 | 0.52 | 0.49 |
| Mean | 0.57 | 0.58 | 0.64 | 0.61 | 0.59 |
| SD | 0.12 | 0.06 | 0.08 | 0.08 | 0.12 |

Table 4-2: Free fraction of 17-BMP in 100% rat plasma

| Concentration (ng/ml) | 1 | 2 | 5 | 20 | 40 |
|---|---|---|---|---|---|
| 1 | 0.008 | 0.017 | 0.012 | 0.018 | 0.012 |
| 2 | 0.020 | 0.014 | 0.023 | 0.020 | 0.025 |
| 3 | 0.015 | 0.011 | 0.020 | 0.011 | 0.010 |
| Mean | 0.014 | 0.014 | 0.018 | 0.016 | 0.015 |
| SD | 0.006 | 0.003 | 0.005 | 0.005 | 0.008 |

**Non-compartmental Pharmacokinetic Analysis**

The areas under the concentration curve versus time (AUC) for 17-BMP after

intravenous administration of BDP (BDPIV) and after intravenous administration of an

equimolar dose of 17-BMP (BMPIV) were summarized in Table 4-3. The fraction (Fr) of

BDP that is converted to 17-BMP was estimated to be 0.89, suggesting that a certain

percentage of BDP is converted to 21-BMP or other minor metabolites in rats.

Table 4-3: AUC(0, ∞) of 17-BMP after different routes of administration

| Day | BDPIV | BMPIV | BDPIT | BMPIT |
|---|---|---|---|---|
| 1 | 52.1 | 49.2 | 31.9 | 42.7 |
| 2 | 41.3 | 56.1 | 35.6 | 44.5 |
| 3 | 43.2 | 48.8 | 31.5 | 45.8 |
| Mean (SD) | 45.5 (5.7) | 51.3 (4.1) | 32.3 (2.3) | 44.3 (1.6) |

The areas under the concentration curve versus time (AUC) for BDP after

intravenous administration of BDP (BDPIV) and after intratracheal administration of an

equimolar dose of BDP (BDPIT) were summarized in Table 4-4. The percentage of intact

BDP (F) that is absorbed into the systemic circulation after inhalation of BDP was

estimated to be 53%, suggesting that a significant percentage of BDP is directly absorbed

into the systemic circulation without activation after inhalation of BDP dry powder in

rats. If complete absorption from lung is assumed, the other 47% of BDP is absorbed into

the systemic circulation in the form of various metabolites. Since 89% of the metabolite

is 17-BMP, 42% of BDP is expected to be in the form of 17-BMP, which is confirmed by

the comparison of AUCs for 17-BMP after BDPIT and after BMPIV (Table 4-3).

Therefore, the lung absorption of inhaled BDP dry powder is relatively complete.

Similarly, the lung absorption of inhaled 17-BMP dry powder was also found to be

relatively complete based on the AUCs for 17-BMP after intratracheal administration of

17-BMP (BMPIT) and after BMPIV (Table 4-3).

Table 4-4: AUC(0, ∞) of BDP after different routes of administration

| Day | BDPIV | BDPIT |
|---|---|---|
| 1 | 5.7 | 2.3 |
| 2 | 4.9 | 3.6 |
| 3 | 7.3 | 3.7 |
| Mean (SD) | 6.0 (1.2) | 3.2 (0.8) |

**Compartmental Pharmacokinetic Analysis**

The plasma concentration of 17-BMP or BDP after IV administration of 17-BMP

or BDP was first modeled by pooling all the animal data together to obtain the initial

estimates for mean parameters. Then the plasma concentrations of BDP and 17-BMP

after intravenous administration of BDP and 17-BMP were modeled simultaneously

(Appendix B for NONMEM code). The fitting results were shown in Figure 4-2. One-

compartment model (Equation 4-8) was sufficient to describe BDP plasma profile after

IV administration of BDP and 17-BMP plasma profile after IV administration of 17-

BMP.

$$C = \frac{Dose}{V} \cdot e^{-\frac{CL}{V} \cdot t}$$  4-8

Mean estimates and inter-day variability of PK parameters (CL and Vd) are shown

in Table 4-5.  An exponential error model was selected for both CL and Vd (Equation 4-

7). The sampling scheme, however, only allowed accurate estimation of inter-day

variability of CL of BDP. With disposition pharmacokinetic parameters fixed, the plasma

concentrations of BDP and 17-BMP after intratracheal administration of BDP and the

plasma concentration of 17-BMP after intratracheal administration of 17-BMP were

simultaneously modeled to estimate the pulmonary absorption rate constants of BDP and

17-BMP and the proportion of intact BDP that was absorbed into the systemic circulation

(Table 4-5) (Appendix C for NONMEM code). The slightly faster absorption of 17-BMP

than BDP suggested that the larger particle size for 17-BMP dry powder did not lead to

longer dissolution time, a favorable factor for better pulmonary targeting. Therefore the

better pulmonary targeting observed for IT 17-BMP compared with IT BDP could be

mainly due to the negative impact of BDP's prodrug property on pulmonary targeting.

The proportion of intact BDP (F) that was absorbed into the systemic circulation was

estimated to be 56%, which is quite consistent with the result from non-compartmental

analysis. The fitting results were shown in Figure 4-3.
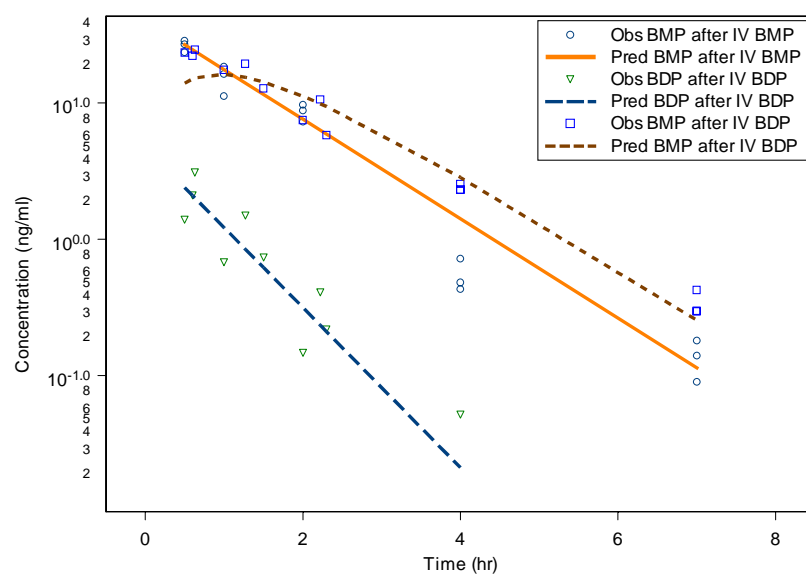


Figure 4-2. The plasma concentrations of 17-BMP and BDP after IV 17-BMP or IV BDP
administration (Obs: observed, Pred: Predicted).
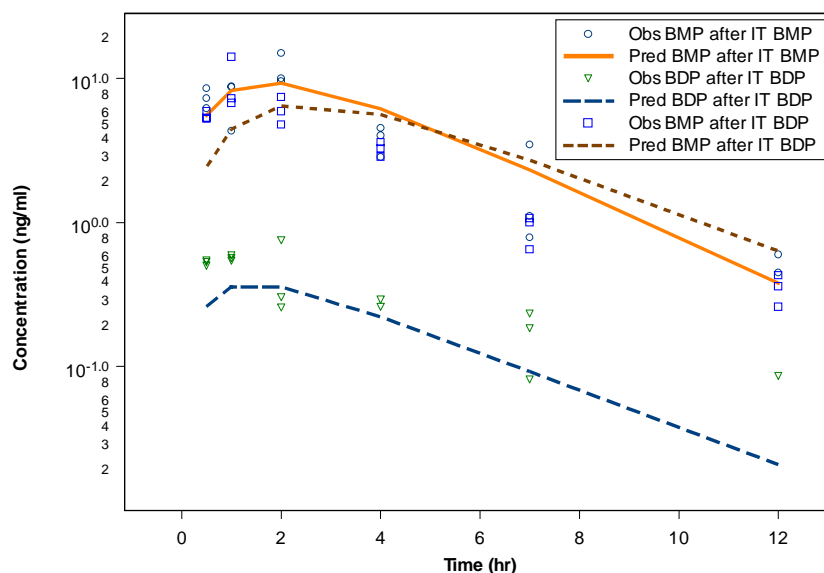
Figure 4-3. The plasma concentrations of 17-BMP and BDP after IT 17-BMP or IT BDP administration (Obs: observed, Pred: Predicted).

