Analytical Studies Concerning the Stability of Betamethasone Valerate and Fusidic acid Hemihydrate in Topical Pharmaceutical Preparations

A thesis submitted to Maynooth University in fulfilment of the requirements for the degree of

Doctor of Philosophy

by

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Abbreviations

°C	Degrees Celsius
%	Percent
FA	Fusidic acid
BV	Betamethasone valerate
MRSA	Methicillin-Resistant *Staphylococcus aureus*
EF-G	Elongation factor G
GCC	Glucocorticoid
C-n	Carbon atom number n
GCC-R	Glucocorticoid receptor
TLC	Thin Layer Chromatography
GC	Gas Chromatography
LC	Liquid Chromatography
HPLC	High Performance Liquid Chromatography
UPLC	Ultra-High Performance Liquid Chromatography
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
N	Number of theoretical plates
α	Selectivity factor
k	Capacity factor
Rs	Resolution
$w_{1/2}$	Peak width at half peak height
HETP	Height equivalent of a theoretical plate
A	Eddy diffusion term
B	Longitudinal diffusion term
u	Linear velocity in m s^{-1}
C	Mass transfer term
m/sec	Metres per second
ml/min	Millilitres per minute
t_0	Dead time
t_R	Retention time
RRT	Relative retention time
UV-Vis	Ultra–violet / visible
RI	Refractive index
DAD	Diode Array Detector
ELSD	Electronic Light Scattering Detector

MS	Mass spectrometry
XRD	X-Ray Diffraction
XRPD	X-Ray Powder Diffraction
λ	Wavelength
CCD	Charged Coupled Device
mg/min	Milligrams per minute
$mg/min/cm^2$	Milligrams per minute per square centimetre
Ph. Eur.	European Pharmacopeia
Rpm	Revolutions per minute
ml	Millilitre
µl	Microliter
mg	Milligram
µm	Micrometre
d	Density
g/L	Grams per litre
M	Molarity of a solution
nm	Nanometre
PL	Polylactide
PCL	Poly(caprolactone)
PLGA	Poly (lactic-*co*-glycolic acid)
M_n	Molecular mass range
w/w	Weight per weight in %
Ga.	Gauge
kV	Kilovolt
IDR	Intrinsic Dissolution Rate
API	Active Pharmaceutical Ingredient
pKa	Acid dissociation constant
LOD	Limit of detection
LOQ	Limit of quantitation
L	Length
ID	Internal diameter
C18	Octadecylsilyl silica gel
O/W	Oil-in-Water Emulsion System
ICH	International Conference on Harmonisation
r	Correlation coefficient

r^2	Coefficient of determination
S-20	Macrogolstearylether-20
S-21	Macrogolstearylether-21
CSA	Cetostearyl alcohol
HPMC	Hydroxypropylmethyl cellulose
RH	Relative Humidity
HLB	Hydrophilic Lipophilic Balance
Σ	Sum of
®	Registered Trade Mark
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
PA	Peak Area

Declaration

I hereby certify that all research presented in this thesis was, unless otherwise stated, performed in the laboratories of mibe GmbH Arzneimittel in Brehna, Germany and that this thesis has not been submitted before, in whole or in part, to this or any other university for any degree and is, except where otherwise stated, the original work of the author.

Signed: _____ Date:_____

Abstract

The research work presented in this thesis describes studies concerning the stability of the drug substances betamethasone 17-valerate and fusidic acid hemihydrate in topical pharmaceutical formulations. In particular, the work concentrates on formulation factors affecting the intra-molecular isomerisation of betamethasone 17-valerate to 21-valerate in a developmental topical cream for treatment of atopic dermatitis and psoriasis. Appropriate analytical tools for the simultaneous analysis of both drug substances have been developed and their suitability for use in the study demonstrated by validation experiments. The results of the isomerisation study indicate that the concentration of the emulsifier macrogolstearylether-21 significantly influences the isomerisation rate of the glucocorticoid. 5 % (w/w) of this substance in the formulation resulted in complete isomerisation within a number of months, whereas 1.5 % (w/w) gave rise to less than 3 % isomerisation after 3 years storage. The study data was used to develop a new topical drug formulation containing both betamethasone 17-valerate and fusidic acid which went to market in early 2017. Additionally, the influence of the polymorphic form of fusidic acid on its stability was investigated. Fusidic acid is known to exist in 4 polymorphic modifications and this study has shown that polymorphic forms I and III are currently available on the commercial market. Intrinsic dissolution – and *in-vitro* permeation studies demonstrate that both polymorphs have similar intrinsic dissolution rates, as well as comparable *in-vitro* release rates from the developmental cream. This indicates that both forms may be used interchangeably without affecting the safety and efficacy of concerned drug products. Finally, the plausibility of incorporating both drug substances into electrospun poly(caprolactone) (PCL) microfibers for potential use as an occlusive medicated dressing was examined. The effect of addition of the novel low molecular weight gelator, Fmoc-OH-C18, on the manufacture of the microfibers as well as on their *in-vitro* drug release rates was studied. The gelator was found to have a profound effect on the morphology of the resulting fibers but had little effect on the release rate of the active drug substances. The results suggest that the use of a medicated electrospun dressing containing betamethasone valerate and fusidic acid might be a viable treatment alternative to the current topical dosage forms. However, significant further work is needed in this regard.

CHAPTER 1

Introduction

1 CHAPTER I – Introduction

1.1 *Pharmaceutical stability testing*

The purpose of pharmaceutical stability testing is to investigate how the quality of a drug substance or drug product varies over time under the influence of a variety of environmental factors such as temperature, light, and humidity; and to use this data to establish a suitable retest period for the drug substance or appropriate shelf-life and storage conditions for the drug product under study [1]. Stability testing therefore forms an integral part of the product development process, beginning with the manufacture of the very first trial formulations and continuing to the stage of marketing authorization and beyond. The stability data generated during the development phase forms a major part of the product quality dossier, and indeed, even after marketing authorization has been granted, the stability of the product must be monitored on an on-going basis, in order to ensure consistent product safety and efficacy.

The goal of any drug product development is to obtain a stable product which does not degrade to a significant degree upon storage. Drug degradation may have several unwanted effects on the finished product, including loss of active substance leading to a corresponding loss of potency of the drug product; generation of potentially toxic impurities; changes to the aesthetic characteristics of the product, such as colour or odour; or degradation of preservatives, possibly leading to microbial contamination. The degradation of drug substances in finished product formulations is usually the result of undesired chemical reactions. Such reactions include hydrolysis of esters; reaction of amines with reducing sugars (Maillard reaction); as well as transesterification and oxidation reactions, to name but a few. Examples of such unwanted side-reactions are described in the following 3 real-life case studies from the pharmaceutical industry.

Case 1: Dexpanthenol, a wound healing compound, is susceptible to hydrolysis in tablet formulations and degrades to form pantoic acid and 3-aminopropanol [2] (Figure 1). If the pH conditions are suitable then pantoic acid may subsequently undergo an intra-molecular lactonisation forming pantolactone. This degradation reaction is extremely sensitive to the water content of the tablets and degradation rates of up to 10 % have been observed by this author in tablets stored

over 36 months at 25 °C/60 % relative humidity. The rate of degradation may be minimized by controlling the content of water in the product at the granulation and drying stages of manufacture.

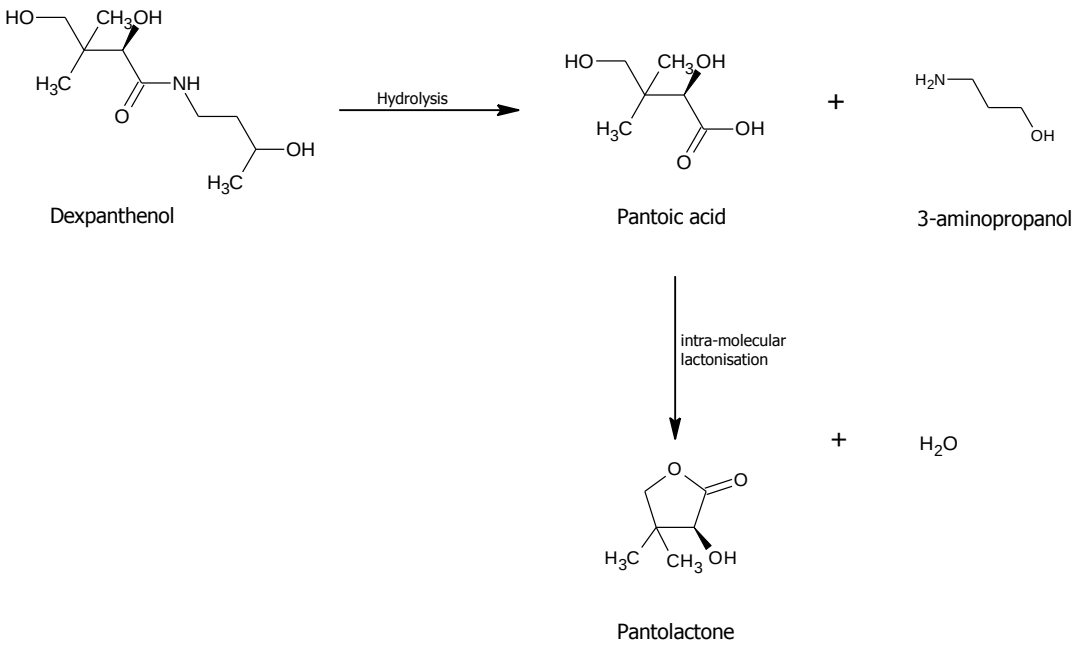


Figure 1 *Schematic of the degradation of Dexpanthenol in Panthenol tablets*

Case 2: The analgesic compound acetylsalicylic acid, commonly sold under the brand name Aspirin®, is available in tablet form for the treatment of mild to acute pain. Stability studies have shown that if the water content of the tablets is sufficiently high, then the drug substance may be hydrolysed to salicylic- and acetic acid [3] (Figure 2) and it is not uncommon for aspirin tablets to smell of acetic acid after long-term storage.

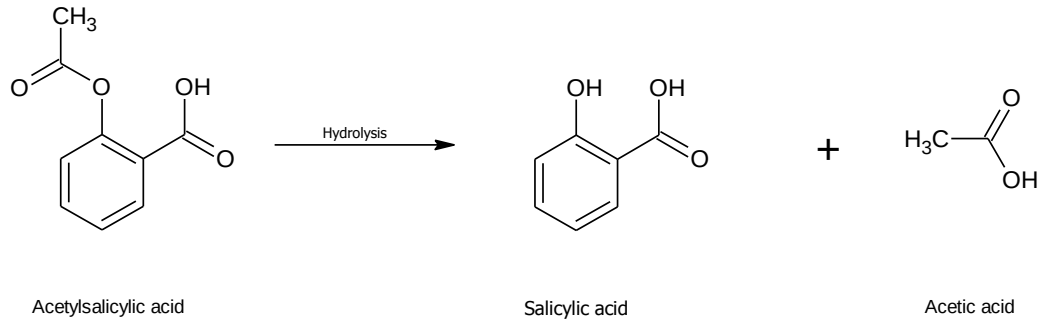


Figure 2 *Hydrolysis of acetylsalicylic acid in a tablet formulation*

Case 3: An example of a drug substance/excipient interaction involves the anti-viral compound acyclovir which is known to undergo a Maillard-type-reaction in tablet formulations containing the reducing sugar lactose [4]. The interaction of both of these compounds results in the formation of an acyclovir-lactose condensation product (Schiff's Base, Figure 3).

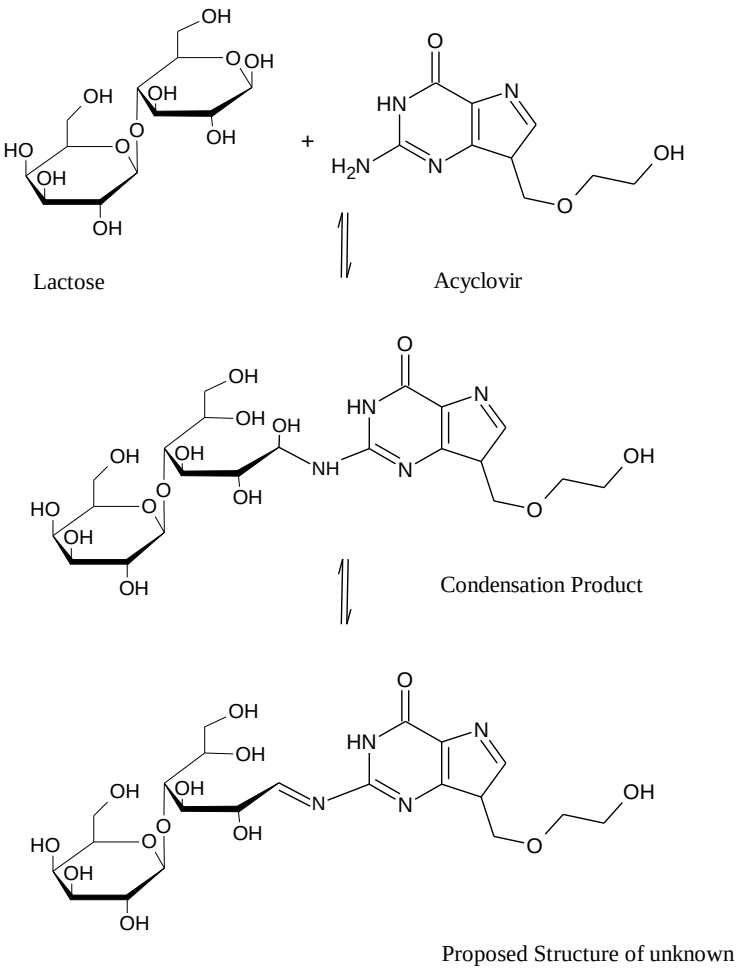


Figure 3 *Reaction of acyclovir with lactose in a tablet formulation*

It is evident from the above examples that a multitude of potential degradation pathways and drug-drug or drug-excipient interactions may be possible for particular drug substances and products. It is the job of the development chemist to sufficiently characterize the degradation pathways of the drug substance and to develop strategies to prevent significant degradation in the drug product formulation. Examples of such strategies include the development of stability-indicating analytical procedures, the use of forced-degradation studies to investigate the intrinsic stability of the drug substance under a multitude of possible stress conditions (e.g. heat, light, pH, oxidation and humidity) as well as the

performance of drug-excipient interaction studies, whereby mixtures of the active drug substances and selected excipients are prepared and stressed in such a manner as to determine their likely compatibility in potential formulation candidates.

In Europe, Japan and the United States the requirements for the design and implementation of stability studies is governed by ICH Guideline Q1A (R2) [5]. Stability studies of drug substances in new drug products are classified into long-term, intermediate and accelerated studies. In general cases, where studies are being performed in climatic zones I and II, long term stability studies should be performed at 25 °C/60 % relative humidity, intermediate studies at 30 °C/65 % relative humidity and accelerated studies at 40 °C/75 % relative humidity. For some products for which special storage conditions are required, e.g. refrigeration, the stability conditions may vary from those described above.

1.2 *Fusidic acid – an antibiotic agent*

1.2.1 *General Characteristics*

Fusidic acid (FA) is a tetracyclic triterpenoid antibiotic chemically related to the steroids [6,7] but not showing any of the corticosteroid like activity associated with the former compounds [8]. It was first isolated from the fermentation broth of the microorganism *Fusidium coccineum* by Godfredsen *et al.* at Leo Pharmaceutical Products in the early 1960s [9–11] and belongs to the small class of antibiotic substances known as the Fusidanes [12]. These compounds are of fungal origin and are characterized by a common 29-*nor* Protostane structure (Figure 4) [12]. To date, 18 naturally occurring Fusidanes have been reported [12] with FA being the only representative of this group to have been used clinically [13]. The substance has a limited spectrum of activity but is particularly effective for treatment of Gram-positive bacterial infections; particularly those caused by *Staphylococcus* species [14–16]. *In-vitro* experiments have also shown it to be effective against co-agulase negative staphylococci, cornyebacteria and gram-positive anaerobes [10,17]. It shows little activity against streptococci and enterococci and most Gram-negative species show complete resistance [18].

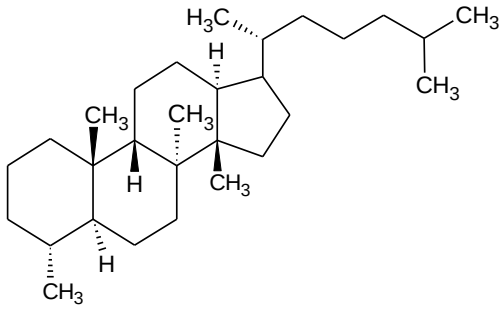


Figure 4 *Chemical structure of 29-nor Protostane*

1.2.2 ***Clinical use and side-effects***

FA is primarily used for the treatment of Gram-positive bacterial infections [10,19] and may be administered through several different routes. Available dosage forms include: creams and ointments for topical treatment of secondary skin infections [13,16,20]; tablets for the systemic treatment of conditions such as prosthetic joint- and bone infections [21–23], septic arthritis [24] or *Staphylococcal* bacteraemia [25]; as well as parenteral dosage forms, e.g. for the treatment of *Staphylococcal* endocarditis [26,27]. Eye drops are also available for the treatment of ocular infections caused by FA sensitive microbes [28].

FA is licenced for use in Europe but has never been approved in the United States [29], although there are currently on-going efforts to attain its approval, given the ever growing problem of bacterial resistance to older antibiotics and the lack of new alternatives coming out of the development pipeline [30]. The substance has regained popularity in recent years due to its low toxicity [31,32] and effectiveness in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) [14,15,17,33], which is becoming and will continue to become a major problem in both clinical and community care in future years [34,35]. In contrast to the high levels of resistance observed for many of the other widely used antibiotics, such as vancomycin and daptomycin [36], only relatively low levels of resistance to FA has been observed thus far in *Staphylococcal* isolates [8,13]. This can be partially attributed to the low level use of this antibiotic since its discovery and its administration together with other anti-*Staphylococcal* agents, helping to minimize the formation of resistant clones [17]. Recent studies however indicate that resistance is on the increase, partially due to the inappropriate use of FA in topical ointments and creams as a monotherapy for the treatment of acute skin infections [17,37].

Side effects of fusidic acid treatment may include jaundice due to impaired liver function [25], as well as upper gastrointestinal discomfort and diarrhoea when administered orally. Several case studies have reported the development of sensitisation during topical administration [31,38].

1.2.3 *Mechanism of action and Structure-activity relationships*

FA blocks bacterial protein synthesis by binding to prokaryotic elongation factor G (EF-G) on the bacterial ribosome [19,39]. This binding prevents the subsequent release of the EF-G complex which results in the stalling of the elongation process, leading to a shut-down of the protein synthesis machinery of the bacterial cell [40–42]. Structure-activity studies (Figure 5) have demonstrated that a number of structural features of the fusidic acid molecule are critical for its antimicrobial activity. It has been shown that, for example, the correct geometry of the $\Delta^{17(20)}$ double bond [43], the presence of hydroxyl groups at both C-3 and C-11 as well as the presence of an ethoxy group at C-16 are required, since modification of these groups leads to a much reduced activity [44]. Simultaneous oxidation of the hydroxyl groups at C-3 and C-11 to form keto moieties leads to a reduction in activity to 1% of the original structure [44]. It has also been shown that a free carboxylic acid group is essential since derivatization of this group leads to a complete loss of activity [44]. In contrast, the lipophilic side chain seems to be least sensitive to modifications, e.g. reduction of the Δ^{24} double bond leads to compounds with a similar activity to FA [44]. FA is metabolised and excreted in the liver [45].

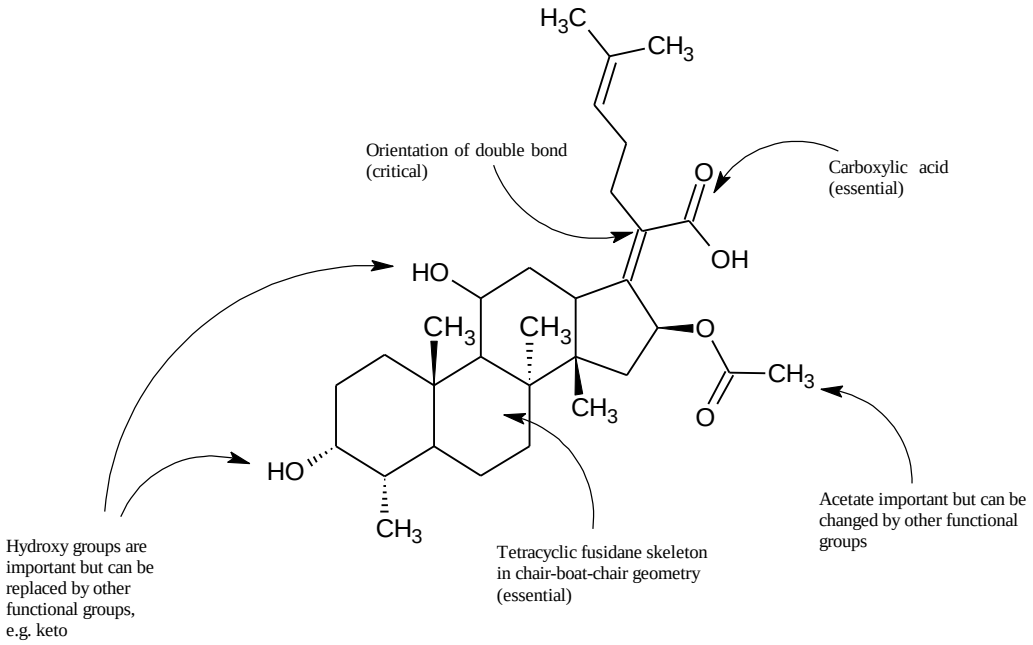


Figure 5 Essential features of the Fusidic acid molecule required for its antimicrobial activity

1.3 *Betamethasone – a topical glucocorticoid*

1.3.1 *General Characteristics and History*

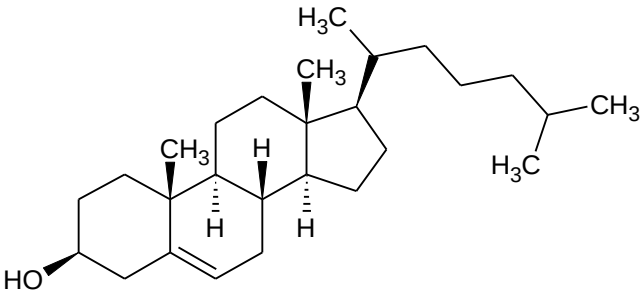
Glucocorticoids (GCCs) are substances of both natural and synthetic origin which are considered to be chemical analogues of cholesterol [46] and whose existence in nature can be traced as far back as the pre-Cambrian period around 2.7 billion years ago [47]. They have strong immunosuppressive, anti-mitotic and anti-inflammatory actions [48–50] and all possess a common cyclopentahydrophenanthrene skeleton, differing only in the substituents linked to the 17 carbon atoms, or in their stereochemical configuration. Miniscule quantities of active steroids were first isolated in the 1930s from adrenal glands [51]. Among the isolated substances were the hormones cortisone and cortisol (hydrocortisone, Figure 6) which were later demonstrated by Hench *et al.* [47] to possess strong anti-arthritic properties. After this discovery many medicinal chemistry groups immediately sought suitable starting materials and synthetic processes that could be used for producing large amounts of cortisone, which went on to become the first steroid hormone to be produced on a large scale commercial basis [51] . The early success of cortisone was however short lived since it was subsequently discovered that long-term use led to severe side-effects, including increased sodium retention, muscular atrophy, osteoporosis and hypertension [52–55].

In subsequent years attempts were made to synthesise new steroid variants with increased glucocorticoid and reduced mineral-corticoid activity, but no synthetic glucocorticoid more active than cortisone was discovered in the early years. This led to the widely held perception that it would be impossible to improve on the potency of the naturally occurring substances [56]. In later years, however, several synthetic glucocorticoids were produced which showed much superior efficacy to cortisone.

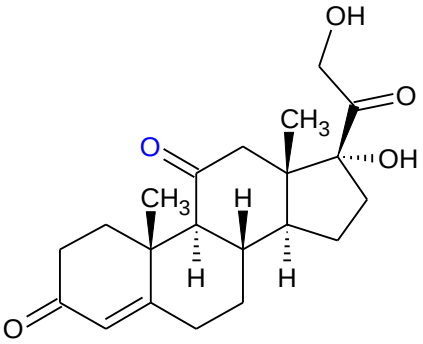
1.3.2 *Structure-Activity Relationships*

After many years of experimentation it was discovered that modification of the type and stereochemistry of the substituents of the steroid backbone could lead to vastly improved glucocorticoid potency (Figure 7). In particular, it was found that modification of the C 1,2 single bond to a double bond led to a 4-fold increase in potency and reduced unwanted side-effects [51]. This eventually led to the development of the compounds prednisone and prednisolone which quickly became the drugs of choice for the systemic treatment of inflammatory disease and remain so today. Addition of a C-16-α-hydroxyl- and a C9-α-fluoro group increased potency by a factor of 5 and helped to reduce the unwanted sodium retention properties. This variant was successfully marketed as triamcinolone [51]. Replacing the C-16-α hydroxyl group with an α-methyl group further increased anti-inflammatory effects, leading to the eventual development of dexamethasone [51].

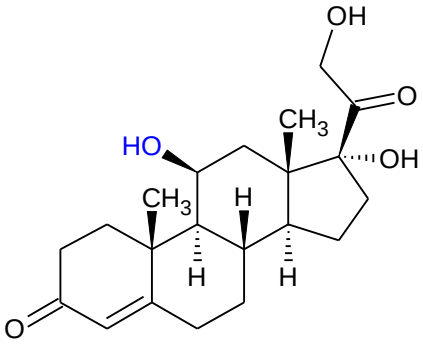
Surprisingly, many of the newly synthesised steroids, although possessing potent systemic action, were found to be topically inactive [47,51]. It was later recognized that in addition to biological activity the drug substances needed to have the appropriate physical properties such as solubility and lipophilicity in order to penetrate the skin and be topically effective [51].



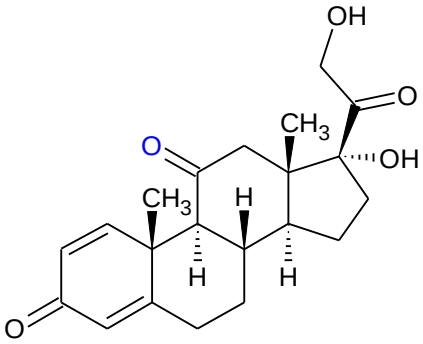
Cholesterol



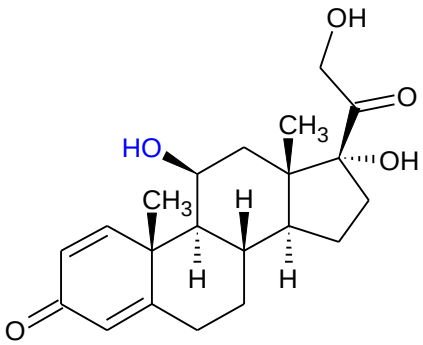
Cortisone



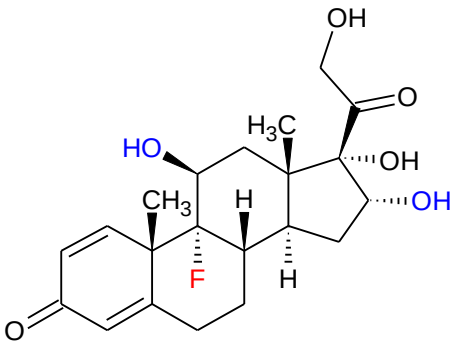
Hydrocortisone (Cortisol)



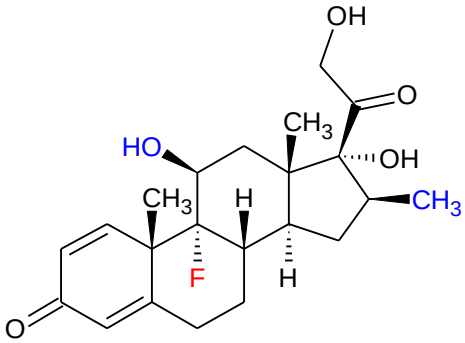
Prednisone



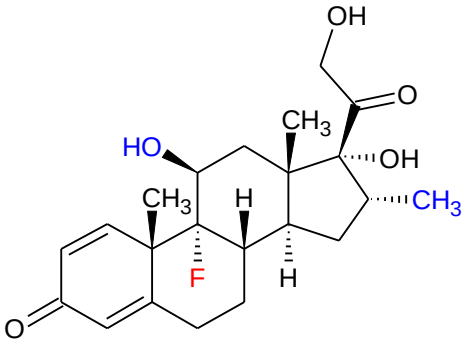
Prednisolone



Triamcinolone



Betamethasone



Dexamethasone

Figure 6 *Chemical structures of some common glucocorticoids*

Experiments showed that a C-16, C-17 acetonide group was found to dramatically reduce the unwanted electrolyte retention properties of the fluorine groups and was subsequently discovered to increase skin permeability properties, resulting in improved percutaneous absorption [51]. Further substitution of the hydroxyl group at C-21 by an acetate led to substances with even greater potential, e.g. hydrocortisone acetate. These esters were more resistant to hydrolysis and showed increased activity [51]. Modern structure-activity models indicate that glucocorticoid binding affinity is dramatically increased by 6-α or 9-α halogenation and tends to increase with increasing lipophilicity [51]. Fluorination also tends to protect the steroid ring from metabolic conversion. Models show good fitting of glucocorticoid 17-esters and unfavourable stearic interactions for C-21 esters with substituents larger than propionate [51].

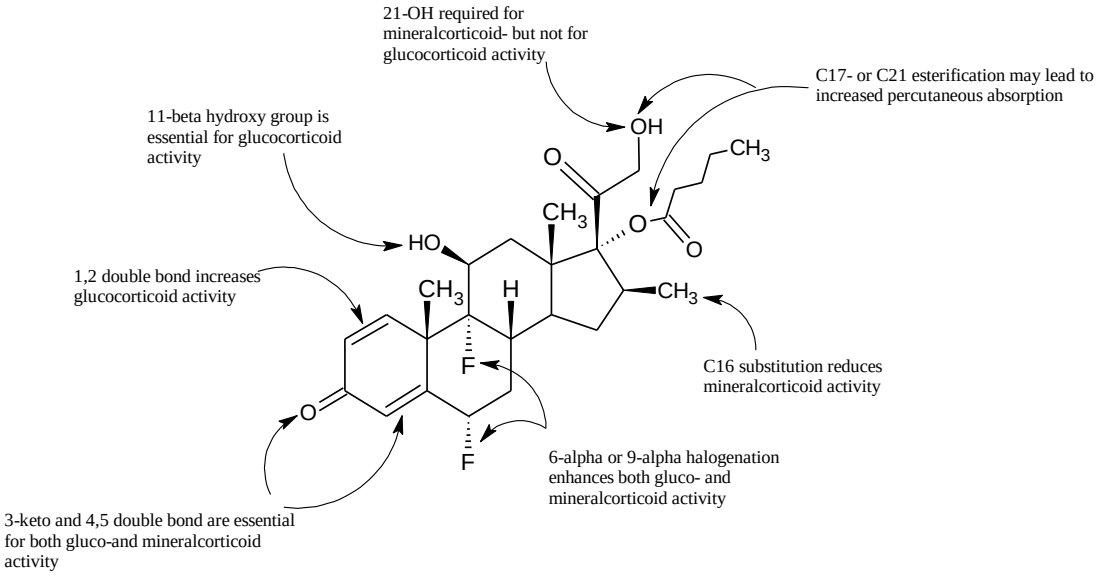


Figure 7 Essential features of glucocorticoids required for their gluco- and mineralcorticoid activity

1.3.3 *Mechanism of Action*

In vivo, approximately 95 % of GCCs are bound to plasma proteins including the specific steroid binding globulin, *transcortin*, which facilitates the transfer of GCCs to target tissues via the blood stream or tissue fluids [57]. Once they have entered the target tissue GCCs function by binding to the glucocorticoid receptor (GCC-R) which belongs to the steroid-hormone-receptor family of proteins and which is found in the cytoplasm of almost all cell types [58]. Once bound, the GCC-R-complex is translocated to the nucleus where it binds to specific target sites on

the genome, thereby initiating the synthesis of various proteins responsible for the observed anti-inflammatory effect. GCCs decrease the initial anti-inflammatory action by reducing capillary permeability, resulting in less leakage of proteins and fluids into the affected tissue and consequently less swelling. Exudation of macrophages and various other immune cells is also reduced by altering the endothelial sticking properties of these cells and reducing their ability to move through the capillary wall.

1.3.4 *Betamethasone-17-valerate*

Betamethasone is a synthetic glucocorticoid which is topically inactive and must therefore be administered in the form of one of its esters, such as betamethasone-17-valerate (BV, Figure 8) or betamethasone-17,21-dipropionate. The esterified forms generally have increased lipophilic nature allowing for better penetration of the drug molecule across the lipid membranes of the skin, thus enhancing the local bioavailability of the substance [59]. BV was discovered in the 1960s using the McKenzie-Stoughton vasoconstriction assay [60], which is a screening method which classifies steroid potency based on their ability to cause vasoconstriction, i.e. narrowing of the blood vessels in the skin. BV has a potency of approximately 25 – 30 times that of cortisol (hydrocortisone) and is classified as a moderate to high potency glucocorticoid depending on how it is formulated [61]. When formulating BV the choice of vehicle used is extremely important since the substance is susceptible to an acid and base catalysed isomerisation in aqueous and semi-solid formulations, resulting in an acyl group migration from position C-17 to the more stable C-21 position of the steroid ring system [62–64]. This isomerisation process is clinically relevant since betamethasone-21-valerate demonstrates only a fraction the potency of its 17-valerate counterpart [60].

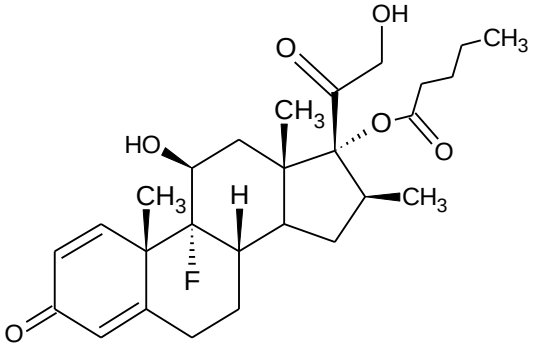


Figure 8 Chemical structure of betamethasone 17-valerate

1.4 *Psoriasis and Atopic Dermatitis*

1.4.1 *Psoriasis*

Psoriasis is a lifelong, chronic, immune-mediated skin disease (Figure 9) which is auto-inflammatory in nature and affects approximately 1- 3 % of the Caucasian population [55]. It may appear at any age but appears before the age of 40 in an estimated 75% of affected individuals [65]. The disease occurs to a similar degree in both men and women and symptoms generally include itchy, round or oval scaly plaques frequently located on the scalp, lower back, umbilical region, intergluteal cleft, knees and elbows [66]. Contributing factors to the disease include predisposing genetic factors, stress, excessive alcohol consume, smoking and obesity [65]. Some medications, such as ß-blockers, malaria tablets and lithium may cause the condition to flare [65].

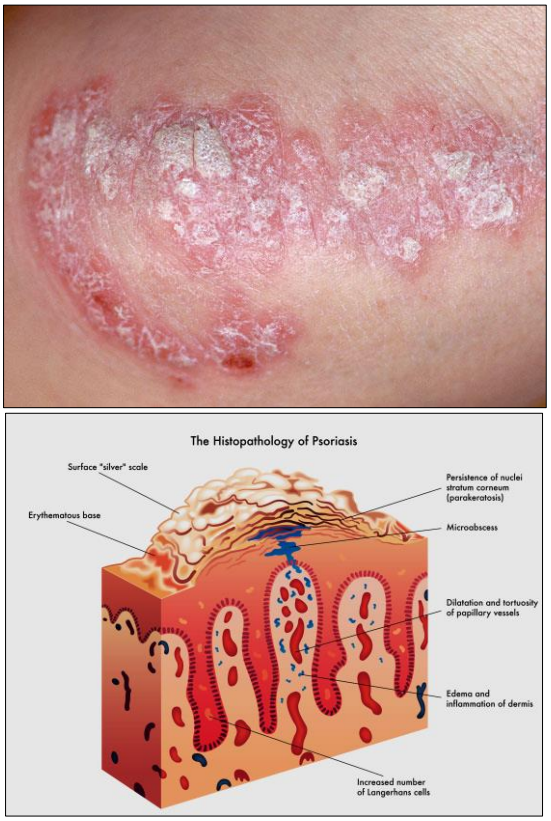


Figure 9(a) Example of psoriasis of the elbow showing the characteristic plaque formation; (b) graphical representation of the histopathology of psoriasis

1.4.2 *Atopic Dermatitis*

Atopic dermatitis, otherwise known as atopic eczema, is a complex skin disorder (Figure 10) caused by the interplay between multiple genetic and environmental factors [67]. The term atopic is used to describe a group of conditions including asthma, eczema and hay-fever which show increased activity of the allergic component of the condition [68]. It is caused by several factors including defects in the skin barrier and abnormalities in the normal inflammatory and allergic responses [69]. The disease has a large genetic component and is linked to a defect in a gene responsible for producing the protein *filaggrin*, which is responsible for providing structural stability to the skin and thereby helping to ensure that the skin barrier remains intact [70,71]. When defects in the skin barrier occur, the immune cells of the dermis are exposed to external antigens and initiate an anti-inflammatory response. The condition occurs in up to 20 % of children [72] and 1 % - 3 % of adults, with 40 % - 60 % of patients also having respiratory allergies [69]. Due to the genetic involvement of the condition, atopic dermatitis tends to run in families. It is usually detected before the age of 2, with only 10 % of cases being detected after the age of 5.

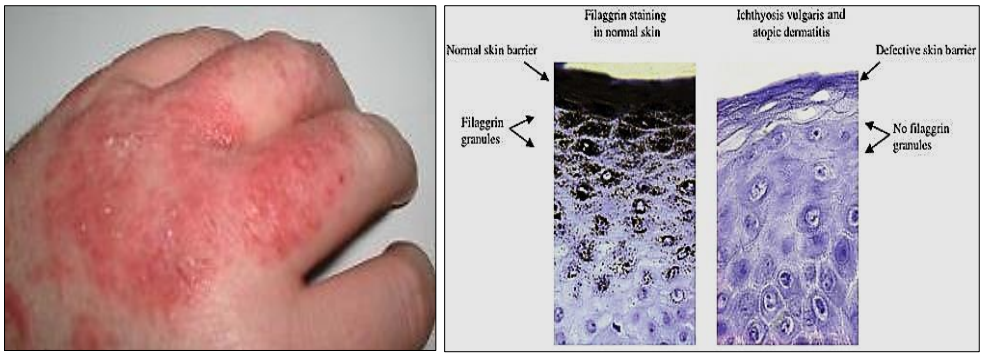


Figure 10 (a) Example of atopic dermatitis of the hand in a young child; (b) micrograph of healthy skin showing the staining of filaggrin compared to diseased skin such as found in atopic dermatitis [reproduced from Irvine et al [70]]

Symptoms include extremely itchy, scaly skin lesions often located on the flexural surfaces of the body. In severe cases weeping and blistering of lesions may occur [73]. Picking and scratching of the skin may lead to scarring and/or to secondary infections, for which an antibiotic such as fusidic acid may need to be prescribed [69]. The condition is often associated with sleep disturbances.

1.4.3 *Treatments and Associated Side-Effects*

The first line of treatment for both psoriasis and atopic dermatitis is topical therapy, including the application of emollients (e.g. E45 cream), calcineurin inhibitors (e.g. tacrolimus), keratolytics (e.g. salicylic acid), topical vitamin D analogues (e.g. calcipotriol) as well as topical corticosteroids, which are by far the most common treatment for both conditions. Such treatments may also be used complimentary to any systemic treatment, e.g. together with oral prednisone or dexamethasone.

In Europe, glucocorticoids are classified into 4 sub-groups depending on their relative potencies. Low potency steroids are suitable for application to sensitive areas such as the face, armpits, and groin as well as being suitable for use by infants and children. Compounds belonging to this group include hydrocortisone and hydrocortisone acetate. Superpotency GCCs on the other hand (e.g. clobetasol 17-propionate) are used for stubborn, cutaneous plaques or lesions on the palms, soles and scalp. Moderate potency (e.g. prednicarbate) and high potency GCCs (e.g. mometasone furoate) are generally used for the remaining areas of the body in adult patients.

Long-term glucocorticoid treatment is associated with a number of severe side-effects which include skin- and muscle atrophy (Figure 11), the formation of cutaneous striae, cataracts as well as the development of osteoporosis and hypertension [53,52]. The skin- and muscle atrophy can be attributed to the reduced vascular blood supply and anti-mitotic effects of GCCs, whereas hypertension is exacerbated by the sodium retaining properties of some of the non-fluorinated steroid compounds, such as prednisolone and prednisone. GCCs also tend to increase intra-ocular pressure which may result in damage to the optic nerve, potentially leading to the development of glaucoma [52].

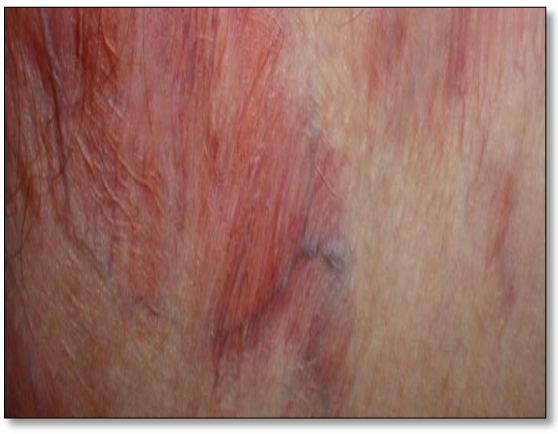


Figure 11 Atrophic skin caused by the long-term use of topical steroids

1.5 *Aims of Research*

The primary aim of the work in this thesis is to study the influence of drug product formulation on the stability of the drug substances betamethasone 17-valerate (BV) and fusidic acid hemihydrate (FA) in a developmental semi-solid cream formulation. In particular, the work will focus on factors affecting the intra-molecular isomerisation of BV, since this process presents a significant challenge to the formulation chemist and the influence of the formulation on this process is not fully understood for such systems. The isomerisation of the 17-valerate ester is of clinical significance since the corresponding 21-valerate ester demonstrates only approximately 1/15th of the potency of the C-17 ester [60]. Much of the literature on this topic tends to concentrate solely on the role of pH in the isomerisation process. The work in this thesis studies the influence of factors other than formulation pH, such as for example, (i) the types and concentrations of excipients; (ii) the influence of oxidative processes and (iii) the effect of trace metals.