Table 4-5: Mean estimates and inter- and intra-day variability of pharmacokinetic parameters for BDP and 17-BMP

| Drug | Parameter | Mean estimates (RSE%) | Inter-day variability (CV%) |
|------|-----------|------------------------|------------------------------|
| BDP | Vd | 21.3 L/kg (19.9%) | - |
| | CL | 28.8 L/kg/hr (23.1%) | 21.2 |
| | $k_{a1}$ | 0.297 $hr^{-1}$ (7.6%) | - |
| | $k_{e1}$ | 1.35 $hr^{-1}$ | - |
| | $t_{1/2}$ | 0.51 hr | - |
| | F | 0.557(7.1%) | |
| 17-BMP | Vd | 2.22 L/kg (8.1%) | - |
| | CL | 1.86 L/kg/hr (6.5%) | - |
| | $k_{a2}$ | 0.369 $hr^{-1}$ (3.3%) | - |
| | $k_{e0}$ | 0.84 $hr^{-1}$ | - |
| | $t_{1/2}$ | 0.83 hr | - |

Simultaneous modeling of the plasma and various tissue concentrations of 17-BMP after IV administration of 17-BMP showed the relative tissue binding with plasma protein binding being the reference (Appendix D for NONMEM code). The absolute tissue bindings in various tissues can therefore be calculated based on the plasma protein binding of 17-BMP measured (Table 4-6). The high plasma protein binding and high tissue binding have very important implication. Since no data is available about the

plasma protein binding of 17-BMP for rat or human, this result suggested that the low

systemic side effects of long term BDP treatment in asthma could be due to the high

protein binding and the high tissue binding of 17-BMP, the active metabolite. The fitting

results were shown in Figure 4-4.

Table 4-6: Plasma protein binding and tissue bindings for 17-BMP

| Tissue | Binding fraction (%) |
|--------|----------------------|
| plasma | 98.4 |
| lung | 98.3 |
| liver | 99.4 |
| kidney | 98.6 |
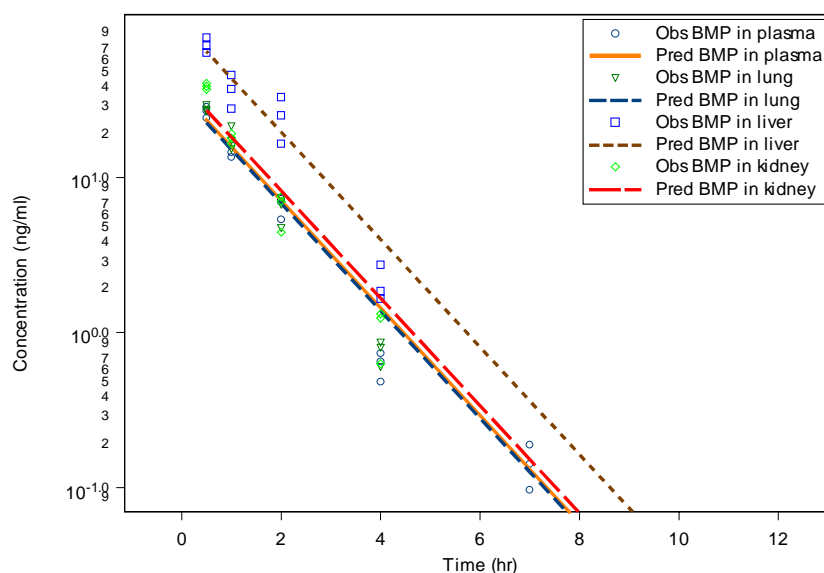


Figure 4-4. The concentrations of 17-BMP in different matrices after IV 17-BMP
administration (Obs: observed, Pred: Predicted).

**Pharmacodynamic Analysis**

The receptor occupancies in different tissues were treated as the pharmacodynamic

maker for 17-BMP since early work in cell systems found a close correlation between the

extent of receptor occupancy and the extent of the biological response. In order to have

accurate estimates of Emax and, especially, $EC_{50}$ in Equation 4-3, the receptor

occupancies in different tissues were pooled to have a wider coverage of tissue 17-BMP

concentration as shown in Figures 4-5, 4-6 and 4-7. The receptor occupancies in the lung

from IT 17-BMP were not included because the measured lung concentration of 17-BMP

included the non-absorbed 17-BMP in the airway. The tissue concentrations of 17-BMP

were converted from ng/g to ng/ml based on the densities of different tissues (0.72g/ml

for lung, 1.08g/ml for liver and 1.07g/ml for kidney [95]).  Assuming linear tissue

kinetics for 17-BMP, the tissue concentrations of 17-BMP after IV administration of 9

µg/kg 17-BMP were simulated based on the parameter estimates of one-compartment

developed for 90 µg/kg 17-BMP IV administration and were also used in modeling fitting

for Equation 4-3. The estimates for Emax and $EC_{50}$ ($EC_{50est}$) in different tissues were

listed in Table 4-7. The actual $EC_{50}$ values were obtained after correcting $EC_{50est}$ with the

tissue binding fractions obtained in Table 4-4 according to Equation 4-4. The estimated

EC50 values are consistent among different tissues, confirming that there are no subtypes

of glucocorticoid receptors in different tissues.  The estimated Emax values, however,

may underestimate the true Emax because of the variability of the assay per se since

many receptor occupancy values were observed to be higher than the estimated Emax for

each tissue. It also indicated a certain degree of replacement of 17-BMP from the

receptor-substrate complex by the radiolabelled tracer during incubation even though it

was assumed that the dissociation at 4 °C is negligible in the ex-vivo receptor binding

assay.
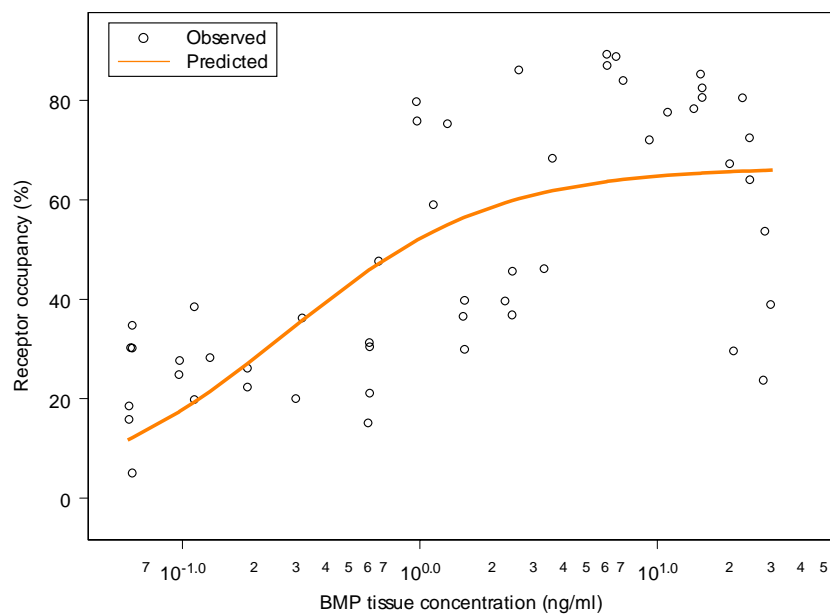
Figure 4-5. The receptor occupancy in lung tissue versus 17-BMP lung concentration.
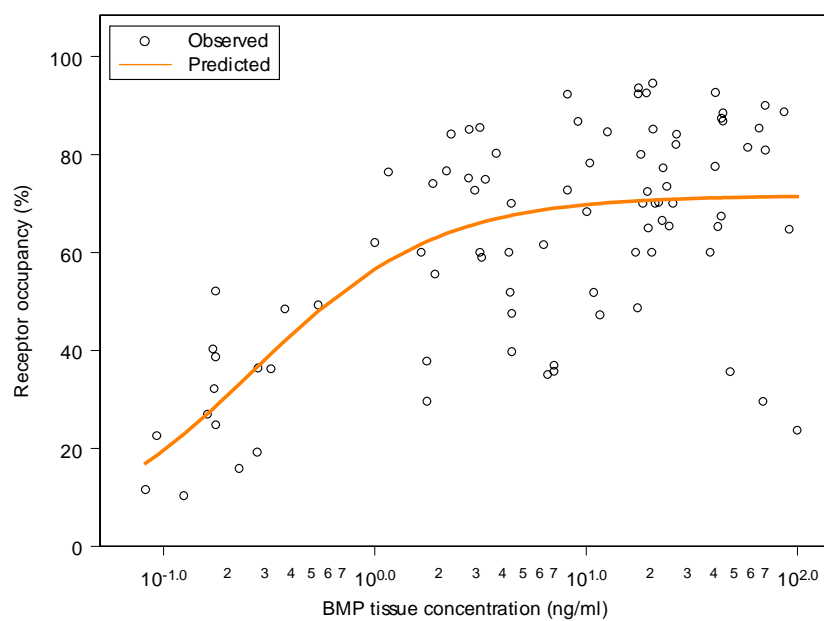


Figure 4-6. The receptor occupancy in liver tissue versus 17-BMP liver concentration.
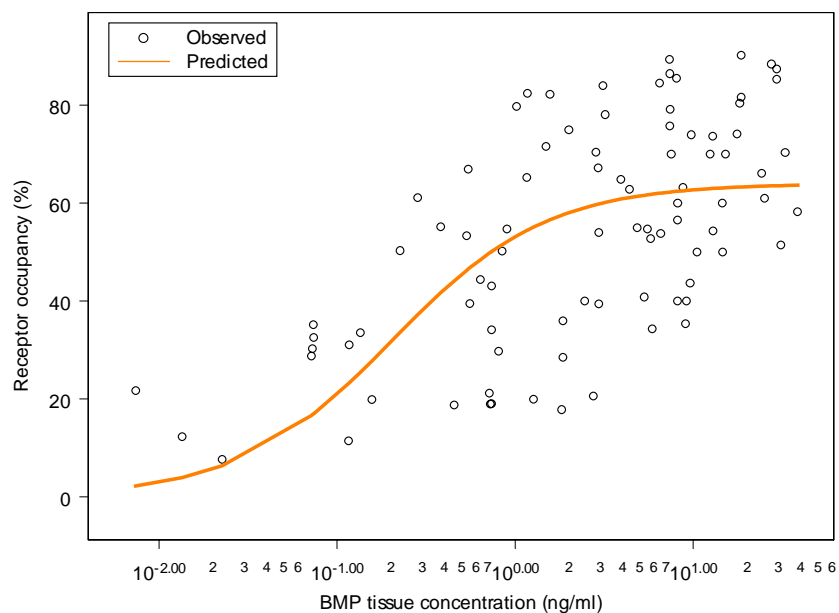
Figure 4-7. The receptor occupancy in kidney tissue versus 17-BMP kidney
concentration.

Table 4-7: Pharmacodynamic parameters for 17-BMP

| Tissue | Emax (%) (95% CI) | $EC_{50est}$ (95%CI) | $EC_{50}$ (95%CI) |
|---|---|---|---|
| lung | 67 (59, 75) | 0.20 (0.06, 0.34) | 0.0033 (0.0010, 0.0056) |
| liver | 70 (66, 74) | 0.41 (0.19, 0.63) | 0.0024 (0.0011, 0.0037) |
| kidney | 64 (58, 70) | 0.22 (0.08, 0.36) | 0.0030 (0.0011, 0.0050) |

**Conclusion**

Both non-compartmental and compartmental analysis suggested that a significant

portion of pulmonarily deposited BDP is absorbed into the systemic circulation in the

form of intact BDP when BDP is administered in micronized dry powder formulation to

rats. Despite the existence of 21-BMP and other metabolites, majority of BDP is

metabolized into 17-BMP. As the active metabolite, 17-BMP showed very high plasma

protein binding and tissue bindings in liver, lung and kidney, suggesting a possible reason

for the low systemic side effect of long term BDP treatment for asthma. The similarity

among the $EC_{50}$ values estimated for rat liver, lung and kidney tissues confirmed the homogeneity of glucocorticoid receptor in different tissues.

CHAPTER 5
PHARMACOKINETIC AND PHARMACODYNAMIC COMPARISON BETWEEN
CHLOROFUOROCARBON AND HYDROFLUOROALKANE METERED-DOSE
INHALERS OF BECLOMETHASONE DIPROPIONATE

**Introduction**

In agreement with the Montreal Protocol [96], chlorofluorocarbon (CFC), the

propellant used in metered dose inhalers (MDIs) for asthma treatment, is being phased

out due to its ozone-depleting effect, necessitating reformulation of MDIs with non-CFC

propellants.   Hydrofluoroalkane-134a(HFA) has been shown to be a safe replacement for

CFC as a pharmaceutical propellant, with the advantage that it has no ozone-depleting

potential [97].  One of these recently developed products is HFA-beclomethasone

dipropionate (HFA-BDP) extrafine aerosol. Different from the traditional CFC-BDP, this

new formulation contains BDP in HFA propellant as a solution, rather than in CFC-BDP

as a suspension.   This dramatic formulation change, combined with the improvement of

delivery devices, leads to a much greater portion of fine-particle mass (60%) compared to

that for CFC-BDP (10-30%), which results in less drug impacting in the throat and being

swallowed and more drug deposited in the large and small airways than with CFC-BDP

[Leach, 1998 #21].

**Hypothesis**

Numerous pharmacodynamic studies have been conducted to compare the clinical

efficacy and side effects of HFA-BDP and CFC-BDP with consistent outcomes that

suggested HFA-BDP could achieve the same level of efficacy at half the dose of CFC-

BDP with similar or even less systemic side effects [Harrison, 1999 #16; Gross, 1999

#129; Ayres, 1999 #138; Ayres, 2000 #137; Busse, 1999 #127; Davies, 1998 #130;

Demedts, 1999 #131; Fireman, 2001 #132; Juniper, 1999 #133; Goldin, 1999 #134;

Marshall, 2000 #135; Thompson, 1998 #136; Lipworth, 2000 #139]. The lack of a

sensitive bioanalytical assay has caused the delayed clarification of BDP's

pharmacokinetic properties. And the fact that BDP was proven to be a poorly active

prodrug [Chanoine, 1991 #53,Wurthwein, 1990 #56] more than one decade after it

became clinically available further complicated its pharmacokinetic studies. Some

pharmacokinetic studies have been conducted to assess and compare the pharmacokinetic

properties of these two formulations [Harrison, 1999 #1; Harrison, 1999 #16; Seale, 1998

#22; Lipworth, 1999 #6]. The unanimous conclusion of these studies was that at the

same dose level, HFA-BDP gave 2-3 fold greater area under the plasma concentration vs

time curve (AUC) and $C_{max}$ than CFC-BDP. In some of these studies [Harrison, 1999 #1;

Harrison, 1999 #16; Seale, 1998 #22], total beclomethasone (BOH), the metabolite of

BDP, was used as the representative of the active metabolite, beclomethasone 17-

monopropionate (17-BMP), to investigate the pharmacokinetic properties of BDP due to

the lack of a sensitive analytical method for 17-BMP. However, estimates of oral

bioavailability and pulmonary deposition based on total BOH were approximately half

those found for 17-BMP based on the recent study by Daley-Yates et al. in which 17-

BMP was quantified by a sensitive HPLC/MS/MS assay [30]. But this study only

investigated the pharmacokinetics of CFC-BDP. 17-BMP was also directly measured in

another comparative study [Lipworth, 1999 #6], but BDP was not quantified to clarify

whether the higher systemic availability of 17-BMP for HFA-BDP was partially due to a

higher percentage of intact BDP being absorbed after inhalation since it was suggested

that the higher peripheral lung deposition and the expected increase in absorption rate for HFA-BDP could lead to greater absorption of intact BDP and partially offset the benefits of higher pulmonary deposition [30].

## Rationale

In the therapy of inflammatory diseases of the respiratory tract inhaled glucocorticoids are employed primarily in order to attain high drug levels in the lung while simultaneously minimizing systemic drug load. BDP is a commonly used inhaled corticosteroid. A new CFC-free, breath actuated formulation, HFA-BDP, has been developed which seems to enable a higher pulmonary deposition. The purpose of this study is to examine the pharmacokinetic profile and systemic side effects of twice daily inhaled BDP comparing two different devices: Sanasthmax[®] (CFC MDI, Glaxowellcome) vs QVAR[®] (HFA MDI, 3M pharmaceuticals), in the same subjects after a single dose of 1000μg.