Appropriate analytical tools for the simultaneous characterization of BV and FA in semi-solid formulations have not been published in the current literature. These must first be developed and their appropriateness for use in the study demonstrated by suitably designed validation experiments. These analytical procedures will then be employed to investigate the formulation factors influencing the stability of the aforementioned drug substances in semi-solid formulations. The ultimate goal is to use the knowledge gained through the study to develop a stable and marketable drug product formulation containing both FA and BV for the topical treatment of infected inflammatory skin conditions, including atopic dermatitis and psoriasis.

Additionally, the influence of the polymorphic form of FA on its stability in bulk form will be investigated. FA is known to exist in 4 polymorphic modifications [74] and at least 2 of these polymorphs are currently available on the commercial market. The influence of the polymorphic form of commercially available FA on the intrinsic dissolution rate of the bulk substance as well as its release rate from a topical cream formulation will be investigated.

Finally, the release rates of both BV and FA from electrospun poly(caprolactone) (PCL) microfibers will be studied with a view to investigating the plausibility of incorporating these drug substances into an electrospun bandage

for use in the treatment of patients for which a cream or ointment may not be practicable. The release characteristics of fibres with different drug loadings and with or without chemical modifiers will be investigated.

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CHAPTER 2

Experimental

2 CHAPTER 2 - Experimental

This thesis has been structured such that each chapter has its own short introduction and experimental section. Consequently, the main experimental chapter focuses predominantly on the theory and application of the analytical methods employed in the research and the analytical equipment used for the respective measurements.

2.1 *High-Performance Liquid Chromatography*

2.1.1 *Introduction*

In an analytical chemistry setting it is often necessary to separate mixtures of chemical substances in order to identify, quantify or isolate one or more of the components of the mixture. The analytical discipline concerned with chemical separation is termed *'chromatography'*, derived from the Greek '*chroma'*, meaning colour, and '*graphein'*, meaning to-write. The field of chromatography encompasses several different chromatographic techniques, including paper chromatography, thin-layer chromatography (TLC), gas chromatography (GC) and liquid chromatography (LC). Despite these different classifications, all chromatographic techniques are essentially based on a similar principle, which involves a mixture of analytes being transferred via a carrier through a stationary phase which retards the movement of the analyte in some fashion. Through the retardation of analytes, differences in both their physical and chemical characteristics are exploited. This results in analytes travelling at different rates through the stationary phase and leads to their separation. The degree of separation is dependent on the analyte-, stationary phase- and mobile-phase characteristics, e.g. hydrophobicity, pH or particle size.

In gas chromatography, for example, the mobile phase is a high-purity inert gas, usually Helium or Nitrogen, with the stationary phase being a solid coated with a thin layer of a high boiling-point liquid, e.g. dimethylpolysiloxane, which is responsible for retardation of the analytes. In the case of liquid chromatography, the stationary phase comprises of a solid substance, often porous silanised silica gel, and the carrier (mobile-phase) is a liquid. The mobile phase may be aqueous, organic or a mixture of both, depending on the mode of chromatography being used, i.e. normal or reversed-phase. Many chromatographic techniques are performed at atmospheric pressure; High-Performance Liquid Chromatography (HPLC), however, is a high pressure analytical technique operating at pressures of up to 400 bar. A modern variant of HPLC, namely

UPLC (ultra-high-pressure liquid chromatography), can be operated at even higher pressures of up to 1000 bar or more. Operating at high pressure results in faster and more efficient separations and it is for this reason that HPLC is the most widely used chromatographic technique in modern analytical laboratories. A high-performance liquid chromatograph comprises essentially of a mobile phase, a pumping system, a degassing unit, an injection system, an analytical column and its associated thermostatic oven, a detector, and a data management system. The function of each of these components is described in Table 1.

Table 1 *Basic components of a modern high-performance liquid chromatograph*

Component	*Function*
Mobile phase	transfers the analyte through the chromatographic system
Pump	pumps the mobile phase at a constant rate through the chromatographic system
Degassing unit	removes dissolved gases from the mobile phase
Injection system	transfers an exact volume of the analyte solution into the chromatograph
Analytical column	retards the movement of the analyte
Column-oven	heats the analytical column to a required temperature
Detector	measures the analyte signal
Data Management System	converts the electrical signal coming from the detector to a visual output

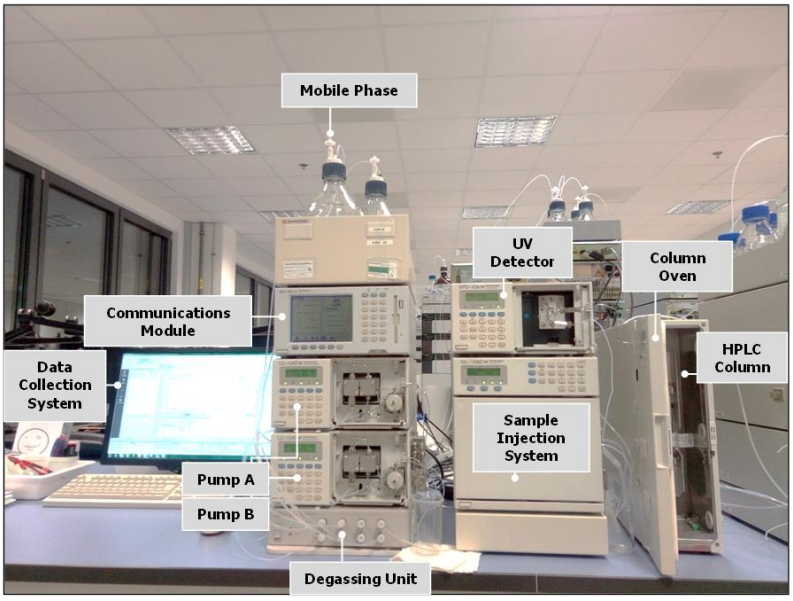

Figure 12 *Basic components of a high-performance liquid chromatograph*

The objective of any HPLC separation is to obtain the best resolution between peaks in the shortest possible time. The resolution (*Rs*) between 2 peaks is governed by *Equation 1* below, where *N* is the theoretical plate count, α is the selectivity factor and *k* is the retention factor.

Equation 1 $$Rs = \frac{1}{4}\sqrt{N} \times \left(\frac{\alpha-1}{\alpha}\right) \times \left(\frac{k}{1+k}\right)$$

The theoretical plate count, *N*, is a measure of the efficiency of a chromatographic separation. In simple terms it is the relationship between the retention time (t_R) and the width of the analyte peak measured at half peak height ($w_{1/2}$). Efficient separations are those which give rise to sharp, narrow peaks. The theoretical plate count can be calculated using *Equation 2*.

Equation 2 $$N = 5.54 \left(\frac{t_R}{w_{1/2}}\right)^2$$

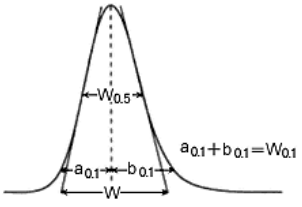

Figure 13 *Peak width at base and half peak height* [1]

When an aliquot of sample is injected into the HPLC system it is distributed on the stationary phase as a narrow band which gradually broadens as it travels through the analytical column. The degree of broadening may be modelled according to the Van-Deemter equation, which describes the relationship between the height equivalent of a theoretical plate (HETP) and the linear velocity of the mobile phase. The equation is made up of the terms A, B, C and u (*Equation 3*, Figure 14), where the A term is known as the eddy-diffusion term, the B term is known as the longitudinal diffusion term, the C term is the mass transfer term and u is the linear velocity of the mobile phase in m s^{-1}.

Equation 3 $$H = A + \left(\frac{B}{u}\right) + (C \,.\, u)$$

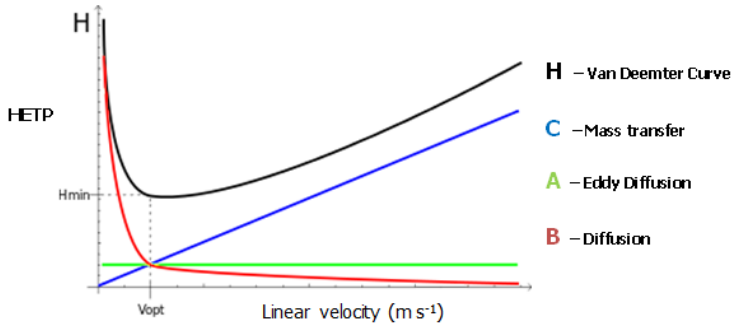

Figure 14 *Van-Deemter Plot showing the influence of the A, B and C terms* [2]

The eddy diffusion term is related to the fact that not all analyte molecules take the same path through the stationary phase. Some molecules take a longer path and others a shorter path (Figure 15 a). Eddy diffusion leads to a broadening of analyte peaks because molecules that travel a shorter path through the stationary phase reach the detector quicker than those which take a longer path. This term is independent of the mobile phase flow rate.

The B term represents longitudinal diffusion within the stationary phase. Since analyte molecules are free to move both with and against the direction of mobile phase flow, they may move in opposite directions resulting in peak broadening (Figure 15 b). The contribution of the B term is more significant at very low flow rates and decreases with increasing linear velocity. The B term does not make a significant contribution to peak broadening at normal flow rates, i.e. above 0.5 ml/min.

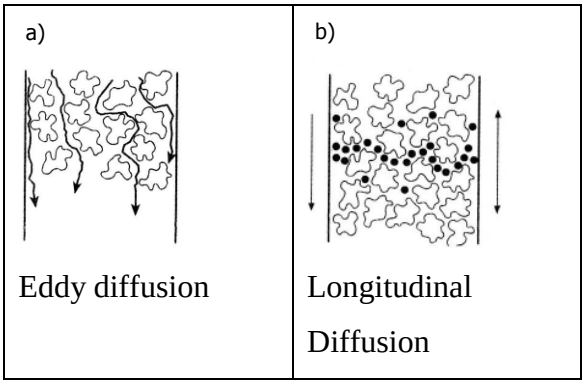

Figure 15 *Eddy-diffusion (a) and Longitudinal-diffusion (b)*

The C term is the mass transfer coefficient and describes the actual chromatographic process within the stationary phase, i.e. the interactions between the analyte molecules and the bonded phase. These interactions are enhanced at low flow rates because the analyte molecules have longer to interact with the stationary phase.

This suggests that lowering the flow rate should increase the plate count and provide a better resolution. However, the benefit obtained at lower linear velocity through an increased mass transfer coefficient is often offset by an increase of the diffusion term B, which increases significantly at very low flow rates.

The retention factor (capacity factor), *k*, is a measure of the degree to which the analyte interacts with the stationary phase. If *k* is a low value then the analyte does not interact significantly with the stationary phase. In contrast, if *k* is a large value the interaction between the analyte and stationary phase is strong. Generally, chromatographers aim for a *k* value of between 2 and 5 for a target analyte. *k* values below 2 will not provide sufficient separation of analyte components and values above 5 will unnecessarily increases the analysis time without bringing a significant improvement in resolution. The capacity factor is described by *Equation 4*, where t_R is the retention time of the analyte and t_0 is the dead time, i.e. the time required for an unretained substance, e.g. uracil, to travel through the HPLC system.

Equation 4 $$k = \frac{t_R - t_0}{t_0}$$

The selectivity factor, α, is a measure of the ratio of the retention factors of 2 substances in a HPLC separation. A selectivity factor of 1 indicates co-elution of both analytes. The larger the α-value, the further away the apices of both peaks are from each other. It may, however, not be presumed that both peaks are baseline resolved, since this is also dependent on the peak width, and therefore on the theoretical plate count.

Equation 5 $$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

On examination of *Equation 1* it is clear that doubling the theoretical plate count (*N*) will not double the resolution between 2 peaks, since the efficiency is governed by the term $\sqrt{N}/4$. In practical terms this means that the resolution cannot be doubled by doubling the length of the analytical column. It is also clear that doubling the retention factor (*k*) brings increasingly less benefit with each doubling of the retention time. The most efficient way to increase the resolution between 2 peaks is to increase the selectivity factor, α, by altering analyte/stationary phase chemistry. This may be achieved by employing an alternative stationary phase or by modifying the composition of the mobile phase.

After separation of the analyte peaks in the stationary phase the analytes are carried via the mobile phase to the detector. The most commonly used detector is the UV-Vis detector which detects analytes based on their absorption of electromagnetic radiation of ultra-violet and/or visible wavelength (190 - 800 nm). For compounds which do not sufficiently absorb such wavelengths, a fluorescence detector may provide an alternative, provided that the compounds of interest fluoresce. Fluorescence detectors may be several orders of magnitude more sensitive than standard UV-Vis detectors. Some analyte molecules do not contain a chromophore and can't be detected using either UV-visible or fluorescence spectrophotometry. In such cases it may be possible to use a non-specific detector, such as a refractive-index (RI), light-scattering (ELSD), conductivity, electro chemical (ECD), or mass spectrometry (MS) detector.

2.1.2 *General Procedures*

2.1.2.1 *Sample preparation*

The following procedure was employed for the preparation of cream samples:

1	Weigh 1.00g of cream into a 50.0 ml volumetric flask
2	Add 30 ml of acetonitrile and shake vigorously by hand. Suspend thoroughly using a vortexer
3	Place in a water bath at 65°C for 5 minutes, shaking at regular intervals
4	Shake for 10 minutes on a flat-bed mechanical shaker and make up to volume with acetonitrile
5	Place in a freezer at approx. -20°C for 20 minutes
6	Centrifuge approx. 15 ml of the cold solution at ca. 3000 rpm for 5 minutes and transfer a portion of the cold supernatant to a volumetric flask. Allow to warm to room temperature
7	Make a 1:2 (v/v) dilution with purified water, e.g. 5.0 ml to 10.0 ml
8	Filter a portion of the solution through a 0.45 µm regenerated cellulose filter into a HPLC vial

2.1.2.2 *Stress-testing of BVand FA*

The procedures described below were used for stressing BV. In the case of FA the procedure remained largely unchanged with only the sample weight being increased from 5.0 mg to 80 mg.

Preparation of the analysis solution – *Acidic Stress*

- Weigh 5.0 mg of the BV into a 50.0 ml volumetric flask
- Add 30.0 ml of acetonitrile, 5.0 ml water and 1.0 ml of 1.0 M HCl
- Shake on a flat-bed mechanical shaker for 4 hours
- Adjust the pH to 7.0 ± 0.2 with 1.0 M NaOH and make up to volume with purified water
- Dilute 1:2 (v/v) with purified water

Preparation of the analysis solution – *Alkaline Stress*

- Weigh 5.0 mg of BV into a 50.0 ml volumetric flask
- Add 30.0 ml of acetonitrile, 5.0 ml of purified water and 1.0 ml of 1.0 M NaOH
- Shake on a flat-bed mechanical shaker for 4 hours
- Adjust the pH to 7.0 ± 0.2 with 0.1 M HCl and make up to volume with purified water
- Dilute 1:2 (v/v) with purified water

Preparation of the analysis solution – *Oxidative stress*

- Weigh 5.0 mg of BV into a 50.0 ml volumetric flask
- Add 30 ml of acetonitrile, 5 ml water and 0.5 ml of 30 % H_2O_2
- Shake on a flat-bed mechanical shaker for 4 hours and make up to volume with purified water
- Dilute 1:2 (v/v) with purified water

Preparation of the analysis solution – *Influence of Ultrasonic Treatment*

- Weigh 5.0 mg of BV into a 50.0 ml volumetric flask and add 35 ml of acetonitrile
- Shake on a flat-bed mechanical shaker for 20 minutes and place in a USB bath for 1 hour (35 Hz, 120 W), making sure to change the water at regular intervals to compensate for temperature increases
- Make up to volume with acetonitrile
- Dilute 1:2 (v/v) with purified water

Preparation of the analysis solution – *Temperature Stress*

- Heat a 1 g sample of bulk BV for 24 hours at 105 °C in an appropriate oven
- Weigh 5.0 mg of the stressed BV into a 50.0 ml volumetric flask and add 35 ml of acetonitrile
- Shake on a flat-bed mechanical shaker for 20 minutes
- Make up to volume with acetonitrile
- Dilute 1:2 (v/v) with purified water

Preparation of the analysis solution – *Daylight Stress*

- Transfer 1 g of BV into a clear glass GC vial, cover the sample with argon and leave standing on a window sill in direct sunlight for 72 hours
- Weigh 5.0 mg of the stressed sample into a 50.0 ml volumetric flask and add 35 ml of acetonitrile
- Shake on a flat-bed mechanical shaker for 20 minutes and make up to volume with acetonitrile
- Dilute 1:2 (v/v) with purified water

Preparation of the analysis solution – *Stability in solution*

- Weigh 5.0 mg of BV into a 50.0 ml volumetric flask and add 35 ml of acetonitrile
- Shake on a flat-bed mechanical shaker for 20 minutes
- Make up to volume with acetonitrile and dilute 1:2 (v/v) with purified water
- Allow to stand at room temperature and for 7 days

Preparation of the analysis solution – *UV 254 nm*

- Place 1 g of BV on an appropriate petri-dish and spread thinly over the complete surface
- Place the sample under a UV lamp set at 254 nm for 72 hours
- Weigh 5.0 mg of the stressed BV into a 50.0 ml volumetric flask and add 35 ml of acetonitrile
- Shake on a flat-bed mechanical shaker for 20 minutes and make up to volume with acetonitrile
- Dilute 1:2 (v/v) with purified water

Preparation of the analysis solution – *Control*

- Weigh 5.0 mg of unstressed BV into a 50.0 ml volumetric flask and add 35 ml of acetonitrile
- Shake on a flat-bed mechanical shaker for 20 minutes and make up to volume with acetonitrile
- Dilute 1:2 (v/v) with purified water

Preparation of the analysis solution – *pH-Stability*

- Weigh 25.0 mg of the active into a 50.0 ml volumetric flask
- Add 15.0 ml of acetonitrile and 35.0 ml of the appropriate buffer solution
- Measure the pH-value and, if necessary, adjust with dilute phosphoric acid or potassium hydroxide solution
- Allow to stand at room temperature for 48 hours (protected from light)
- Dilute 1:10 (v/v) with 50:50 acetonitrile/purified water (v/v)
-

2.1.2.3 ***Preparation of Buffer Solutions used for Stress Testing***

The buffer solutions employed in the pH-stability tests were prepared as described below using the following stock solutions:

- 0.2 M hydrochloric acid solution / 0.2 M sodium hydroxide solution
- 0.2 M potassium biphthalate comprising of 40.85 g of potassium biphthalate dissolved in 1000 ml of purified water
- 0.2 M potassium monophosphate comprising of 22.22 g of potassium dihydrogen phosphate dissolved in 1000 ml of purified water
- 0.2 M boric acid solution comprising of 12.37 g of boric acid + 14.91 g of potassium chloride dissolved in 1000 ml of purified water
- 0.2 M potassium chloride solution comprising of 14.91 g of potassium chloride dissolved in 1000 ml of purified water

Buffer pH 1.2

Transfer 50 ml of 0.2 M potassium chloride solution to a 200.0 ml volumetric flask and add 85 ml of 0.2 M hydrochloric acid solution. Make up to volume with purified water.

Buffer pH 2.2

Transfer 500 ml of 0.2 M potassium chloride solution to a 200.0 ml volumetric flask and add 7.8 ml of 0.2 M hydrochloric acid solution. Make up to volume with purified water.

Buffer pH 3.2

Transfer 50 ml of 0.2 M potassium biphthalate solution to a 200.0 ml volumetric flask and add 15.7 ml of 0.2 M hydrochloric acid solution. Make up to volume with purified water.

Buffer pH 4.2

Transfer 50 ml of 0.2 M potassium biphthalate solution to a 200.0 ml volumetric flask and add 3.0 ml of 0.2 M hydrochloric acid solution. Make up to volume with purified water.

Buffer pH 5.2

Transfer 50 ml of 0.2 M potassium biphthalate solution to a 200.0 ml volumetric flask and add 28.8 ml of 0.2 M hydrochloric acid solution. Make up to volume with purified water.

Buffer pH 6.2

Transfer 50 ml of 0.2 M potassium dihydrogen phosphate solution to a 200.0 ml volumetric flask and add 8.1 ml of 0.2 M sodium hydroxide solution. Make up to volume with purified water.

Buffer pH 7.2

Transfer 50 ml of 0.2 M potassium biphthalate solution to a 200.0 ml volumetric flask and add 34.7 ml of 0.2 M hydrochloric acid solution. Make up to volume with purified water.

Buffer pH 8.2

Transfer 50 ml of 0.2 M boric acid/potassium chloride solution to a 200.0 ml volumetric flask and add 6.0 ml of 0.2 M hydrochloric acid solution. Make up to volume with purified water.

2.1.2.4 *Equipment*

All HPLC experiments described in this thesis were performed on Shimadzu *Prominence* HPLC systems (Shimadzu, Tokyo, Japan). The systems were equipped with a binary pump (LC-20AD), a temperature-controlled auto-sampler (SIL-20AC$_{HT}$), a temperature-controlled column compartment (CTO-20AC) as well as an on-line degasser (DGU-20A$_5$). A SPD-M20A diode-array detector (DAD) was employed for detection. The software packages used were SHIMADZU LCsolution version 1.24 SP1 and SHIMADZU Class-VP version 6.14 SP2A.

2.2 *X-Ray Powder Diffraction*

2.2.1 *Introduction*

X-ray diffraction, commonly abbreviated as XRD, is an analytical technique used to obtain information about the structural arrangement of atoms and molecules within a crystal lattice. Comprehensive structural data may be obtained using the single-crystal x-ray diffraction technique which involves irradiation of a high quality crystal of the analyte with a beam of x-rays and recording the resulting diffraction pattern. Since the x-ray diffraction pattern is highly correlated with the 3 dimensional arrangements of atoms within the crystal lattice, it can be used to determine the particular crystal class and space group of the crystal lattice and can even be used to determine the exact molecular structure and stereochemistry of chemical substances. In many cases, however, it may be extremely difficult, or impossible, to obtain crystals of suitable quality and as such this technique is often not practicable for routine analysis in a pharmaceutical setting.

X-ray powder diffraction, or XRPD, provides a much quicker and routine-friendlier alternative to single-crystal XRD. An XRP diffractometer comprises in principle of an x-ray source, a sample holder, a detector, and a goniometer for rotation of the sample within the x-ray beam (Figure 16 a). X-rays are produced by application of a 15 – 60 kV potential to a tungsten filament (cathode) within a sealed tube under vacuum. Electrons are ejected from the filament and strike an Anode producing x-rays according to an “inverse” photoelectric effect. The Anode is made of either Cr, Fe, Co, Cu, Mo or Ag; with Cu and Mo being the most common materials. The resulting x-rays are generally not monochromatic, instead comprising of both kα und kβ radiation (Figure 17). A beta filter, often comprising of an element just below the target material

on the periodic table, can be used to remove unwanted x-ray wavelengths. For example, if using a Copper anode, a Nickel beta filter may be used which has the potential to remove 50 % of kα and 99 % of the kβ radiation. Further selection may be obtained using a monochromator.

The sample is irradiated with a beam of monochromatic x-rays at an angle, θ, to the sample surface and the scattered x-rays are detected at an angle equivalent to 2θ of the angle of the incident beam (Figure 16 b). XRPD is based on the premise that a powder sample will present an infinite and entirely random selection of all possible crystal faces at the powder interface. In order to observe all potential lattice planes the sample is rotated within the x-ray beam whilst maintaining the detector angle 2θ to the incident beam. In single-crystal measurements x-rays are diffracted by the individual atoms in the crystal, with each atom being represented by a single point. In powder diffraction, however, x-rays are scattered not from a single crystal but from a theoretically infinite number of crystallites. This leads to the formation of a characteristic diffraction cone as illustrated in Figure 18 b.

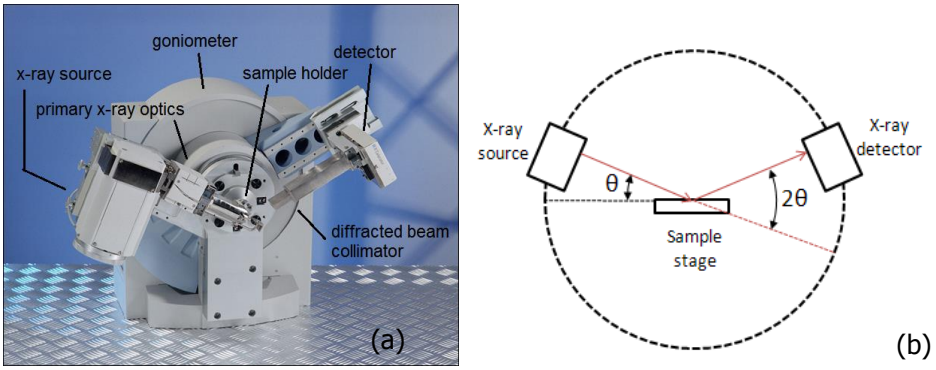

Figure 16 Example of the construction of a typical XRP diffractometer (a); illustration of the incident angle, θ, and diffraction angle, 2θ, of the employed x-ray beam relative to the sample [3]

The reflected x-rays can be interpreted according to Bragg's law provided below, where n is an integer (= 1,2,3,…) related to the order of the diffracted beam, λ represents the wavelength of the incident x-ray beam, d is the interplanar distance between lattice planes (d-spacing) and θ is the angle of the incident x-ray beam.

Equation 6 $n\lambda = 2d \sin\theta$

Bragg's law allows for the calculation of the angle where constructive interference from x-rays scattered by parallel planes of atoms will produce a diffraction peak. Since the

wavelength λ is fixed, a family of planes produces a diffraction peak only at a specific angle 2θ. The resulting diffracted x-ray intensity is measured using a suitable detector and the resulting signals undergo mathematical treatment, including Fourier transformation. The mathematical calculations used to obtain a diffractogram from the detected signals are complex and must be performed by a computer. The end result is a typical diffractogram as exemplified in Figure 19.

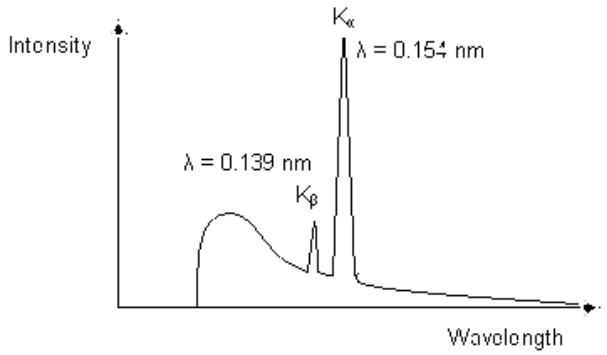

Figure 17 *Spectrum of x-rays produced by bombardment of a copper anode with high energy electrons* [4]

XRP detectors may be of the point- or position sensitive type. Point detectors observe one point in space at a time whereas position sensitive detectors cover a wider 2θ range. Position sensitive detectors include charged coupled devices (CCD) and solid state semiconductor strips which observe all photons over a certain range. In practice, a single point scintillation detector is most commonly used. Such detectors work by converting diffracted x-rays to visible light by fluorescence. This is achieved with the aid of a NaI or YAP (Yttrium Aluminum Perovskite) crystal. The resulting light impulses are then detected and enhanced using a photomultiplier.

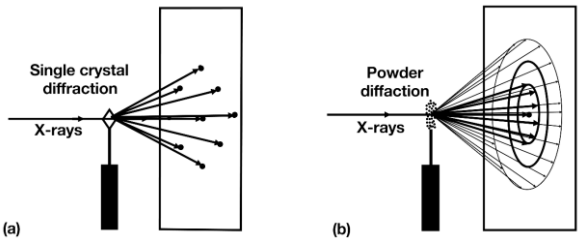

Figure 18 *Typical diffraction patterns of a single-crystal (a) and powder sample (b)* [5]

Powder diffractograms can provide information about the phase identity, phase composition and degree of crystallinity of a powder sample and this technique is the "gold standard" for the determination of polymorphism in pharmaceutical substances.

Polymorphism can be described as the occurrence of different physical modifications of the same chemical substance which differ in the 3 dimensional packing of atoms and/or molecules within the crystal lattice. Nowadays, regulatory authorities place particular emphasis on the study of polymorphism in pharmaceutical substances since experience has shown that different polymorphs of the same substance very often possess different chemical and physical characteristics which can lead to a different "*in-vivo*" performance of a drug product.

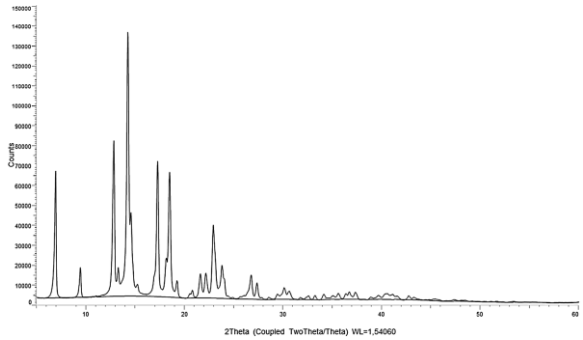

Figure 19 Example XRD diffractogram of a powder sample

Since the XRP diffraction pattern of a substance is correlated to the 3 dimensional arrangements of atoms and molecules within its crystal lattice, different polymorphs will inherently produce different XRP diffraction patterns. This property is made use of in the pharmaceutical industry as a tool to compare the crystalline structures of different batches of a chemical substance in order to ensure consistent batch-to-batch reproducibility.

An amorphous substance is a substance which, in contrast to a crystalline substance, does not possess any long-range structural order of atoms and/or molecules. For this reason, amorphous compounds do not produce diffraction peaks in XRD. Instead, they give rise to a characteristic "halo", as illustrated in Figure 20 below. This fact is useful when determining the crystallinity of a substance using XRPD data.

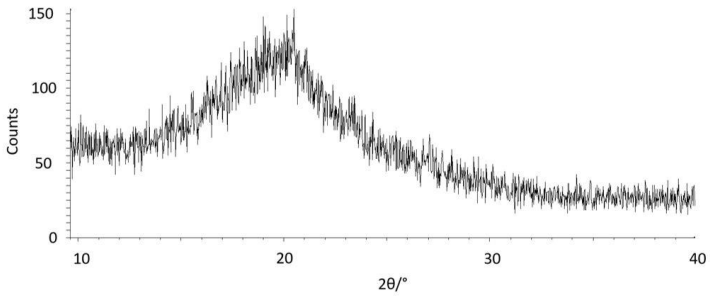

Figure 20 Characteristic "halo" in the XRP diffractogram of an amorphous substance

By obtaining both purely amorphous and purely crystalline samples of the substance of interest and preparing mixtures with different ratios of amorphous to crystalline substance, a calibration curve of the sum of the area of the diffraction peaks versus crystalline content can be generated. Pure crystalline substance can be obtained through recrystallisation and amorphous substance could be generated, for example, through freeze-drying of a solution of the substance.

2.2.2 *General Procedures*

All XRD experiments presented in this thesis were performed by the company Jesalis Pharma GmbH (Jena, Germany) using a Bruker D8 Advance (Bruker, Massachusetts, USA) diffractometer with parallel beam geometry; a Cu/Cu kα radiation source; and a LynxEye position sensitive detector with silicon strip detection technology.

2.3 *Intrinsic Dissolution Rate*

2.3.1 *Introduction*

The solubility of pharmaceutical drug substances may be influenced by several factors including: the degree of crystallinity, the particle size and/or the specific surface area of the drug substance. The presence of polymorphism and pseudopolymorphism is another critical aspect to be considered; and it has been extensively documented in the literature that different polymorphs of a substance may possess different solubility properties, which may give rise to differences in their *in-vivo* bioavailability. During drug product development it is often necessary to compare the relative solubilities of several polymorphs of a substance so that an informed decision can be made regarding the polymorph to be used in the final drug product formulation, so far as different polymorphs exist. The goal of such experiments is to compare the intrinsic solubility of the different polymorphs independent of any differences in their specific surface area and particle size distribution. Both these characteristics are unlikely to be identical between polymorphs and may have a large effect on the dissolution rate. It must be ensured in all experiments that the influence of surface area and particle size on the dissolution rate is the same for all polymorphs; since only then can a true comparison of the intrinsic solubility differences between polymorphs be made.

The intrinsic solubility of a substance may be evaluated by measuring its intrinsic dissolution rate. The intrinsic dissolution rate is defined as the rate of dissolution of a pure, compacted sample of a substance from a defined surface area. The procedure involves preparation of a compact of the substance in a hydraulic press

(Figure 21 *A, B*) and measurement of the dissolution rate of this compact in a suitable fluid medium at a specific pH, ionic strength, rotation speed and temperature, which are chosen based on the physico-chemical characteristics of the drug molecule. Since not all powders are suitable for compaction, the compactability and required press tonnage must be investigated individually for each substance prior to any quantitative measurement. The preparation of a compact ensures that neither the particle size nor the surface area have an influence on the dissolution rate, since inter-particle spaces are removed on compaction. When choosing a suitable dissolution medium for the test, it must be ensured that sink conditions are present. Sink conditions are said to be present if a maximum of 1/3 of the saturation concentration of the analyte were reached, if the complete sample of the drug substance were dissolved in the required volume of dissolution medium. The choice of the pH value of the dissolution medium is likely to be critical for ionisable substances. The intrinsic dissolution rate (mg/min/cm^2) is calculated as the slope of the regression line (mg/min) obtained by plotting the amount of dissolved drug substance versus time; divided by the exposed surface area of the drug substance compact, which is a constant 0.5 cm^2.

2.3.2 *General Procedures*

Intrinsic dissolution experiments described in this thesis were performed using an ERWEKA DT800 dissolution apparatus (ERWEKA, Germany) equipped with 6 rotating cylinders as described in Ph. Eur. 2.9.29. A rotation speed of 50 rpm and a medium comprising of 500 ml of 0.01M sodium hydroxide were used. Each compact was prepared by compacting 100 mg of sample at a force of 5 tonnes using a suitable hydraulic press. The experiment was performed at 37 °C. Preliminary experiments were performed in order to investigate the effect of rotation speed (50, 100 and 150 rpm) and press tonnage (3, 5 and 7 tonnes) on the release rate of the substance. The rotation speed was found to have a significant effect; with an increased rotation speed leading to a more rapid release rate. The press tonnage had no effect on the release rate. Suitable conditions were chosen which would lead to approximately 10 - 20 % release of the drug substance over a reasonable time period. For the final analysis, samples were taken at 10, 20, 30, 40, 50 and 60 minute intervals with a total release of ca. 15 % of the drug. Samples were measured by UV-visible spectrophotometry at a wavelength of 240 nm using a Specord205 spectrophotometer (AnalytikJena, Germany).

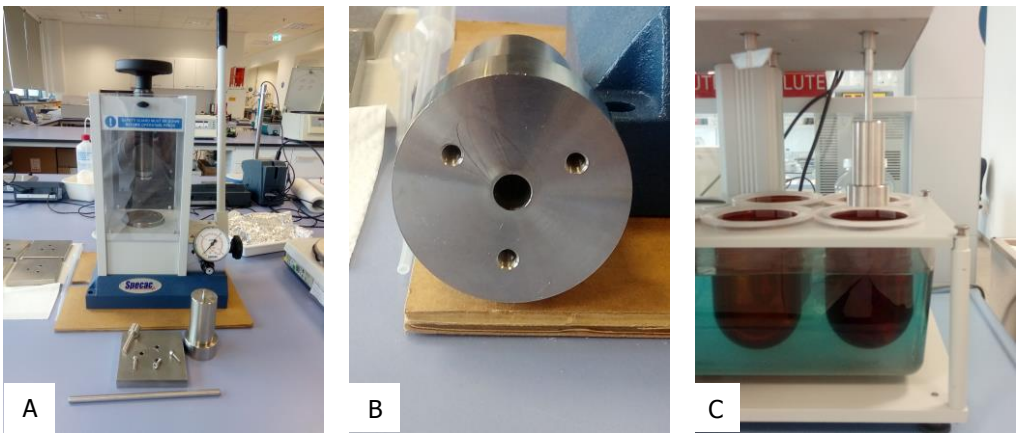

Figure 21 Hydraulic press and utensils for compact preparation (a), sample holder (b), dissolution set-up (c)

2.4 *In-Vitro Membrane Permeation Study*

2.4.1 *Introduction*

A membrane permeation study is an *in-vitro* study designed to mimic the diffusion of a drug substance out of topically applied pharmaceutical drug products, such as creams and ointments, across the epidermal-barrier to the target skin layers. Such tests are primarily used to compare the release rates of drug substances from different formulation candidates during product development and also as a quality control tool to monitor the batch-to-batch reproducibility of the release rate. The most common apparatus used for this test is the so-called "Franz-Cell" apparatus which is a vertical diffusion cell as shown in Figure 22/ Figure 23. The Franz-cell apparatus comprises of 6 jacketed, diffusion cells connected by suitable tubing to a heated reservoir of fluid kept at a constant temperature of 32 °C using a suitable temperature bath. A sample of the cream or ointment to be measured is placed in a specially designed donor compartment above the synthetic membrane (or skin sample). The reverse side of the membrane is in contact with the receptor medium, the composition of which is chosen based on the solubility properties of both the drug substance and drug product matrix. The receptor cell contains a magnetic stirrer which ensures that the correct hydrodynamic environment is present for an optimal drug diffusion rate. Samples are removed at predefined intervals through a sampling port and measured using an appropriate analytical procedure, e.g. HPLC.

2.4.2 *General Procedures*

For the experiments performed in this thesis a Franz cell apparatus (Hanson Research, Chatsworth, USA) comprising of 6 vertical diffusion cells was employed. Each diffusion cell contained a magnetic stirring bar and approximately 7 ml of a collector medium comprising of a mixture of 50:50 methanol/water (v/v). Porafil® membranes (Macherey-Nagel, Germany) made from regenerated cellulose and having a porosity and diameter of 0.45 µm and 25 mm, respectively, were employed in all tests. 300 mg of the cream sample were used for each diffusion cell. Sampling was performed at intervals of 60, 120, 180, 240 and 300 minutes and the samples were measured by HPLC. The HPLC method comprised of a Spherisorb ODS 2, 150 x 4.6 mm column packed with 5 µm particles and a mobile phase comprising of methanol, 10 g/L phosphoric acid, purified water and acetonitrile at a ratio of 10:20:20:50 v/v/v/v. The flow-rate, column-oven temperature and detector wavelength were 2.0 ml/Min, 25 °C and 235 nm, respectively. The method was run isocratically for 15 minutes and an injection volume of 15 µl was used.

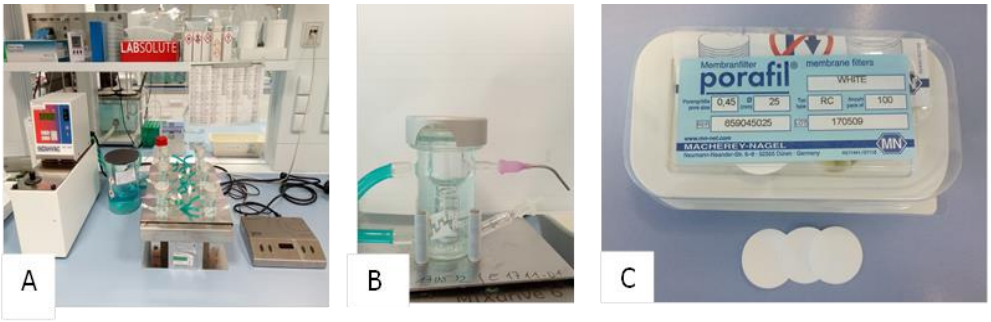

Figure 22 Franz-cell apparatus with thermostat and stirring unit (a), single vertical diffusion cell (b), and artificial membrane (c)

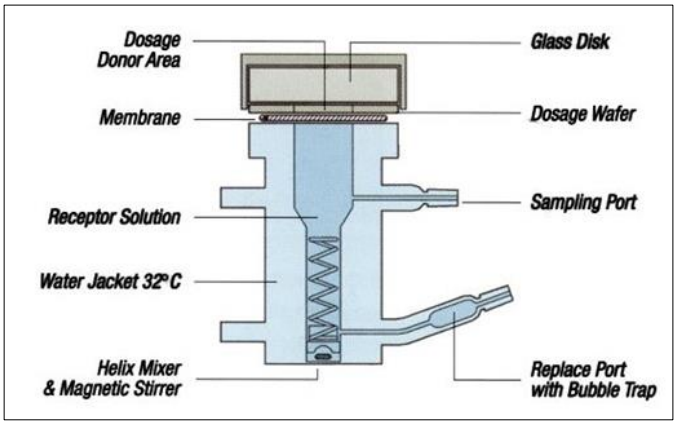

Figure 23 Detailed illustration of a vertical diffusion cell [6]

2.5 *Particle Size Determination by Laser Diffraction*

2.5.1 *Introduction*

Laser diffractometery is an analytical technique designed to measure the size distribution of particles in a given sample matrix, e.g. solid particles suspended in a liquid; where a particle may include: drops in a gas or liquid, solid particles in a gas or liquid, or gas bubbles in a liquid medium. Since individual particles may possess very different morphologies, e.g. spherical, plate or long needle-like forms, it is not possible to define the exact size of a real particle with a single number. Instead, particle size measurements are reported, not as absolute values, but as sphere-equivalent results, e.g. sphere equivalent volume. This is illustrated in Figure 24 a, where both the cylinder and sphere have the same volume but different morphologies. It is clear that at least 2 values are required to describe the size of the cylinder, but only 1 value is required to describe the sphere, namely the diameter. Particle size results are always to be understood as sphere equivalent approximations, regardless of the morphology of the particular particles.

A laser diffractometer works by illuminating a sample of particles with light from a laser source (HeNe gas laser, 466 / 633 nm) and measuring the scattering angles of that light after interaction with the particles (Figure 24 b). The optical system is constructed in such a manner that particles with the same size will scatter light onto the same position of the detector (Figure 24 c). In general, smaller particles give rise to larger scattering angles than larger particles; and it is this property which allows the scattering angle to be used to estimate the particle size. The detector is made up of multiple detection modules which are arranged such that a wide range of scattering angles may be detected, e.g., in the case of the Mastersizer 2000, which was used for experiments in this thesis, there are 52 detection modules. Subsequent to detection, the measured detector intensities are Fourier transformed and fitted to a suitable mathematical model.

There are currently 2 popular models in use which describe the light scattering characteristics of particles; these are the Fraunhofer- und the Mie Model. The Fraunhofer model is an approximation and is generally considered to be less accurate than Mie model. Mie theory provides a more accurate prediction of the scattering intensities for all incident wavelengths and scattering angles than the Fraunhofer model, particularly when dealing with particles below 50 µm in size. Particle size results are generally reported in the form of a particle size distribution (see Figure 24 d) which plots the volume percent (y-axis) against the particle size (x-axis, µ).

2.5.2 *General Procedures*

All particle size measurements in this thesis were performed using a Malvern Mastersizer 2000 laser diffractometer with a Hydro 200S wet dispersion unit (Malvern Instruments Limited, UK). Purified water was obtained from the in-house purification system at mibe GmbH (Brehna, Germany). Fusidic acid hemihydrate was of Ph. Eur. grade and was obtained from OJSC Biosintez (Penza, Russia) and Ercros SA (Madrid, Spain). Betamethasone valerate was obtained from Crystal pharma (Valladolid, Spain). Polysorbat 80 was of synthesis grade and was purchased from Merck (Darmstadt, Germany).