## Materials and Methods

### Materials

The following BDP formulations were supplied by GlaxoWellcome Clinical Trial Supplies: BDP aqueous nasal spray, Beconase[®]; CFC-BDP MDI, Sanasthmax[®]. HFA-BDP MDI was obtained from 3M. Activated charcoal (Medicoal[®]) was administered orally for the charcoal block procedure.

### Subjects

The study was conducted at the University Clinic "Bergmannsheil" in Bochum, FRG in accordance with the revised Declaration of Helsinki (Hong Kong Revision 1989) and to Good Clinical Practice guidelines. A total of 6 healthy male volunteers entered the

study.  They had a mean age of 26.5 (range 22-30) years, height of 186.2 (range 180-198) cm and weight of 78.7 (range 70-85) kg.  The protocol was approved by the Ethics Committee of Ruhr University, Bochum and all subjects gave written informed consent.

**Study Design**

The study was an open, randomized, 8-way crossover design with a washout period of at least 1 week between each of the treatments.  The eight treatments comprised of 1a) 1000µg Sanasthmax inhaled with charcoal for 6 subjects 1b) 1000µg Sanasthmax inhaled without charcoal for 6 subjects (both doses delivered via CFC MDI, GlaxoWellcome) 2a) 1000µg Qvar inhaled with charcoal for 6 subjects 2b) 1000µg Qvar inhaled without charcoal for 6 subjects 3a) 1000 µg Beconase oral with charcoal for 1 subject 3b) 1000 µg Beconase oral without charcoal for 3 subjects (2ml Beconase® was put in a teaspoon and drunk by the subjects) 4a) 1000 µg Qvar oral with charcoal for 3 subjects 4b) 1000 µg Qvar oral without charcoal for 2 subjects (The subjects received 10 puffs of Qvar on their tongues and swallowed the drug with liquid).  A single dose of the test substance was administered at 8 a.m. to allow for a complete pharmacokinetic/pharmacodynamic evaluation over 24 hours and blood samples for drug, cortisol, lymphocyte and granulocyte analysis in plasma were collected at frequent intervals.

**Investigational Procedures**

The subjects were instructed and trained to use the inhalation technique given in the package insert for each of the drugs/devices.  Subjects were fasted from midnight before each study day until 12 p.m. of the study day.  The subjects were required to arrive at the Clinical Pharmacology Unit of the University Clinic "Bergmannsheil" at 7:30 a.m. of study day and they stayed at the unit for the entire 24-hour study period.  Cannulation of a forearm vein was performed at 7:45 a.m. on study day.  The cannula was kept patent by

means of a mandrin. Blood samples were taken via the cannula before drug administration and 10, 20, 30, 60 minutes as well as 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24h after dosing. Blood was drawn into potassium oxalate/sodium fluoride tubes in order to inhibit further esterase hydrolysis, mixed immediately and placed on ice. Within 15 minutes, the sample was centrifuged at 1000g for 15 min in a refrigerated centrifuge. The plasma samples were stored at -30°C prior to determination of BDP, 17-BMP and BOH. The sample handling and storage procedures were shown to prevent the hydrolysis of BDP and its metabolites.

For the dynamic evaluation, differential blood cell counts were performed before and after drug administration at the following time points: 1, 2, 3, 4, 6, 8, 10, 14, 16, 18, 20, 22, 24 hours and endogenous cortisol was determined by following the same time schedule.

**Bioanalytical Methods**

Plasma concentrations of BDP, 17-BMP and BOH were determined by LC-MS-MS. The plasma samples were mixed thoroughly and centrifuged prior to solid phase extraction (SPE) on $C_{18}$ 50 mg Microlute II 96 well blocks. The procedure was fully automated using a custom built Zymark robotic 96 well solid phase extraction system. The resulting extracts were evaporated to dryness under a stream of heated nitrogen (nominally 40°C) and reconstituted with 75 μl of 40/60 (v/v) acetonitrile/25 mM ammonium formate pH 5 buffer. The method employed a 150×2.1 mm i.d. $RPC_8$ 5μm Symmetry Shield (Waters Corps.) analytical column and a Perkin Elmer API-3000 mass spectrometer. A 6 min gradient elution profile was used with a flow rate of 0.3 ml min-1 and no splitting (Solvent A: 25 mM ammonium formate pH5.0 and B: 100% acetonitrile-

start 55% A and 35% B, end 10% A and 90% B). Protonated molecules, MH+, were used as precursor ions with selected reaction monitoring of the following transitions for BDP, 17-BMP and BOH respectively: m/z 521→ 319, m/z 465→279 and m/z 409→279. The calibration range for this method was 50-3000 pg ml$^{-1}$ from 0.6 ml plasma for BDP and 17-BMP and 50-1000pg ml$^{-1}$ for BOH. The coefficients of variation for the three analytes were as follows: BDP 7.9%, 17-BMP 5.9%, BOH 8.1%. The limit of quantification for each analyte was 50 pg ml$^{-1}$.

Lymphocyte and granulocyte number were determined by Coulter-Counter and blood smear.

Plasma cortisol concentrations were determined with a competitive solid-phase radioimmunoassay, applicable for measurements in plasma (Coat-A-Count, Diagnostic Products Corp., obtained through H. Biermann GmbH, Bad Nauheim, FRG). The unknown samples (25µL of plasma) were incubated for 45 min (plasma) with $^{125}$I-labled cortisol in tubes coated with anticortisol antibodies. After aspirating the liquid from each tube, the radioactivity in the tubes was measured with a gamma counter (Multi-Crystal Counter LB 2104; Berthold, Bad Wildbad, FRG). The between–assay's coefficient of variation was 6.3% in plasma at a mean cortisol concentration of 48 µg/L. This assay showed no cross-reactivity towards BDP and 17-BMP in the concentration range observed in the clinical study.

**Pharmacokinetic Analysis**

Noncompartmental PK analysis was performed using the WinNonlin® software (Pharsight, CA, USA). Where possible the following pharmacokinetic parameters were calculated: the area under the plasma concentration-time-curve (AUC) extrapolated to

infinity where the extrapolated AUC was estimated from Computed$C_{last}$/terminal

elimination rate constant, the mean residence time (MRT), elimination half-life ($t_{1/2}$)

based on log-linear regression, maximum plasma concentration ($C_{max}$) and the time to

$C_{max}$ ($t_{max}$).

**Pharmacodynamic Analysis**

Twenty-four hour plasma cortisol concentrations were used to assess the effects of

single dose of BDP on the basal hypothalamic-pituitary-adrenal (HPA) axis.  Since there

was no placebo group as the reference, the areas under the cortisol concentration-time

curve (AUC), calculated by the linear trapezoidal rule over 24 hours, for the two

formulations were directly compared for possible difference in their effects on the basal

hypothalamic-pituitary-adrenal (HPA) axis.

In addition, the twenty-four hour blood lymphocyte and granulocyte counts were

used to compare the effects of these two formulations on inflammation related cells.  As a

cumulative measure of pharmacodynamic activity on the blood cells, the area under the

effect-time curve ($AUC_E$) will be calculated using linear trapezoidal rule over 24 hours

with the effect defined as the difference between number of cells (N, in percent of pre-

dose) (Equation 5-1).

$$AUC_E = \int_0^{24}(100\% - N)dt \qquad\qquad 5\text{-}1$$

**Statistical Analysis**

The primary pharmacodynamic parameters of interest are $AUC_{24}$ for plasma

cortisol, lymphocyte and granulocyte.  Treatment comparisons were made after log

transformation using analysis of variance. The analysis was performed using SAS[®]

software (SAS, NC, USA).

## Results and Discussions

**Efficiency of Charcoal Block Procedure**

Two different approaches were used to orally administrate 1000µg BDP: (a) The subjects received 10 puffs of Qvar® on their tongues and swallowed the drug with liquid, (b) 2ml Beconase® was put in a teaspoon and drunk by the subjects. Either with or without charcoal, no intact BDP was detected in plasma. The mean plasma concentration profile for 17-BMP following oral dosing (a) is shown in Figure 5-1. It is obvious that the use of the charcoal block procedure resulted in very low area under the plasma concentration vs. time curve ($AUC_{with}$ =417.2±2.4, $AUC_{with}$: AUC with charcoal) for 17-BMP. By comparing this value with $AUC_{w/o}$ (2090.2±95.7, $ACU_{w/o}$: AUC without charcoal) for 17-BMP following oral dosing without charcoal, the efficiency of the charcoal block procedure was estimated to be 80% for (a) and 100% for (b).

Among the 3 subjects who orally received Qvar with Charcoal, AUC calculated for subject 2 was unusually high compared to the other two subjects and even higher than his corresponding AUC without charcoal treatment. Further outlier test (Dixon's criteria) showed that it was an outlier ($p<0.01$). As a result, this subject's data were not included for statistical analysis.
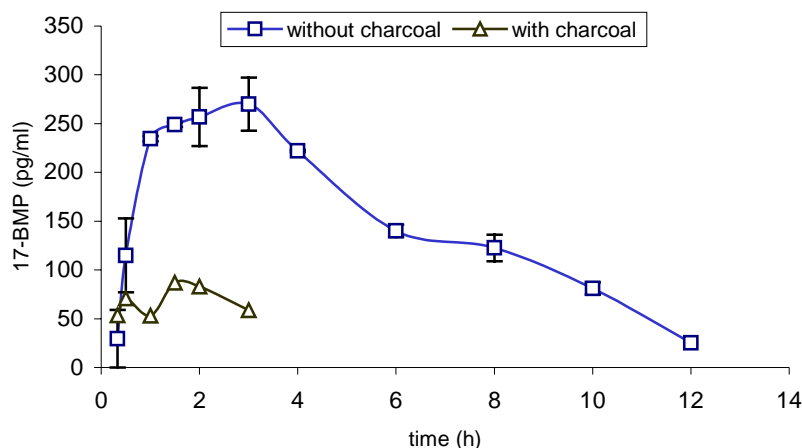
Figure 5-1. Plasma concentration of 17-BMP following oral dosing of Qvar® (HFA-BDP) with or without charcoal

**Pharmacokinetics**

Following inhalation of BDP, both BDP and 17-BMP were detectable. The mean

plasma concentration profiles for BDP and 17-BMP with and without charcoal block are

shown in Figures 5-2 and 5-3. Table 5-1 and 5-2 summarize the results of non-

compartmental pharmacokinetic analysis for BDP and 17-BMP after single dose of 1000

µg BDP inhaled via Qvar® and Sanasthmax® metered-dose inhalers.
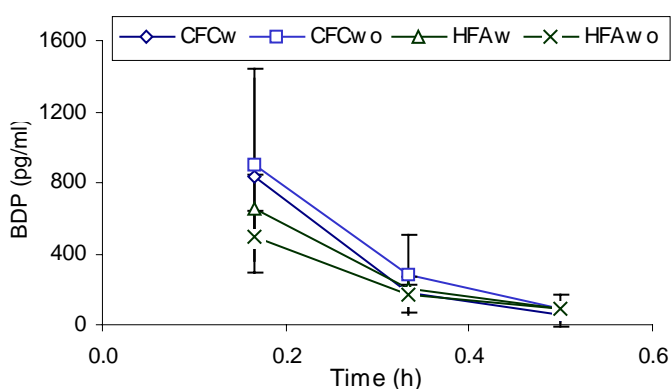


Figure 5-2. Plasma concentration of BDP following inhalation of Qvar® (HFA-BDP) and Sansthmax® (CFC-BDP) with or without charcoal (Sw: Sansthmax® with charcoal , Sw/o: Sansthmax® without charcoal , Qw: Qvar® with charcoal, Qw/o: Qvar® without charcoal))
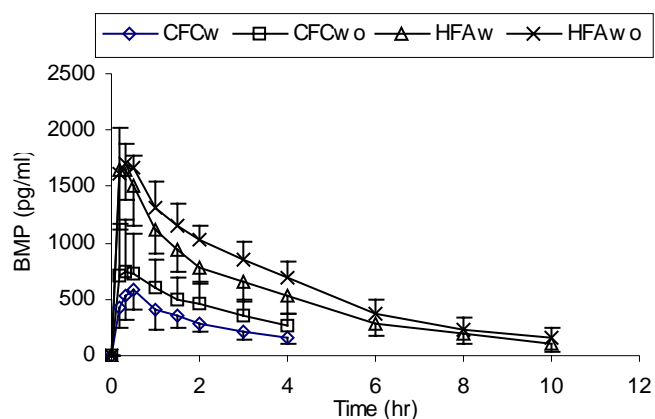
Figure 5-3. Plasma concentration of 17-BMP following inhalation of Qvar® (HFA-BDP) and Sansthmax® (CFC-BDP) with or without charcoal (Sansthmax® with charcoal, Sansthmax® without charcoal, Qvar® with charcoal, Qvar® without charcoal)

For BDP, there were no significant differences between Qvar® and Sanasthmax® in terms of several important pharmacokinetic parameters. In contrast, plasma levels of 17-BMP were significantly greater following Qvar® than following Sanasthmax® (Figure. 5-3) with the $C_{max}$ ratio being 2.4 to 3.7 for with charcoal method and 1.9 to 2.9 for without charcoal method at 90% confidence. The time to reach the peak 17-BMP levels ($t_{max}$) was significantly earlier with Qvar®. All pharmacokinetic parameters in Table 5-2 between these two formulations were significantly different if BDP is administered with charcoal. Without charcoal block, only $C_{max}$ and AUC are significantly different between these two formulations.

The calculation of the pharmacokinetic parameters for BDP was based on very few points that were detectable. As a result, it is very likely that the early differentiating points for these two formulations were missed during the sampling process due to the rapid metabolism of BDP in lung and circulating system. This could be the reason why no significant differences were detected between these two formulations in terms of the

pharmacokinetic performance of BDP despite the obvious differences for 17-BMP.  The

earlier $t_{max}$ for 17-BMP following HFA-BDP with charcoal block indicted a faster

Table 5-1: Pharmacokinetic parameters of BDP after inhalation of Qvar® and Sanasthmax®

| Treatment | Formulation | Cmax (pg/ml) | tmax (hr) | AUC (pg h/ml) | MRT(hr) |
|---|---|---|---|---|---|
| With charcoal | Qvar® | 634 (473, 852) | 0.17 (0.17) | 158 (121, 208) | 0.22 (0.21, 0.23) |
| | Sanasthmax® | 612 (226, 1660) | 0.17 (0.17) | 294 (193, 449) | 0.20 (0.19, 0.21) |
| Without charcoal | Qvar® | 479 (338, 680) | 0.17 (0.17) | 130 (93, 182) | 0.23 (0.22, 0.24) |
| | Sanasthmax® | 684 (254, 1842) | 0.17 (0.17) | 229 (136, 387) | 0.21 (0.20, 0.22) |

Geometric mean (95% CI) n=6

Table 5-2: Pharmacokinetic parameters of 17-BMP after inhalation of Qvar® and Sanasthmax®

| Treatment | Formulation | Cmax (pg/ml) | tmax (hr) | AUC (pg h/ml) | MRT(hr) | t1/2(h) |
|---|---|---|---|---|---|---|
| With charcoal | Qvar® | 1787 (1336, 2392) | 0.33 (0.13, 0.5) | 5568 (3853, 8048) | 3.3 (2.5, 4.4) | 3.0 (2.1, 4.2) |
| | Sanasthmax® | 564 (372, 854) | 0.5 (0.33-0.5) | 1576 (997, 2491) | 2.0 (1.6, 2.5) | 1.9 (1.7, 2.2) |
| | P-value | 0.005 | <0.01 | 0.003 | 0.004 | 0.015 |
| | Ratio (90% CI) | 3.2 (1.7, 5.9) | - | 3.5 (1.9, 6.6) | - | - |
| Without charcoal | Qvar® | 1848 (1629, 2096) | 0.17 (0.17, 0.5) | 7070 (5587, 8946) | 3.7 (2.9, 4.7) | 2.9 (2.2, 3.9) |
| | Sanasthmax® | 678 (387, 1187) | 0.42 (0.33, 0.5) | 2678 (1613, 4445) | 2.7 (1.8, 4.0) | 2.6 (1.9, 3.7) |
| | P-value | 0.007 | 0.19 | 0.008 | 0.03 | 0.23 |
| | Ratio (90% CI) | 2.7 (1.5, 4.9) | - | 2.6 (1.5, 4.7) | - | - |