2.5.2.1 *Preparation of the sample solution – betamethasone valerate*

Weigh 100 mg of sample into a suitable glass beaker and add 3 drops of polysorbate 80. Mix with the aid of a spatula until no further dry particles are observed and slowly add 10 ml of purified water. Mix with the aid of a vortexer and sonicate for 60 seconds in an ultrasonic bath. Transfer the sample dropwise with the aid of a Pasteur pipette to the dispersion unit until an obscuration value of between 10 and 20 is reached. Start the measurement. The mean value of 6 single measurements should be reported. The results should be reported as a % volume distribution.

2.5.2.2 *Preparation of the sample solution – fusidic acid hemihydrate*

Weigh 100 mg of sample into a suitable glass beaker and add 3 drops of polysorbate 80. Mix with the aid of a spatula until no further dry particles are observed and slowly add 10 ml of purified water. Mix with the aid of a vortexer and sonicate for 60 seconds in a ultra-sonic bath. Transfer the sample dropwise with the aid of a Pasteur pipette to the disperging unit until an obscuration value of between 10 and 20 is reached. Start the measurement. The mean value of 6 single measurements should be reported. The results should be reported as a % volume distribution.

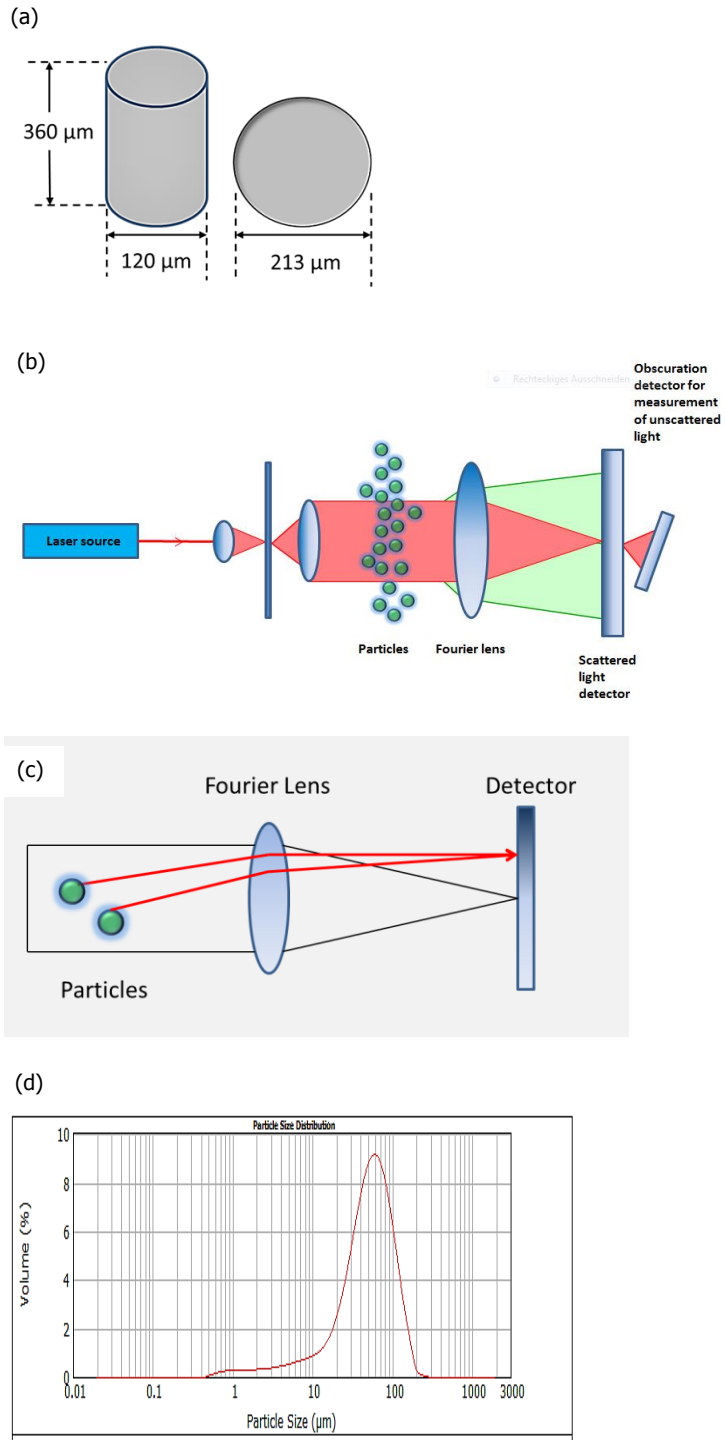

Figure 24 (a) Sphere equivalent volume, (b) diffractometer setup, (c) all particles of the same size are focused onto the same detector cell by the fourier lens, (d) example of particle size volume distribution

2.6 *Electrospinning*

2.6.1 *Introduction*

Electrospinning is a procedure for producing spun polymer threads or membranes, by application of a high voltage electric field to a drop of polymer fluid, usually comprising of a mixture of the required polymer, such as Polylactide (PLA) or Poly(caprolactone) (PCL), in a suitably volatile solvent. Application of the electric field causes the solution to become charged which in turn leads to droplet deformation (Taylor-cone formation). A charged jet is ejected from the tip of the cone which accelerates towards the counter electrode, leading to the formation of continuous fibers. Electrospun fibres have huge potential for use in biomedical applications, including tissue engineering and as bioresorbable wound dressings. One particularly interesting potential use of electrospun microfibers is in the topical- and systemic delivery of drug substances. Electrospun fibres have a large surface area allowing for relatively high drug loading capacities and have the added advantage of being modifiable, i.e. fibre thickness, solubility and pore size may be modified allowing for a tailored release rate of the drug substance.

2.6.2 *General Procedures*

2.6.2.1 *Drug-Polymer Solution Preparation*

Electrospun fibres were prepared by dissolving the required quantity of drug substance in chloroform (HPLC grade, Sigma-Aldrich) and diluting this solution to the required concentration with a pre-prepared PCL solution comprising of 1 g of PCL (M_n 70.000 – 90.000, Sigma-Aldrich) in a mixture of 7.5 ml of chloroform and 2.5 ml of ethanol (HPLC grade, Sigma-Aldrich). For example, co-loaded fibres containing 20 % FA (w/w) and 1.21 % BV (w/w) were prepared by dissolving 200 mg of FA in 1.0 ml of chloroform and mixing this solution with 1.0 ml of a solution comprising of 12.1 mg BV in 1.0 ml of chloroform. This mixture was then diluted to 10.0 ml with PCL solution. For fibres containing the gelator, Fmoc-C18-OH, an extra solution comprising of 1.5 mg gelator in 1.0 ml of ethanol was prepared, mixed with both drug substance solutions, and then made up to 10.0 ml with 7.0 ml of PCL solution. Appropriate control samples were also prepared.

2.6.2.2 *Electrospinning Set-Up*

The electrospinning set up employed in all experiments consisted of a 10 ml syringe and stainless steel blunt-ended needle (18 Ga. 51 mm / pst3. Hamilton), a syringe pump (IVAC P3000) with a variable flow rate from 1 ml to 6 ml, a ground electrode and a high voltage power supply (0-20 kV, (PLS K007-20, Spraybase, Ireland))

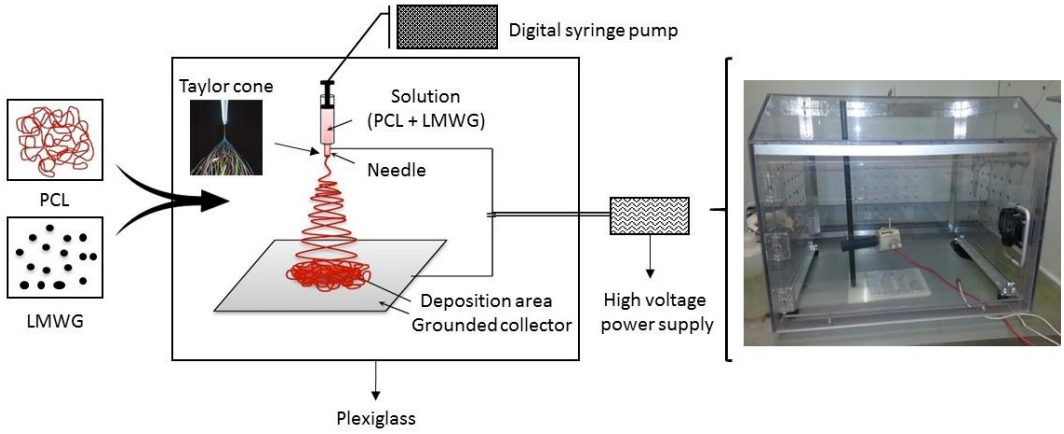

Figure 25 *Schematic of the electrospinning set-up used in this study*

2.6.2.3 *Analysis of the release rate of drug substance*

The analysis of the release rates of FA and BV from the electrospun fibres was performed using the same Franz-Cell apparatus, experimental setup and analysis procedure as described in section 2.4.

Agar diffusion experiments were performed using the *Staphylococcus aureus* bacterial strains: ATCC 6538P, ATCC 6538 and ATCC 29213. Additional tests were performed

2.7 *In-Vitro Release – Agar Diffusion Test*

using the ATCC 12228 strain of *Staphylococcus epidermidis*. These strains were chosen because they are the designated reference strains as required by the European Pharmacopeia and used frequently in relevant literature [26]. For safety reasons it was not possible to use clinical strains of MRSA. All microorganisms were obtained from the Leibnitz-Institut DSMZ - German collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). The test was performed by placing small discs of electrospun membrane, as well as diverse controls, on the surface of 4-5 mm thick agar plates (Tryptic Soy Agar, Merck/Millipore, pH 7.3 ± 0.2) inoculated with 24 hour cultures of the test microorganisms. Each strain was inoculated separately. Reference

solutions of fusidic acid prepared in buffer pH 6.0, which were required for comparative purposes, were placed in 6 mm cavities on separate agar plates which had been pre-prepared using an appropriate sterile, stainless-steel punch. The concentration of fusidic acid applied to these cavities was equivalent to the concentration present in the equivalent area of electrospun disc and based on the minimum inhibitory concentration (MIC) of 1 mg/L for *Staphylococcus* sp. as detailed in the literature [26,27]. Zones of inhibition were evaluated using a calibrated calliper (INSIZE, China) after incubation of the plates at 37 °C for 24 hours.

Table 2 Overview of samples employed in the Agar Diffusion Test

FUSIDIC ACID - Sample: Fusidic acid (Mic.); OJSC Biosintez; Lot: 831113					
FA, Filterpaper saturated with 25 µl of solution			50 µl FA solution filled into cavity		
Sample Name	Quantity of Fusidic acid	Quantity comparable with Sample	Sample Name	Quantity of Fusidic acid	Quantity comparable with Sample
G	200 µg	B	K	200 µg	B
H	350 µg	C	L	350 µg	C
I	450 µg	D	M	450 µg	D
			N	0.25 µg	< B, C & D
			O	2.5 µg	< B, C & D
			P	25 µg	< B, C & D
			R: Control Buffer pH 6.0	0 µg	

2.7.1 *In-Vitro Antimicrobial Efficacy Testing*

Numerous methods are described in the literature for determining the antimicrobial efficacy of wound bandages [23,28–37]. On review of this literature it was decided to employ 2 separate procedures (Methods A and B) to evaluate the antimicrobial effectiveness of the electrospun membranes. The procedures were designed to mimic the conditions present in an infected wound, i.e. moderate temperature and high humidity. All microbiological experiments were performed in the microbiology laboratory of mibe GmbH Arzneimittel by Ms. Sylvia Noll. The procedures were as follows:

2.7.1.1 *Method A (Membrane Filtration Method)*

Three 2 x 2 cm pieces of each of the electrospun membranes to be examined (n=3, B, C and D) were placed on sterile petri dishes and 20 µl of inocula (equivalent to 10^9 CFU), prepared using *Staphylococcus aureus* strains ATCC 6538 and ATC 29213 as well as *Staphylococcus epidermidis* strain ATCC 1228, were placed in the middle of each piece of membrane. Each membrane was subsequently covered with a 1.5 x 1.5 cm piece of parafilm (microbiologically inert) in order to prevent it from drying out. The petri dishes were incubated at 20 – 25 °C in an 80 % humidity environment in order to simulate wound conditions [38]. After 24 hours the number of viable microorganisms was determined by transfer of the membrane samples into 100 ml of a recovery diluent and extracting by shaking and vortexing followed by appropriate serial decimal dilution. The membrane filtration method (according to Ph. Eur.2.6.12) was used to count the number of surviving bacteria (CFU/ml). The decimal log reductions in the number of viable micro-organisms were calculated against the value for the inoculum. The above mentioned diluent was phosphate buffer saline pH 7.2 with 3 % polysorbate.

2.7.1.2 *Method B (Pour Plate Method)*

Small discs of the electrospun membranes containing fusidic acid as well as diverse controls (diameter ca. 5 mm) were cut aseptically from the bulk material. Each piece of membrane was incubated for 48 hours at 35 – 37 °C with 1 ml of inoculum (equivalent to 10^{10} CFU) in 48 well cell culture multiwall plates (CellStar, Greiner Bio-One). The number of viable microorganisms was determined after 5, 24 and 48 hours by transferring 0.2 ml aliquots of the incubated inoculum into 1.8 ml of a phosphate buffered saline pH 7.2 recovery diluent and performing serial decimal dilution. The pour plate method (Ph. Eur., 2.6.12) was used for counting the number of surviving microorganisms (CFU/ml). The decimal log reductions in the number of viable micro-organisms were calculated after 24 hours against the value for the inoculum.

2.8 *References*

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CHAPTER 3

Development of Analytical Procedures

3 CHAPTER 3 - Development of Analytical Procedures

3.1 *Introduction*

In order for active drug substances to reach their target sites they need to be formulated and administered in a suitable pharmaceutical vehicle, e.g. cream, tablet or injection solution. Two requirements of any drug vehicle are (i) that the drug substance is sufficiently stable in the chosen vehicle and (ii) that the drug substance is released at an appropriate rate so as to guarantee that the efficacy of the drug product is acceptable and reproducible. The development of an appropriate formulation requires the employment of a stability-indicating analytical procedure which can quantify the active substances and preservatives, as well as impurities and degradation products, in relevant formulation candidates.

Current international guidelines [1,2] require that the content and purity of active pharmaceutical ingredients (API) are analysed during both release and shelf-life testing of marketed drug products. This requirement ensures that the API does not degrade to such an extent that the efficacy of the product is diminished and also, that the levels of potentially toxic impurities, arising through degradation of the API, are maintained below specified limits [3].

In the past, it was common in analytical laboratories to have several individual HPLC procedures for the separate analysis of APIs and preservatives in the same finished formulation. In recent times, however, there appears to be a trend towards developing a single procedure for the analysis of all components in a single chromatographic run. Examples include Shaikh *et al* [4], who developed a procedure for the simultaneous quantitation of chlorocresol, mometasone furoate and fusidic acid in a topical cream, and Goswami *et al* [5], who developed a procedure for the analysis of halometasone, fusidic acid, methyl paraben and propyl paraben in a single run. This trend has most likely been driven by advances in analytical technology such as Ultra-Performance Liquid Chromatography (UPLC), as well as the availability of modern stationary phases with multiple selectivity modes and increased efficiency, e.g. mixed mode phases and sub 3 µm shell-core particle technologies. Cost efficiency and environmental awareness issues are also likely to have played a significant role.

3.2 *Aim*

The principle objective of the work in this chapter was to develop analytical procedures based on HPLC and a suitable detection method (e.g. UV, ELSD, MS, RI) which could be employed for the stability-indicating content and purity analysis of formulation candidates of a developmental cream containing the drug substances BV and FA. The procedures were required to be based on HPLC since this technique is the most widely used for the analysis of pharmaceutical compounds and is available in most analytical laboratories, making transfer of the procedures between laboratories significantly easier. From the outset the objective was to employ a single assay procedure for the selective quantitative analysis of the two drug substances BV and FA as well as the preservative compounds methyl paraben, propyl paraben and potassium sorbate. The simultaneous measurement of all analytes of interest in a single HPLC run would enable a more cost-effective assay analysis of formulation candidates. It was also envisaged to develop a separate procedure for the purity analysis of both APIs.

The development of a single procedure for both assay and purity was initially considered but not deemed practicable since it was clear, due to the complexity of the samples, that a relatively long run time would be needed for the purity measurement. A long run-time for assay would, however, have been counterproductive, since in-process samples need to be measured quickly so that the manufacturing process can continue on to the next phase. It was envisaged that the developmental cream would be an oil-in-water based emulsion system and contain the drug substances BV and FA suspended in a matrix of excipients, such as vaseline, liquid paraffin, gelling agents, emulsifiers and preservatives.

3.3 *Results and Discussion*

The initial physico-chemical characterisation and early analytical development were performed using a model cream which had the same qualitative formulation as the final product but whose quantitative formulation was chosen based on the experience of the formulation chemist rather than on relevant stability data (see Chapter 5), i.e. the active substances and excipients were known but the quantities of each required to achieve a stable formulation were not. Nothing was known about the stability of the APIs in the cream base. The first task was to develop a basic sample preparation procedure. In order to do this, the solubility of the active substances and the cream base in different solvents used routinely in HPLC analysis was first investigated.

3.3.1 *Investigation of the Solubility of the Active Substances*

The solubility of the active substances was investigated by adding 20 mg of each substance to 50.0 ml of selected solvents or solvent mixtures in an appropriate glass beaker and stirring for 10 minutes using a magnetic stirrer. The solubility was then determined visually at room temperature. The degree of solubility was assessed using criteria such as ‘completely dissolved after X minutes’, ‘does not dissolve well’ and ‘insoluble’. The dissolution time was recorded as well as any relevant observations, e.g. aggregation, flocculation. The above procedure can be viewed as a “Quick-Test” which allows for a fast qualitative analysis of the solubility but without actual quantitative data. In most cases this test is sufficient to obtain a basic idea of the solubility of the API and/or drug product. The results indicated that both active substances are insoluble in purified water and soluble in common high polarity organic solvents such as methanol and acetonitrile which are relatively cheap solvents used frequently in RP-HPLC analysis. A summary of the data is provided in Table 3.

3.3.2 *Investigation of the Solubility of the Cream Base*

The solubility of BV/FA cream base was investigated by suspending 500 mg of the cream in 20.0 ml of solvent and stirring for 10 minutes at room temperature. The same solvents as used for testing the solubility of the APIs were employed. The results were again evaluated visually. The purpose of the experiment was to gain information about which solvents were likely to be suitable for extracting the active substance from the cream base without dissolving unwanted matrix components. From the data presented in Table 3 and Table 4 it was concluded that acetonitrile was a suitable solvent candidate since both active substances dissolve well in this solvent but the matrix components remain largely undissolved. This is to be preferred since large quantities of dissolved matrix components could potentially cause problems, such as peak tailing, double- or extraneous peaks, during HPLC analysis.

Table 3 Solubility of the active substances in selected solvents

Solvent	**Observation** BV	**Observation** FA
Purified water	Insoluble	Insoluble
Methanol	Completely dissolved after 2 minutes	Completely dissolved after 3 minutes
Acetonitrile	Dissolved immediately	Completely dissolved after 3 minutes
2-Propanol	Completely dissolved after 3 minutes	Completely dissolved after 2 minutes
Ethanol	Completely dissolved after 2 minutes	Completely dissolved after 2 minutes
Ethanol/1-Butanol (50:50 v/v)	Completely dissolved after 3 minutes	Completely dissolved after 3 minutes
Dichloromethane	Dissolved immediately	Dissolved immediately
Ethanol/ Dichloromethane (50:50 v/v)	Dissolved immediately	Dissolved immediately

Table 4 Solubility of BV/FA cream in selected solvents

Solvent	**Observation**
Purified water	Milky, well dispersed suspension without agglomerates
Methanol	Slow, incomplete dissolution
Acetonitrile	Does not dissolve well
2-Propanol	Slow, incomplete dissolution
Ethanol	Dissolves completely
Ethanol/1-Butanol (50:50 v/v)	Slow, incomplete dissolution
Dichloromethane	Fast, complete dissolution
Ethanol/Dichloromethane (50:50 v/v)	Fast, complete dissolution

3.3.3 ***Sample Preparation Procedure***

Using the information obtained from the solubility tests and experience with other semi-solid dosage forms, the initial sample preparation procedure provided in Table 5 was devised.

Table 5 Sample preparation procedure

	Procedure	Purpose of step
1	Weigh 1.00g of cream into a 50.0 ml volumetric flask	*Weighing of sample*
2	Add 30 ml of acetonitrile and shake vigorously by hand. Suspend thoroughly using a vortexer	*Suspension of sample*
3	Place in a water bath at 65°C for 5 minutes, shaking at regular intervals	*The increased temperature allows for complete suspension of the sample by facilitating the melting of the fatty components, e.g. vaseline*
4	Shake for 10 minutes on a flat-bed mechanical shaker and make up to volume with acetonitrile	*Extraction of the analytes from the matrix*
5	Place in a freezer at approx. -20°C for 20 minutes	*'Freezing out' of any partially soluble matrix components which may have dissolved on heating of the sample*
6	Centrifuge approx. 15 ml of the cold solution at ca. 3000 rpm for 5 minutes and transfer a portion of the cold supernatant to a volumetric flask. Allow to warm to room temperature	*The cold solution is centrifuged in order to remove unwanted matrix components. The cold solution is used because the unwanted matrix components are less soluble at lower temperature. Allowing the supernatant to warm to room temperature before dilution helps to prevent temperature-related dilution errors*
7	Make a 1:2 dilution with purified water, e.g. 5.0 ml to 10.0 ml	*This step is performed in order to increase the polarity of the sample solution. This ensures good peak shape during HPLC analysis. The injection of a sample in pure acetonitrile combined with an aqueous mobile phase would lead to severe peak fronting. The dilution with purified water also helps to remove unwanted matrix components*
8	Filter a portion of the solution through a 0.45 µm regenerated cellulose filter into a HPLC vial	*Filtration of the sample to remove unwanted particulates. A regenerated cellulose filter was used because it is standard in the laboratory and is compatible with the employed solvents. Recovery rates were acceptable.*

3.3.4 ***Development – Purity Method***

As previously mentioned, current guidelines governing the quality of marketed drug products require that the degradation of active drug substances is monitored throughout the shelf-life of the product. A literature search of published analytical methods which might be suitable for the required analysis was performed but none of the reported methods were suitable for the simultaneous analysis of impurities of both BV and FA in a single chromatographic run.

Analytical procedures have been published for the analysis of the single APIs in topical formulations. For example, Po *et al.* [6] developed a HPLC procedure for the

analysis of BV and its degradation products in topical preparations. However, the vast majority of published methods were only suitable for the analysis of the main component, i.e. the API, and did not consider potential impurities. Furthermore, many of the published procedures were often poorly selective, i.e. could not measure the analyte of interest unequivocally without interferences from other sample components. Such methods were often based on UV-Vis spectrophotometry [7] or atomic absorption spectrometry [8] which are no longer considered to be state-of-the-art or stability-indicating. Shaikh *et al.* [4] published a method for the simultaneous quantitation of chlorocresol, mometasone furoate and FA in creams which is capable of separating and quantifying the impurity 3-didehydrofusidic acid (3-ketofusidic acid), which is one of the main impurities of FA. However, 14 other impurities related to FA have been described, many of which are potential degradation products [9] (Figure 26). These impurities have not been considered in any of the peer-reviewed literature methods found. Consequently, it was necessary to develop a novel procedure for the selective analysis of impurities of both active substances.

3.3.5 *Initial Chromatographic Conditions*

FA has a single carboxylic acid functional group which has a pK_a of 5.3 [10]. Consequently, the retention time of FA in an RP-HPLC system will be influenced by the pH of the mobile phase [11]. A suitable mobile phase should have a pH of 2 units below the pK_a of the acidic group [12], ensuring that the molecule remains completely protonated in solution and therefore enhancing hydrophobic interactions between the analyte and the stationary phase. The impurities of fusidic acid (see Table 8) are either acidic, as FA, or neutral compounds. For this reason a mobile phase acidified with phosphoric acid was chosen. BV and its impurities betamethasone and betamethasone 21-valerate are neutral compounds and their retention on the analytical column should not be affected by pH.

The chosen starting conditions comprised of mobile phase A made up of a 20:20:40:40 (v/v/v/v) mixture of methanol, acetonitrile, purified water and a 10 g/L solution of phosphoric acid (85 % w/w) and mobile phase B comprising of a 22:70:4:4 (v/v/v/v) mixture of the same solvents. The gradient program started with 100 % mobile phase A held for 3 minutes followed by an increase to 100 % mobile phase B over the next 25 minutes. 100 % B was then held for a further 10 minutes before returning to the start conditions. The total run time was 60 minutes. The initial flow-rate was 1.0 ml/min

and 20 µl of sample were injected. Detection was performed at 235 nm using a UV-Vis detector.

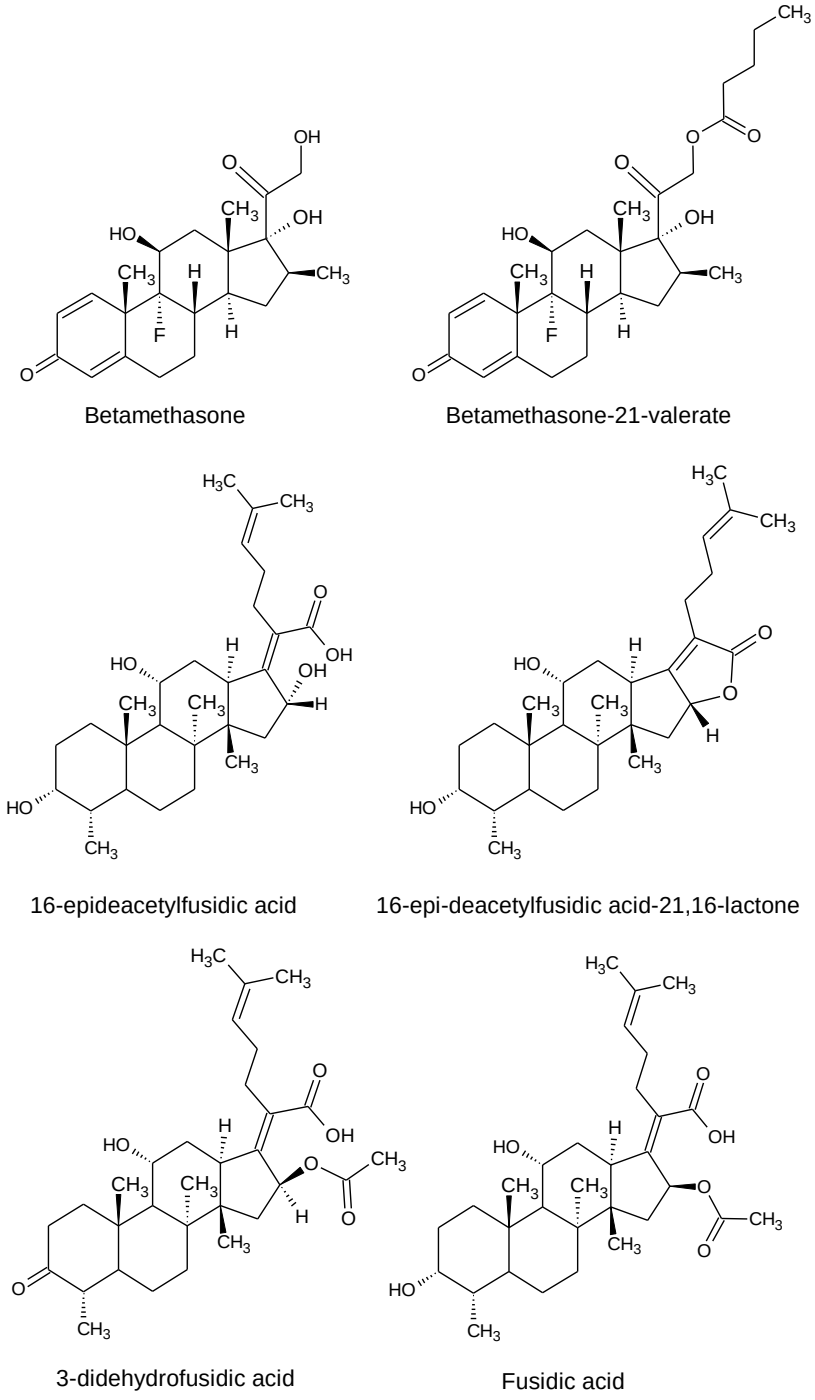


Figure 26 Structures of some of the major degradation products of BV and FA

Through column screening experiments it was found that a YMC-Pack Pro C18, 150 mm x 4.6 mm column with 3.5 µm particles provided the best overall separation of all impurities, although several impurities were still not satisfactorily separated. In order to achieve an acceptable separation, the gradient program, column-temperature, flow-rate and mobile phase composition required optimisation.

3.3.6 *Optimisation*

3.3.6.1 *Optimisation – Gradient program*

A combination of modification of the initial part of the gradient program from 100 % A for 3 minutes to 95:5 (v/v) A/B for 20 minutes and reduction of the flow rate to 0.7 ml/min was found to increase resolution between all of the peaks eluting in the early part of the chromatogram. It was also found that reducing the quantity of methanol in mobile phase A from 200 ml to 170 ml per litre enabled a better separation between the impurities betamethasone-21-valerate and 26-oxofusidic acid as well as between the impurities (24R)-24,25-dihydroxyfusidic acid-21,24-lactone and 26-hydroxyfusidic acid. The above optimisation steps enabled an acceptable separation of the 2 previously co-eluting peak pairs as demonstrated by Figure 27 and Figure 28.

3.3.6.2 *Optimisation - Sample weight / injection volume*

Very early on during method development it became apparent that an injection volume of 20 µl was not sufficient to achieve a limit of quantitation (LOQ) of below 0.1 %, which is the reporting threshold required by the relevant guideline [3]. The injection volume was increased to 150 µl and the limits of detection and quantitation were re-calculated using Equation 7 and Equation 8. The LOQ may be defined as the lowest concentration of analyte which can be accurately and precisely quantified by the analytical system and is related to the limit of detection (LOD) which is the lowest quantity of analyte which can be detected by the analytical system.

Equation 7 $$LOD = \frac{H(N) \times 3 \times c(S)}{H(S) \times 2}$$

Equation 8 $$LOQ = \frac{H(N) \times 10 \times c(S)}{H(S) \times 2}$$

Where,

H(N) = Height of noise in millivolts (mV) or absorption units (AU)

c(S) = Concentration of test solution in %

H(S) = Height of signal in mV or AU

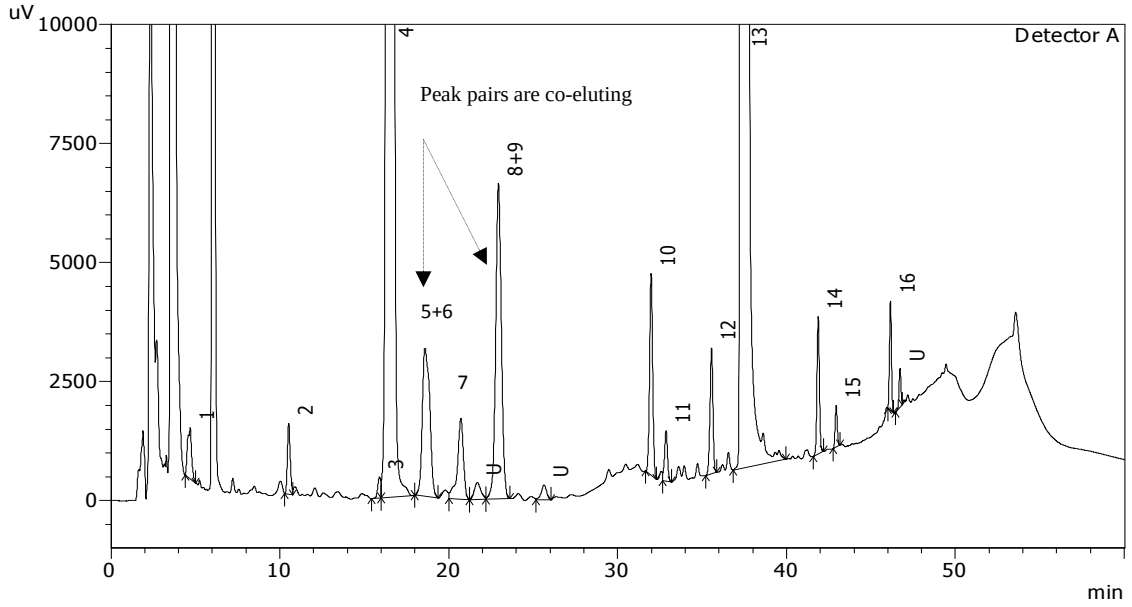


Figure 27 Chromatogram of BV/FA cream measured using the initial procedure and demonstrating the co-elution of peaks 5 and6 as well as 8 and 9. The HPLC conditions are provided in section . The sample was prepared as described in Table 5.

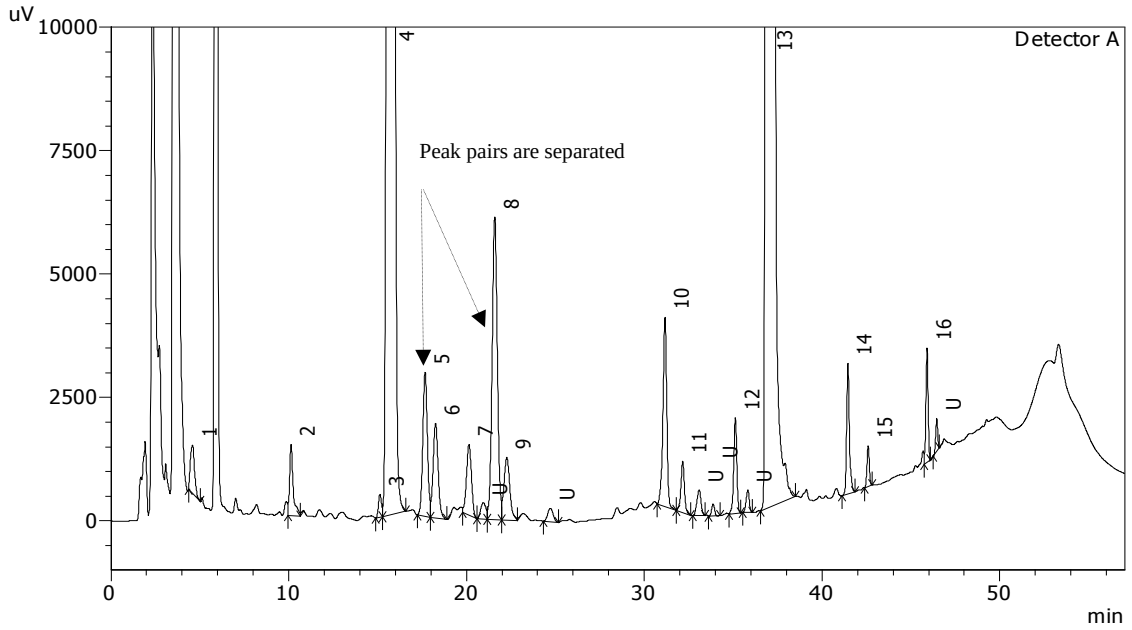


Figure 28 Chromatogram of BV/FA cream measured with the optimized procedure. The peak pairs which were co-eluting in Figure 27 are now separated. The HPLC conditions are provided in section 3.3.6.1. The sample was prepared as described in Table 5.

1. Betamethasone
2. 24,25-dihydro-24,25-dihydroxyfusidic acid
3. 24,25-dihydro-24,25-dihydroxyfusidic acid-21,25-lactone
4. Betamethasone-17-valerate
5. (24R)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone
6. 26-hydroxyfusidic acid
7. (24S)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone
8. Betamethasone-21-valerate
9. 26-oxofusidic acid
10. 3-didehydrofusidic acid
11. 11-didehydrofusidic acid
12. 16-epideacetylfusidic acid
13. Fusidic acid
14. 16-epideacetylfusidic acid-21,16-lactone
15. Deacetylfusidic acid-21,16-lactone
16. 11-deoxyfusidic acid

U = unknown impurity

Table 6 *LOD and LOQ values with 1 g of sample and 150 µl injection volume*

Substance	*LOD, %*	*LOQ, %*
FA	0.0079	0.0264
BV	0.0633	0.2110

The LOQ value of 0.21 % for BV did not meet the required specification of ≤ 0.1 %. There were 2 practicable solutions for decreasing the LOD, i.e. the sensitivity, of the procedure. These were 1) to further increase the injection volume and/or 2) to increase the analyte concentration in the sample solution. The injection volume of 150 µl was already too large for many of the HPLC instruments in the laboratory, which were only fitted with 50 µl sample loops. A reduction of the injection volume to 50 µl had previously been envisaged in order to increase flexibility within the laboratory by enabling the method to be run on all HPLC systems. Additionally, a further increase of injection volume was likely to lead to peak deformation due to overloading of the HPLC column, leaving an increase of the analyte concentration in the sample solution as the only practicable option.

The analytical procedure was modified by increasing the sample weight from 1.0 g to 5.0 g and reducing the injection volume from 150 µl to 50 µl. The LOD and LOQ values were recalculated under the optimised conditions (Table 7). With the revised sample weight and injection volume the required LOQ level of ≤ 0.1 % was obtained.

Table 7 *LOD and LOQ values with 5 g of sample and 50 µl injection volume*

Substance	*LOD, %*	*LOQ, %*
FA	0.0033	0.0109
BV	0.0291	0.0972

3.3.7 ***Identification of Impurities***

The available impurities of BV (namely, betamethasone and betamethasone 21-valerate) were identified by dissolving an appropriate quantity of each compound in the sample solvent and injecting them into the HPLC. Their respective retention times and relative retention times were recorded and compared to the peaks in the sample solution. For the known impurities of FA, which were not available as pure substances, the EP standards *fusidic acid for peak identification CRS* and *fusidic acid impurity mixture CRS* were used. EP and CRS refer to European Pharmacopeia and Chemical Reference Substance, respectively. A summary of the investigated relative retention times (rrt) of the BV and FA impurities is provided in Table 8.

Table 8 Nomenclature and rrt values of BV and FA impurities

Nomenclature according to the current Ph. Eur. monograph	*Impurity Label*	**rrt value (relative to the fusidic acid peak)**
Betamethasone	-	0.11
Betamethasone-17-valerate	***API***	-
Betamethason-21-valerate	-	0.43
24,25-dihydro-24,25-dihydroxyfusidic acid	*Impurity A*	0.26
24,25-dihydro-24,25-dihydroxyfusidic acid 21,25-lactone	*Impurity B*	0.31
(24R)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone	*Impurity C*	0.36
26-hydroxyfusidic acid	*Impurity E*	0.38
(24S)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone	*Impurity D*	0.41
Impurity N	*Impurity N*	0.42
26-oxofusidic acid	*Impurity F*	0.46
3-didehydrofusidic acid	*Impurity G*	0.68
11-didehydrofusidic acid	*Impurity H*	0.73
16-deacetylfusidic acid	*Impurity O*	0.84
16-epideacetylfusidic acid	*Impurity I*	0.92
Fusidic acid	***API***	-
16-epideacetylfusidic acid 21,16-lactone	*Impurity J*	1.19
Deacetylfusidic acid 21,16-lactone	*Impurity K*	1.25
9,11–anhydrofusidic acid	*Impurity L*	1.31
11-deoxyfusidic acid	*Impurity M*	1.36

3.3.8 *Final HPLC Method*

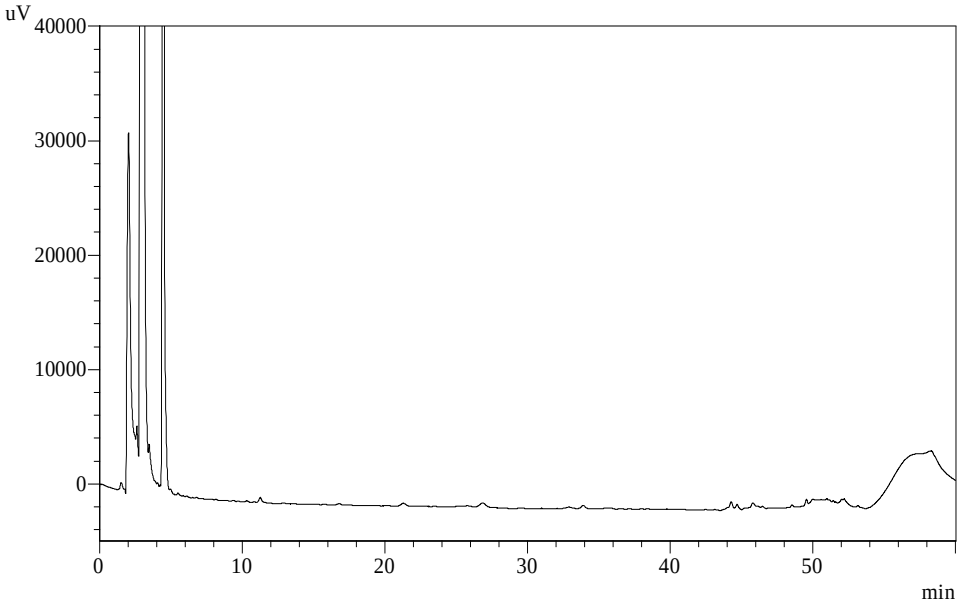
After the method optimisation studies, the conditions used in the final HPLC method for the analysis of BV/FA Cream are detailed in Table 9.

Table 9 Final HPLC Conditions

HPLC Column	-	YMC-Pack Pro C18, 150 mm L x 4.6 mm ID, 3 µm particles			
Guard Column	-	C18, 4.0 mm L x 3.0 mm ID			
Flow rate	ml/min	0.7			
Mobile Phase	A	170 ml methanol + 215 ml 10 g/L H_3PO_4 + 215 ml purified water + 400 ml acetonitrile			
	B	220 ml methanol + 40 ml 10 g/L H_3PO_4 + 40 ml purified water + 700 ml acetonitrile			
Wavelength	nm	235			
Column temperature	°C	40			
Auto-sampler temperature	°C	4			
Gradient	-	Time	Event	% A	% B
		0	Pump A B	95	5
		20	Pump A B	95	5
		45	Pump A B	0	100
		50	Pump A B	95	5
		60	Pump A B	95	5
Injection volume	µl	50			

3.3.9 *Example Chromatograms*

3.3.9.1 *Placebo (cream without the active substances)*



3.3.9.2 *BV/FA Cream*

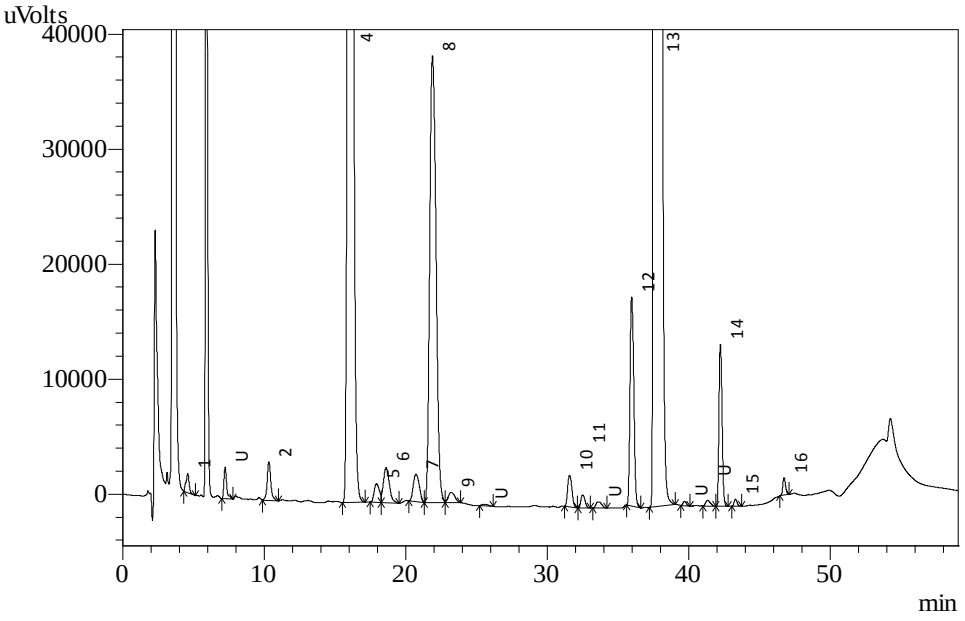


Figure 29 (a) Placebo without the active substances (b) Chromatogram of a degraded cream sample analysed after stressing under the conditions of 40 °C/75 % RH at pH 7 for 2 months. The sample demonstrated significant degradation. HPLC conditions as provided in section 3.3.8. Peak 3 was not present in this particular sample

1. Betamethasone
2. 24,25-dihydro-24,25-dihydroxyfusidic acid
3. 24,25-dihydro-24,25-dihydroxyfusidic acid-21,25-lactone
4. Betamethasone-17-valerate
5. (24R)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone
6. 26-hydroxyfusidic acid
7. (24S)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone
8. Betamethasone-21-valerate
9. 26-oxofusidic acid
10. 3-didehydrofusidic acid
11. 11-didehydrofusidic acid
12. 16-epideacetylfusidic acid
13. Fusidic acid
14. 16-epideacetylfusidic acid-21,16-lactone
15. Deacetylfusidic acid-21,16-lactone
16. 11-deoxyfusidic acid

U = unknown impurity

3.4 *Development – Assay Method*

The target analytes for the assay method were the 2 active substances BV and FA as well as the preservative compounds potassium sorbate, methyl paraben and propyl paraben. The chemical structures of theses analytes are provided in Figure 30.

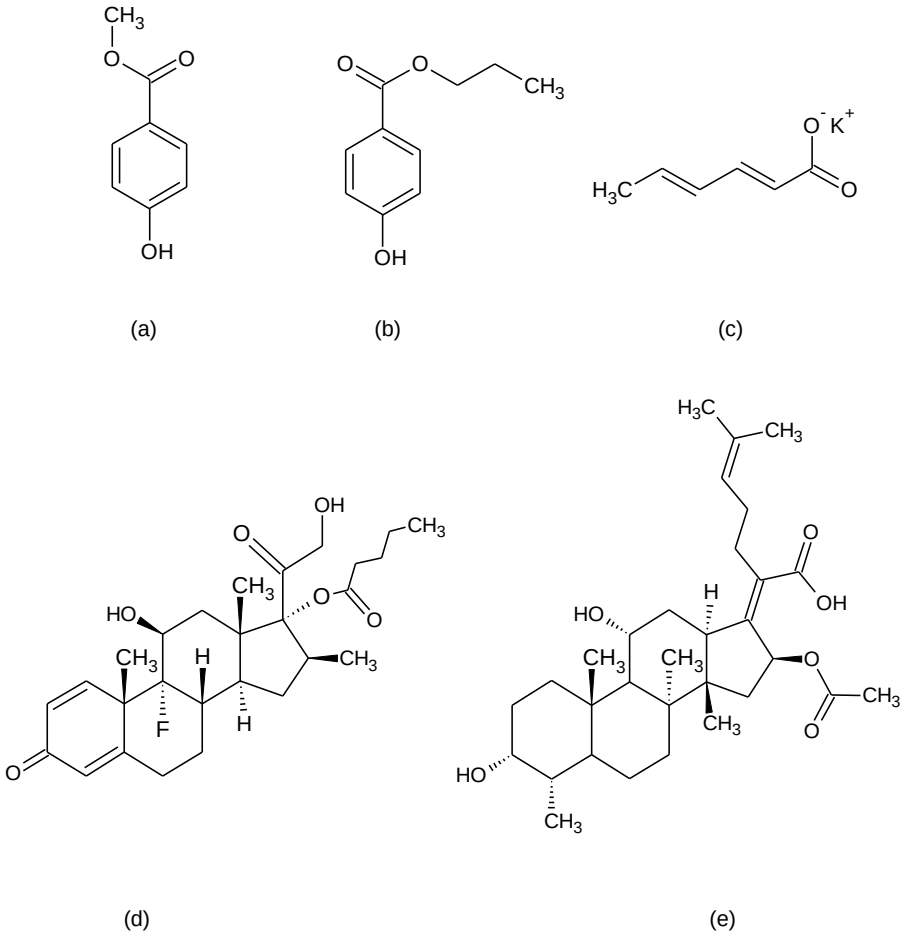


Figure 30 Chemical structures of methyl paraben (a), propyl paraben (b), potassium sorbate (c), BV (d) and FA (e)

The first step of the development process involved a search of the current literature for appropriate published methods which could be used or adapted to tackle the current analysis needs. No analytical procedures were found which were suitable for the simultaneous analysis of all 5 analytes of interest in a single chromatographic run. As already mentioned in section 3.3.4, the majority of reported methods were only suitable for the analysis of a maximum of 1 or 2 of the compounds of interest [6,7,8,13] and were often based on poorly selective procedures [7,8]. It was therefore necessary to develop a novel procedure specific to the required analysis.

As previously discussed, the acidic nature of FA means that its retention on the HPLC column will be influenced by the pH of the mobile phase. To achieve an

acceptable retention of this compound on a C18 column, it needs to remain in its protonated form in solution so that it can interact sufficiently with the stationary phase. BV, as well as methyl- and propyl paraben are neutral compounds whose retention times will remain largely unaffected by pH. Potassium sorbate is the potassium salt of sorbic acid and may be present in either ionised- or non-ionised form in aqueous solution. The retention time of the dissociated form will be influenced by the pH of the mobile phase since the sorbate anion may become protonated depending on its pKa value and on the pH of the sample solution/mobile phase [11,12]. The compound will only be reproducibly retained in its protonated form, i.e. as sorbic acid, which has a pKa value of 4.8 [14], necessitating the use of a mobile phase with a pH of 2.8 or lower, i.e. 2 pH units below the pKa of the strongest acidic group in the analytes of interest.

3.4.1 *Initial Chromatographic Conditions*

After consideration of the points discussed in the previous section, an acidic mobile phase containing 10 g/L phosphoric acid (85 % w/w) and having a pH of approximately 1.6 was chosen as mobile phase A. Mobile Phase B comprised of an 83:17 (v/v) mixture of acetonitrile and methanol. For the initial experiments a Nucleosil® C18 100-5, 250 mm L x 4.6 mm ID column with 5 µm particles was chosen. The oven-temperature; detector wavelength; and injection volume used were 25 °C; 240 nm and 50 µl, respectively.

It was considered unlikely that an isocratic run with the above mobile phases would be sufficient to provide a stability-indicating separation of such a highly complex mixture of analytes with very different polarities (2 APIs + 3 preservatives + 16 main impurities + matrix). It was decided to perform an initial 60 minute gradient run beginning with a high proportion of aqueous phase followed by a gradual increase of the proportion of organic solvent. The initial conditions chosen for the gradient program were 60 % mobile Phase A (aqueous) and 40 % mobile phase B (organic) held for 10 minutes, after which the percentage of B was increased to 100 over the next 30 minutes and held at this concentration for a further 10 minutes. After returning to the initial conditions over a period of 5 minutes, the system was equilibrated for a further 5 minutes giving a total run time of 60 minutes. The initial flow rate was 1.0 ml/min (Table 10).

A sample of BV/FA cream was prepared according to the procedure described in section 3.3.3 and analysed according to the described method. As demonstrated by Figure 31, all target peaks eluted before 30 minutes, indicating that a run time of 60

minutes was unnecessarily long. It was also evident that several of the early eluting peaks were insufficiently resolved (0 - 10 minutes), as in the case of potassium sorbate and methyl paraben which were co-eluting. The same experiment was repeated with a more hydrophobic Gemini C18 column (250 mm L x 4.6 mm ID, 5 µm) and a more polar Spherisorb ODS 2 (250 mm L x 4.6 mm ID, 5 µm) column, but neither column provided an acceptable separation.

Table 10 Initial chromatographic conditions

Column description	-	Nucleosil C18 100-5 (250 mm L x 4.6 mm ID)			
Flow rate	ml/min	1.0			
Mobile Phase	A	500 ml 10 g/L phosphoric acid + 500 ml purified water			
	B	833 ml Acetonitrile + 167 ml Methanol			
Wavelength	nm	240			
Gradient Program		Time (Min.)	Event	% A	% B
		START	Pump A B	60	40
		10.0	Pump A B	60	40
		40.0	Pump A B	0	100
		50.0	Pump A B	0	100
		55.0	Pump A B	60	40
		60.0	STOP	-	-
Injection volume	µl	50 µl			

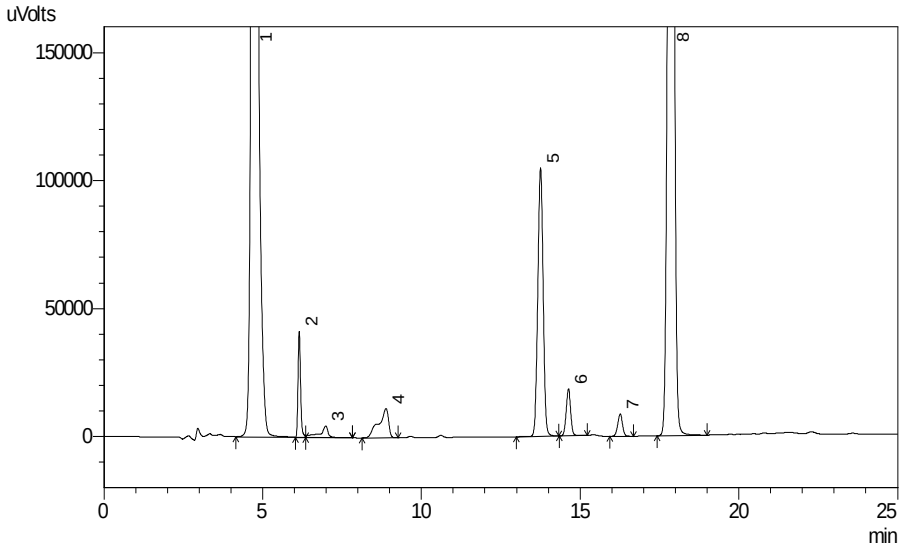


Figure 31 Chromatogram of BV/FA cream spiked with the impurities betamethasone, betamethasone-21-valerate and 3-didehydrofusidic acid. Chromatographic conditions as provided in Table 10. Peak assignment: coelution of potassium sorbate and methyl paraben (1), betamethasone (2), Unknown (3), Unknown (4), BV (5), betamethasone 21-valerate (6), 3-didehydrofusidic acid (7) and FA (8)

3.4.2 *Optimisation*

3.4.2.1 *Optimisation – Run time*

The initial experiments showed that a run time of 60 minutes was not necessary since all target analytes eluted within 30 minutes. The mobile phase flow-rate and gradient program were therefore optimised as detailed in Table 11 with the aim of reducing the run time.

Table 11 *Modified Gradient I*

Flow rate ml/min		1.5			
		Time (Min.)	Event	% A	% B
Gradient program		START	Pump A B.	60	40
		20.0	Pump A B.	10	90
		25.0	Pump A B.	10	90
		27.0	Pump A B.	60	40
		35.0	Pump A B.	60	40
		35.0	STOP	-	-

Using the modified gradient program a sample of BV/FA cream, which had been spiked with the impurities betamethasone, betamethasone-21-valerate and 3-didehydrofusidic acid, was analysed. All peaks of interest eluted within 27 minutes. To aid identification of the impurities, active substances and preservatives, standard solutions of the substances were also injected. The results indicated that the potassium sorbate and methyl paraben peaks continued to co-elute.

3.4.2.2 *Optimisation - Column temperature*

In an attempt to enhance analyte-stationary phase interactions, the column-oven temperature was reduced from 25 °C to 20 °C. This would normally lead to a longer retention time of the analyte which can, in some circumstances, help to improve resolution. On this occasion it proved to be unsuccessful. An increase of the column temperature was not considered at this point because this would have reduced the level of interaction between analyte and stationary phase, leading to shorter retention times and reduced selectivity.

3.4.2.3 *Optimisation – Gradient program*

The above experiments indicated that a significant change of chemistry would be required to separate the potassium sorbate and methyl paraben peaks. The analytes were not being retained sufficiently long on the analytical column to allow for interaction with the stationary phase, indicating that the mobile phase composition was too strong, i.e. too much organic solvent was being employed at the beginning of the gradient program. There were 2 possible approaches to solve this problem. The first approach was to select an alternative column with a more hydrophobic character, which would help to increase the strength of the analyte-stationary phase interaction of the early eluting substances. This option was considered unlikely to bring improvement. Alternatively, the gradient program could be modified by increasing the proportion of aqueous phase at the beginning of the program, leading to an increased affinity of the analytes for the C18 phase. The later approach was chosen. The proportion of mobile phase A was increased from 60 % to 70 % and a standard solution containing potassium sorbate, methyl paraben and propyl paraben was analysed with the gradient program detailed in Table 12.

Table 12 *Modified Gradient II*

	Time (Min.)	Event	% A	% B
Gradient program	START	Pump A B.	70	30
	16.0	Pump A B.	70	30
	25.0	Pump A B.	10	90
	27.0	Pump A B.	70	30
	35.0	Pump A B.	70	30
	35.0	STOP	-	-

The potassium sorbate and methyl paraben peaks began to separate at a mobile phase composition of 70:30 (v/v) A/B. Further increasing the percentage of A to 82 % provided a complete baseline separation of both peaks (*Rs* = 1.5). At 85 % A the resolution between the 2 peaks increased further to 1.8 (Figure 32). An increased proportion of aqueous phase lead to an increased system pressure which was counteracted by increasing the column oven temperature from 25 °C to 30 °C. The increased temperature did not adversely affect the peak resolution.

3.4.2.4 *Optimisation - Column dimensions and flow rate*

Since it had been observed that the separation of potassium sorbate and methyl paraben was more dependent on the polarity of the mobile phase and the structure of the gradient program than on the minor selectivity differences between different C18 stationary phases (i.e. the mobile phase chemistry plays a more significant role than the column chemistry), it was attempted to reduce the column dimensions and optimise the flow rate in order to save mobile phase and make the separation more cost effective. For this purpose a Supelco Ascentis® column packed with 2.7 µm fused core particles and having the dimensions 100 mm L x 3.0 mm ID was chosen. This column was relatively new on the market and the fused core technology provided high column efficiency (theoretical plate count) with a relatively low backpressure (≈ 200 – 300 bar) at normal flow-rates (≈ 1 – 2 ml/min). This allows the column to be used with conventional HPLC systems (up to ca. 400 bar) whilst providing an efficiency approaching that of UPLC [15].

3.4.2.5 *Optimisation of the injection volume*

On injecting 50 µl of a mixed standard solution, poor peak shape for the early eluting compounds was observed due to overloading of the smaller HPLC column which had an ID of 3 mm as opposed to 4.6 mm for the original column. Reduction of the injection volume from 50 µl to 20 µl and then to 10 µl resulted in a significant improvement of peak shape (Figure 33). An injection volume of 10 µl was chosen for the final method.

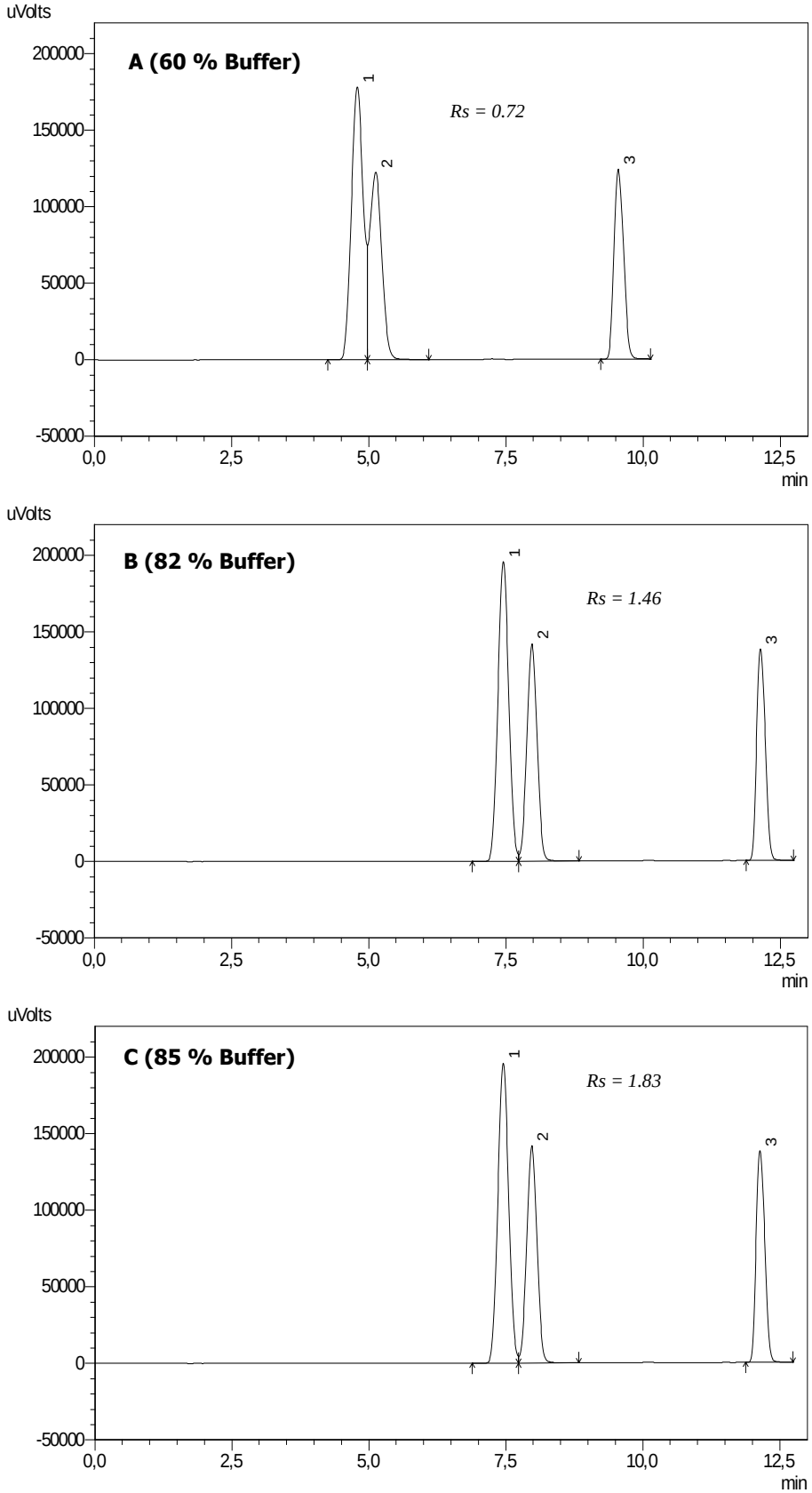


Figure 32 (a), (b) and (c) Optimisation of the separation of potassium sorbate and methyl paraben. Baseline separation (resolution, Rs = 1.5) is obtained with 82 % buffer. Peak assignment: PS (1), MP (2) and PP (3)

3.4.3 *Final HPLC Method*

After the method optimisation studies, the conditions used in the final HPLC method for the analysis of BV/FA Cream are detailed in Table 13

Table 13 Final HPLC Conditions

Column description	-	Supelco Ascentis C18, 100 mm x 3.0 mm with 2.7 µm Fused Core™ particles			
Guard column	-	C18, 4.0 mm L x 3.0 mm ID with Phenomenex® Security Guard™ Cartridge system			
Flow rate	ml/min	0.75			
Mobile Phase	A	833 ml acetonitrile + 167 ml methanol			
	B	10 g phosphoric acid (85 % m/m) in 1000 ml of purified water			
Wavelength	nm	240			
Column temperature	°C	30			
Auto-sampler temperature	°C	25			
Gradient	-	Time	Event	% A	% B
		0.0	Pump A B.	18	82
		16.0	Pump A B.	48	52
		25.0	Pump A B.	90	10
		27.0	Pump A B.	90	10
		27.1	Pump A B.	18	82
		35.0	STOP	-	-
Injection volume	µl	10			

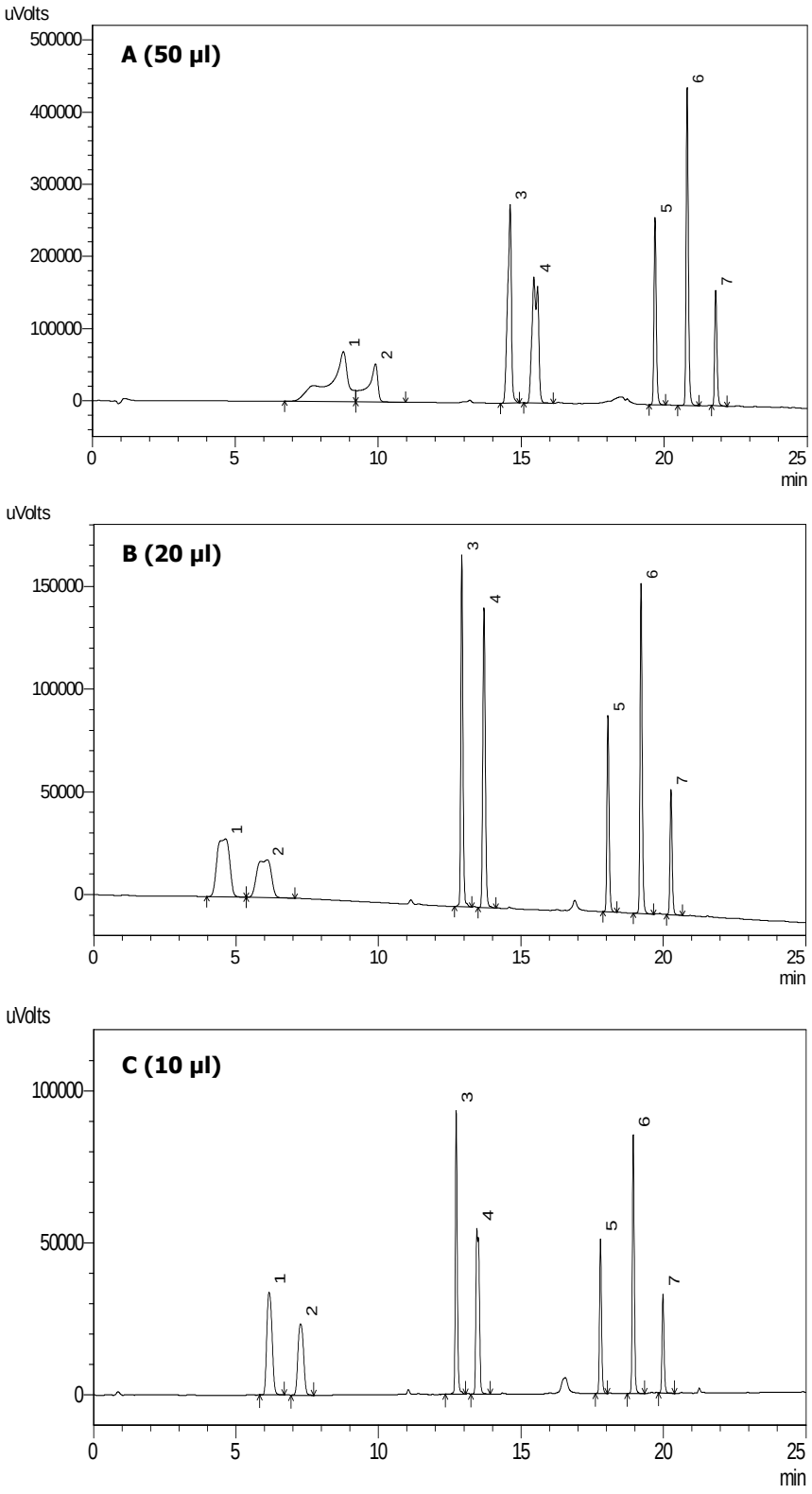
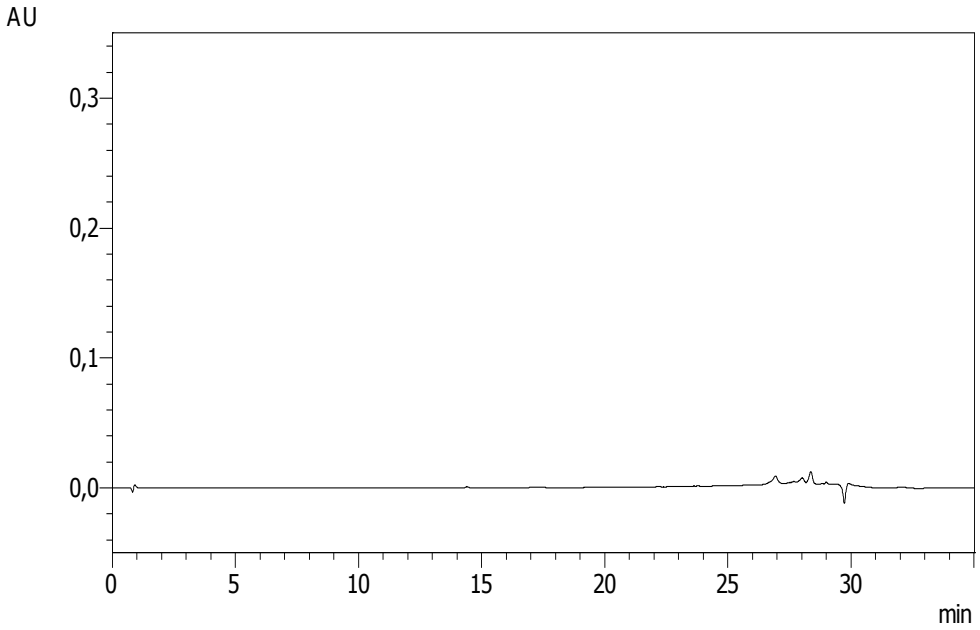


Figure 33 Effect of injection volume on the peak symmetry of early eluting compounds. A (50 µl) = complete peak deformation leading to double peaks, B (20 µl) = broad, deformed peaks, C (10 µl) = sharp, symmetrical peaks. Peak assignment: PS (1), MP (2), betamethasone (3), PP (4), BV (5), betamethasone-21-valerate (6) and FA (7).

3.4.4 *Example Chromatograms*

3.4.4.1 *Placebo (cream without active substances)*



3.4.4.2 *BV/FA Cream*

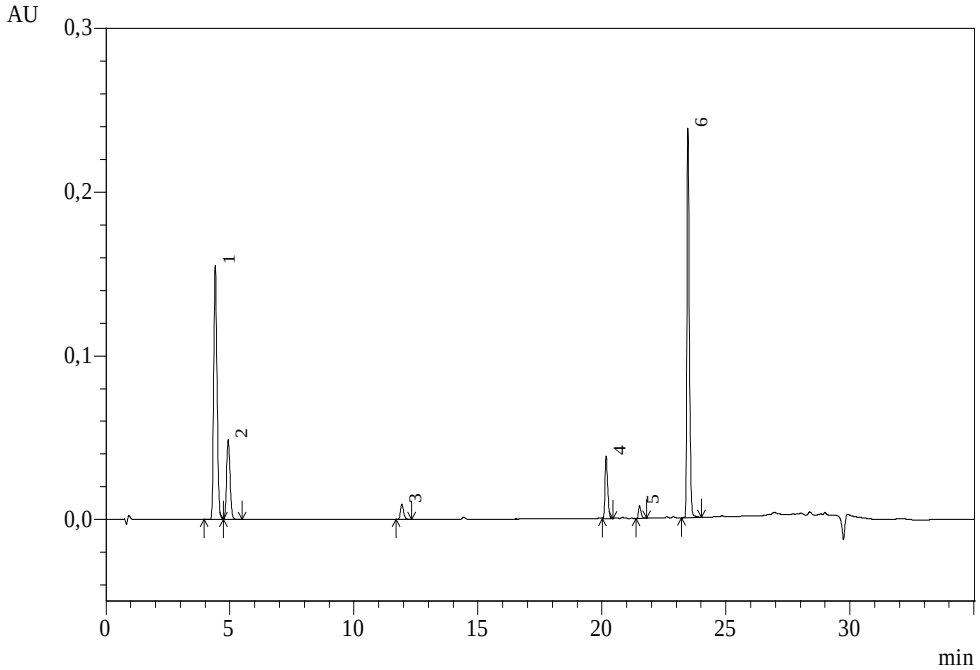


Figure 34 (a) Placebo (b) A chromatogram of topical cream after storage at 40 °C/75 % relative humidity for 9 months. The HPLC conditions are the same as those provided in Table 13. Peak assignment: PS (1), MP (2), PP (3), BV (4), Degradant betamethasone-21-valerate (5) and FA (6)

3.5 *Stress-Testing of the APIs*

3.5.1 *Introduction*

Stress testing is an important part of both the analytical- and formulation development processes. It is primarily used as a tool to investigate the intrinsic stability of a drug substance, thus providing information about likely degradation pathways for this substance in the finished product. It is also used to check the stability-indicating properties of an analytical procedure in respect of likely degradation products of the active substances [16,17]. Stress-testing of FA and BV was performed under the following conditions:

1. Acidic (hydrochloric acid)
2. Basic (sodium hydroxide)
3. Oxidative (hydrogen peroxide)
4. Ultrasonic bath
5. Temperature (105 ºC)
6. Exposure to daylight
7. Stability in solution (acetonitrile/water)
8. Exposure to UV radiation (254 nm)
9. Control (non-stressed)
10. pH profile (pH 1 – pH 8)

The acidic, basic and oxidative stress-tests were performed using dilute solutions of hydrochloric acid, sodium hydroxide and hydrogen peroxide, respectively. In each case, an initial investigation was performed in order to determine the concentration of each reagent required to provide sufficient, but not complete, degradation of the active compounds. A description of the sample preparation procedure is provided in Chapter 2. Only then could useful information be attained which would allow identification of likely degradation products/mechanistic pathways of degradation for the active substances. Under some conditions little or no degradation was observed. In such cases, it was not attempted to increase the harshness of the stress conditions further, since any impurities generated were unlikely to be relevant for finished product analysis since the cream would never be exposed to such harsh conditions during manufacture, packaging or storage. The percentage of impurities arising through the stressing procedures was measured according to the HPLC method described in section 3.3.8.

3.5.2 *Results*

3.5.2.1 *Fusidic acid hemihydrate*

As demonstrated by the results presented in Table 14 and Figure 37, FA degrades significantly under basic pH and high temperature conditions and to a lesser extent under acidic and oxidative conditions. The substance is relatively stable at pH values between 3 and 7, having a pH optimum of approximately 4.2 in this experiment. At pH values below 3, FA begins to degrade rapidly (see Figure 35).

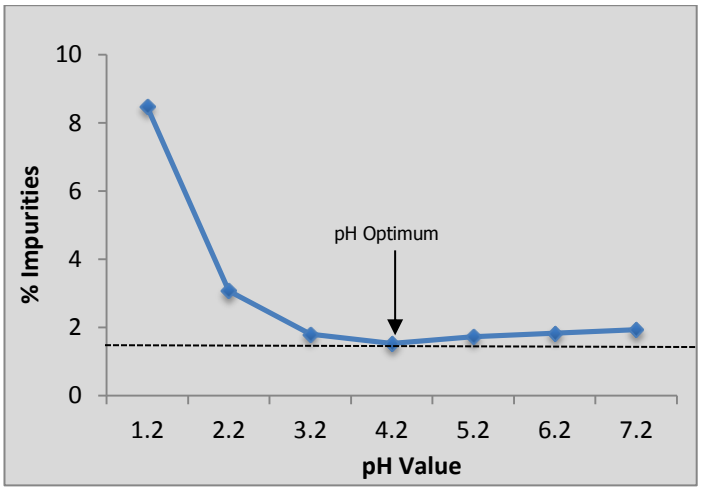


Figure 35 *pH stability profile of FA over the range 1.2 – 7.2*

3.5.2.2 *Betamethasone-17-valerate*

BV is extremely sensitive to basic pH conditions. In the presence of the prescribed volume of 1.0 M sodium hydroxide there was complete degradation of the active substance as well as of its primary degradation products betamethasone-21-valerate and betamethasone (Table 15 (a)). On employing an equal volume of a weaker 0.1 M sodium hydroxide solution, complete degradation was also observed with betamethasone as the main degradation product (≈ 70 %). A further reduction of the base concentration to 0.001 M resulted in a lower degree of degradation of approximately 2.2 %, with betamethasone-21-valerate being the major degradation product. No betamethasone was detected. BV also proved to be unstable under acidic conditions with again, betamethasone-21-valerate and betamethasone being the major degradation products. The substance remained stable under all other stress conditions investigated (see also Table 15 (b)).

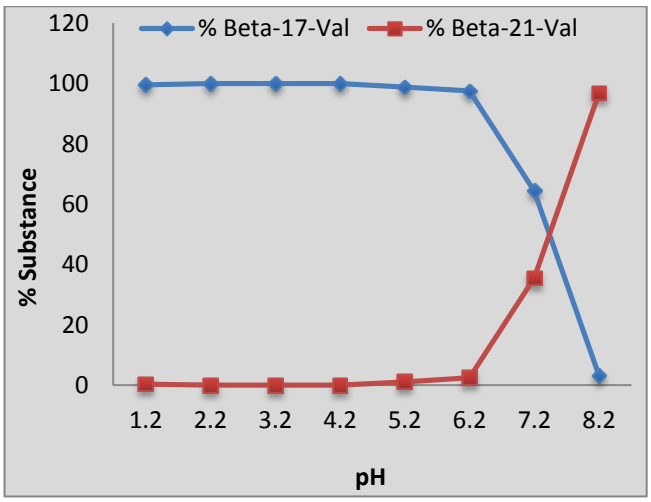


Figure 36 Correlation between BV loss (blue line) and the corresponding increase of betamethasone 21 valerate (red line) over the pH range 1.2 - 8.2

The results confirm that the rearrangement reaction is both acid and base catalysed but that the acyl group migration occurs much more readily under basic pH conditions. It also confirms that the acyl migration dominates over the hydrolysis reaction, i.e. that the acyl group migrates first to the 21 position and is then hydrolysed to betamethasone base. The above data is in good agreement with literature studies [18]. Figure 36 shows that BV is relatively stable between pH 1 and 5 but that its stability decreases rapidly at pH values > 5.

3.5.2.3 *General Conclusions from the Stress Testing*

The results of stress-testing indicate that pH and temperature are likely to be critical factors with regard to the stability of BV and FA in any potential cream formulation (Figure 37 and Figure 38). The results also indicate that the developed analytical procedures are stability-indicating with regard to the two active substances since they are capable of detecting any loss of content of the active substances as well as any increase of likely degradation products in stressed samples. One indicator of the stability-indicating nature of an analytical procedure is the so called "mass-balance ratio", which is obtained by adding the content value of the API (in %) and the sum of all impurities (in % relative to the API). Ideally, if the content of the API drops by 5 %, then an equivalent 5 % increase of degradation products would be expected, which would provide a sum of 100 %. However, in reality 100 % is seldom reached and values between 95 - 105 % are generally considered acceptable. In the present case the mass-balance ratios are acceptable.

Table 14 Assay and purity results from stress testing of FA

FUSIDIC ACID HEMIHYDRATE	**RRT**	**Response factors**	**Control**	**Acid**	**Base**	**Oxidative**	**Daylight (3 days)**	**UV-Light (254nm)**	**Ultrasonic Bath**	**Temp.105°C/ 24h**	**Stability in Solution (10 days)**
Assay (Content value, %)	--	--	97.64	97.80	92.57	96.63	98.02	97.46	97.57	88.83	94.91
24,25-dihydro-24,25-dihydroxy-Fusidic acid (**Imp. A**)	0.27	--	<0.05	0.091	0.082	0.050	<0.05	<0.05	<0.05	<0.05	<0.05
24,25-dihydro-24,25-dihydroxy-Fusidic acid 21,25-lactone (**Imp. B**)	0.43	--	0.079	<0.05	<0.02	0.150	0.090	0.095	0.090	0.079	0.091
(24R)-24,25-dihydro-24,25-Dihydroxyfusidic acid 21,24-lactone (**Imp. C**)	0.49	0.7	0.083	0.186	0.179	0.126	0.114	0.120	0.110	0.485	0.145
(24S)-24,25-dihydro-24,25-Dihydroxyfusidic acid 21,24-lactone (**Imp. D**)	0.54	0.7	0.099	0.144	0.081	0.142	0.116	0.120	0.117	0.425	0.154
Imp. N	0.56	--	0.052	<0.05	<0.05	0.060	0.058	0.124	0.058	<0.05	<0.05
26-oxofusidic acid (**Imp. F**)	0.60	0.3	0.063	0.056	0.046	0.071	0.069	0.079	0.070	0.310	0.233
3-didehdrofusidic acid (**Imp. G**)	0.83	--	0.254	0.251	0.237	0.253	0.255	0.260	0.255	0.409	0.254
11-didehydrofusidic acid (**Imp. H**)	0.86	--	0.090	0.204	5.887	0.090	0.090	0.089	0.091	0.205	0.205
16-*epi*-deacetylfusidic acid (**Imp. I**)	0.95	0.6	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Deacetylfusidic acid 21,16-lactone (**Imp. O**)	1.14	0.6	0.042	0.098	0.281	<0.05	<0.05	<0.05	<0.05	0.623	0.077
9,11-anhydrofusidic acid (**Imp. L**)	1.17	--	0.072	0.083	0.073	0.074	0.074	0.072	0.074	0.942	0.070
11-deoxyfusidic acid (**Imp. M**)	1.27	--	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.245	<0.05
Single unknown (max.)	0.28	--	0.087	0.080	0.067	0.086	0.056	0.057	0.056	0.105	0.084
Sum of unknown impurities (%)	--	--	0.138	0.193	0.067	0.192	0.159	0.161	0.159	0.218	0.142
Sum of all impurities (%)	--	--	1.23	1.64	7.40	1.56	1.35	1.12	1.02	3.94	1.37
Mass Balance Ratio (%)	--	--	98.87	99.45	100.22	98.32	99.37	98.58	98.59	92.77	96.28

Table 15 (a) Assay and purity results from stress testing of BV

BETAMETHASONE-17-VALERATE	**RRT**	**Response factors**	**Control**	**Acid 1.0 M**	**Acid 0.01 M**	**Base 1.0 M**	**Base 0.1 M**	**Base 0.01 M**	**Base 0.001 M**	**Oxidative**
Assay	--	--	99.04	1.22	100.32	Complete Degradation	0.00	2.50	99.24	98.89
Betamethasone, %	0.30	1.154	<0.05	57.340	<0.05		70.329	10.178	<0.05	<0.05
Betamethasone-21-valerate, %	1.35	0.930	0.942	42.483	0.092		0.884	92.723	2.208	0.933
Sum of unknown impurities, %	--	--	<0.05	<0.05	<0.05		11.318	<0.05	<0.05	0.316
Sum of all impurities, %	--	--	0.94	99.82	0.09		82.53	102.90	2.20	1.24
Mass balance Ratio (%)	--	--	99.98	101.04	100.41		82.53	105.40	101.44	100.13

Table 15 (b) Assay and purity results from stress testing of BV

BETAMETHASONE-17-VALERATE	**RRT**	**Response factors**	**UV-Light (254nm)**	**Ultrasonic Bath**	**Temp. 105 °C / 24h**	**Solution Stability (8 days)**	**Daylight (8 days)**
Assay	--	--	99.25	98.53	99.51	98.88	99.63
Betamethasone, %	0.30	1.154	<0.05	<0.05	<0.05	<0.05	0.207
Betamethasone-21-valerate, %	1.35	0.930	0.451	1.302	0.375	0.284	0.307
Sum of unknown impurities, %	--	--	<0.05	<0.05	<0.05	<0.05	<0.05
Sum of all impurities, %	--	--	0.45	1.30	0.38	0.28	0.51
Mass balance Ratio (%)	--	--	99.70	99.83	99.89	99.16	100.14

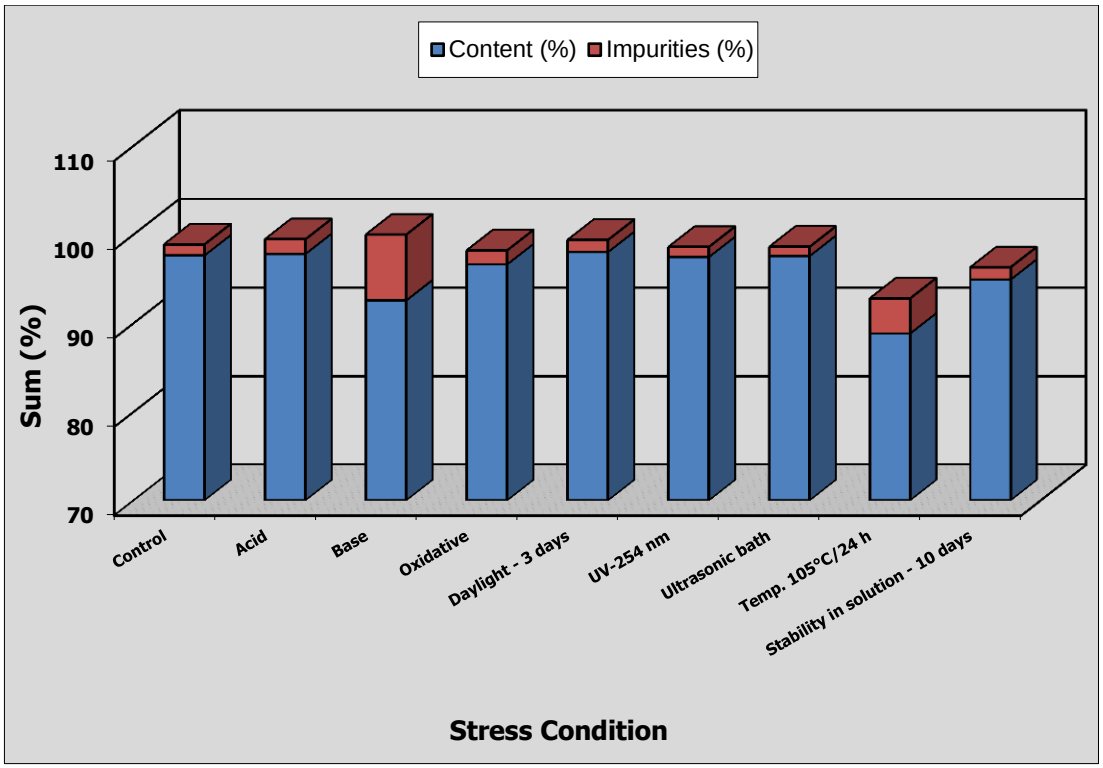


Figure 37 *Stress testing of FA – results*

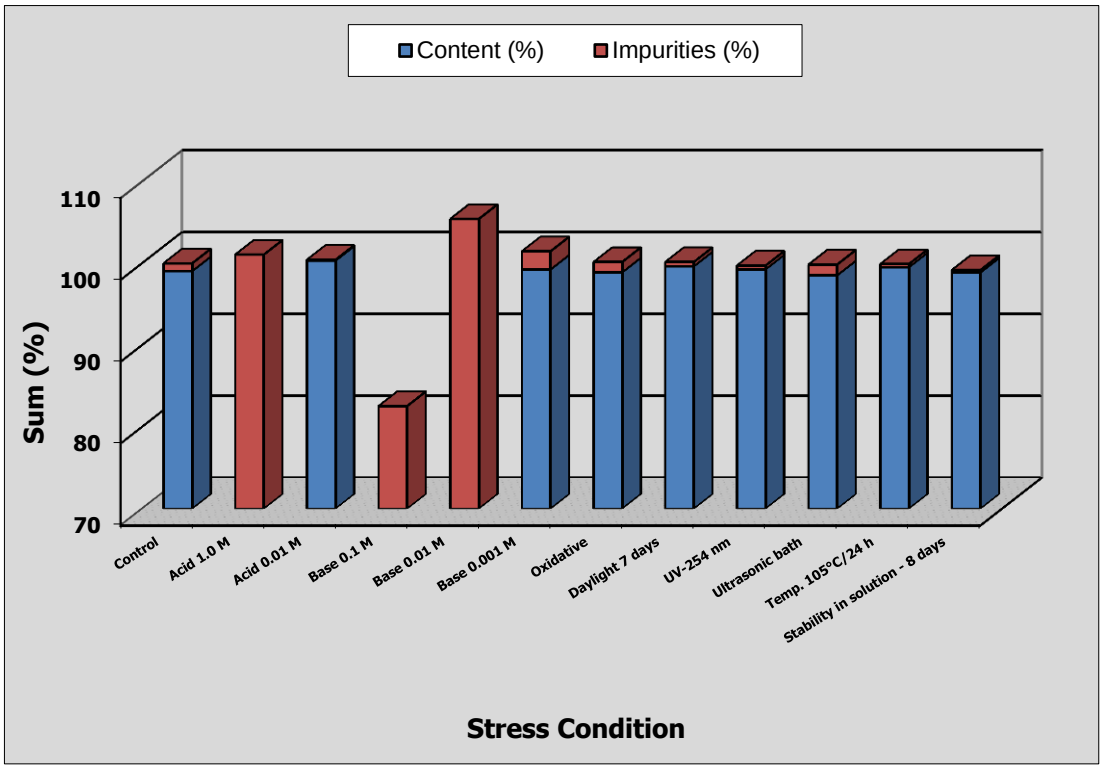


Figure 38 *Stress testing of BV – results*

3.6 *Discussion and Conclusions*

A primary aim of the work in this chapter was to develop an analytical procedure which could be employed for the stability-indicating analysis of the content of the active substances betamethasone 17-valerate and fusidic acid hemihydrate as well as the preservative compounds potassium sorbate, methyl paraben und propyl paraben in candidate formulations of a developmental hydrophillic cream. One of the main challenges encountered in developing such a procedure was the difference in polarity between the 5 analytes of interest. Potassium sorbate is the potassium salt of sorbic acid and is highly water soluble. In contrast, the more lipophilic compounds betamethasone-17-valerate and fusidic acid hemihydrate are practically insoluble in water but are soluble in high polarity organic solvents, such as acetonitrile and ethanol. The challenge was to develop a procedure which would allow sufficient extraction of both the hydrophilic and hydrophobic analytes but without extracting large quantities of the hydrophobic matrix components of the cream, since these usually collect at the HPLC column-inlet frit or adsorb onto the stationary phase. This can cause problems with backpressure, peak shape as well as with retention time, since the irreversible adsorption of matrix components will inevitably have an influence on the chemical interactions between the analyte and the stationary phase.