Geometric mean (95% CI) n=6

absorption rate for HFA-BDP than CFC-BDP.   The longer $MRT_{inh}$ for HFA-BDP than

that for CFC-BDP, however, implied a longer mean absorption time (MAT) in lung since

the HFA-BDP and CFC-BDP have the same $MRT_{i.v}$, which was against the faster

absorption rate for HFA-BDP.   Also the terminal elimination half-lives of these two

formulations were found significantly different when charcoal was coadministered.  This

controversy could be explained by the significant difference between the plasma 17-BMP

concentrations for two formulations. Due to the relatively low plasma concentration of 17-BMP for Sanasthmax®, the terminal points that were used to calculate the terminal elimination rate constant in WinNonlin® could not be the real elimination terminal points, but the points before the elimination phase. In contrast, the high concentration of 17-BMP for Qvar® guaranteed more points to be monitored to a later time and hence the terminal phase could be sufficiently characterized. As a result, if there exits a fast distribution process during drug disposition, the estimated terminal $t_{1/2}$ for Sanasthmax® will be shorter than its real elimination $t_{1/2}$. This limitation on terminal points could cause a similar error in estimating $MRT_{inh,}$ underestimating $MRT_{inh}$ for Sanasthmax® when extrapolating from the last time point to infinity. A more sensitive assay would clarify this. Despite these differences, our $t_{1/2}$ (1.9-3.7hr) and $MRT_{inh}$ (1.8-4.0 hr) for 17-BMP after Sanasthmax® inhalation without charcoal are consistent with those (2.3-2.7 hr for $t_{1/2}$ and 3.5-4.1 hr for $MRT_{inh}$) recently reported by Daley-Yates [30] in a study involving only CFC-BDP. The significantly higher $C_{max}$ and AUC values for 17-BMP following HFA-BDP inhalation compared with CFC-BDP inhalation implied a significantly higher deposition percent of inhaled HFA-BDP in the lung, which agrees with the results of scintingraphic methods and other pharmacokinetic studies [32, 84]. The ratio of 2.0-3.0 between HFA and CFC for AUC suggested a dramatic dose adjustment for HFA-BDP at an equivalent efficacy level. A relative potency ratio of 2.6 for HFA vs CFC-BDP was reported for Qvar® MDI as their effects on $FEV_1$ in patients with moderate to severe asthma were compared [98]. Several other studies have already shown that half the dose of HFA-BDP was as effective as CFC-BDP in the control of moderately severe asthma [99-101].

There is always a concern about the source of the higher 17-BMP exposure following HFA-BDP inhalation. It is known that the pulmonary deposition of HFA-BDP is significantly higher than CFC-BDP based on the results from scintingraphic methods and other pharmacokinetic studies [32, 84]. But is 17-BMP generated after BDP is absorbed into the systemic circulation or activated in the lung before BDP is absorbed into the systemic circulation? It is believed that the higher 17-BMP systemic exposure is partially due to more intact BDP that is directly absorbed into the circulation [30]. The results in this study, however, suggested that even with a higher pulmonary deposition, HFA-BDP showed similar BDP systemic exposure to CFC-BDP as indicated by the AUCs for BDP following HFA-BDP and CFC-BDP inhalation with charcoal. Given the much higher 17-BMP systemic exposure for HFA-BDP, the activation efficiency of HFA-BDP in the lung is clearly higher than that of CFC-BDP. To be more conservative about this conclusion due to the small number of points for AUC estimation for BDP, a worst scenario simulation was conducted to assess the impact of missing early BDP concentration points on this conclusion. Basically, HFA-BDP was treated as if it was administered by intravenous bolus route and CFC-BDP was treated as if the first order absorption process was so slow that the first concentration was $C_{max}$ for BDP (Figure 5-4). Even with this most extreme assumption, the BDP exposure from HFA-BDP is only 1.3 times that from CFC-BDP based on AUCs of BDP, still suggesting a more efficient activation of HFA-BDP in the lung given the ratio of 3.5 for 17-BMP between HFA-BDP and CFC-BDP.  The reason for this phenomenon is not clear, but clarification of the distribution of esterase in the lung should provide valuable information because it is known that inhaled HFA-BDP is mainly deposited in the peripheral airways while most

inhaled CFC-BDP is deposited in the main bronchi according to their mean mass

aerodynamic diameters (MMAD) [102, 103].
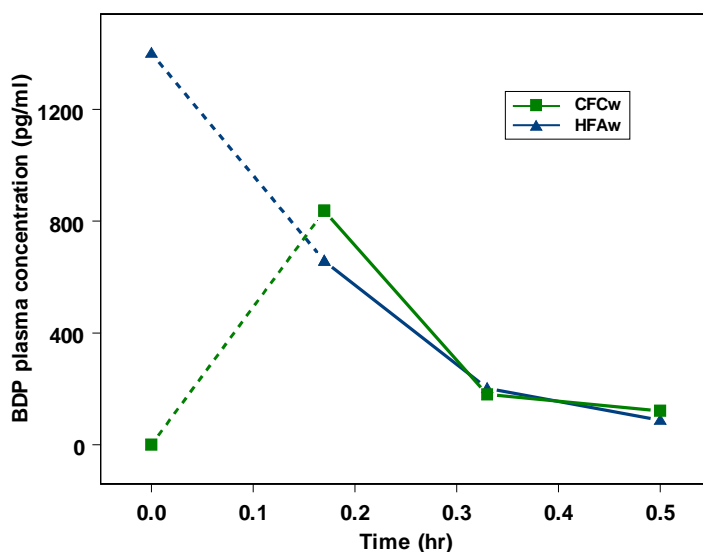


Figure 5-4: Simulated BDP plasma profile (dashed line) following inhalation of Qvar[®] (HFA-BDP) and Sansthmax[®] (CFC-BDP) with charcoal (CFCw: Sansthmax[®] with charcoal, HFAw: Qvar[®] with charcoal)

Prior to the present work, we had hypothesized that the pulmonary deposition

percentage could be calculated based on the differences in AUCs after oral and inhalation

administrations with and without charcoal block, assuming the pulmonary bioavailability

is 100%. However, the limited data from oral study invalidated this approach. Without

any doubt, though, the much higher $C_{max}$ and AUC are mainly due to the higher

pulmonary deposition of HFA-BDP.

**Pharmacodynamics**

Plasma cortisol, lymphocyte and granulocyte concentration-time profiles for both

formulations with charcoal or without charcoal block are shown in Figures 5-5, 5-6 and

5-7.

Figure 5-5: 24-hour plasma concentration of cortisol following inhalation of Qvar[®] (HFA-BDP) and Sansthmax[®] (CFC-BDP) with or without charcoal (Sw: Sansthmax[®] with charcoal, Sw/o: Sansthmax[®] without charcoal, Qw: Qvar[®] with charcoal, Qw/o: Qvar[®] without charcoal)
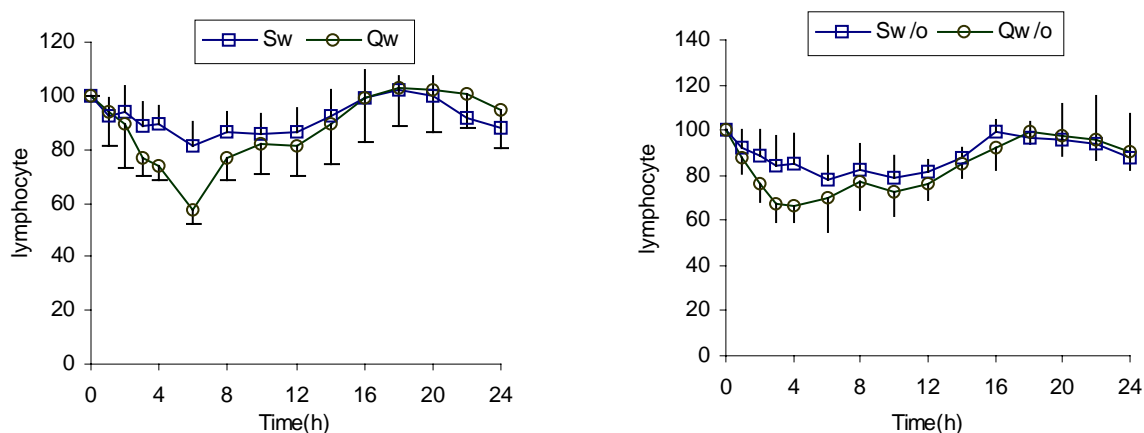