Another aspect of the work was to develop a selective HPLC procedure for the purity analysis of the active substances in the cream preparations. The main challenge here was to develop a procedure which could separate the 2 active substances as well as 2 known degradation products of betamethasone valerate and 15 known impurities of fusidic acid. A suitable separation was achieved by beginning isocratically for the first 20 minutes, allowing for the elution of hydrophilic components, and then increasing the proportion of the organic phase to 92 % in order to elute the more hydrophobic analytes. The procedure also needed to be sensitive enough to analyse impurities at low concentrations, which proved to be a problem for betamethasone 17-valerate due its low concentration in the cream (1.2 mg/g). Optimisation of the sample preparation procedure was viewed to be the most efficient way to increase sensitivity. By increasing the concentration of analytes in the sample solution 5 fold, a suitable level of sensitivity was obtained.

Both of the developed analytical procedures were checked for selectivity, sensitivity, reproducibility, accuracy and robustness. Both procedures were found to be

fit-for-purpose and sufficiently robust to be suitable for application in a routine quality control laboratory.

A final aspect of the work in this chapter was the performance of stress testing of the drug substances in order to elucidate possible degradation pathways for these substances in the finished product and to demonstrate the stability-indicating nature of the developed procedures. It has been shown that both APIs are labile under extreme pH conditions and at elevated temperature. Betamethasone 17-valerate was shown to be unstable under both strong acidic and basic pH conditions, being substantially less stable with regard to basic conditions than acidic. The required pH for optimum stability was found to be approximately 3.5. Fusidic acid was also found to be unstable under strong acidic and basic conditions, being less stable at lower pH than at higher pH. The pH optimum with regard to stability lay at ca. 4.2. The calculated mass-balance values from these experiments demonstrate that both analytical procedures are stability-indicating.

The analytical procedures must be validated according to the relevant ICH guidelines [19] for the parameters, selectivity, linearity, precision, accuracy, sensitivity and robustness. This has been addressed in Chapter 4.

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CHAPTER 4

Validation of Analytical Procedures

4 CHAPTER 4 – Validation of Analytical Procedures

4.1 *Introduction*

Current legislation governing the licencing of new pharmaceutical products requires that analytical procedures used for quality control- and stability testing of these products are validated before the drug product application can be approved. The process of validation may be described as the verification, through appropriately designed analytical experiments, that an analytical procedure is suitable for its intended purpose. In Europe, relevant validation characteristics are defined in ICH guideline Q2R1 – Validation of Analytical Procedures: Text and Methodology [1], which was published in its revised format in November 2005. According to this guideline, typical validation characteristics which should be considered include: Accuracy, Precision, Selectivity, Limit of Detection, Limit of Quantitation, Linearity and Range. System suitability and Robustness are 2 further parameters which are generally considered important for validation. Each of the validation characteristics can be defined as follows:

The *Selectivity* of an analytical method can be defined as the ability of the method to accurately measure the analyte or analytes of interest without interference from other components of the sample solution, e.g. solvent, matrix or other analyte components.

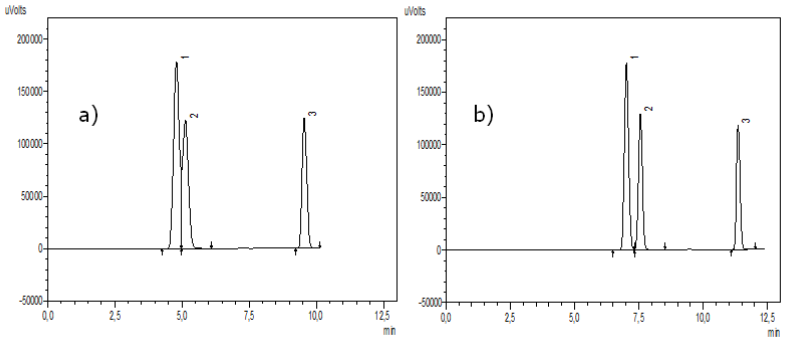

Figure 39 *Insufficient selectivity (a) vs. sufficient selectivity (b)*

System suitability, as the name suggests, is the experimental confirmation that the analytical system to be employed during the validation study is suitable to perform the required task. Testing system suitability usually involves multiple injections of a suitable standard solution and analysis of the precision of both the retention times and peak areas of all resulting chromatograms. *Accuracy*, (sometimes termed Recovery), can be defined as a measure of the closeness of the obtained result to an agreed

theoretical value. *Precision*, on the other hand, is defined as the closeness of the measured results to each other, i.e. the scatter between multiple measurements of the same homogenous sample. An analytical procedure may be accurate but have a low precision, or vice-versa. In an ideal situation a procedure should be both accurate and precise (Figure 40).

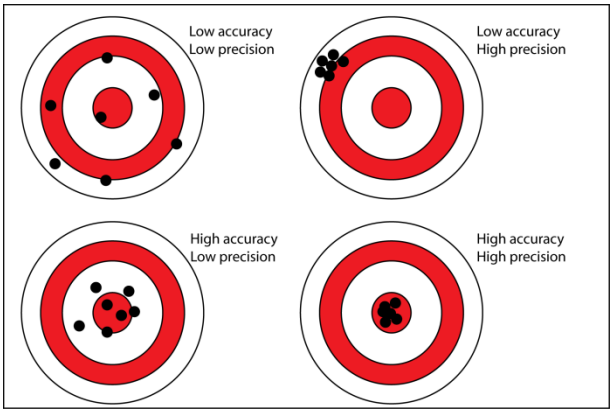

Figure 40 *Definition of accuracy and precision in analytical measurements*

The *Limit of Detection* (LOD) is the lowest concentration of analyte which can be detected by the analytical system. It is normally defined as the concentration of analyte required to provide a signal-noise-ratio of 3, where the signal- and noise heights are defined as *H* and *h* (Figure 41) and the signal-to-noise-ratio is *2H/h*. The *Limit of Quantitation* (LOQ) is the lowest concentration of analyte which can be accurately quantified by the analytical system. It is normally defined as the concentration of analyte required to provide a signal-to-noise-ratio of 10. The *Linearity* of an analytical procedure describes the degree to which a linear relationship exists between the concentration of the analyte and the recorded response. It is usually expressed in the form of the correlation coefficient, *r*, which ranges from 0 to 1, where a correlation coefficient of 1.000 indicates a perfect linear relationship with a positive slope. Some analytical procedures may not show a linear relationship between analyte concentration and response, e.g. biological immunoassays [2].

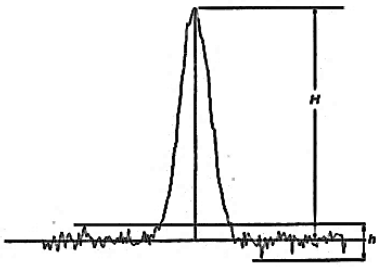

Figure 41 *Signal (H) to Noise (h) Ratio for determination of LOD and LOQ*

Non-linear processes may however show linear like behaviour over narrow concentration ranges, leading to the misinterpretation of the concentration/response model as being linear. It is therefore important to check the residual values from the linearity experiment for any obvious trends. This is performed by generation of a residual plot, which is a scatter plot of the individual differences between the values predicted by the best-fit-line and the actual observed values. If the analytical procedure is truly linear then the residuals should be distributed randomly about 0, as in Figure 42 (a) below.

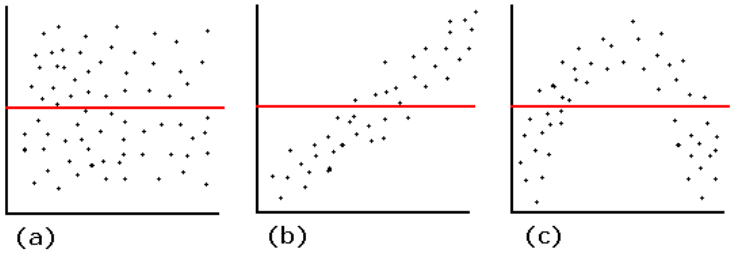

Figure 42 *Residual distributions: random (a), non-random (b + c)*

The *Robustness* of an analytical procedure is a measure of the ability of the procedure to remain unaffected by small changes to the chromatographic conditions. It provides an indication of how the method is likely to perform in the laboratory on a day-to-day basis where human, technical and environmental variation come into play. Examples of changes to the chromatographic conditions include increasing or decreasing the column temperature or the percentage of organic modifier in the mobile phase. The goal of the analytical chemist is to develop a procedure which is not significantly influenced by minor changes to the chromatographic conditions, or if the method is affected that these effects are known and documented. Procedures lacking robustness may cause problems during routine analysis leading to reduced sample through-put and increased analysis costs.

4.2 ***Aim***

The purpose of the work in this chapter is to demonstrate the validity of the analytical procedures developed in Chapter 3 for use in the stability-indicating HPLC analysis of betamethasone-17-valerate and fusidic acid hemihydrate in developmental cream formulations. The procedures will be validated according to current ICH guidelines for

the parameters selectivity, linearity, precision, accuracy, sensitivity and robustness within the appropriate concentration range for each analyte.

4.3 *Setting Appropriate Acceptance Criteria*

Before validation of any analytical procedure, it is first necessary to define suitable acceptance criteria for each of the parameters to be validated. The selection of suitable criteria will depend on several factors, the most important of which will include: (i) the concentration level of the analyte in the sample, (ii) the required specification for the analyte in the drug product and (iii) the nature of the analytical procedure, e.g. assay by HPLC or "loss on drying" test by gravimetric analysis.

In order to set appropriate acceptance criteria it is also essential to have a basic understanding of the statistical concepts surrounding the probability distribution of analytical measurements. In general, it is to be expected that the results of analytical measurements will demonstrate a normal distribution around a certain mean value (Figure 43), although there are some methods, e.g. microbiological assays, where this is not the case. The normal distribution is described by the equation [3] :

$$y = \frac{1}{\sigma\sqrt{2\pi}} e^{\frac{-(x-\mu)^2}{2\sigma^2}}$$

Where,

y	=	vertical height on a point on the normal distribution
x	=	point on the horizontal axis
σ	=	standard deviation of the data
μ	=	mean of the data distribution
e	=	exponential constant, 2.71828…
π	=	3.14159…

Using this equation it can be shown that in a normally distributed population of values, 68 % of the values will lie with ± 1 standard deviation and 95 % will lie within ± 2 standard deviations of the population mean (1.96 to be exact). This must be considered when setting acceptance criteria. Otherwise, the precision of the analytical procedure may not be compatible with the product specification. If for example, the content of a drug substance in a tablet is defined as 100 % and the specification for that substance is 95 – 105 %, then it is obvious that it would make no sense to employ a

procedure which has a standard deviation (precision) of 5 %, since a large number of the measured values would lie outside the specification just by chance alone, despite the fact that the content of drug substance in the tablet batch was acceptable.

The normal distribution assumes a sufficiently large sample size and can generally be applied as a model when sample sizes are close to or above n = 60. During routine analysis and method validation, however, it is not feasible to perform 60 or more separate measurements of a sample. Usually a maximum of 6 measurements are made. Since a sample size of 6 is considerably lower than 60, it is statistically unsafe to presume that the arithmetic mean of 6 values represents the true mean of the entire sample population. To compensate for this lack of confidence in the mean, a variant of the normal distribution model, the t-distribution, is instead used. The mean is then reported together with its associated confidence interval which varies depending on sample size. If the sample size is low the mean is reported with a wide confidence interval indicating that the confidence that the calculated mean represents the true mean is lower than if the sample size were high, where the mean value would have a narrower confidence interval. At a sample size of approximately 60 the t-distribution resembles the normal distribution. In precision and accuracy experiments all mean values should be reported with their corresponding confidence intervals.

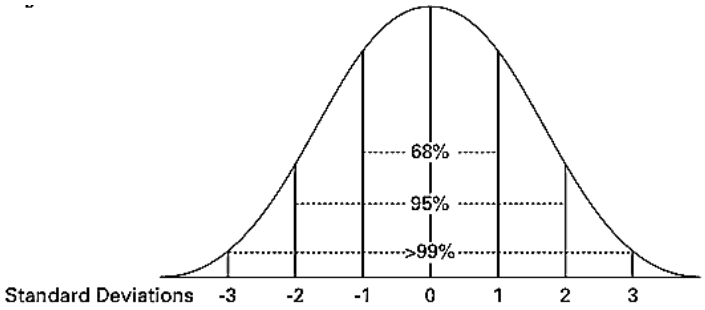

Figure 43 Normal distribution curve

On consideration of the above points, the acceptance criteria described in Table 16 were set for the validation of the assay and purity methods.

Table 16 Validation specifications for the assay and purity methods

Validation Parameter	**Specification Assay Method**	**Specification Purity Method**
Range	50 – 150 %	0.1 – 8.0 %
Selectivity	No interferences from placebo, solvent, matrix or related substance components. Peak purity demonstrated by DAD analysis	No interferences from placebo, solvent, matrix or related substance components.
Precision - Repeatability - Intermediate precision	 RSD ≤ 2 % RSD ≤ 2 %	 RSD ≤ 10 % RSD ≤ 10 %
Accuracy - Mean value - RSD of all values	API Preservatives 98.0 – 102.0 % 97.0 – 103.0 % ≤ 2.5 % ≤ 3.5 %	 90 – 110 % ≤ 15 %
Linearity	$r \geq 0.995$ Residuals show no trend y-intercept passes through “0”	$r \geq 0.995$
Sensitivity - LOQ	Not relevant	≤ 0.1 %
Robustness	The following changes will be made to the chromatographic conditions and any effects noted: – Column temperature ± 5 °C – Mobile phase flow rate ± 0.2 ml/Min. – Acetonitrile in mobile phase A ± 10 ml/L – Methanol in mobile phase A ± 10 ml/L – Detector wavelength ± 2 nm – Mobile phase B at start of gradient ± 4 %	The following changes will be made to the chromatographic conditions and any effects noted: – Column temperature ± 5 °C – Mobile phase flow rate ± 0.2 ml/Min. – Acetonitrile in mobile phase A ± 20 ml/L – Methanol in mobile phase A ± 20 ml/L – Buffer in mobile phase A ± 20 ml/L – Detector wavelength ± 2 nm – Mobile phase B at start of gradient ± 5 %

4.4 *Validation of the Assay Method*

4.4.1 *Selectivity*

The selectivity of the analytical method for each of the analyte components was demonstrated by measuring the following solutions on the chromatograph:

- Solvent , comprising of acetonitrile/purified water (50:50 v/v)
- Placebo (without actives and preservatives)
- Fusidic acid hemihydrate identification standard
- Betamethasone-17-valerate identification standard
- Potassium sorbate identification standard
- Methyl paraben identification standard
- Propyl paraben identification standard
- Mixed standard solution
- Betamethasone-17-valerate/Fusidic acid cream, lot: 110101, stored under ambient conditions
- Betamethasone-17-valerate/Fusidic acid cream, lot: 110101, stored under accelerated conditions (40°C/75 % RH) for 9 months
- Betamethasone-17-valerate/Fusidic acid cream, lot: 110101, stored under stress conditions (Light stress - exposure to daylight for 3 days)
- Betamethasone-17-valerate/Fusidic acid cream, lot: 110101, stored under stress conditions (Oxidative stress – exposure to air for 3 days)

The stressed samples were analysed in order to demonstrate the selectivity of the analytical method for the analytes of interest in the presence of possible degradation products and matrix components. The stressed conditions chosen, i.e. 9 months at 40°C/75 % relative humidity, as well as 3 days exposure to light and to air, were considered to be ‘worst-case’ since the product is highly unlikely to be exposed to such conditions during manufacture, transport or long-term storage (the product is stored protected from air and light in aluminium tubes).

Analysis of the resulting chromatograms demonstrates that there are no apparent interferences from either the solvent, placebo, preservative or related substance components.

The purity of each of the relevant analyte peaks was further examined using a diode-array detector, whereby UV-spectra (190 nm – 400 nm) were recorded and compared

across the whole of the analyte peak. Using this technique, co-eluting impurities are detected as differences in the recorded spectra. The purity graph consists of a single trace that represents the peak purity index for each point (Figure 44). The peak purity analysis demonstrated that each of the analyte peaks was pure, indicating that there are unlikely to be co-eluting impurities. It should be noted that this technique can only be considered to be an estimate of the peak purity and may not be suitable for detecting co-eluting impurities present in low concentrations and/or having very similar spectral properties to the main peak, e.g. structurally related compounds. However, the combination of this technique and visual examination of real chromatograms provides a reasonable level of certainty that the examined analyte peaks are pure.

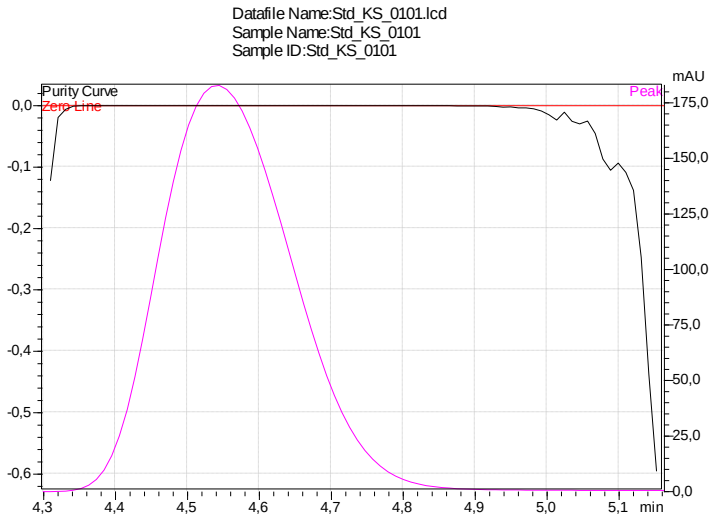

Figure 44 *Example of a peak purity measurement output*

4.4.2 ***Linearity***

Linearity of the detector response for potassium sorbate

The determination of the linearity of the detector response for potassium sorbate was performed over 8 concentration levels within the range 46 % to 153 % of the normal standard solution concentration (20 µg/ml = 100 %). The solutions were prepared by diluting a stock standard.

Table 17 *Linearity data – potassium sorbate*

Concentration level	Average PA	Conc. (µg/ml)	Conc. (%)
1	612907	9.201	46.01
2	818408	12.268	61.34
3	1027464	15.335	76.68
4	1219448	18.402	92.01
5	1428276	21.469	107.35
6	1634448	24.536	122.68
7	1818586	27.603	138.02
8	2025814	30.670	153.35

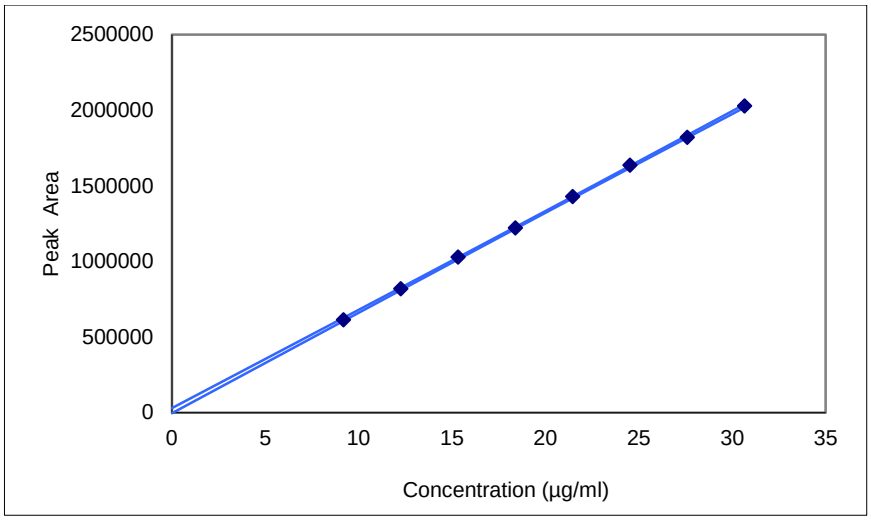

Figure 45 *Linearity of the detector response for potassium sorbate with 95 % confidence interval*

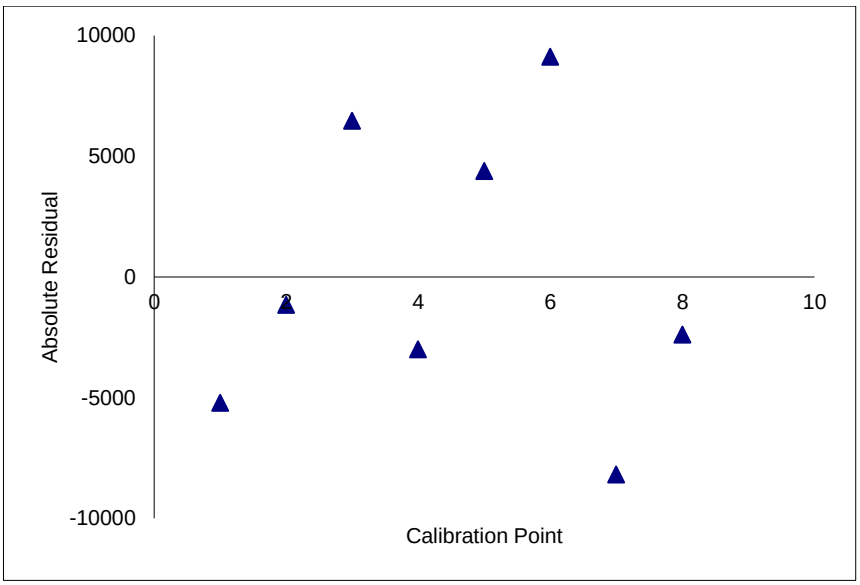

Figure 46 *Plot of the residuals versus calibration point*

Linearity of the detector response for MP, PP, BV and FA

The linearity experiments for the remaining analytes were performed analogous to the experiment for potassium sorbate. The correlation coefficient, *r*, for each of the linearity experiments meets the acceptance criterion of ≥ 0.995. The scatter plots of the residuals, i.e. the difference between the values predicted by the best fitting line and the actual observed values, show that they are spread about zero with no definitive trend. This indicates that the best-fitting line is a good fit for the observed data and is strong evidence that the relationship between concentration and detector response is a linear one. The calculated confidence interval for each of the y-intercepts contains the point "0", indicating that the y-intercept passes through the origin. The use of a single-point calibration during routine analysis is therefore justified.

4.4.3 *Precision*

The analysis of method precision was performed on 2 separate days, whereby 6 test solutions were prepared on each day by a different analyst and measured using a different HPLC machine and reagents, e.g. mobile phase. The intermediate precision was assessed by statistically comparing the data sets from both days. The calculated rsd values from both the repeatability and intermediate precision experiments meet the prescribed criterion of ≤ 2 %. The precision of the analytical method is considered to be satisfactory.

Table 18 Intermediate precision

Sample No.		Content (mg/g)				
		Potassium sorbate	**Methyl paraben**	**Propyl paraben**	**Betamethasone 17-valerate**	**Fusidic acid Hemihydrate**
Day 1 Analyst 1 HPLC 1	**1**	1.73959	0.80609	0.16150	1.23536	19.89318
	2	1.74070	0.80541	0.16185	1.23488	19.88125
	3	1.74443	0.80481	0.15984	1.23180	19.84768
	4	1.76891	0.80963	0.16169	1.24056	20.05848
	5	1.75867	0.80986	0.16159	1.24120	20.00365
	6	1.76984	0.80795	0.16040	1.23551	19.99061
Day 2 Analyst 2 HPLC 2	**7**	1.77157	0.82353	0.16475	1.25067	20.10262
	8	1.76523	0.82114	0.16533	1.25356	20.14918
	9	1.77287	0.82315	0.16359	1.25168	20.11890
	10	1.76646	0.82039	0.16340	1.25576	20.18444
	11	1.76934	0.82122	0.16408	1.24959	20.08537
	12	1.76190	0.81571	0.16302	1.25877	20.23292
m.v.		1.76079	0.81407	0.16258	1.24494	20.04569
s.d.		0.01228	0.00747	0.00170	0.00939	0.12419
rsd, %		0.70	0.92	1.05	0.75	0.62
Confidence (95%)		0.00780	0.00475	0.00108	0.00596	0.07890

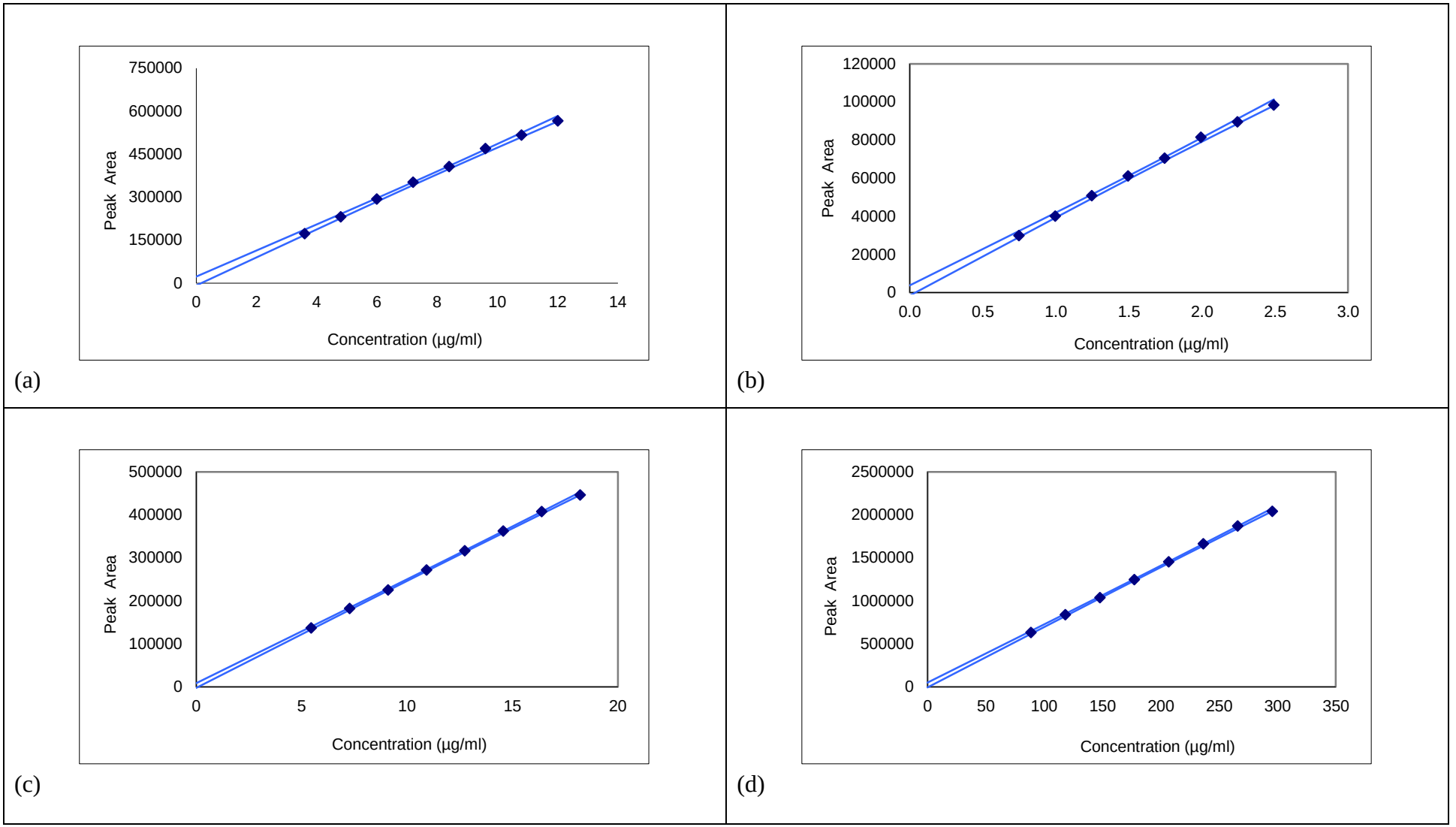

Figure 47 *Linearity plots for (a) methyl paraben, (b) propyl paraben, (c) betamethasone 17-valerate, (d) fusidic acid hemihydrate*

Table 19 Summary of data from the repeatability experiment

Sample No.	Content (mg/g)				
	Potassium sorbate	**Methyl paraben**	**Propyl paraben**	**Betamethasone-17-valerate**	**Fusidic acid hemihydrate**
1	1.73959	0.80609	0.16150	1.23536	19.89318
2	1.74070	0.80541	0.16185	1.23488	19.88125
3	1.74443	0.80481	0.15984	1.23180	19.84768
4	1.76891	0.80963	0.16169	1.24056	20.05848
5	1.75867	0.80986	0.16159	1.24120	20.00365
6	1.76984	0.80795	0.16040	1.23551	19.99061
m.v.	1.75369	0.80729	0.16115	1.23655	19.94580
s.d.	0.01393	0.00217	0.00082	0.00362	0.08320
rsd, %	0.79	0.27	0.51	0.29	0.42
Confidence (95%)	0.01462	0.00228	0.00086	0.00380	0.08731

Table 20 Summary of statistical data from linearity experiments

Substance	y = mx + c	*r*	r^2	Residuals	Concentration Range, %	Concentration Range, µg/ml	95 % confidence interval of y-intercept	
							Lower confidence limit	Upper confidence limit
Potassium sorbate	y = 65680x + 13804	0.9999	0.9999	No trend	46.1 – 153.4	9.2 – 30.7	- 3140	30749
Methyl paraben	y = 47190x + 7311	0.9992	0.9984	No trend	45.0 – 150.1	3.6 – 12.0	- 8149	22770
Propyl paraben	y = 39561x + 1108	0.9992	0.9985	No trend	46.7 – 155.8	0.75 – 2.49	- 1565	3781
Betamethasone-17-valerate	y = 24520x + 2978	0.9998	0.9997	No trend	45.0 – 150.0	5.5 – 18.2	- 2508	8465
Fusidic acid hemihydrate	y = 6896x + 20841	0.9998	0.9996	No trend	44.3 – 147.8	88.7 – 295.6	- 7218	48900

4.4.4 *Accuracy*

The accuracy (recovery rate) of the analytical method was demonstrated by preparing 9 solutions comprising of placebo spiked with each analyte at 50 %, 100 % and 150 % concentrations. Three solutions were prepared at each concentration level. The measured content values of these solutions were then compared to the theoretical value (spiked amount) in order to determine the percentage recovery. The percentage-recovery values were used to calculate the mean recovery value, standard deviation, relative standard deviation and confidence intervals for the mean (95 %).

Table 21 Data from the accuracy experiment

Sample No.	% Recovery				
	Potassium sorbate	**Methyl-paraben**	**Propyl-paraben**	**Betamethasone 17-valerate**	**Fusidic acid hemihydrate**
50 % - 1	101.78	102.89	102.24	98.98	98.59
50 % - 2	102.97	102.59	101.43	99.53	98.21
50 % - 3	102.17	103.20	102.51	101.42	100.58
100 % - 1	101.37	102.48	101.37	100.57	98.16
100 % - 2	101.90	100.18	98.81	100.93	98.81
100 % - 3	103.46	100.26	99.75	100.77	98.43
150 % - 1	101.93	100.79	100.19	101.03	99.34
150 % - 2	102.41	100.30	99.33	102.11	100.14
150 % - 3	103.66	101.60	100.81	101.38	99.17
m.v.	102.41	101.59	100.72	100.75	99.05
s.d.	0.78	1.23	1.29	0.97	0.85
rsd, %	0.77	1.21	1.28	0.96	0.86
Confidence (95 %)	0.60	0.95	0.99	0.74	0.65

The recovery and rsd values for potassium sorbate, methyl paraben and propyl paraben meet the specifications of 97.0 – 103.0 % for the mean recovery and ≤ 3.5 % for the rsd value. The acceptance criteria were set under consideration of the release specifications for the 3 components which is 90 – 110 % in each case. The recovery and rsd values for betamethasone-17-valerate and fusidic acid hemihydrate meet the specifications of 98.0 – 102.0 % for the mean recovery and ≤ 2.5 % for the rsd value. The acceptance criteria were set under consideration of the release specification for the 2 components which is 95 – 105 % in each case. The accuracy of the analytical method is considered acceptable.

4.4.5 *Robustness*

Two approaches were taken to the analysis of method robustness. The first approach involved an analysis of the stability of the sample and standard solutions over a fixed time period, whilst the second approach involved an analysis of the effects of changes of the mobile phase composition and system parameters on the chromatography and overall performance of the method.

4.4.5.1 *Stability of the sample and standard solutions*

The stability of the sample and standard solutions was analysed over a period ranging from 0 to 47 hours, while stored in darkness, e.g. the vial compartment of the HPLC at 25 ºC.

Table 22 Stability of solutions – Potassium sorbate

Time [h]	Sample 1	Sample 1	Standard 1	Standard 2
0	1173397	1152873	1349187	1330137
5	1175022	1154385	1349764	1329214
9	1177216	1158589	1353369	1334547
14	1179516	1160979	1355412	1336915
19	1181037	1161760	1357758	1339205
23	1182162	1164788	1359775	1341063
28	1184383	1165120	1360362	1343507
33	1186804	1167379	1364901	1344529
37	1189244	1170600	1365825	1349926
42	1190644	1171464	1371741	1352831
47	1191725	1172458	1372808	1354715
m.v.	1180966	1165538	1360082	1341508
s.d.	10797	6183	8073	8615
rsd	0.91	0.53	0.59	0.64
% Deviation over 47 hours	+ 1.56	+ 1.70	+ 1.75	+ 1.85

The peak area plots of each of the analytes from the sample and standard solutions show no degradation trend over the 47 hour test period. Instead, the values appear to increase with time. This increase of peak area over time has been observed in all samples and standards and the rate of increase in both the sample and standard solutions appears to be similar. The increase of peak area can therefore most likely be attributed to normal system drift. Since both the sample and standard solutions show similar trends, there is unlikely to be a significant effect on assay results. The peak area values of any relevant degradation products of the active substances did not increase significantly over the measurement period, indicating that the APIs did not degrade

appreciably. The sample and standard solutions are considered to be stable for up to 47 hours when stored in darkness at 25 °C.

4.4.5.2 *Robustness of the HPLC method*

In order to investigate if the analytical method was robust enough to withstand small changes to the make-up of the mobile phase as well as adjustments to the instrument set-up parameters, several modifications/adjustments were made to both and the results were recorded. The changes that were studied were as follows:

- The column-oven temperature was adjusted by ± 5 ºC.
- The mobile phase flow rate was adjusted by ± 0.2 ml/min
- The volume of methanol in mobile phase A was adjusted by ± 10 ml/L
- The detector wavelength was adjusted by ± 2 nm
- The proportion of mobile phase B at the beginning of the gradient run was adjusted by ± 4 %

The quality of the chromatography obtained after these changes was monitored with the aid of a solution prepared from a sample which had been stressed under the conditions of 50 °C/75 % RH for 8 weeks and contained all of the relevant degradation products of betamethasone-17-valerate and fusidic acid hemihydrate. A review of the resulting chromatograms led to the following conclusions:

– An increase of the column-oven temperature by 5 °C leads to a shortening of the retention times of all peaks and to a reduction of the resolution between the potassium sorbate and methyl paraben peaks. A reduction of the column-oven temperature by 5 °C has the opposite effect.

– An increase of the flow-rate by 0.2 ml/min. leads to a shortening of the retention time of all peaks. A decrease of the flow-rate by 0.2 ml/min. leads to a lengthening of the retention time of all peaks. In both instances the resolution between the potassium sorbate and methyl paraben peaks remains largely unaffected.

– Increasing/decreasing the volume of methanol in mobile phase A by ± 10 ml/L has no significant effect on the chromatography. The resolution between potassium sorbate and propyl paraben remains unaffected.

– In order to evaluate the effect of small changes of wavelength on the content results, an assay test was performed on a sample of Betamethasone-17-valerate/Fusidic acid cream and the content values were calculated at 238 nm, 240 nm and 242nm.

Table 23 Robustness of the assay method to wavelength changes

Compound Name	Assay value at 238 nm (mg/g)	Assay value at 240 nm (mg/g)	Assay value at 242 nm (mg/g)
Potassium sorbate	1.7594	1.7593	1.7595
Methyl paraben	0.8040	0.8037	0.8040
Propyl paraben	0.1656	0.1657	0.1655
Betamethasone-17-valerate	1.2375	1.2394	1.2384
Fusidic acid	20.1866	20.0818	20.0926

As demonstrated by the above results, varying the wavelength by ± 2 nm has no significant effect on the assay results. The analytical method is deemed to be robust with respect to minor changes of wavelength.

– An increase of the proportion of mobile phase B at the beginning of the gradient by 4 % (82 % -> 86 %) leads to a lengthening of the retention times of the early eluting peaks. The resolution between the potassium sorbate and methyl paraben peaks increases considerably from 2.4 to 3.3. A decrease of the proportion of mobile phase B at the beginning of the gradient by 4 % (82 % -> 78 %) leads to a shortening of the retention times of the early eluting peaks and to a reduced resolution between the potassium sorbate and methyl paraben peaks from 2.4 to 1.4.

The resolution between the critical peak pair of potassium sorbate and methyl paraben is dependent on a number of factors such as the proportion of organic modifier in the mobile phase and the steepness of the gradient program. The resolution between these peaks will affect the quantitative results due to possible integration errors when the peaks are not fully resolved. Consequently, it will be specified as a system suitability criterion for routine analysis that the resolution between these 2 peaks must be greater than 1.5.

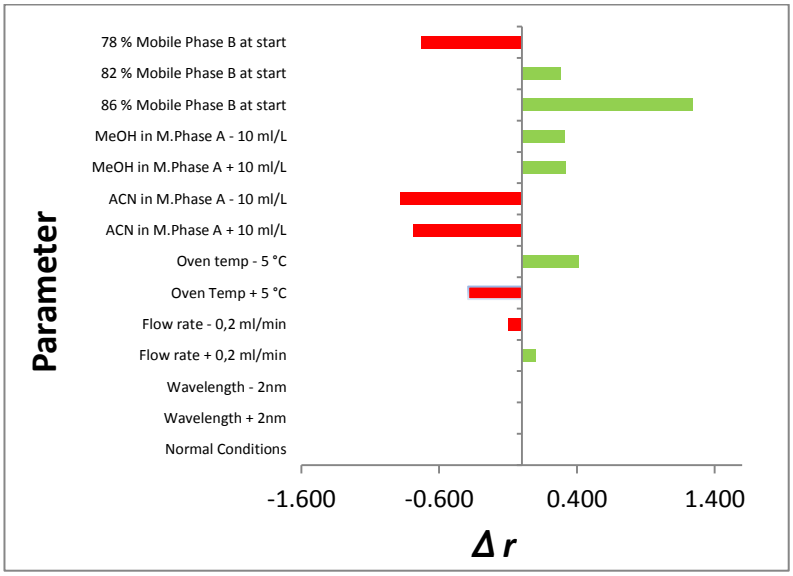

Figure 48 Graphical representation of the effect of changes of the analytical parameters on the resolution between the critical pair KS and MP. Green indicates an increase, and red a decrease of resolution between both peaks

4.5 *Validation of the Purity Method*

4.5.1 *Methodology*

The analytical procedure for the determination of the purity of betamethasone-17-valerate and fusidic acid hemihydrate in the developmental cream was validated for selectivity, linearity, precision, accuracy and robustness according to ICH guideline Q2 (R1) in a similar fashion to the assay method. Additionally, the sensitivity of the procedure as well as the response factors for each of the impurities were evaluated. The response factors for betamethasone and betamethasone-21-valerate were calculated by dividing the slope of the best-fitting line from the linearity experiment of each impurity with the slope obtained for the best-fitting line of the betamethasone-17-valerate (API) linearity experiment. The response factors for the impurities of fusidic acid monohydrate were taken directly from the EP 7.3 monograph for the drug substance.

4.5.2 *Results*

The results of the validation experiments are provided in Figure 25 - Figure 28*Table 28*. The analytical procedure has been demonstrated to be selective, linear, precise, accurate and robust within the investigated concentration range. The procedure can be considered fit-for-purpose.

Table 24 Impurity response factors

Impurity/Active	**Response Factor**
Betamethasone-17-valerate	1.000
Betamethasone-21-valerate	0.930
Betamethasone	1.154
Fusidic acid	1.000
3-Didehydrofusidic acid	1.074
(24R)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone	1.429
(24S)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone	1.429
26-Oxofusidic acid	3.333
16-epideacetylfusidic acid	1.667
Deacetylfusidic acid 21,16-lactone	1.667

4.6 *Discussion and General Conclusions*

The aim of the work in this chapter was to demonstrate that the analytical procedures developed in Chapter III are fit-for-purpose, i.e. to demonstrate that the procedures are capable of performing the task for which they were designed. In the case of the assay procedure, it has been shown that the method is capable of a selective separation of the active- and preservatives compounds without interference from the excipient or solvent components of the cream preparation, sample or mobile phase. The suitability of the procedure to accurately and precisely determine the content of the analytes of interest has been demonstrated through appropriately designed experiments, the results of which adhere to well established and accepted pharmaceutical industry standards. It has been demonstrated that there is a linear correlation between the analyte concentration and the detector response. The use of a single point calibration (single concentration) for quantitation is justified, since the 95 % confidence interval of the y-intercept of the best-fit line contains the value "zero". This indicates that the y-intercept is not significantly different from "zero" and it can therefore be presumed that the regression line goes through the origin.

In Chapter III a separate procedure was also developed for the analysis of the purity of the drug substances in formulations candidates. This procedure was validated in an analogous fashion to the assay procedure, with additional experiments being performed for the determination of the limits of detection and quantitation. As previously discussed, the sensitivity of the purity procedure proved to be suboptimal during development due to the low concentration of the glucocorticoid in the candidate

cream formulations (1.2 mg/g). The sensitivity was increased by increasing the analyte concentration in the sample solution. An increase of the injection volume was not feasible due to constraints of the analytical equipment and the requirement for the method to be capable of being operated on all HPLC machines within the laboratory. During validation the limits of detection and quantitation were determined and found to be below the required reporting threshold of (0.1 %).

In conclusion, the analytical procedures have been demonstrated to be fit-for-purpose and may be employed in the analysis of formulation candidates of the developmental cream. This is the subject of Chapter V.

Table 25 Summary of the statistical data from linearity experiments

Substance	y = mx + c	r	r^2	Residuals	Concentration Range, %	Concentration Range, µg/ml	95 % confidence interval of y-intercept	
							Lower confidence limit	Upper confidence limit
Betamethasone	y = 92932x + 8269	0.9999	0.9999	No trend	0.09 – 8.71	0.05 – 5.3	6392	10146
Betamethasone-21-valerate	y = 75300x - 514	0.9999	0.9999	No trend	0.06 – 5.82	0.04 – 3.5	-1276	248
Betamethasone-17-valerate	y = 80537x + 895	0.9999	0.9999	No trend	0.09 – 8.68	0.05 – 5.3	-354	2145
3-didehydrofusidic acid	y = 23072x + 3252	0.9999	0.9999	No trend	0.06 – 6.05	0.6 – 60.5	-7015	511
Fusidic acid hemihydrate	y = 29387x + 8134	0.9999	0.9999	No trend	0.08 – 7.84	0.8 – 78.4	-671	16893

*Specification: $r > 0.995$

Table 26 Summary of the data from the repeatability experiment

Sample No.	Content (%)					
	Betamethasone-21-valerate	3-didehydrofusidic acid	16-epideacetylfusidic acid-21,16-lactone	16-epideacetyl-fusidic acid	Sum of all impurities Betamethasone valerate	Sum of all impurities Fusidic acid
1	19.193	0.433	0.333	0.376	19.367	3.296
2	19.197	0.428	0.334	0.378	19.372	3.298
3	19.110	0.425	0.332	0.376	19.297	3.390
4	19.162	0.429	0.331	0.384	19.343	3.324
5	18.976	0.422	0.327	0.382	19.161	3.304
6	19.113	0.426	0.329	0.384	19.300	3.322
m.v.	19.125	0.427	0.331	0.380	19.307	3.322
s.d.	0.082	0.004	0.002	0.004	0.078	0.035
rsd, %	0.43	0.92	0.75	0.98	0.41	1.06
Confidence (95%)	0.086	0.004	0.003	0.004	0.082	0.037

*Specification: rsd ≤ 10 %

Table 27 Summary of the data from the intermediate precision experiment (intra-laboratory precision)

Sample No.		Content (%)					
		Betamethasone-21-valerate	3-didehydrofusidic acid	16-epideacetylfusidic acid-21,16-lactone	16-epideacetylfusidic acid	Sum of all impurities Betamethasone valerate	Sum of all impurities Fusidic acid
Day 1 Analyst 1 HPLC 1	1	19.193	0.433	0.333	0.376	19.367	3.296
	2	19.197	0.428	0.334	0.378	19.372	3.298
	3	19.110	0.425	0.332	0.376	19.297	3.390
	4	19.162	0.429	0.331	0.384	19.343	3.324
	5	18.976	0.422	0.327	0.382	19.161	3.304
	6	19.113	0.426	0.329	0.384	19.300	3.322
Day 2 Analyst 2 HPLC 2	1	19.945	0.447	0.325	0.419	20.283	3.489
	2	18.966	0.435	0.300	0.404	19.323	3.435
	3	18.946	0.429	0.304	0.401	19.273	3.298
	4	19.452	0.440	0.317	0.401	19.778	3.371
	5	20.178	0.454	0.326	0.427	20.529	3.637
	6	20.296	0.453	0.332	0.417	20.633	3.540
m.v.		19.379	0.435	0.324	0.396	19.638	3.392
s.d.		0.483	0.011	0.011	0.018	0.534	0.112
rsd, % (*)		2.5	2.5	3.5	4.6	2.7	3.3
Confidence (95%)		0.307	0.007	0.007	0.012	0.339	0.071

*Specification: rsd ≤ 10 %

Table 28 *Summary of the data from the accuracy experiment*

Sample No.	% Recovery				
	Betamethasone-21-valerate	Betamethasone	Betamethasone-17-valerate	Fusidic acid	3-didehydrofusidic acid
0.1 % - 1	93.14	83.94	86.62	104.71	95.73
0.1 % - 2	97.55	74.74	90.72	105.55	102.12
0.1 % - 3	98.90	95.35	91.98	105.27	94.48
1 % - 1	108.30	111.86	100.72	100.01	98.18
1 % - 2	111.50	109.40	103.70	100.88	100.40
1 % - 3	112.98	110.63	105.07	103.03	103.87
8 % - 1	111.76	111.40	103.94	102.12	104.79
8 % - 2	111.01	111.00	103.24	101.56	104.47
8 % - 3	112.80	112.16	104.91	103.02	105.46
m.v. (*)	106.44	102.27	98.99	102.91	101.06
s.d.	7.70	14.19	7.16	1.96	4.10
rsd, % (**)	7.2	13.9	7.2	1.9	4.1
Confidence (95%)	5.92	10.91	5.50	1.51	3.15

*Specification: average recovery value 90 – 110 %, **rsd ≤ 15 %

4.7 *References*

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CHAPTER 5

Formulation factors affecting the isomerisation rate of betamethasone 17-valerate in a developmental hydrophilic cream - A HPLC and microscopy based stability study

5 CHAPTER 5 – Isomerisation of Betamethasone 17-valerate

5.1 *Introduction*

Betamethasone is a synthetic corticosteroid with anti-inflammatory properties which is employed in the treatment of inflammatory skin disorders such as eczema and psoriasis [1,2]. Topical application of betamethasone base is not feasible since only 12 – 14 % of the administered dose of the drug is absorbed through the skin [3]. To help improve local bioavailability the substance is normally administered in the form of one of its esters, such as betamethasone-17-valerate (BV) or betamethasone-17,21-dipropionate, which have increased lipophilic nature allowing for better penetration of the drug molecule across the lipid membranes of the skin [4,5]. BV is one of the more potent and commonly used esters of betamethasone and is available in several marketed products for topical use, such as Betnovate® Cream (GlaxoSmithKline) for monotherapy, or together with clioquinol in Betnovate™-C (GlaxoSmithKline) for combinational therapy of inflammatory skin-conditions with mild secondary infections.

The formulation of BV into a suitably stable vehicle is often problematic. The substance is susceptible to an acid and base catalysed isomerisation in aqueous and semi-solid formulations resulting in an acyl group migration from position C-17 to the more stable C-21 position of the steroid ring system [6] (Figure 49). This isomerisation process is of significant clinical relevance since betamethasone-21-valerate demonstrates only $\approx 1/15^{th}$ of the potency of its 17-valerate counterpart [5]. In order to guarantee the safety and efficacy of drug products containing BV, the rate of isomerisation must be kept to a minimum.

In a highly regulated environment such as the pharmaceutical industry, the stability of pharmaceutical preparations is ensured through extensive analytical testing. The data generated through this testing is used as a basis for justifying the shelf-life of a pharmaceutical product. In a clinical setting such as in a hospital pharmacy, however, physicians often like to prescribe dilutions of topical corticosteroids in order to reduce possible side-effects associated with their use, such as skin atrophy and pigmentation loss [5]. Very often, such dilutions do not undergo the same rigorous analysis as the original product and the short- or long-term stabilities of these dilutions may not be known. The practice of dilution of marketed corticosteroids is widespread and appears in principle to be justified, since numerous studies have shown that the relationship between concentration and activity of topically applied glucocorticoids is non-linear [5,7],

with much weaker dilutions often demonstrating similar activity to the undiluted original product in vasoconstriction assays [2,7–9]. However, due to the lack of stability data there may be potential issues regarding the safety and efficacy of such dilutions.

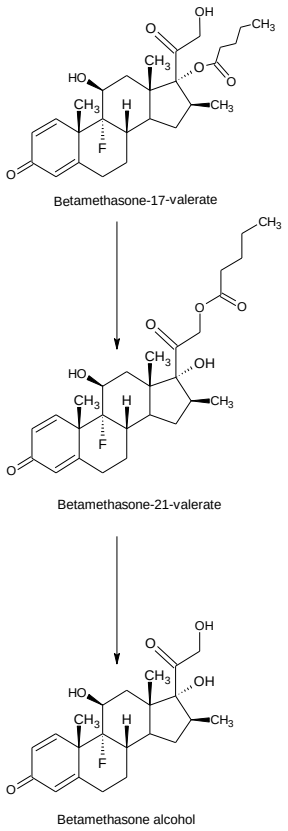


Figure 49 *Schematic of the isomerisation of betamethasone 17-valerate*

Previously published studies on this topic have demonstrated that the dilution of semi-solid formulations containing BV can affect the stability of the drug substance. It has been shown that the choice of diluent plays a crucial role with regard to the isomerisation process. For example, 1:4 dilutions of Betnovate® in Unguentum Merck and white soft paraffin were stable over 12 months [5], whereas dilutions in Emulsifying Ointment showed 50 % degradation within several hours [10]. Many of the published studies offered little explanation as to why the diluent was influencing the isomerisation rate [5,6,9–11]. In most cases, the increased rate of isomerisation was attributed to an increased pH of the diluted preparation, without consideration of other factors. Boonsaner *et al* [8] studied the stability of betamethasone 17-valerate in Beeler's Basis and Cold Cream® and came to the conclusion that the degradation rate cannot be solely dependent on the pH alone but that other unknown factors must be involved. It was demonstrated that preparations with similar pH values can show very different stabilities. No conclusive evidence was provided as to what these unknown factors may

have been. During the early stages of formulation development it became apparent that other factors not related to the pH were affecting the isomerisation rate of BV in the initial trial formulations. Since the above mentioned literature studies did not provide a plausible explanation with regard to the isomerisation process in semi-solid dosage forms, it was necessary to study this process more extensively.

5.2 *Aim*

The primary aim of the research described in this chapter was to investigate which formulation factors influence the isomerisation rate of BV in a model hydrophilic cream. Different variables, such as, (i) the types and concentrations of excipients; (ii) the influence of oxidative processes and (iii) the effect of trace metals, were investigated. It was intended that the information gained through these studies would then be used to develop a stable, marketable topical medication containing both BV and FA for the treatment of inflammatory skin disorders with secondary infections.

5.3 *Results*

5.3.1 *Initial trial batches*

The necessary excipients for the manufacture of the cream were chosen based on the knowledge and expertise of the responsible formulation chemists as well as on regulatory requirements. An overview of these components is presented in Table 29.

A number of trial formulations were prepared with different quantities of the excipients Stearate-20 (S-20), Cetylstearyl alcohol (CSA) and Hydroxypropylmethyl cellulose (HPMC), the proportions of which are also provided in Table 29. These trial formulations were the first test formulations which were physically stable enough to be placed on long-term and accelerated stability studies, i.e. the respective oil-in-water (O/W) emulsions demonstrated no significant phase separation. The batches were labelled 18I, 18II, 19 and 20 (see Table 29). In contrast to the other formulations, variant 18I contained the antioxidant alpha-tocopherol, which was added to investigate if oxidative processes were likely to affect the stability of the drug substance. The quantities of all other ingredients of the cream as well as the manufacturing procedure and equipment were identical for all batches. In each case, the pH was adjusted to 5.0 ± 0.2 and the active pharmaceutical ingredients were initially present in suspended form in each of the cream formulations.

The stability of each of the trial formulations was determined by HPLC analysis [12,13] after storage for 1 month under real-time (25 °C/60 % RH) and accelerated (40 °C/75 % RH) stability conditions. The results indicated that variant 20 was markedly more stable than any of the other variants with regard to the isomerisation rate (Figure 50 a). Variants 18I, 18II and 19 contained approx. 3 % - 4 % betamethasone-21-valerate after storage for 1 month at 25 °C/60 % RH. This level of degradation was considered unacceptable since this quantity of impurity would normally be expected to appear after 24 – 36 months storage in a comparable pharmaceutical product. Furthermore, current guidelines [14,15] require that the content of the drug substance should lie between 95 – 105 % or, if justified, between 90 – 105 % of the label claim throughout the shelf-life of the product. The above results indicated that, in variant 18 for example, the API would have been completely degraded after 36 months, if the isomerisation had continued at a linear rate.

Table 29 *Formulations of the first experimental batches*

		Var. 18I with tocopherol	Var. 18II	Var. 19	Var. 20
Article	Description	Proportion in % mass	Proportion in % mass	Proportion in % mass	Proportion in % mass
1	Macrogolstearylether-20 (S-20)	5.0000	5.0000	5.0000	3.6500
2	Cetylstearyl alcohol (CSA)	5.0000	5.0000	5.0000	6.3500
3	Paraffin, liquid	21.8800	21.8800	21.8000	21.8800
4	White Vaseline	9.8200	9.8200	9.8200	9.8200
5	Water, purified	21.5050	21.5050	11.5060	18.0000
6	Methyl paraben	0.1000	0.1000	0.1000	0.1000
7	Propyl paraben	0.0200	0.0200	0.0200	0.0200
8	Potassium sorbate	0.2500	0.2500	0.2500	0.2500
9	Water, purified	15.0000	15.0000	25.0000	18.5050
10	Citric acid-monohydrate	0.1000	0.1000	0.1000	0.1000
11	Hypromellose (HPMC)	1.0000	1.0000	2.0000	0.5000
12	Paraffin, liquid	13.3000	13.3000	13.3010	13.3010
13	Fusidic acid $0.5H_2O$	2.4000	2.4000	2.4000	2.4000
14	Betamethasone 17-valerate	0.1214	0.1214	0.1214	0.1214
15	Paraffin, liquid	4.5000	4.5000	5.0000	5.0000
16	Tocopherol, alpha-	0.0004	0.0000	0.0000	0.0000

The data also seemed to indicate that reducing the quantity of HPMC to 0.50 % may slow the rate of isomerisation, whereas in contrast, increasing the quantity of HPMC from 1.00 % to 2.00 % did not affect the stability of BV. Decreasing the

quantity of S-20 to 3.65 % in combination with an increase of CSA to 6.35 % may also slow the rate of isomerisation. As evident by a comparison of the data from variants 18I and 18II, the addition of alpha-tocopherol to the formulation has no effect on the rate of isomerisation. It was therefore concluded that oxidative degradation processes are unlikely to play a role in the observed degradation of BV in the investigated hydrophilic formulation.

5.3.2 *Optimisation I*

5.3.2.1 *Methodology*

Despite the encouraging data from variant 20, the stability of the first trial batches proved to be unacceptable, requiring optimisation of the formulation. Further experimental batches were manufactured and subjected to stability studies/HPLC analysis in a similar fashion to the initial formulations. The compositions of these optimised formulations are provided in Table 30. Some of the optimisation steps included (i) varying the concentrations of S-20 and HPMC; (ii) use of an alternative emulsifier, Macrogolstearylether-21 (S-21); (iii) addition of disodium edetate in order to remove metal contaminants; as well as the addition of the API to the aqueous phase of the cream rather than the oil phase (iv).

5.3.2.2 *Effect of S-20, CSA and HPMC concentrations*

The data generated after 5 months real time storage and 1 month accelerated storage is presented in Figure 50 (c/d) and demonstrates that increasing the content of HPMC from 0.50 % to 1.00 % increases the isomerisation rate. In contrast, a reduction of the quantity of S-20 from 5.00 % (variant 18II) to 2.14 % (variant 14) coupled with an increase of the quantity of CSA from 5.00 % (variant 18II) to 7.86 % (variant 14) appeared to slow the rate of isomerisation. Replacing S-20 with Macrogolstearylether-21 (S-21) also appeared to slow the rate of isomerisation, whereas suspending the API in the aqueous phase of the cream (as opposed to the oil phase) did not improve stability.

HPMC is present in the formulation as a thickening agent which in theory should not affect the stability of BV. However, according to the Technical Handbook for METHOCEL Cellulose Ethers from the DOW chemical company [16], Methocel (HPMC) also possesses emulsifying properties and can act as a surfactant in solution. It was therefore considered possible that HPMC may help to increase the solubility of BV in the aqueous phase of the cream, leading to an increased rate of isomerisation. Despite

being theoretically possible, it seemed unlikely that an increase of the content of HPMC by 0.50 % could lead to such a dramatic increase of the isomerisation rate from 10 % (0.50 % HPMC) to 35 % (1.00 % HPMC) after 1 month storage. Furthermore, the data from the initial trial batches did not support this hypothesis, since variant 19 contained 2.00 % HPMC and did not show such as marked increase of isomerisation as compared to variant 18. After further review of the formulations and analytical data it was noticed that variant 18 with 1.00 % HPMC had a pH value of 6.0 in contrast to the other variants which had pH values of 5.0 ± 0.2. Since it was known from the studies of Bundgaard and Hansen [17] that the kinetics of the transesterification of BV to the 21-valerate ester in aqueous solution is pH dependent, it was considered likely that the shift in pH from 5 to 6 was responsible for the increased isomerisation rate. Repeat experiments confirmed this hypothesis. When the pH of the cream was kept constant the concentration of HPMC had no influence on the isomerisation rate. Experiments in aqueous media later showed that the pH increases with increasing HPMC concentration.

5.3.2.3 *Effect of disodium edetate, S21 and O/W phase*

Literature studies indicate that the presence of trace metals in pharmaceutical preparations can cause degradation of glucocorticoids [18,19]. For this reason the influence of the chelating agent disodium edetate on the degradation rate of BV was investigated (variant 18 with EDTA). The results showed little improvement in stability as compared to variant 18 without EDTA, e.g. variant 18 with 0.5 % HPMC. It could be concluded that metal ions are unlikely to influence the isomerisation rate of BV in the cream. Likewise, the suspension of the API in the aqueous phase did not significantly influence the isomerisation rate, indicating that the presence of water alone is not sufficient to initiate the isomerisation process. This was perhaps to be expected since BV is practically insoluble in water [20]. This observation, however, confirmed that the API must be in solution for the isomerisation to take place and, furthermore, that some component of the cream must be enhancing the solubility of the initially suspended BV crystals in the aqueous phase of the cream. Since it had been observed that a reduction of the concentration of S-20 leads to an increased stability, it was suspected that this emulsifying compound was aiding the dissolution of the suspended BV crystals into the aqueous phase of the drug formulation.

In the initial experiments, replacing S-20 with S-21 appeared to decrease the isomerisation rate of BV. The chemical structures of S-20 and S-21 differ in the number of oxyethylene ($-OC_2H_4$) groups attached to the stearic acid chain. Both substances have

very similar HLB values (Hydrophilic-Lipophilic-Balance) of 15.2 and 15.5 according to the manufacturer [21], indicating that they are likely to show very similar solubilities in aqueous solution. The HLB value is a measure of the relative hydrophilicity or lipophilicity of a surfactant and higher values indicate increased water solubility. Due to the similarity of the HLB values it would be expected that both substances would influence the stability of the cream formulation to a similar extent. Repeat experiments could not confirm the initial observation and it was finally concluded that no major differences exist between these substances with regard to their influence on the isomerisation process. Due to commercial and regulatory considerations, however, S-21 was employed in all further experiments.

5.3.3 *Optimisation II*

The results of the initial and optimisation I experiments demonstrated that reducing the content of S-20/21 from 5.00 % (variant 18) to 3.65 % (variant 20) and further to 2.14 % (variant 14) significantly slowed the rate of isomerisation. In order to investigate this effect in more depth, further trial formulations were produced containing less S-21 and with varying quantities of CSA. The goal was to find the optimum concentration of these components which would provide the lowest rate of isomerisation without compromising the physical stability of the cream. The proportions of liquid paraffin and Vaseline were also adjusted with the aim of optimising the physical stability of the preparation. The accelerated stability studies demonstrated that maximum physical- and chemical stability was achieved with 1.5 % S-21 and 5.5 % CSA (variant 36, Figure 52 a/b)). Reducing the S-21 concentration to 1.00 % did not appear to bring further improvement. Stability studies of the final formulation (variant 36) which were performed under real time conditions showed no apparent increase of betamethasone-21-valerate content after 1 month and < 3 % increase after a period of 36 months storage.

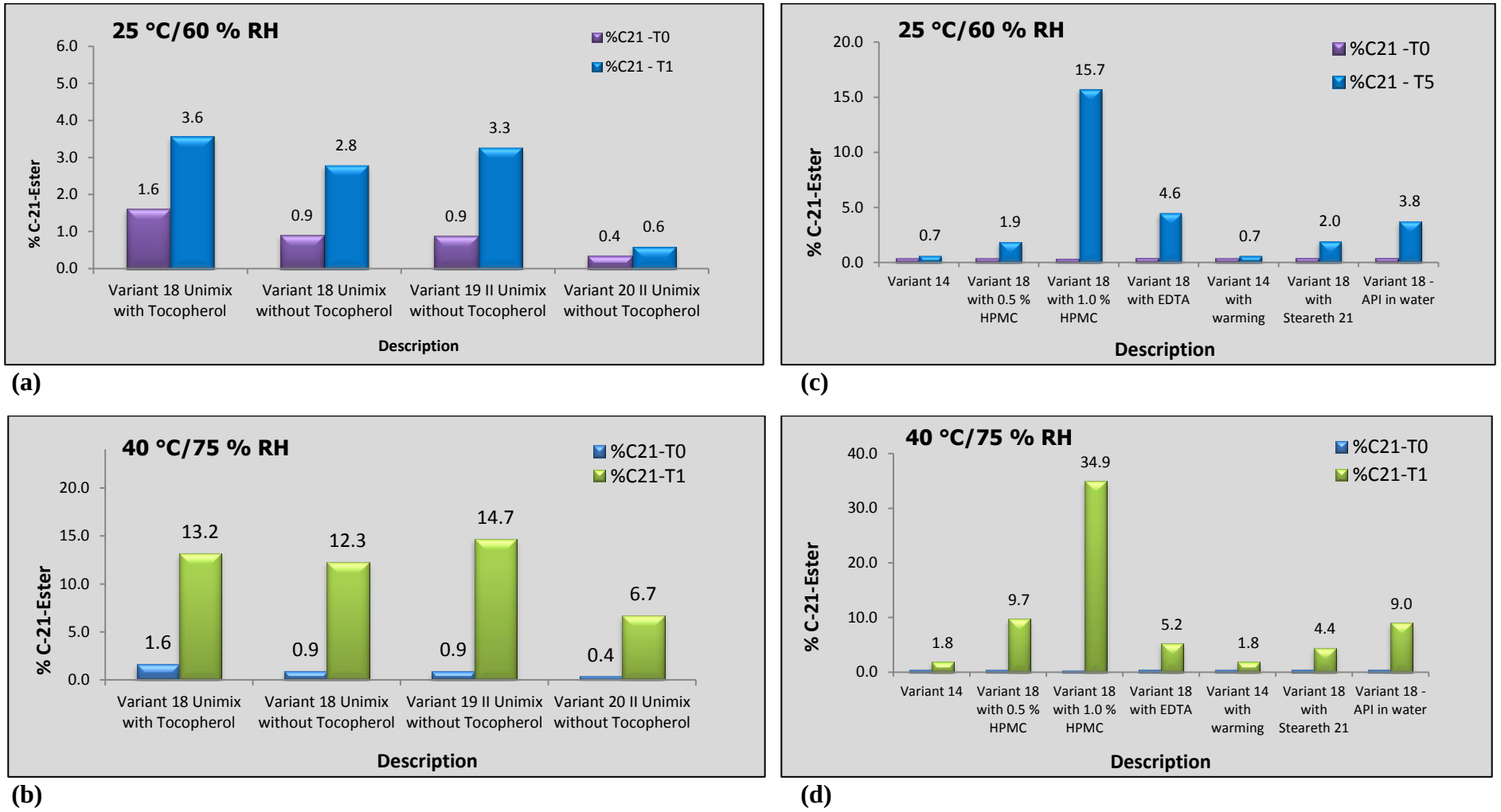


Figure 50 Graphs showing the stability data of the initial- (a/b) and optimised trial formulations (c/d) at 25 °C (upper graphs) and 40 °C (lower graph). Displayed results are an average of n=2 measurements

Table 30 Quantities of S-20, CSA and HPMC in the optimised test formulations

	Var. 14	*Var. 14 with warming*	*Var. 18 with 0.5 % HPMC*	*Var. 18 with 1.0 % HPMC*
Article	Proportion in mass %	Proportion in mass %	Proportion in mass %	Proportion in mass %
Macrogolstearylether-20/21 (S-20/21)	2.14	2.14	5.00	5.00
Cetylstearyl alcohol (CSA)	7.86	7.86	5.00	5.00
Hypromellose (HPMC)	0.50	0.50	0.50	1.00

	Var. 18 with 0.1 % Na_2EDTA	*Var. 18 with Steareth-21*	*Var. 18 API in H_2O*
Article	Proportion in mass %	Proportion in mass %	Proportion in mass %
Macrogolstearylether-20/21 (S-20/21)	5.00	5.00	5.00
Cetylstearyl alcohol (CSA)	5.00	5.00	5.00
Hypromellose (HPMC)	1.00	1.00	2.00

5.3.4 *Confirmational study*

As previously mentioned, optimisation of the concentrations of S-21 and CSA resulted in an increased chemical stability of BV, whilst optimisation of the proportions of Vaseline and liquid paraffin lead to an increased physical stability of the cream preparation. In order to demonstrate definitively that the reduction of the concentration of the emulsifier gives rise to the increased stability of BV, and not, for example, the adjustment of the Vaseline and paraffin quantities, the concentration of S-21 in optimised variant 36 was again increased from 1.5 % to the initial concentration present in variant 18, i.e. 5.00 %. The concentrations of all other components of the cream remained equivalent to the optimised variant 36. The HPLC stability results confirmed unequivocally that an increase of the concentration of the primary emulsifier, S-21, leads to an increase of the isomerisation rate.

5.3.5 *Effect of pH on the isomerisation rate*

The effect of pH on the isomerisation rate of BV was investigated in both aqueous solution and in the cream preparation itself. The substance is stable in aqueous solution at pH values between 1.2 and 4.2 with an optimum of approx. 3.2 in this experiment. This is in good agreement with the reported literature value of 3.5 [11]. Above pH 4.2 the

rate of isomerisation increases gradually, then increasing very rapidly above pH 6.2. Complete isomerisation of BV was observed after 48 hours at pH 8.2 (see section 3.5).

A similar trend was observed in the cream preparation (Figure 52 (c)). At pH values between 2.7 and 5.3 the drug substance is relatively stable, although some degradation still occurs. At higher pH values, e.g. > 6, the isomerisation rate increases very rapidly giving rise to complete isomerisation within 1 month at pH 7.6 and 40°C/75 % RH. A pH range of between 5.0 ± 0.5 was chosen for the cream since this range is a compromise between skin tolerability and drug stability. Lower pH values are generally considered too acidic for topical treatment (in particular for the treatment of damaged skin where the healing process may be affected by the pH [22]) and higher pH values would compromise the stability of the API, as demonstrated by the analytical results.

5.3.6 *Experiments in liquid paraffin*

During manufacture, BV is suspended in liquid paraffin before being added to the rest of the ingredients during the last stage of the process. In order to confirm that the isomerisation only occurs when an aqueous medium is present, the stability of BV in liquid paraffin was investigated by stressing under conditions which were known to give rise to isomerisation in aqueous solution. No isomerisation was observed, indicating that water is necessary for this process to occur. Since the API crystals are initially suspended in the cream, the BV must first dissolve into the aqueous (continuous) phase of the O/W emulsion for isomerisation to occur. This hypothesis was further investigated by experimentation in aqueous solution.

5.3.7 *Effect of excipients on the aqueous solubility of BV*

Since it was known that water is necessary for the isomerisation to occur, it seemed plausible to suggest that the cream preparation might be stabilised by decreasing the quantity of water in the formulation. However, from the very beginning of the formulation development process it had been decided that the finished formulation should contain between 60 – 70 % water, in order to ensure that the required physico-chemical properties of the drug product were achieved. Furthermore, in a hydrophilic cream the quantity of water is likely to be vastly in excess of the minimum quantity required to dissolve the API in the presence of emulsifier. Small reductions of the water quantity are therefore highly unlikely to influence the isomerisation rate. More significant reductions of the water quantity would result in the manufacture of a cream

with different physico-chemical characteristics as required. This is not preferred since such characteristics, e.g. viscosity, are considered to influence the *in-vitro* and *in-vivo* release rates of drug substances from cream matrices [23]. For this reason a reduction of the water content of the formulation was not further pursued. The most feasible option available was to try to reduce the solubility of the drug substance through modification of the quantities of non-aqueous excipient components in the cream matrix.

5.3.8 *Effect of S-21 concentration on the solubility and stability of BV*

The relationship between the isomerisation rate of BV and the concentration of S-21 was investigated over the range 0.0 % - 1.0 % of S-21. The influence of pH was removed by adjusting the pH of all solutions to 5.3 ± 0.2 during preparation. The solubility of BV was measured by HPLC using the purity procedure developed in Chapter 3. Figure 51 shows how the solubility of BV increases with increasing concentration of S-21. In contrast, the rate of isomerisation does not increase proportionally. This is because of the relatively favorable pH of 5.3 which prevents a rapid isomerisation from occurring (section 5.3.5). It was concluded that S-21 increases the solubility of BV in aqueous media, suggesting that the increased emulsifier concentration also leads to an increased solubility of BV in the drug product formulation.

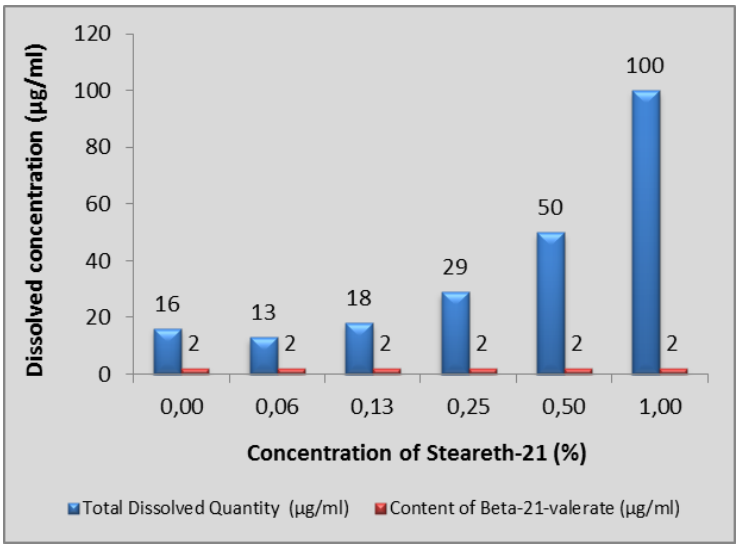
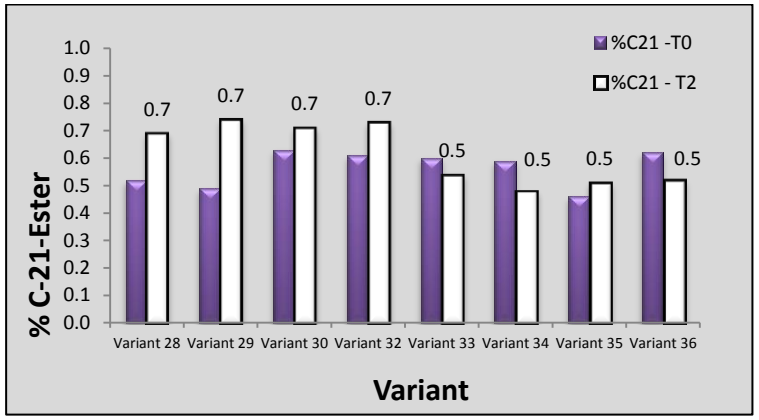
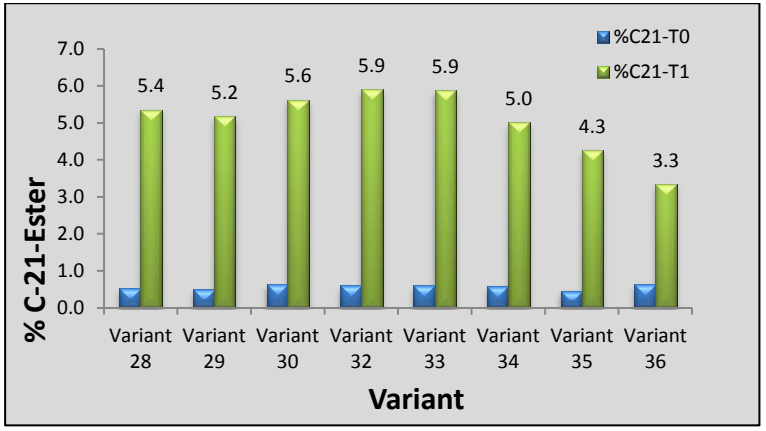


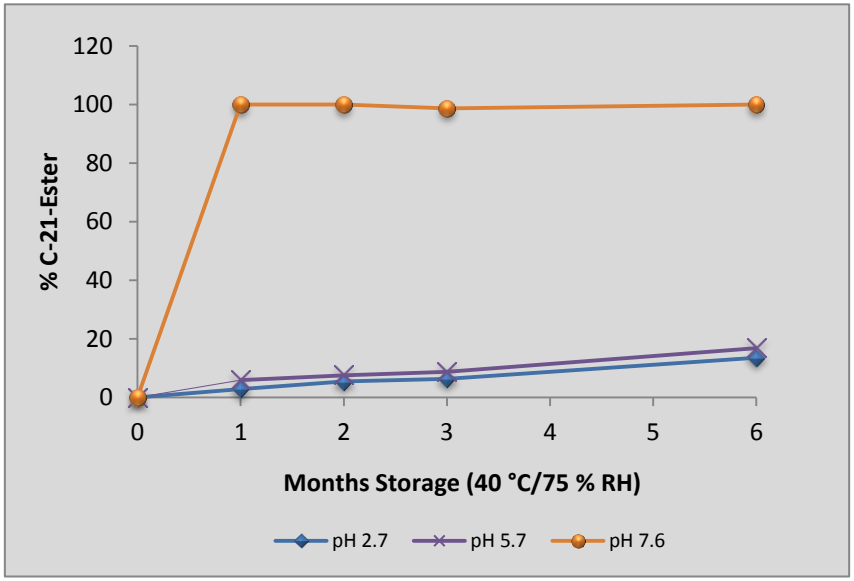
Figure 51 Effect of emulsifier concentration on the solubility and isomerisation rate of betamethasone-17-valerate in aqueous solution at pH 5.3. Displayed results are an average of n=2 measurements



(a)



(b)



(c)

Figure 52 Stability data from round II of optimisation, (a) 2 months at 25 °C/60 % RH, (b) 1 month at 40 °C/75 % RH, (c) demonstrates the influence of pH on the isomerisation rate of BV in the cream formulaton. Displayed results are an average of n=2 measurements

5.3.9 *Effect of variation of both S-21 concentration and pH*

The effect of simultaneously varying both S-21 concentration and pH was investigated by preparing samples in the same fashion as in section 5.3.8, the only difference being that appropriate buffers were employed over the pH range 3.2 to 7.2. Buffers were required due to the need to achieve accurate pH values over the specified range. The results (Figure 53) demonstrate that at higher pH values, e.g. 7, and low concentrations of S-21, the rate of isomerisation remains low, indicating that the BV must be solubilised into solution before the isomerisation can take place. At lower pH values, e.g. pH 3, and higher concentrations of S-21, the rate of isomerization remains low, indicating that the isomerization only takes place when the pH conditions allow. The results indicate that the BV must first be solubilised into the aqueous phase where the rate of isomerization is then pH dependent. This means that in the cream formulation higher concentrations of emulsifier more rapidly solubilize the BV thus increasing the overall concentration of drug substance present in the aqueous phase. Whether the BV then isomerizes is solely dependent on the pH of the surrounding matrix.

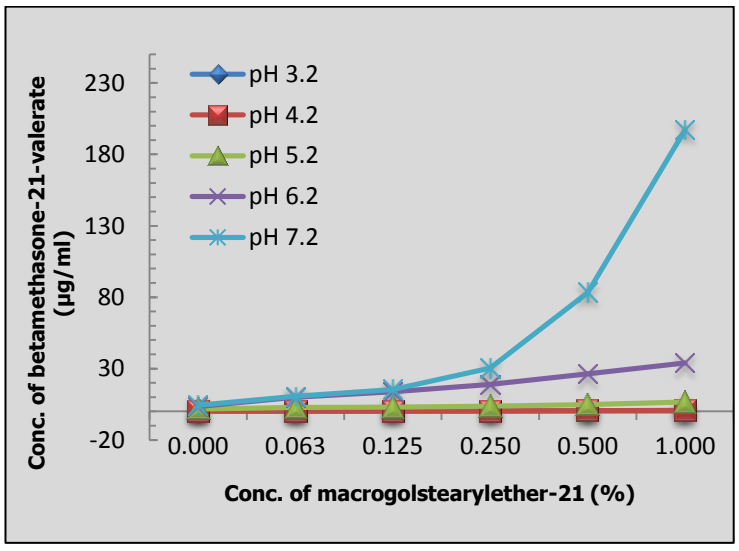


Figure 53 Effect of both pH and emulsifier concentration on the isomerisation rate of BV in aqueous solution (pH 3.2 – 7.2). Displayed results are an average of n=2 measurements

5.3.10 *Effect of Ethoxy-Chain Length of the Emulsifier on the Isomerisation rate*

Since the initial experimental data had suggested a correlation between the HLB value of the emulsifier (which is determined primarily by the ethoxy-chain length) and the rate of transesterification of BV, it was decided to investigate this effect further. Test formulations containing mixtures of emulsifiers with different ethoxy chain lengths

were manufactured and the stability of BV in these formulations was investigated over time. The compounds steareth-10 (S-10), S-21 and steareth-100 (S-100) were chosen for this purpose. Test batches were prepared by adding a further 3.5 % of each of the chosen emulsifiers to variant 36 which already contained 1.5 % of S-21. The batches were subjected to stability studies and tested at intervals of 1, 2, 3 and 6 months.
The following mixtures were prepared:

- Cream product with 1.5 % S-21 and 3.5 % S-10 HLB ≈ 13
- Cream product with 5.0 % S-21 HLB ≈ 15
- Cream product with 1.5 % S-21 and 3.5 % S-100 HLB ≈ 18
- Cream product with 1.5 % S-21 HLB ≈ 15

The results presented in Table 31 and Figure 54 show that the addition of a further 3.5 % of emulsifier leads to a marked increase of the isomerisation rate. There does, however, not appear to be a relationship between the HLB value of the emulsifier mix and the isomerisation rate. If, as hypothesised, an increase of the HLB value of the emulsifier results in a reduction of the isomerisation rate (this is what the initial data seemed to suggest), then it would be expected that the steareth-10 mixture would show the highest rate of isomerisation (HLB ≈ 13) and the steareth-100 mix the lowest (HLB ≈ 18). This is not the case. It appears that the overall mass of emulsifier incorporated into the formulation is more important than its HLB value, as demonstrated by the very similar isomerisation rates observed for each of the macrogolstearylether variants. Note: all variants had pH values of 5.0 ± 0.2, ensuring that none of the results were affected by the pH.