Figure 5-6: 24-hour blood lymphocyte count following inhalation of Qvar[®] (HFA-BDP) and Sansthmax[®] (CFC-BDP) with or without charcoal (Sw: Sansthmax[®] with charcoal, Sw/o: Sansthmax[®] without charcoal, Qw: Qvar[®] with charcoal, Qw/o: Qvar[®] without charcoal)

With charcoal block, there was no statistically significant difference between the AUCs' of all three surrogate markers for two formulations (Table 5-3). The lack of a placebo group hinders further conclusion on whether a significant suppression was induced or not. But the significant difference in AUCs of 24-hour cortisol levels without
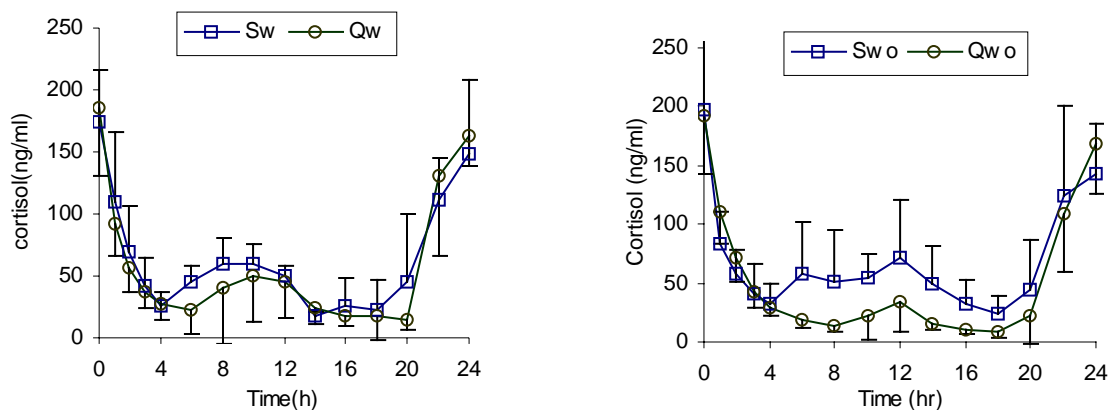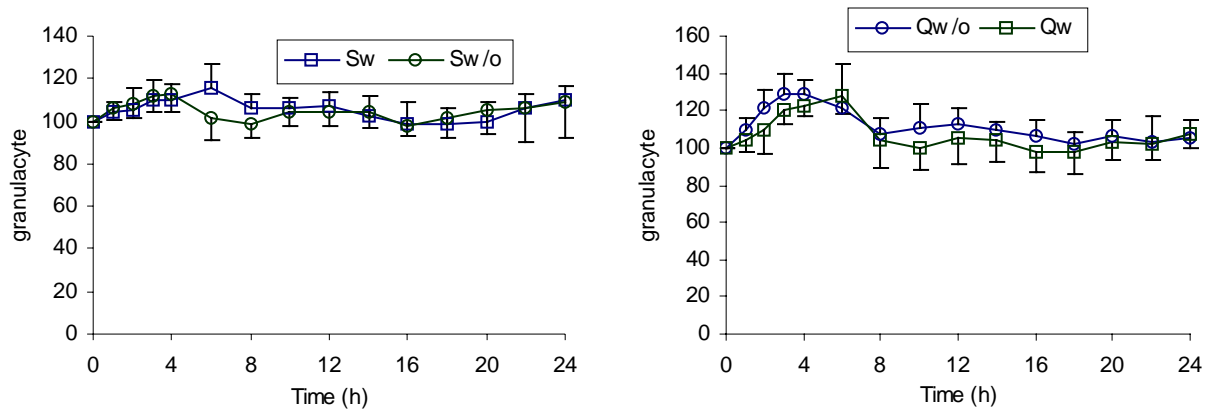
Figure 5-7: 24-hour plasma granulocyte count following inhalation of Qvar$^®$ (HFA-BDP) and Sansthmax$^®$ (CFC-BDP) with or without charcoal (Sw: Sansthmax$^®$ with charcoal, Sw/o: Sansthmax$^®$ without charcoal, Qw: Qvar$^®$ with charcoal, Qw/o: Qvar$^®$ without charcoal)

charcoal block indicated that at least Qvar$^®$ caused significant cortisol suppression. No statistically significant differences were detected in AUCs of lymphocyte and granulocyte levels for the two formulations even though there was a trend that Qvar had more lymphocyte suppression effect and granulacyte induction effect.

Table 5-3: AUC$_{24}$ for three PD markers after inhalation of Qvar$^®$ and Sanasthmax$^®$

| Treatment | Cortisol (ng*h/ml) | Lymphocyte (%*h) | Granulocyte (%*h) |
|---|---|---|---|
| Qvarwo | 1012 | 1997 | 2657 |
| | (994, 1031) | (1983, 2010) | (2641, 2673) |
| Sanasthmaxwo | 1436 | 2113 | 2496 |
| | (1406, 1467) | (2100, 2125) | (2483, 2508) |
| p-value | 0.02 | 0.18 | 0.14 |

Geometric mean (95% CI) n=6

Several methods are currently used to assess the effect of inhaled corticosteroids on the HPA axis function. The most common include spot morning (eg. 8 a.m.) cortisol measurements, urinary cortisol excretion, the ACTH stimulatory test and the 24-hour plasma cortisol AUC evaluation [55]. The present study employed the 24-hour plasma cortisol AUC method because of its ability to detect small changes that may be seen with low dose inhaled corticosteroid therapy [55]. No significant difference in cortisol

suppression was detected after a single dose of 1000μg Qvar® and Sansthmax® with charcoal block despite the much higher plasma concentration of 17-BMP for Qvar®. Even though Qvar showed more significant cortisol suppression than Sansthmax without charcoal block, there is enough room for dose adjustment for Qvar® to reduce cortisol suppression and still be as effective as Sansthmax®. The 24-hour AUCs of the lymphocyte and granulocyte levels did not show significant difference between these two formulations.

## Conclusion

In conclusion, present study has shown that unchanged BDP has negligible oral bioavailability with limited absorption after inhalation regardless of the formulations, which indicated the extensive metabolism of BDP in both liver and lung. The higher bioavailability of 17-BMP for inhaled HFA-BDP (Qvar®) than for CFC-BDP (Sansthmax®) is due to the fact that more percentage of inhaled HFA-BDP is deposited in lung even though there is still a portion of plasma 17-BMP that is due to gastrointestinal absorption. An interesting finding is that despite the higher pulmonary deposition and fast absorption, HFA-BDP showed better activation efficiency in the lung than CFC-BDP. Combined with other efficacy studies, the high bioavailability supports a significant dose reduction for HFA-BDP with the potential to reduce side effects while being equally effective to treat asthma as CFC-BDP at a higher dose.

CHAPTER 6
CONCLUSIONS

The overall objective of this thesis is to evaluate the pharmacokinetics and

pharmacodynamics of BDP. First, a simple, sensitive and selective LC-(ESI+)-MS-MS

method was developed for simultaneously quantifying BDP and its active major

metabolite, 17-BMP, in rat plasma and tissue samples.  Validation results have shown

that the method is robust and meets the requirements of the pharmacokinetic investigation

of inhaled BDP.  Then the pulmonary targeting of BDP after intratracheal administration

of micronized BDP dry powder was evaluated with an ex-vivo receptor binding assay in

rats.  The prodrug property of BDP and the fast pulmonary absorption of BDP were

found to affect its pulmonary targeting in a negative way even though the high

lipophilicity of BDP and the resulting longer dissolution time contributed to longer

pulmonary residence time, a beneficial factor for better pulmonary targeting. With non-

compartmental analysis and compartmental modeling, it was found that almost 90% of

BDP was metabolized to its active metabolite, 17-BMP, in rat systemic circulation and a

significant portion of pulmonarily deposited BDP is absorbed into the systemic

circulation in the form of intact BDP when BDP is administered in micronized dry

powder formulation to rats.  The impact of prodrug property of BDP on its pulmonary

targeting was quantitatively assessed by the activation portion of BDP into 17-BMP in

the lung.  As the active metabolite, 17-BMP showed very high plasma protein binding

and tissue bindings in liver, lung and kidney, suggesting a possible reason for the low

systemic side effect of long term BDP treatment for asthma. The similarity among the

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$EC_{50}$ values estimated for rat liver, lung and kidney tissues confirmed the homogeneity of glucocorticoid receptor in different tissues. The clinically study comparing HFA-BDP (Qvar[®]) and CFC-BDP (Sansthmax[®]) has shown very low unchanged BDP systemic exposure due to negligible oral bioavailability and limited absorption after inhalation for both formulations. The higher pulmonary deposition of HFA-BDP (Qvar[®]) led to higher bioavailability of 17-BMP compared with CFC-BDP (Sansthmax[®]) even though a small portion of systemic 17-BMP is due to gastrointestinal absorption. Despite the higher pulmonary deposition and fast absorption, higher percentage of BDP was activated into 17-BMP in the lung for HFA-BDP (Qvar[®]) than for CFC-BDP (Sansthmax[®]). These results supported a significant dose reduction for the new formulation with the potential to reduce side effects while being equally effective to treat asthma as the traditional formulation at a higher dose.