Table 31 *Effect of the ethoxy chain length of the emulsifier on the isomerisation rate of BV*

Emulsifier Mixture	% betamethasone-21-valerate				
Storage period (40 °C/75 % RH)	0 months	1 month	2 months	3 months	6 months
1.5 % S-21 / 3.5 % S-10	0.0	5.45	9.98	12.01	27.40
5.0 % S-21	0.0	5.19	7.89	10.07	25.29
1.5 % S-21 / 3.5 % S-100	0.0	5.99	10.71	13.51	31.90
1.5 % S-21	0.0	1.60	3.77	5.91	12.24

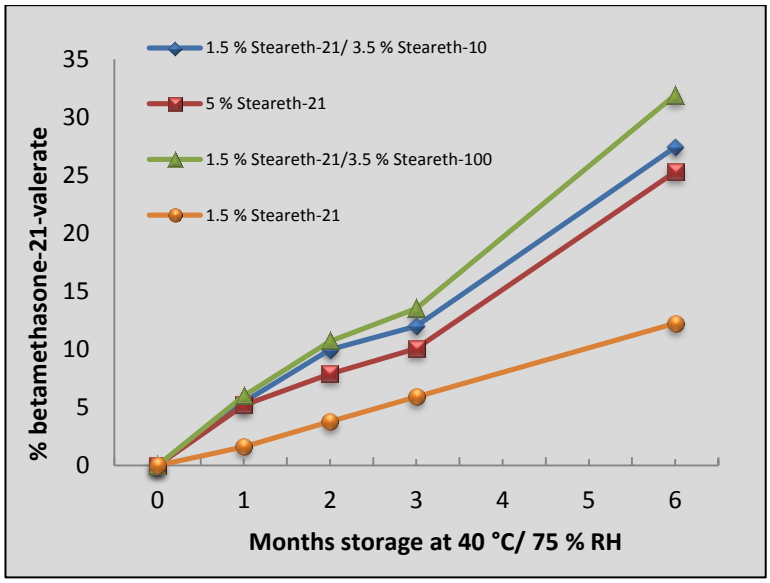


Figure 54 Effect of the ethoxy chain length of the emulsifier on the isomerisation rate of BV in BV/FA cream stored at 40 °C/ 75 % RH. Displayed results are an average of n=2 measurements

5.3.11 *Microscopy*

The effect of modifying the concentration of emulsifier on the isomerisation rate was also examined by microscopy. This technique allowed for the direct observation of any dissolution phenomena of the suspended BV crystals in real time. One drawback of this technique, however, was that the BV employed in the cream formulation was micronised (particle size distribution of 90 % < 5 µm) which made the observation of these minute crystals difficult in such a complex matrix. This problem was overcome by employing non-micronised BV which had a particle size distribution of 90 % < 110 µm, thus making the observation of the dissolution of the crystals considerably easier. One batch of each of variant 18 (the least stable variant) and variant 36 (the most stable variant) were prepared with non-micronised BV and placed on stability for 1 year under accelerated conditions (40 °C/75 % RH). The microscopic profiles and chemical stability of the creams were analysed after 1, 2, 3, 6, 9 and 12 months.

The micrographs presented in Figure 55 demonstrate that the BV crystals in variant 18 (with 5 % S-21) dissolve over time. After 12 months storage at 40 °C/75 % RH there are no visible crystals remaining in the cream. The data from the chemical analysis also indicates that the BV has significantly degraded under these conditions. In contrast, the BV crystals in variant 36 remain largely unchanged, a well-defined crystal structure being present throughout the entire study. The isomerisation rate of BV is also much lower in variant 36 than in variant 18, as shown by the chemical data. This

confirms the hypothesis that increasing the concentration of emulsifier leads to a more rapid dissolution of the suspended crystals.

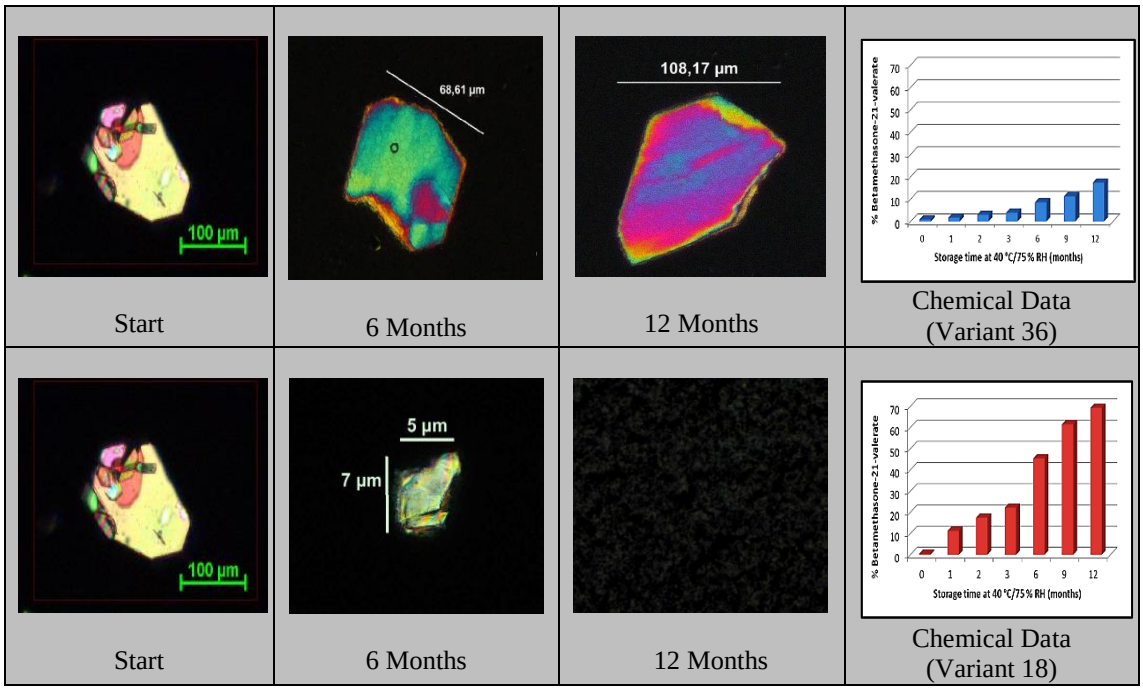


Figure 55 Micrographs and chemical data of BV crystals in variants 36 (top) and 18 (bottom) after storage at 40 °C/75 % RH (x 40, polarisation)

5.4 *Discussion and Conclusions*

The current study has demonstrated that the pH of the pharmaceutical vehicle is not the only factor to be considered when formulating BV into semi-solid drug products. The stability of BV in such products may be influenced by the concentration of the emulsifier to such a degree, that either practically no isomerisation occurs throughout the shelf-life, or alternatively, that complete isomerisation occurs within a number of hours. An increased concentration of S-20 or S-21 has been shown to enhance the solubility and isomerisation rate of BV in the aqueous phase of a hydrophilic cream. Maximum chemical stability was achieved at a concentration of ≤ 1.5 % (w/w) of this substance in the preparation. The data suggests that the co-emulsifier, CSA, does not play a significant role in the isomerisation process. Overall, the factors influencing the rate of isomerisation of suspended BV in a hydrophilic cream can be summarised as follows (Figure 56):

- It is necessary that BV is dissolved in aqueous solution in order for the isomerisation to take place. Transesterification will not occur to a significant extent in a non-aqueous environment, e.g. a fatty, anhydrous ointment.
- At high concentrations of emulsifier such as S-20 or S-21 (e.g. 5 %) and higher pH values (e.g. > 6) there is a rapid rate of isomerisation.
- At high concentrations of emulsifier such as S-20 or S-21 (e.g. 5 %) and lower pH values (e.g. < 4) there is a moderate rate of isomerisation which is pH dependent.
- At low concentrations of emulsifier (e.g. 1 %) and higher pH values (e.g. > 6) there is a moderate rate of isomerisation which appears to be mainly dependent on the solubility of the API in the aqueous phase.
- At low concentrations of emulsifier (e.g. 1 %) and lower pH values (e.g. < 4) there is a slow rate of isomerisation, since very little BV is dissolved in the aqueous phase and the quantity which is dissolved isomerises at a slow rate due to the low pH.

In order to optimise the stability of BV in a hydrophilic cream suspension, both the concentration of emulsifier and the pH value should be kept as low as possible. It seems reasonable to hypothesise that the instabilities observed on dilution of BV creams with emulsifying ointment and other emulsifier containing diluents in previous studies [9,10] may have been due to an increased solubility of BV in the aqueous phase. This study demonstrates that the concentration of emulsifier chosen for a topical vehicle containing BV is likely to be of critical importance when developing a stable formulation. Equally, the diluent used for diluting marketed corticosteroid preparations must be chosen with care, taking not only pH but also emulsifier concentration into account. Based on the observed mechanism, it seems plausible that the findings are also relevant for other topically formulated C-17 esterified glucocorticoids, although this remains to be confirmed. In the case of Betamethasone/Fusidic acid cream a stable formulation was obtained with 1.5 % S-21. The complete formulation of the final cream is presented in Table 32. It should be noted that for reasons of intellectual property the manufacturing process for the cream cannot be disclosed. Fusicutan® plus Betamethason cream (Figure 57) came to market in Germany, Austria, Poland and Croatia in early 2017. The application for a Europe-wide market authorisation is in progress.

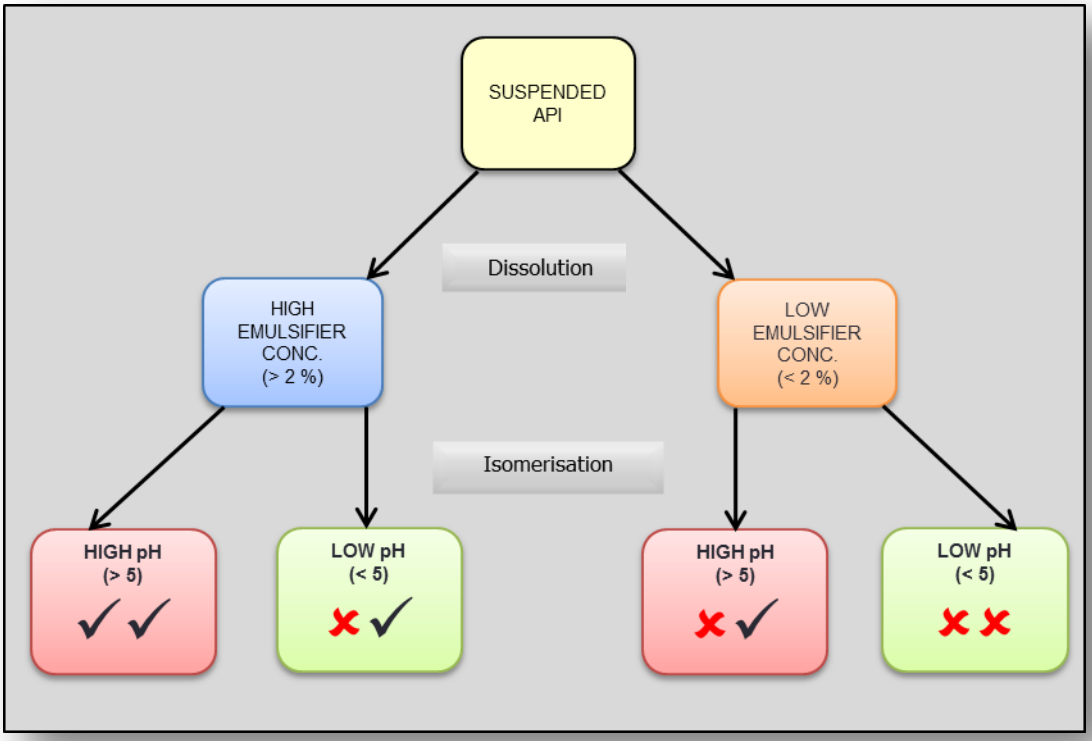


Figure 56 Schematic of the effect of emulsifier concentration and pH on the isomerisation rate of BV in a hydrophilic cream

Figure 57 Fusicutan® plus Betamethason Cream which came to market in early 2017

Table 32 Final formulation of Fusicutan® plus Betamethason Cream

Article	Proportion in mg/g Cream
Macrogolstearylether-21 (S-21)	15.000
Cetylstearyl alcohol (CSA)	55.000
Paraffin, liquid	318.636
White Vaseline	215.000
Water, purified	365.290
Methyl paraben	0.800
Propyl paraben	0.160
Potassium sorbate	2.500
Citric acid-monohydrate	1.000
Hypromellose (HPMC)	5.000
Fusidic acid hemihydrate	20.400
Betamethasone 17-valerate	1.214
Σ	1000.000

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CHAPTER 6

Polymorphism in Commercial Sources of the Antibiotic Fusidic acid – A Study of its Effects on the Stability of the Drug Substance and its Release Rate from a Marketed Topical Drug Product

6 CHAPTER 6 - Polymorphism in Commercial Sources of Fusidic acid

6.1 *Introduction*

Pharmaceutically active substances often exist in a number of different crystalline forms. The existence of two or more crystalline forms of the same substance, which have different arrangements and/or conformations of molecules in their respective crystal lattices, is known as polymorphism [1,2]. Differences in crystal structure may also arise due to incorporation of water (termed *hydration*) or solvent molecules (termed *solvation*) into the crystal lattice of a drug substance, a state known as pseudopolymorphism [3,4]. Apart from existing in several crystalline states, drug substances can also exist in an amorphous state, which differs from the crystalline state in that there is no long range order of molecules within a lattice structure.

As mentioned above, crystalline substances show long range order of the molecules within the crystal lattice. Thus, crystals may be considered as being made up of numerous repetitions of a single 3-dimensional structure, or unit cell [5]. Lattice theory describes the 3-dimensional packing of molecules within a unit cell and according to this theory; any individual crystal may be classified as belonging to one of seven possible crystal classes, which are: cubic, tetragonal, orthorhombic, monoclinic, triclinic, hexagonal and trigonal [5]. Each class is defined by the relationships between the individual dimensions, a, b and c, and the individual angles, α, β and γ, of the unit cell [5] (Figure 58).

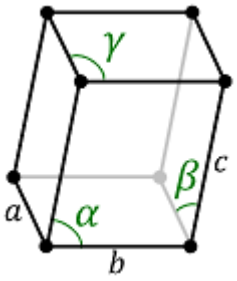

Figure 58 Example of a triclinic unit cell [6]

Further to the assignment to a particular crystal class, a crystal may also be designated to one of 14 Bravais lattices, and further to one of the 230 individual space groups which exist [7]. Consequently, lattice theory allows for a complete description of the relative positions and symmetries of atoms and/or molecules in a unit cell. Since polymorphs of the same substance differ in how the molecules are packed within the

unit cell, two forms of the same substance may belong to completely different crystal classes and/or space groups.

Due to differences in lattice structures and energies, different polymorphs of the same substance may vary in their physical properties, such as stability, solubility, melting point, density, hardness as well as optical and electrical characteristics [8–10]. It is these differences that make polymorphism such an important aspect in drug product development. One high-profile case of crystal polymorphism, involving the HIV protease inhibitor, ritonavir, (marketed as Norvir® in liquid and capsule form by Abbott Laboratories), was reported in the late 1990's. New polymorphs of the drug substance were only detected after the already marketed product began failing dissolution testing [11,12]. It was subsequently discovered that a polymorphic conversion of the drug substance in the capsule preparation during storage was responsible for the failed dissolution results. This discovery eventually led to the removal of the capsule dosage form from the market [11]. In total, five crystal forms of ritonavir were subsequently discovered. Micrographs of Forms III and IV are presented in Figure 59. The above case exemplifies the importance of polymorphism in pharmaceutical development and it is the goal of the development chemist to select the most stable polymorph of a substance for incorporation into the intended drug product. This can be challenging since many polymorphs are metastable and their stability may be dependent on several factors, such as the drug product formulation as well as the environmental and chemical conditions present during manufacture and storage.

During the manufacture of a drug product, the active drug substance may be subjected to both thermal and mechanical stress, which can result in conversion of the

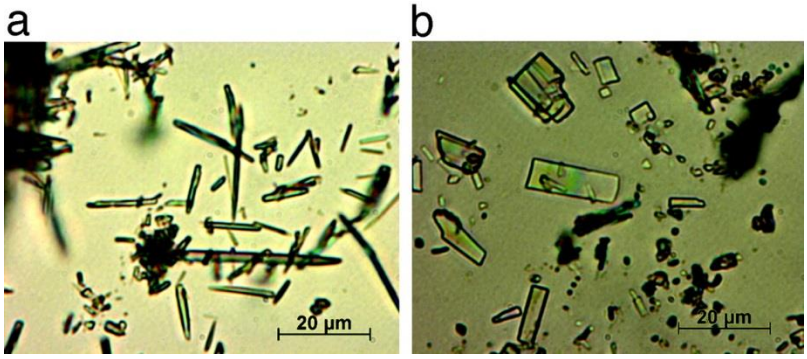

Figure 59 Photomicrographs of different polymorphs of ritonavir, a HIV protease inhibitor (a = Form III, b = Form IV) [11]

desired polymorphic form to a less desirable form, or to the generation of amorphous material within the bulk substance. This is highly undesirable since the presence of a different polymorph could alter the efficacy of the drug product. The presence of

significant quantities of amorphous material in the bulk substance can also influence the stability of the product, since amorphous materials tend to be more soluble and less stable than their crystalline equivalents. Examples of stress factors encountered during the manufacture of drug products include: drying, homogenizing, milling, grinding, wet granulating and compaction.

Often, drug substances must be micronized before they can be incorporated into drug products. Micronisation is a mechanical process for reducing the particle size of bulk drug substances in order to improve their homogeneity and bioavailability in the finished drug product. This is normally performed by jet-milling or grinding. Both of these processes are highly energetic and can lead to polymorphic interconversion, particularly, if the drug product is present in a metastable form. The grinding of the antibiotic substance chloramphenicol palmitate is an example of such a process, where it was observed that Form B converted to the less therapeutically desirable form A during grinding [13]. Further examples of process related polymorphic conversions include the conversion of Form II of the neuroleptic drug substance chlorpromazine hydrochloride [13] to Form I during wet granulation; the conversion of the monohydrate Form C of the barbiturate phenobarbital to the anhydrous Form B during drying at 45 °C [13]; as well as the conversion of the needle-like α-Form of the non-steroidal anti-inflammatory piroxicam to the cubic β-Form during the compaction stage of tabletting [13].

It has been well documented that polymorphs of the same drug substance can possess different stabilities and/or solubility properties, which may lead to variations in local- and/or systemic bioavailability [14–17]. Drug substances which show differences in stability between their respective polymorphs include: Fenretinide, where Form I was shown to be more stable than Form II at all temperatures between 4 °C and 80 °C [18]; Enapril maleate, where in contrast to form I, form II was shown to be unsuitable for tablet production [19]; and furosemide where form II was found to be more photolabile than form I [20]. The above examples demonstrate that polymorphism is a complex theme which can present significant problems when a pharmaceutical manufacturer needs to change supplier of an active pharmaceutical ingredient (API) or add a second supplier in order to ensure a constant supply of a particular drug substance. In such cases, it must be ensured that the alternative drug substance is of the same polymorphic form as the original, which necessitates characterisation studies of the crystalline structure, solubility and *in-vitro*-release properties of the API from the drug product, as well as the performance of suitably designed stability studies [1,3,21].

FA is known to demonstrate polymorphism, with 4 crystalline forms (I – IV) described to-date in relevant literature [22–24]. Despite the fact that FA has been on the market for several decades [25], polymorphism in this substance has only been documented in more recent times and to-date no relevant data has been published with regard to the release rate of different polymorphs of FA from topical pharmaceutical formulations. This is surprising given the strict legislative requirements with regard to the use of different polymorphic drug forms in pharmaceutical products [26,27].

6.2 *Aim*

The goal of the current study is to investigate polymorphism in commercial sources of fusidic acid and to assess its effects on the stability of the bulk drug substance and its *in-vitro* release from the topical pharmaceutical dosage form developed in Chapter 5. This information is of critical importance when considering whether FA from different commercial sources can be used interchangeably in the developed drug product without significant implications for the safety and efficacy of the product. The study will also investigate the influence of micronisation on the stability of the drug substance.

6.3 *Materials and Methods*

Fusidic acid was purchased from Ercros SA (Madrid, Spain, Manufacturer A), Joyang Laboratories (Jiangsu, China, Manufacturer B) and OJSC Biosintez (Penza, Russia, Manufacturer C). Gradient grade methanol and acetonitrile were purchased from VWR International GmbH (Darmstadt, Germany). Purified water was obtained from the in-house purification system at mibe GmbH Arzneimittel (Brehna, Germany). Phosphoric acid (85 % m/m, analysis grade, d = 1.71 g/ml) was purchased from Merck (Darmstadt, Germany). All cream samples were provided by mibe GmbH Arzneimittel (Brehna, Germany).

The materials and methods employed for measurement of the intrinsic dissolution and *In-vitro* release rate are described in sections 2.3 and 2.4. XRPD experiments were performed as described in section 2.2. All stability studies were performed in Binder KBF 720 stability chambers (Binder Inc., USA) according to ICH Guideline Q1A [28]. FTIR analysis was performed on a Perkin-Elmer SpectrumOne spectrophotometer (Perkin-Elmer, Massachusetts, USA)

6.4 *Results*

6.4.1 *FTIR, XRPD and Microscopic Analysis*

The characterisation of FA has been described in several patents [23,24] as well as in mainstream scientific literature [22]. Extensive data has been published, including FTIR-spectra and XRP diffractograms of each of the polymorphic forms of FA discovered to date. The most significant differences between the FTIR spectra of these forms are observed in the region 1650 cm^{-1} to 1750 cm^{-1}. Form I shows a single stretching band at ≈ 1720 cm^{-1}, whilst form II shows a band at ≈ 1720 cm^{-1} and an additional stretch at ≈ 1697 cm^{-1}. Form III shows two very distinct bands at approx. 1748 cm^{-1} and 1688 cm^{-1}. A comparison of the literature data with the FTIR data of FA from the commercial samples shows that FA from Manufacturer A is of Form III, FA from Manufacturer C is of Form I and that FA from Manufacturer B comprises predominantly of Form III but is not of pure crystalline form. It contains significant quantities of either Form I or Form II, which can be seen by the presence of an extra absorption band at ≈ 1720 cm^{-1} (Figure 60).

Powder X-Ray diffraction analysis of FA from manufacturers A, B and C (Figure 62) demonstrates that A and B are of similar crystal form (form III), since both diffractograms are comparable for all significant reflexes. In contrast, the diffractogram of FA from Manufacturer C shows significant differences to A and B and is comparable with the literature data for Form I. The XRPD data agrees with the results of the FTIR analysis. Scanning electron micrographs of FA from each of the manufacturers show differences in morphology (Figure 62). FA from Manufacturer A and B comprises of flat, irregularly shaped particles whilst FA from Manufacturer C comprises of predominantly spherical particles.

6.4.2 *Stability Studies*

Stability studies of micronised and non-micronised FA from each manufacturer were performed according to the relevant ICH Guidelines with a view to investigating if the polymorphic modification of FA influences the stability of the bulk drug substance during storage. It should be noted that the particle size distributions of FA from each manufacturer were comparable. Samples comprising of 5 g of drug substance stored under a nitrogen atmosphere in a suitably sealed polyethylene bag, which was further packaged in a heat sealed aluminium sachet, were placed on stability under the conditions of 5 ± 3 °C, 25 °C/60 % relative humidity (RH), 30 °C/65 % RH and 40 °C/75 % RH for 24, 24, 12 and 6 months, respectively.

The results (Figure 61 a, b and c) demonstrate that the stability of FA is temperature dependent. The greatest stability is observed at 5 ± 3 °C with the stability decreasing with increasing temperature. No significant difference in stability was observed between polymorphic Form I (Manufacturer C) and Form III (Manufacturers A and B) under ICH conditions. However, the data showed that micronisation of the API has a negative effect on the stability of FA, as demonstrated by the higher levels of impurities observed as compared to the non-micronised material. This is likely due to the reduction of the particle size and corresponding significant increase of the specific surface area of the substance after micronisation. XRPD and FTIR analysis confirmed that the polymorphic form of FA does not change during micronisation or storage.

6.4.3 *Stress tests*

The results of the ICH stability studies showed that there are no significant differences in stability between polymorphic forms I and III under the chosen conditions. This however was no guarantee that there are no differences in the intrinsic stabilities of the different modifications under other conditions. In order to examine this further, the effect of humidity and light were investigated under stress conditions. Light and humidity were chosen as stress factors because experience had shown that these factors give rise to degradation in FA. The photo-stability of FA was examined according to ICH guideline Q1B, whereby the samples were illuminated with 1.2 million lux hours at an energy of 200 watt hours/m^2. The humidity test was performed by placing FA in an open container and storing at 60 % relative humidity for 6 months. The results of both tests indicated that FA from Manufacturer B was significantly less stable under these conditions than FA from either of the two other manufacturers (Figure 61 d). This instability may be due to the fact that this product does not consist of a pure polymorphic form, but of a mixture of two or more forms. The further investigation of this point was beyond the scope of this work.

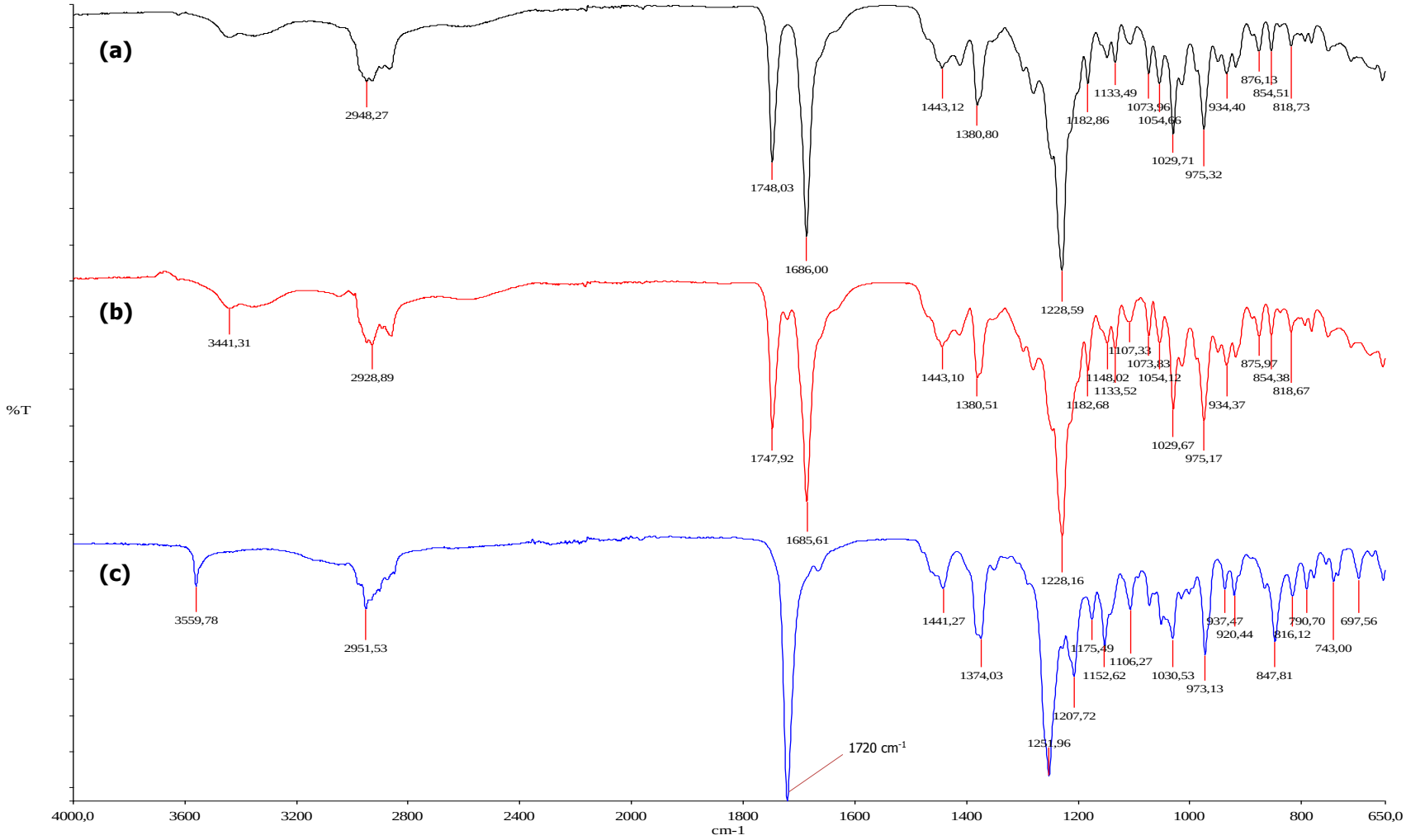

Figure 60 *FTIR spectra of FA from manufacturers A (black), B (red) and C (blue) demonstrating major differences, particularly in the region 1600 cm^{-1} to 1800cm^{-1}*

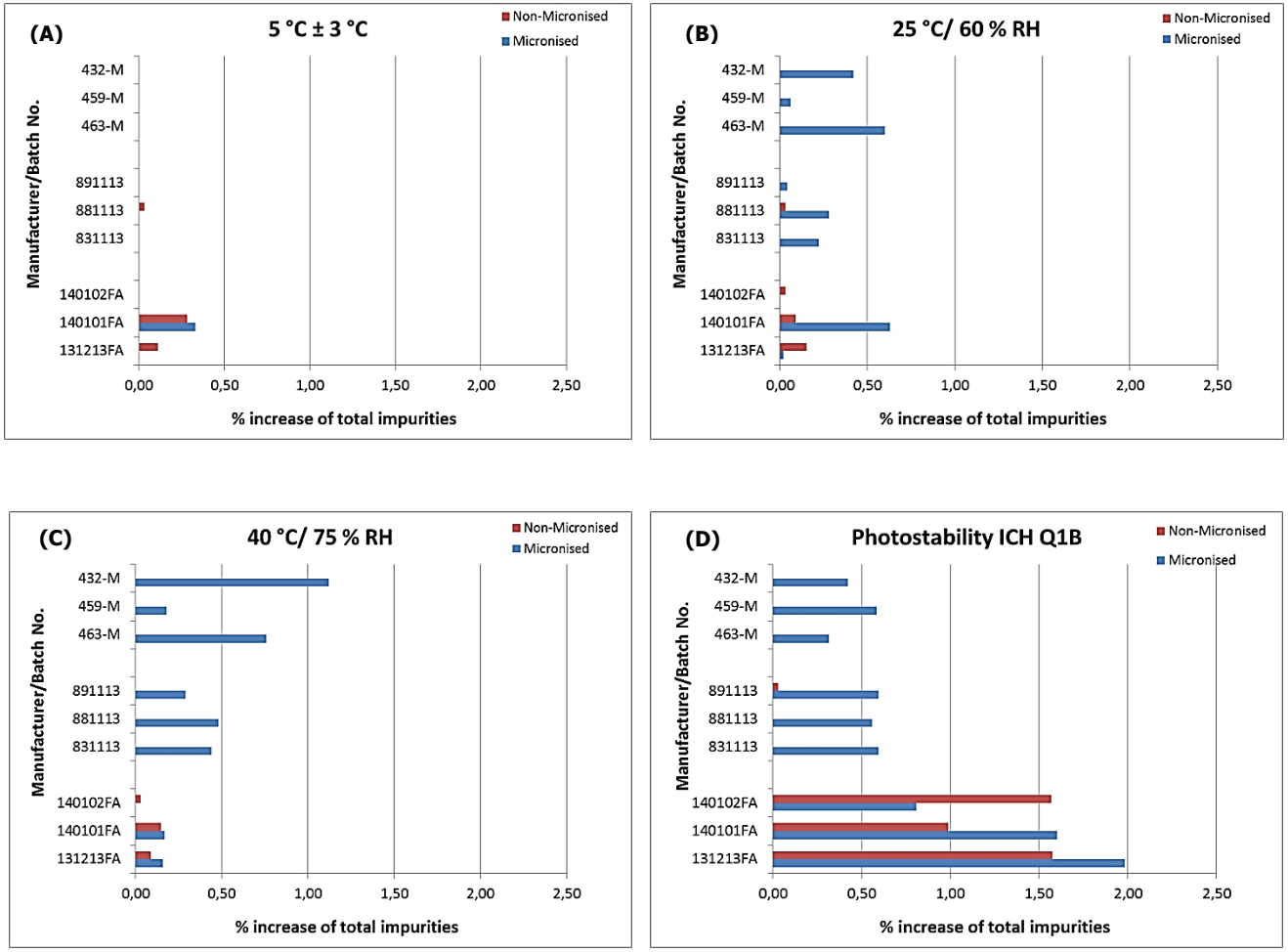

Figure 61 Results of stability studies on FA at 5 °C /24 months (a), 25 °C/60 % RH/24 months, 40 °C /75 % RH/6 months as well as under photolytic stress conditions as described in ICH Q1B(Manufacturer A = 432-M - 463-M; Manufacturer B = 140101FA - 131213FA; Manufacturer C = 89113 - 831113)

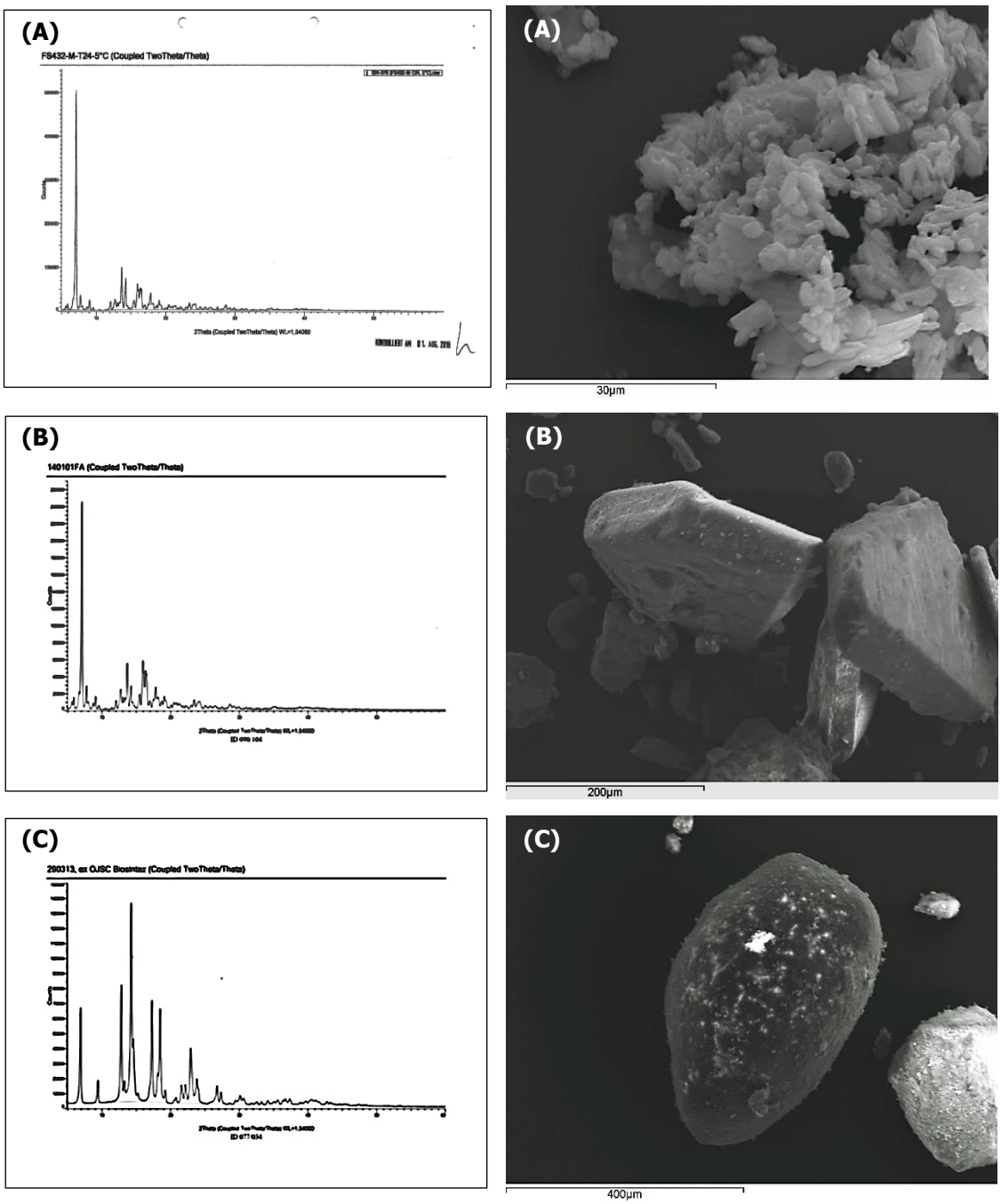

Figure 62 X-ray powder diffractograms and scanning electron micrographs of FA from Manufacturer A (micronised, form III), B (non-micronised, form III) and C (non-micronised, form I) showing differences amongst manufacturers

6.4.4 *Intrinsic Dissolution Rate*

The solubility properties of FA from each of the three manufacturers were compared by measuring the intrinsic dissolution rates of samples from each manufacturer according to Ph. Eur. 2.9.29 using the rotating cylinder apparatus. Samples were taken at 10, 20, 30, 40, 50 and 60 minute intervals and the cumulative release rate in mg was plotted against time. The intrinsic dissolution rate (mg/min/cm^2) was calculated as the slope of

the best-fitting line (mg/min) of this plot divided by the surface area of the drug substance (= 0.5 cm^2). The measured release rates were 0.42, 0.36 and 0.30 $mg/min/cm^2$ for manufacturers A, B and C, respectively (Figure 63, Table 33). Statistical analysis of the data showed no significant difference between the IDR profiles of FA from the different manufacturers, indicating that the samples have comparable intrinsic dissolution rates (see appendix 2 for an example of the statistical analysis procedure).

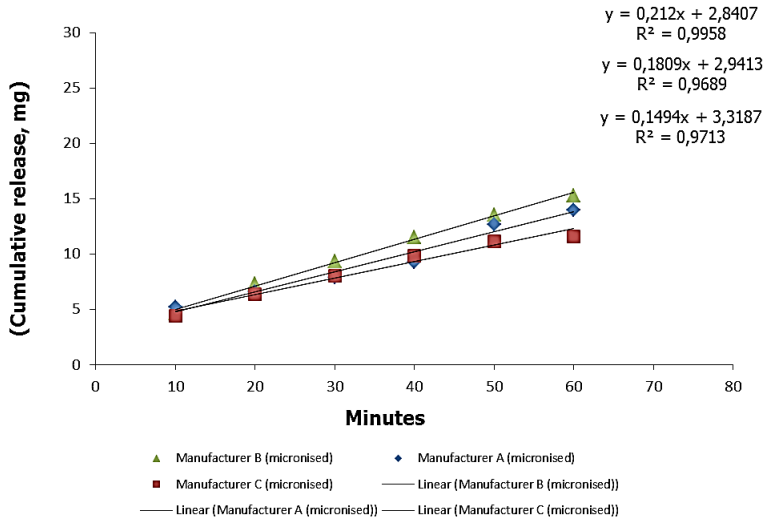

Figure 63 *Intrinsic dissolution data – plot of cumulative release versus time. Displayed values are an average of n=6 measurements*

Table 33 *Intrinsic dissolution rates of FA from manufacturers A, B and C*

	slope	*unit*	*Slope/Surface area*	*unit*
Manufacturer A (Ercros)	0.1809	**mg/min**	0.36	**$mg/min/cm^2$**
Manufacturer B (Joyang)	0.2120	**mg/min**	0.42	**$mg/min/cm^2$**
Manufacturer C (OJSC)	0.1494	**mg/min**	0.30	**$mg/min/cm^2$**

Surface area of disc = 0.5 cm^2

6.4.5 *In-Vitro-Release Profile from Fusicutan® plus Betamethason Cream*

The results of the intrinsic dissolution tests indicated that the investigated samples show comparable dissolution rates in the model system used for this test. This data provides useful clues about the comparability of the solubility properties of the measured samples. It can however not easily be extrapolated to the release rate of the drug substance from a semi-solid drug product. In semi-solid systems the drug substance is very often suspended, with only a fraction of the total quantity of drug substance being

dissolved in solution. In order to compare the release rates of drug substances from such preparations an *in-vitro* penetration test is often employed. One of the most commonly employed versions of such a test uses a vertical diffusion cell, or Franz cell, comprising of a jacketed, vertical diffusion cell connected by appropriate tubing to a heated reservoir of fluid which is continuously pumped around the system to ensure a constant temperature of 32 °C, thus mimicking the temperature at the external skin surface (Figure 64)

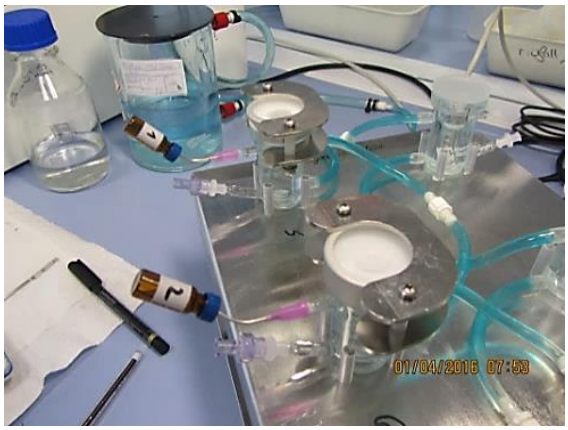

Figure 64 *Franz Cell Apparatus (Vertical Diffusion Cell)*

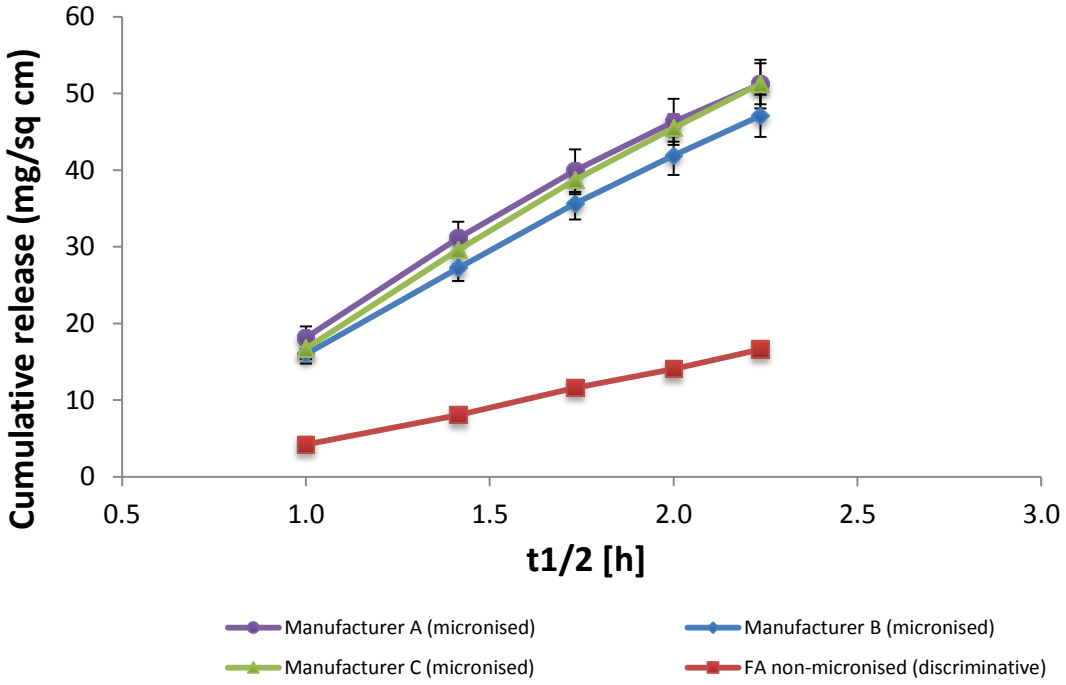

Figure 65 *In-vitro-release profiles of FA from Fusicutan® plus Betamethasone cream*

In order to demonstrate the discriminatory power of the procedure, i.e. the ability of the procedure to detect differences in the release rates of API from different cream formulations, a batch of cream was prepared which contained non-micronised fusidic

acid. The release rate for this batch was significantly slower than for the batches which contained micronised material. The slower release rate can be attributed to the larger

Table 34 Example calculation spreadsheet for the statistical comparison of the intrinsic dissolution rates (part I)

STATISTICAL EVALUATION OF INTRINSIC DISSOLUTION DATA (related to Wilcoxon Rank Sum/Mann-Whitney Rank Test)

Product 1	Time	t1/2	Cumulative Release [mg]							
	[Minutes]	[Minutes]	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	s.d.
Joyang	20	4.47	8.930	8.040	9.340	8.340	7.980	8.860	8.582	0.498
Ch: 140102FA	30	5.48	12.730	10.880	12.910	11.340	12.190	12.340	12.065	0.728
	40	6.32	16.330	16.250	18.360	17.260	18.900	15.690	17.132	1.166
	50	7.07	17.100	17.840	18.860	17.400	18.810	20.260	18.378	1.068
	60	7.75	19.660	20.130	22.610	21.440	21.710	23.670	21.537	
	slope		3.409	3.533	3.686	3.699	3.780	3.851	3.660	
	R^2		0.987	0.984	0.968	0.970	0.966	0.967		
	sd		0.743	0.902	1.088	1.286	1.636	0.792		

Product 2	Time	t1/2	Cumulative Release [mg]							
	[Minutes]	[Minutes]	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Mean	s.d.
OJSC	20	4.47	8.530	8.400	8.430	9.430	8.270	8.170	8.538	0.415
Ch: 891113	30	5.48	12.790	11.770	12.940	14.250	11.600	11.150	12.417	1.038
	40	6.32	15.530	14.680	15.180	16.480	14.550	14.610	15.172	0.681
	50	7.07	18.310	17.510	19.410	20.270	17.830	18.450	18.630	0.942
	60	7.75	20.800	19.920	20.560	21.690	20.190	20.410	20.595	
	slope		3.699	3.614	3.672	3.697	3.683	3.727	3.682	
	R2		0.998	0.991	0.985	0.965	0.965	0.967		
	s.d.		0.281	0.346	0.616	0.267	0.572	0.351		

Table 34 Example calculation spreadsheet for the statistical comparison of the intrinsic dissolution rates (part I)

T/R ratios	RS1	RS2	RS3	RS4	RS5	RS6
TS1	0.9216	0.9432	0.9283	0.9221	0.9257	0.9148
TS2	0.9550	0.9774	0.9620	0.9555	0.9592	0.9479
TS3	0.9965	1.0199	1.0038	0.9971	1.0010	0.9891
TS4	1.0001	1.0236	1.0074	1.0006	1.0045	0.9927
TS5	1.0218	1.0458	1.0292	1.0224	1.0263	1.0142
TS6	1.0411	1.0655	1.0487	1.0417	1.0457	1.0334

Results:

The eighth and twenty-ninth ordered individual ratios are the lower and upper limits of the 90% confidence interval for the ratio of the median in-vitro release rates (slopes) of test- and reference products.