## APPENDIX A
### DERIVATION OF FULL PLASMA FREE FRACTION

At equilibrium, $[D]+[P]=[DP]$, where D is drug, P is protein and DP is the complex. Assuming Kd is the equilibrium constant, $Kd=[DP]/([D]*[P])$

Since $[Dtotal]=[D]+[DP]$ (assuming 1:1 binding), $[DP]=[Dtotal]-[D]$.

Then $Kd=([Dtotal]-[D])/([D]*[P])$ (1)

Define $fu=[D]/[Dtotal]$. Then dividing both the numerator and denominator of (1) by $[Dtotal]$, we get $Kd=(1-fu)/(fu*[P])$. Since only a very small percentage of total protein is bound by drugs, $[Ptotal] \approx [P]$.

Then $Kd=(1-fu)/(fu*[Ptotal])$

Define f' as the unbound fraction at another Ptotal, say $[Ptotal]'$.

Then $(1-fu)/(fu*[Ptotal])=(1-f')/(f'*[Ptotal]')$ (Kd is independent of the concentration of protein).

If $[Ptotal]=10\%$ and $[Ptotal]'=100\%$,

$f'=fu/(10-9*fu)$.

If $[Ptotal]=1\%$ and $[Ptotal]'=100\%$,

$f'=fu/(100-99fu)$.

# APPENDIX B
## NONMEM CODE FOR IV BOLUS ADMINISTRATION OF BDP OR 17-BMP

```
$PROBLEM FIT OF BDP AND 17-BMP AFTER IV OF 1000UG BDP OR IV OF 17-BMP
$INPUT ID TIME DV CMT AMT EVID DRUG
$DATA "c:\yaning\bdp\nm\bdmpIV.csv" IGNORE=#
$SUBROUTINE ADVAN6 TRANS1 TOL=3

$MODEL
COMP=1;BDP
COMP=2;BMP

$PK
TVVP=THETA(1)
TVCLP=THETA(2)
TVVM=THETA(3)
TVCLM=THETA(4)

VP=TVVP
CLP=TVCLP*EXP(ETA(1))
VM=TVVM
CLM=TVCLM

;SECONDARY PARAMETERS
KD=CLP/VP
KM=CLM/VM

IF (VP.LT.0.00001) EXIT 1 100
IF (VP.GT.9000) EXIT 1 200
IF (CLP.LT.0.00001) EXIT 1 300
IF (CLP.GT.9000) EXIT 1 400
IF (VM.LT.0.00001) EXIT 1 500
IF (VM.GT.9000) EXIT 1 600
IF (CLM.LT.0.00001) EXIT 1 700
IF (CLM.GT.9000) EXIT 1 800

S1=VP
S2=VM

$DES
DADT(1)=-(CLP/VP)*A(1)
DADT(2)=0.8925*0.88*CLP/VP*A(1)-CLM/VM*A(2)

$ERROR

Y=F*(1+ERR(1))
IPRED=F

$THETA
(0.001,37.5)    ;VP
```

```
(0.001,18.1)     ;CLP
(0.001,2.24)  ;VM
(0.001,1.86)  ;CLM

$OMEGA
(0.04)

$SIGMA
(0.1)

$EST METHOD=1 NOABORT PRINT=15 MAXEVAL=9999
$COV PRINT=E
$TABLE ID TIME DV CMT AMT EVID DRUG IPRED
     NOPRINT ONEHEADER FILE=bdmpiv.fit
```

# NONMEM CODE FOR INHALATION ADMINISTRATION OF BDP OR 17-BMP

```
$PROBLEM fit of BDP and BMP after iv or inhalation of 1000ug BDP or BMP
$INPUT ID TIME DV CMT AMT EVID DRUG
$DATA "c:\yaning\bdp\nm\data\bdmpinh.csv" IGNORE=#
$SUBROUTINE ADVAN6 TRANS1 TOL=3

$MODEL
COMP=1;BDP
COMP=2;BMP
COMP=3;BDP INHALATION
COMP=4;BMP INHALATION

$PK
TVVP=THETA(1)
TVCLP=THETA(2)
TVVM=THETA(3)
TVCLM=THETA(4)
TVKA1=THETA(5)
TVKA2=THETA(6)
TVFR=THETA(7)

VP=TVVP
CLP=TVCLP*EXP(ETA(1))
VM=TVVM
CLM=TVCLM
KA1=TVKA1;BDP ABSORPTION
KA2=TVKA2;BMP ABSORPTION
FR=TVFR; FRACTION OF INTACT BDP ABSORBED

;SECONDARY PARAMETERS
KD=CLP/VP
KM=CLM/VM

S1=VP
S2=VM

FACTOR=0.8925*0.88

$DES
DADT(1)=-(CLP/VP)*A(1)+KA1*FR*A(3)
DADT(2)=FACTOR*CLP/VP*A(1)+KA1*(1-FR)*FACTOR*A(3)+KA2*A(4)-CLM/VM*A(2)
DADT(3)=-KA1*A(3)
DADT(4)=-KA2*A(4)
$ERROR

Y=F*(1+ERR(1))
IPRED=F
```

```
$THETA
(21.3 FIX)   ;VP
(28.8 FIX)   ;CLP
(2.22 FIX)   ;VM
(1.86 FIX)   ;CLM
(0.3);KA1
(0.3);KA2
(0.0001,0.5, 1);FR


$OMEGA
(0.1);ETA1 CANNOT HAVE BOUNDS

$SIGMA
(0.1);ERR1 CANNOT HAVE BOUNDS

$EST METHOD=0 NOABORT PRINT=15 MAXEVAL=9999 POSTHOC
$COV PRINT=E
;$SIMULATION(12)
$TABLE ID TIME DV CMT AMT EVID DRUG IPRED
NOPRINT ONEHEADER FILE=BDMPALL.TAB
```

# APPENDIX D
## NONMEM CODE FOR TISSUE BINDING OF 17-BMP

```
$PROBLEM SIMULTANEOUS FIT OF 17-BMP IN PLASMA, LUNG, LIVER, KIDNEY
AFTER IV OF 90UG/KG 17-BMP
$INPUT ID TIME DV ORGN AMT EVID MDV
$DATA "c:\yaning\bdp\nm\data\BMPIVNICK.csv" IGNORE=#
$SUBROUTINE ADVAN1 TRANS2

$PK

TVV=THETA(1)
V=TVV*EXP(ETA(1))
TVCL=THETA(2)
CL=TVCL
S1=V
FLUNG=THETA(3)
FLIVER=THETA(4)
FKID=THETA(5)

$ERROR
C=F
IF (ORGN.EQ.2) C=F*FLUNG
IF (ORGN.EQ.3) C=F*FLIVER
IF (ORGN.EQ.4) C=F*FKID
Y=C*(1+ERR(1))
IPRED=C

$THETA
(0.001,2.6)          ;V
(0.001,2.14)         ;CL
(0.001,1)            ;FLUNG
(0.001,2.8)          ;FLIVER
(0.001, 1.3)         ;FKIDNEY

$OMEGA
(0.04) ;V

$SIGMA
(0.129);EPS1

$EST METHOD=0 NOABORT PRINT=15 MAXEVAL=9999 POSTHOC
$COV
$TABLE ID TIME AMT IPRED ORGN EVID
NOPRINT ONEHEADER FILE=bmpadvan1nick.fit
```

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BIOGRAPHICAL SKETCH

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