8th T/R ratio =	0.9549776	lower limit
29th T/R ratio =	1.0263347	upper limit

In terms of percentage. this corresponds to **95.50** **to** **102.63** **%**

Because this 90% confidence interval falls/~~does not fall~~ within the limits of 75% to 133.33%

the test product has **passed** at Stage 1.

Conclusion:

Based on the above results the intrinsic dissolution rate of the Test product 1
is similar to the intrinsic dissolution rate of the Test product 2.

Table 35 Example calculation spreadsheet for the statistical comparison of the intrinsic dissolution rates (part II)

particle size of the FA crystals. This indicates that the analytical procedure is capable of discriminating between batches which exhibit different release rates. Figure 65 shows the results of the in *in-vitro* diffusion test. A statistical analysis of the release rates of FA from each of the test batches of cream (Table 34) was performed using a non-parametric confidence interval procedure related to the Mann-Whitney rank test [29]. The release rates were not significantly different according to this test.

6.5 *Discussion and conclusions*

The goal of the studies performed in this chapter was to investigate polymorphism in commercial sources of fusidic acid and to assess its effects on the stability of the bulk drug substance and its in-*vitro* release from the topical pharmaceutical dosage form developed in Chapter 5. The results indicate that there are at least two polymorphic forms of fusidic acid currently available on the commercial market. FTIR and XRPD experiments have confirmed that 2 of the 3 manufacturers which were investigated produce FA of form III, although manufacturer B does not appear to produce polymorphic pure product. FTIR analysis showed a band at ≈ 1720 cm^{-1}, which is characteristic of either form I or II. The third manufacturer was found to produce FA of form I.

Under refrigerated, real-time and accelerated- stability conditions the API from all three sources was found to have a comparable stability when packaged under an atmosphere of nitrogen in an air-tight, light protective container. The stability of FA was found to be temperature dependent with the greatest stability being observed at 5 °C ± 3 °C. Under stress conditions the API from manufacturer B (form III) was found to be significantly less stable than either of the other sources. This could possibly be due to the lack of polymorphic purity observed in samples from this manufacturer. This issue was, however, beyond the scope of the study and was not pursued further.

The intrinsic dissolution rate of each of the drug substance samples was determined according to Ph. Eur. 2.9.29. Statistical analysis of the resulting dissolution data did not suggest a significant difference between the solubilities of each of measured samples. This was in agreement with previous literature studies. In order to demonstrate that polymorphic forms I and III show no significant difference with regard to their release rate from the drug product (which could lead to differences in the *in-vivo* bioavailability of the drug substance) their diffusion rates from the marketed topical product Fusicutan® plus Betamethason (developed in Chapter 5) were analysed and found to be statistically comparable. The data indicates that polymorphic forms I and III

have similar solubility properties and are therefore likely to demonstrate a similar *in-vivo* bioavailability. Overall, it can be concluded that forms I and III can be used interchangeably in the drug product without affecting the safety or efficacy of that product. This means that each of the manufacturers investigated in this study may be established as a second supplier, ensuring that the drug product can still be produced and marketed even if one of these suppliers ceases its production of FA over the coming years.

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CHAPTER 7

In-vitro-Release and Antimicrobial Efficacy Studies of Electrospun Poly(caprolactone) Membranes containing the Drug Substances Fusidic Acid and Betamethasone 17-valerate as well as the Novel Low Molecular Weight Gelator *FMOC-OH-C18*

7 CHAPTER 7 – *In-vitro* Release Studies of Electrospun Fibers

As already discussed in Chapter 1, atopic dermatitis (also termed "atopic eczema") is a complex genetic skin disorder linked to a defect in a gene known as *FLG*, which is responsible for the production of *filaggrin*, a dermal protein involved in maintaining intactness and structural stability of the skin-barrier [1–3]. In diseased skin the absence of *filaggrin* can lead to barrier defects which expose the immune cells of the dermis to external antigens, resulting in an inflammatory response which is characterised by the typical atopic dermatitis related symptoms, such as itching and swelling. The first line of treatment for atopic dermatitis is topical therapy, including the application of emollients, keratolytics, topical vitamin D analogues as well as topical corticosteroids [4–6]. Due to the allergic component of the disease it is often advantageous to cover the affected area with a wet or dry dressing to avoid contamination by foreign antigens and microbes, which may exacerbate the symptoms or cause secondary infection [7–9]. Such occlusive dressings also have the added advantage of not having to be applied several times daily, as with for example, a topical cream. Additionally, a dressing may help to stop the wound from drying out, which is an important factor for successful healing [10].

7.1 *Introduction*

7.1.1 *Electrospinning for Biomedical Applications*

In the past decade several studies have investigated the possibility of incorporating drug substances, such as antibiotics, into wound dressings [11–14]. One of the most novel types of experimental dressing comprises of membranes manufactured from drug loaded ultra-thin fibers produced by electrospinning [15,16]. Electrospinning is a simple and cost-effective procedure involving application of a high voltage electric field to a drop of fluid, usually comprising of a mixture of a polymer such as Polylactide (PLA) or poly(caprolactone) (PCL, Figure 66) in a suitably volatile solvent. Application of the electric field causes the solution to become charged which in turn leads to droplet deformation (Taylor-cone formation). This results in the ejection of a charged jet from the tip of the cone which accelerates towards the counter electrode, leading to the formation of continuous fibers [17,18]. A schematic of the electrospinning set-up used in the current study is presented in Figure 67.

Electrospun fibres have huge potential for use in biomedical applications, including tissue engineering and as bioresorbable wound dressings [15–17,19,20]. One particularly interesting potential use of electrospun microfibers is in the topical- and

systemic delivery of drug substances. Electrospun fibres have a large surface area allowing for relatively high drug loading capacities and have the added advantage of

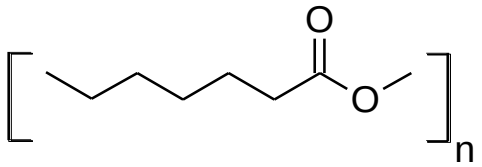


Figure 66 Chemical structure of poly(caprolactone)

being modifiable, i.e. fibre thickness, solubility and pore size may be modified allowing for a tailored release rate of the drug substance. Examples of electrospun fibres as drug delivery systems include the incorporation of the antibiotic Gentamicin into polylactide-based ultrathin fibres for the treatment of musculoskeletal infections [21,22]; the manufacture of fusidic acid and rifampicin co-loaded PLGA nanofibers for the prevention of orthopaedic implant associated infections [23] as well the production of dexamethasone encapsulated coaxial electrospun PCL/Poly(ethylene oxide) hollow microfibers for inflammation regulation [24].

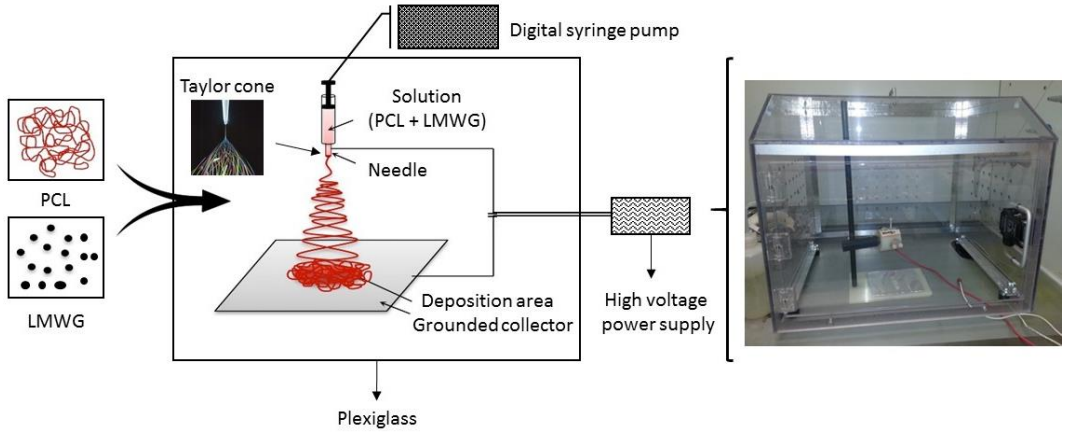


Figure 67 Schematic of the electrospinning apparatus used in the current study

7.1.2 ***Aim***

In this study the feasibility of incorporating the synthetic glucocorticoid BV and the antibiotic compound FA into electrospun PCL fibres as a potential topical therapy for infected atopic dermatitis will be investigated. The morphological, physicochemical, *in-vitro*-release- and antimicrobial properties of the resulting electrospun fibres will be examined. Additionally, the influence of the incorporation of a novel gelator, Fmoc-OH-C18 (General Procedures

Figure 68), on the morphology and manufacturability of the fibres, as well as on their drug release properties will be studied. Fmoc-OH-C18 is an *N*-Fmoc-L-serine lipoamino acid which can function as a low molecular weight gelator (LMWG), having the ability to form gels in a range of organic solvents of different polarity, including ethanol [25].

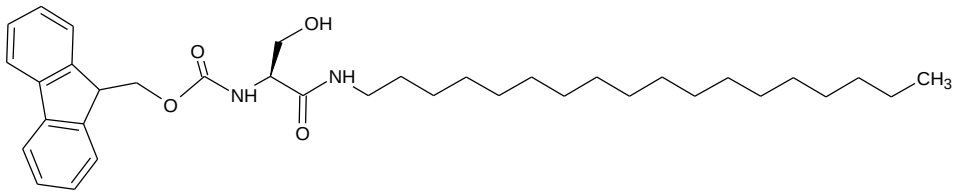


Figure 68 Chemical structure of FMOC-OH-C18

7.2 *General Procedures*

7.2.1 *Reagents*

Fusidic acid and Betamethasone 17-valerate were both of Ph. Eur. Quality and purchased from Ercros SA (Madrid, Spain) and Crystal pharma (Valladolid, Spain), respectively. Fmoc-OH-C18 was synthesised according to the procedure of *Ramos et al* [25]. Gradient grade methanol and acetonitrile as well as HPLC grade chloroform and ethanol were purchased from VWR International GmbH (Darmstadt, Germany) and Sigma Aldrich (Darmstadt, Germany). Purified water was obtained from the in-house purification system at mibe GmbH Arzneimittel (Brehna, Germany). Phosphoric acid (85 % m/m, analysis grade, d = 1.71 g/ml) was purchased from Merck (Darmstadt, Germany). Porafil® membranes made from regenerated cellulose and having a porosity and diameter of 0.45 µm and 25 mm were purchased from Macherey-Nagel (Düren, Germany). Poly(caprolactone) (M_n 70.000 – 90.000) was purchased from Sigma-Aldrich (Darmstadt, Germany). Electrospun fibers were manufactured according to the procedure described in section 2.6.

7.2.2 *Instrumentation*

Differential scanning calorimetry experiments were performed on a DSC Auto Q20 from TA Instruments equipped with TA 40 Refrigerated Cooling System (TA Instruments, Delaware, USA). The evaluation of thermograms was performed using the TA Instruments Universal Analysis 2000 software. Samples were measured by

equilibrating the sample for 5 minutes at 25 °C after which the temperature was ramped to 170 °C at 10 °C/Min. After holding at this temperature for 1 minute the sample was cooled to -30 °C at a rate of -35 °C/Min., held for 5 minutes at this temperature and then reheated to 250 °C at 10 °C/Min. Scanning electron micrographs were obtained using a Hitachi 3200N SEM instrument (Hitachi, Tokyo, Japan). *In-vitro* penetration studies as well as the associated HPLC measurements were performed according to the procedures described in section 2.4. The samples used in the experiment were cut from the original membranes described in section 2.6.2.

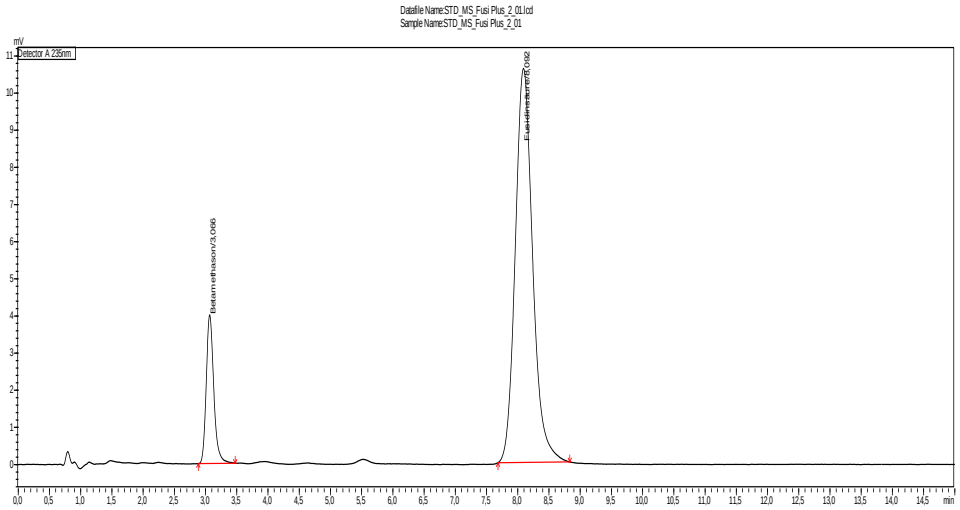


Figure 69 *Example HPLC Chromatogram showing the BV and FA peaks*

7.3 ***Results***

7.3.1 ***Morphology***

Figure 71 shows the morphology of the respective electrospun fibres after single loading with 5 %, 10 %, 15 % and 20 % of each drug substance. The scanning electron micrographs show significant bead formation which may be dependent on drug loading, since bead formation appears to be more frequent at lower loading percentages. The formation of homogenous, bead-free fibres was not possible at any of the drug loading concentrations. The addition of 0.15 % of the gelator FMOC-OH-C18 had a profound effect on the morphology of the resulting fibres. Figure 72 shows release curves and SEM images which compare the fibre morphology of electrospun co-loaded membranes prepared both with- and without the gelator. In the absence of the gelator it is not possible to effectively spin the solution into acceptable fibres and massive bead formation occurs. The presence of the gelator in the initial mixture, in contrast, results

in morphologically homogenous fibres with a diameter of ca. 2 µm. No bead formation was observed.

7.3.2 *DSC*

All membranes displayed a melting peak at 58 °C which could be associated with the melting of PCL. After quench cooling and recrystallisation a doublet peak was observed for PCL at ca. 54 °C in all samples. There was no evidence of thermal events due to either of the drug substances. Due to the rapid loss of solvent during electrospinning the drug substances are unlikely to have time to crystallise and are most likely present in an amorphous state. The addition of the gelator had no effect on the appearance of the thermogram.

7.3.3 *In-Vitro-Release – Membrane Permeation Study*

The results of the membrane permeation study of mono drug-loaded membranes show a typical burst release of the drug substance over the first 30 minutes of the test (Figure 71); followed by a tailing off over the remaining 150 minutes. After 180 minutes the drug substances are completely released from membranes with 5 % - 15 % drug loadings. The compositions of the different fibers are provided in Table 36. At 20 % drug loadings the drug substance was not completely released after 180 minutes and consequently, the measurement time was increased to 300 minutes with sampling intervals of 60 minutes for all subsequent studies. As demonstrated by Figure 72 the addition of the Fmoc gelator to co-loaded fibres does not significantly affect the release rates of either of the drug substances. These fibres also show typical release profiles with an initial burst followed by a slow tailing off. Both drug substances are completely released after 300 minutes.

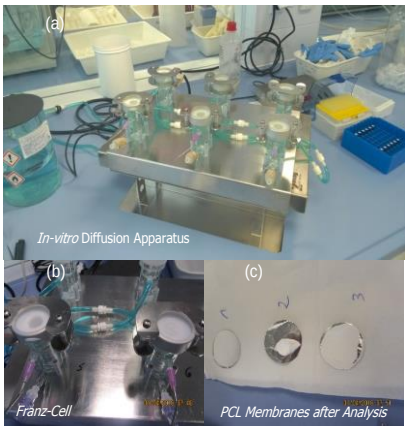


Figure 70 In-vitro membrane diffusion apparatus (a), Single Franz-Cell set-up (b) and membranes after analysis (c)

Table 36 Composition of the electrospun membranes employed in the in-vitro release test

Label	Concentration of FA in polymer solution	Composition	Label	Concentration of BV in polymer solution	Composition
A	5 % (w/w)	50 mg FA dissolved in 2 ml Ethanol + 2 ml of PCL solution comprising of 1 g PCL + 7.5 ml $CHCl_3$ + 2.5 ml Ethanol	E	5 % (w/w)	50 mg BV dissolved in 2 ml Ethanol + 2 ml of PCL solution comprising of 1 g PCL + 7.5 ml $CHCl_3$ + 2.5 ml Ethanol
B	10 % (w/w)	100 mg FA dissolved in 2 ml Ethanol + 2 ml of PCL solution comprising of 1 g PCL + 7.5 ml $CHCl_3$ + 2.5 ml Ethanol	F	10 % (w/w)	100 mg BV dissolved in 2 ml Ethanol + 2 ml of PCL solution comprising of 1 g PCL + 7.5 ml $CHCl_3$ + 2.5 ml Ethanol
C	15 % (w/w)	150 mg FA dissolved in 2 ml Ethanol + 2 ml of PCL solution comprising of 1 g PCL + 7.5 ml $CHCl_3$ + 2.5 ml Ethanol	G	15 % (w/w)	150 mg BV dissolved in 2 ml Ethanol + 2 ml of PCL solution comprising of 1 g PCL + 7.5 ml $CHCl_3$ + 2.5 ml Ethanol
D	20 % (w/w)	200 mg FA dissolved in 2 ml Ethanol + 2 ml of PCL solution comprising of 1 g PCL + 7.5 ml $CHCl_3$ + 2.5 ml Ethanol	H	20 % (w/w)	200 mg BV dissolved in 2 ml Ethanol + 2 ml of PCL solution comprising of 1 g PCL + 7.5 ml $CHCl_3$ + 2.5 ml Ethanol

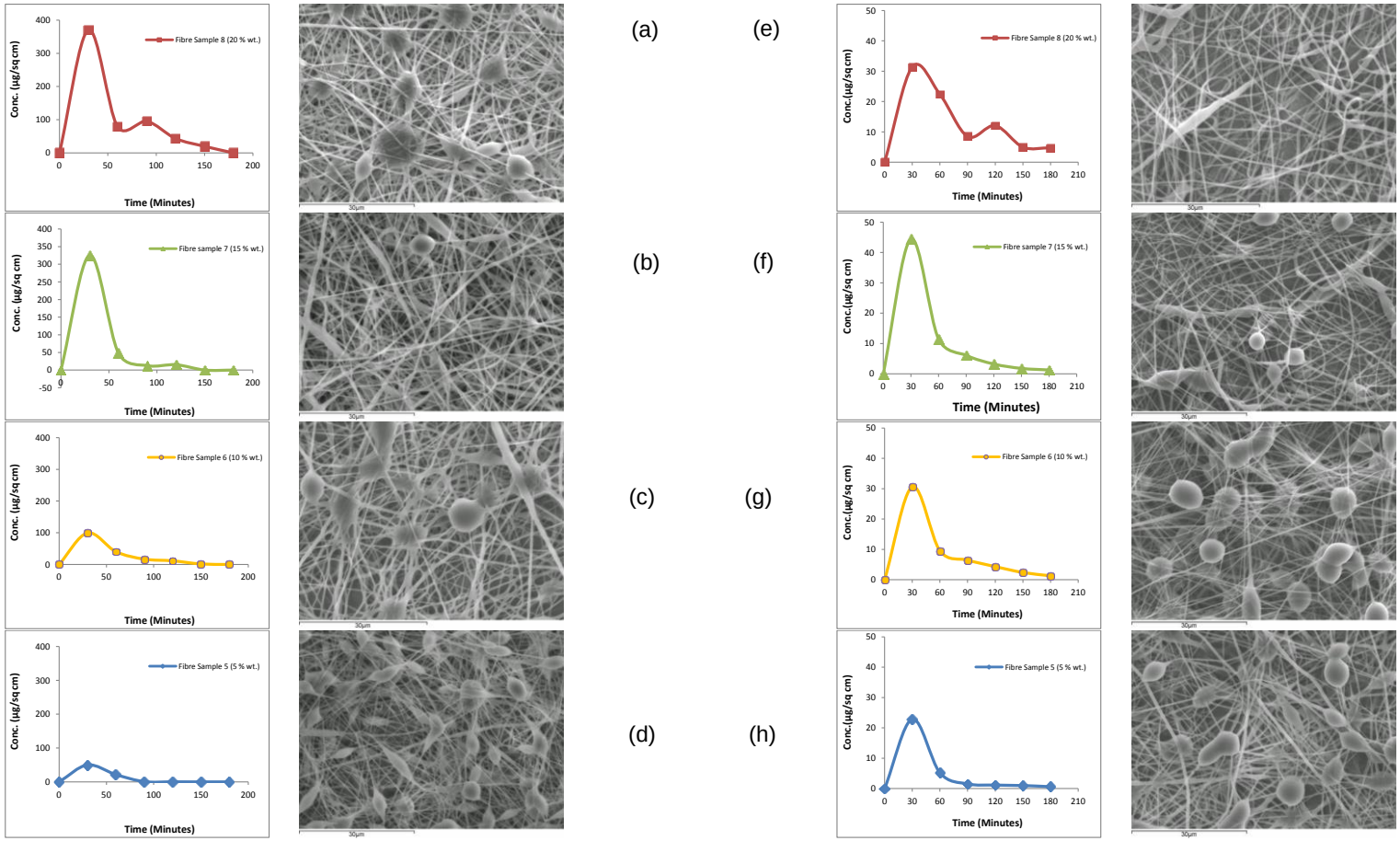


Figure 71 Release profiles and scanning electron micrographs of electrospun fibres with 5 %, 10 %, 15 % and 20 % loadings of FA (a-d) and BV (e-h). The displayed results are the average of n=6 measurements

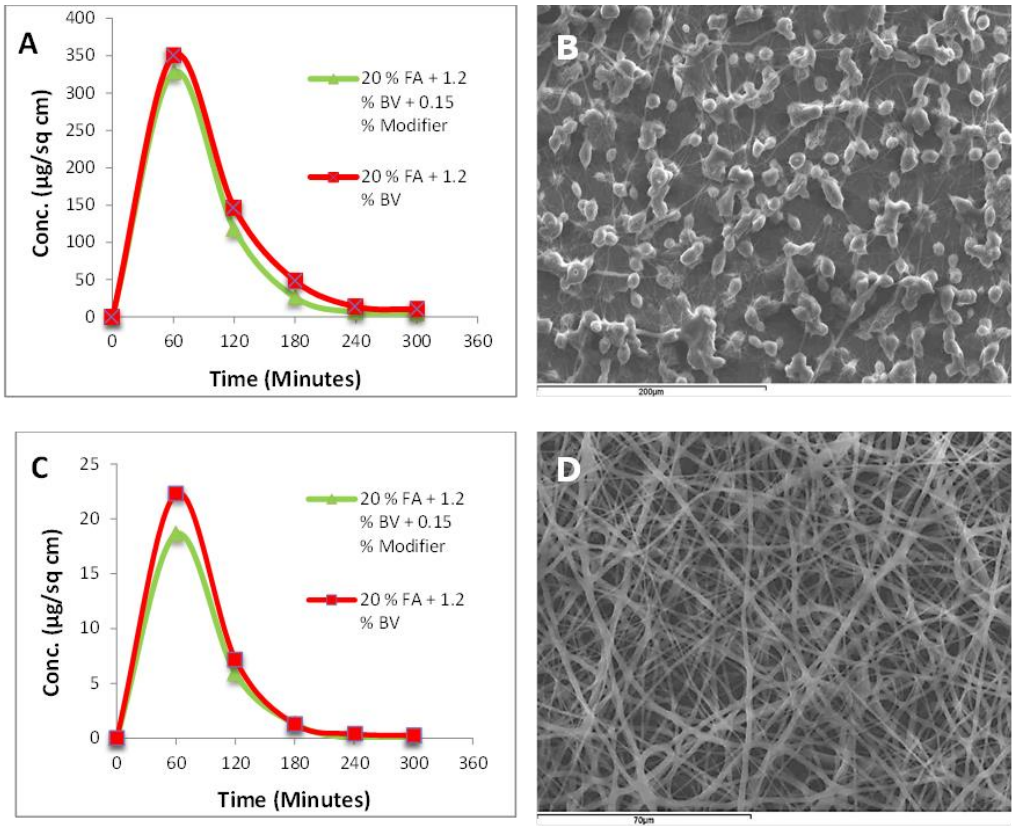


Figure 72 Comparison of drug release profiles of FA (panel A) and BV (panel C) with and without the addition of FMOC-OH-C18. Micrographs B and D show the morphology of fibres manufactured with (D) and without (B) the Fmoc gelator. The compositions of the respective fibers are equivalent to Product C and D in Table 37. The displayed results are an average of n=6 measurements

7.3.3.1 *In-Vitro Release – Agar Diffusion Test*

The pieces of electrospun membranes containing FA (B, C and D in Figure 73) show clear inhibition zones around them, whereas the control samples do not (A, E and F). The diameters of the observed inhibition zones (Hemmhof) are provided in Table 38. The size of the inhibition zones observed for the electrospun fibers B, C and D do not differ visually from each other, which suggests a similar antibacterial activity. Additionally, the size of the inhibition zone of sample D does not differ from that of sample C, indicating that the presence of the Fmoc gelator does not affect the release of FA from the electrospun membrane. This confirms the results of the *in-vitro* permeation study which also showed a similar release rate for membranes with and without the gelator. The results demonstrate that the bioactivities of electrospun fibres containing FA are similar to those observed for solutions with an equivalent concentration of pure FA. This can be observed by comparing the plates P1 and P2 with plate P3 in Figure 73.

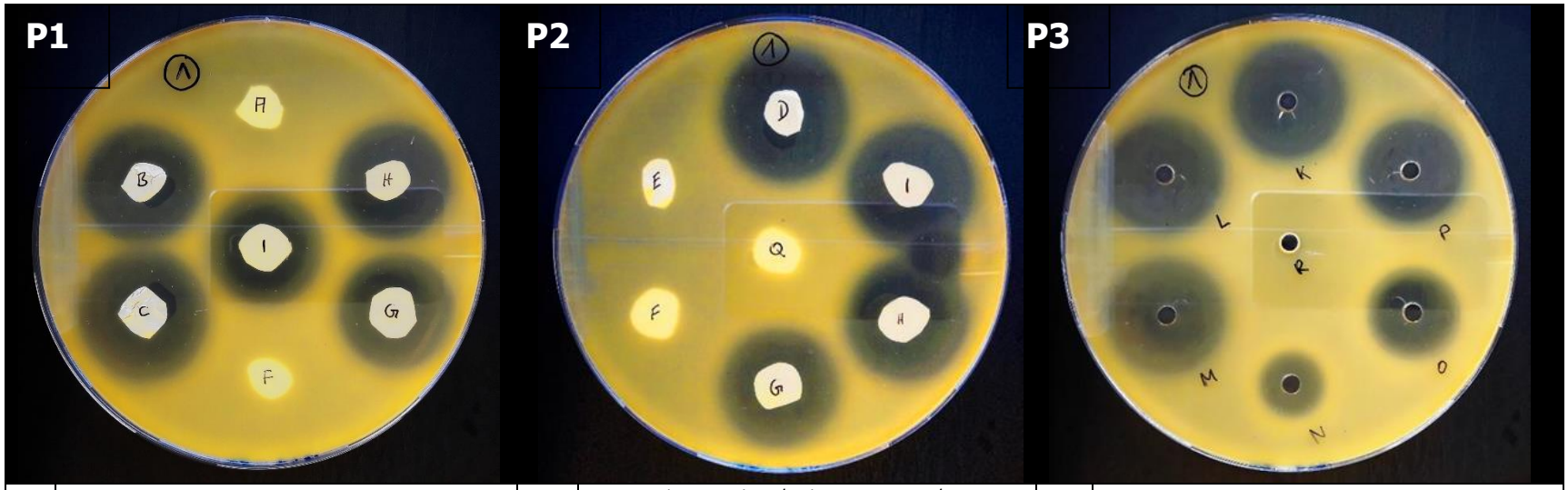
Overall, the results indicate that fusidic acid is released from the electrospun membranes at a sufficient rate and to a sufficient extent as to ensure an adequate antibacterial activity.

Table 37 Composition of the electrospun membranes employed in the agar diffusion test

Quantity of FA (µg) in the portion of membrane used for the experiment	**Composition of polymer solution used for electrospinning**
Control A Quantity of Fusidic acid: 0 µg	(CONTROL 1) 1.5 mg Fmoc-OH-C18 + 1 ml Ethanol + 2 ml PCL Solution (1 g PCL + 7.5 ml chloroform + 2.5 ml Ethanol)
Product B Quantity of Fusidic acid = ca. 230 µg	10 % FA (100 mg in 1 ml chloroform) 0.60 % BV (6 mg BV in 1 ml chloroform) 89.4 % PCL solution (8 ml of PCL solution made with 1 g of PCL, 7.5 ml chloroform, 2.5 ml ethanol)
Product C Quantity of Fusidic acid = ca. 450 µg	20 % FA (200 mg in 1 ml chloroform) 1.21 % BV (12.1 mg BV in 1 ml chloroform) 78.79 % PCL solution (8 ml of PCL solution made with 1 g of PCL, 7.5 ml chloroform, 2.5 ml ethanol)
Product D Quantity of Fusidic acid = ca. 380 µg	20 % FA (200 mg in 1 ml chloroform) 1.21 % BV (12.1 mg BV in 1 ml chloroform) 0.15 % JRO gelator 4 (1.5 mg Fmoc-OH-C18) in 1 ml Ethanol) 78.64 % PCL solution (8 ml of PCL solution made with 1 g of PCL, 7.5 ml chloroform, 2.5 ml Ethanol)
Control E Quantity of Fusidic acid: 0 µg	(CONTROL 2) PCL solution (99.85 %) and JRO gelator 4 (0.125 % Fmoc-OH-C18)

Table 38 Results of the Agar Diffusion Test showing the diameter of the resulting inhibition zone (Hemmhof) in mm for each sample

Sample Name	**Hemmhof [mm]**	**Comparable with sample**	**Hemmhof [mm]**	**Sample Name**	**Hemmhof [mm]**	**Comparable with sample**	**Hemmhof [mm]**
G	26.17	B	28.88	K	22.33	B	28.88
H	27.75	D	30.02	L	23.72	D	30.02
I	29.20	C	31.87	M	24.32	C	31.87
				N	14.90	< B	28.88
				O	19.79	< D	30.02
				P	23.36	< C	31.87



A	Control – Membrane with Fmoc-OH-C18/ without FA	D	PCL Membrane with BV/with Fmoc-OH-C18/ equivalent to 380 µg FA	K	50 µl FA solution equivalent to 200 µg FA
B	PCL Membrane with BV/without Fmoc-OH-C18/ equivalent to 230 µg FA	E	Control – Membrane with Fmoc-OH-C18/ without FA	L	50 µl FA solution equivalent to 350 µg FA
C	PCL Membrane with BV/without Fmoc-OH-C18/ equivalent to 450 µg FA	F	Blank – Filterpaper without FA	M	50 µl FA solution equivalent to 450 µg FA
F	Blank – Filterpaper without FA	G	Filter paper immersed in FA solution / equivalent to 200 µg FA	N	50 µl FA solution equivalent to 0.25 µg FA
G	Filter paper immersed in 25 µl FA solution / equivalent to 200 µg FA	H	Filter paper immersed in FA solution / equivalent to 350 µg FA	O	50 µl FA solution equivalent to 2.5 µg FA
H	Filter paper immersed in 25 µl FA solution / equivalent to 350 µg FA	I	Filter paper immersed in FA solution / equivalent to 450 µg FA	P	50 µl FA solution equivalent to 25 µg FA
I	Filter paper immersed in 25 µl FA solution / equivalent to 450 µg FA	Q	Control – Filter paper with buffer pH 6.0 / without FA	R	Control – buffer pH 6.0 / without FA

Figure 73 Demonstration of the bioactivity of FA released from the drug-loaded electrospun membranes against on example strain b - Staphylococcus aureus ATCC 6538

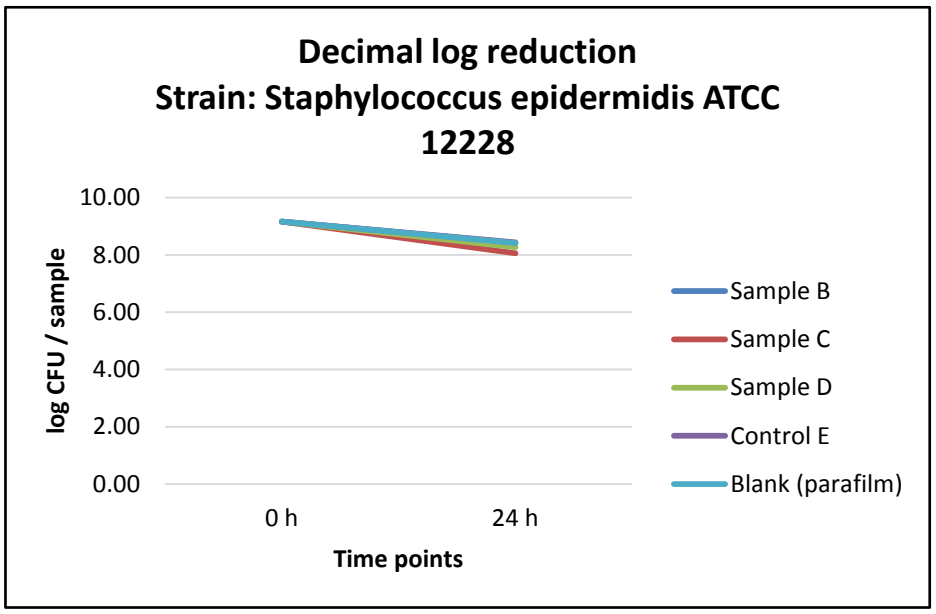


Figure 74 Plot of the log reduction of the bacterial count versus time – Method A. Sample descriptions are provided in 2.7

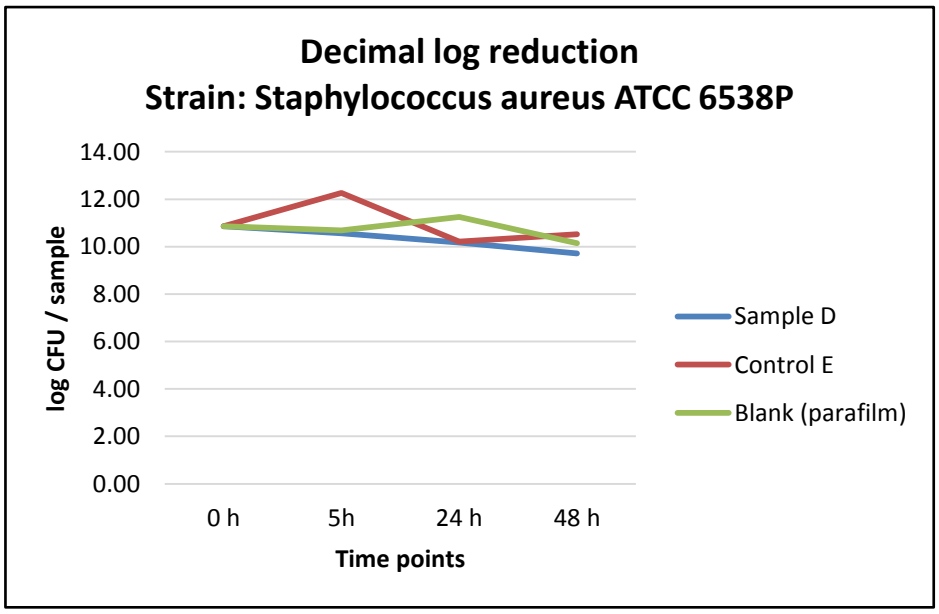


Figure 75 Plot of the log reduction of the bacterial count versus time – Method B. Sample descriptions are provided in 2.7

7.3.3.2 *Antimicrobial Efficacy*

At clinically relevant concentrations fusidic acid works primarily as a bacteriostatic agent, i.e. it prevents growth but doesn't directly kill the bacteria. However, at higher concentrations it may also work bactericidal. The agar diffusion test provides useful information about the release rate and inhibitory action of an antibiotic but it doesn't allow any conclusion to be drawn about the mode of inhibition. As described in section 2.7.1, two methods (A & B) were used to evaluate the antimicrobial efficacy. The

results of both methods show a drop of bacterial count of approximately 1 decimal log reduction. This indicates that FA is functioning predominantly bacteriostatically, and not bactericidal, at the investigated concentration. In order to be considered to have a bactericidal effect, FA would have to demonstrate a minimum of 3 decimal log reductions over 2 days (Ph. Eur. 5.1.3 cutaneous use), which is equivalent to an approximate 100 times greater efficacy than observed. The blank (parafilm) and control samples also showed a log reduction of the bacterial count which was comparable to that observed for the membranes which contained FA. It might be expected that the bacterial count in these samples should have increased as compared to the membranes containing FA. The lack of bacterial growth in these samples may possibly be attributed to innate antimicrobial attributes of parafilm and polycaprolactone filaments or due to a disturbance of the micro environment required for the bacteria to propagate. This phenomenon would need to be investigated in further experiments.

7.4 *Discussion and Conclusions*

The aim of the work in this chapter was to investigate the feasibility of incorporating the antibiotic FA and the glucocorticoid BV into electrospun fibres for use as an occlusive bandage in the treatment of infected atopic dermatitis. It has been demonstrated that it is possible to produce electrospun fibers loaded with clinically relevant concentrations of both drug substances. Membranes were produced containing 20 % (w/w) and 1.21 % (w/w) of FA and BV, respectively. The effects of the addition of the novel gelator Fmoc-OH-C18 on morphology, manufacturability, *in-vitro* release and antimicrobial efficacy of the resulting fibers have been investigated. *In-vitro* membrane permeation studies have demonstrated that both drug substances are released in a rapid initial burst and that the addition of the gelator does not significantly influence the drug release rate. It has been shown that the addition of the Fmoc gelator has a profound effect of the morphology of the resulting fibers. Massive bead formation was observed in the absence of the gelator. It is considered possible that the gelator induces a change of the viscosity and/or surface tension of the polymer solution which in turn affects its spinability. This phenomenon has been described in the literature [39]. This would suggest that a critical surface tension is required in order for fibers to be drawn from the solution. This hypothesis would need to be tested further. Antimicrobial efficacy

studies have demonstrated that FA is released adequately from the electrospun fibers and that the resulting membranes have an efficacy against the chosen test organisms similar to that of a solution of equal concentration of the pure substance.

Based on the initial experimental results it appears plausible that a drug loaded bandage containing FA and BV for the treatment of atopic dermatitis could be manufactured by electrospinning. Significant further development work is however needed before a proper conclusion can be drawn. The work in these studies only investigated the *in-vitro* efficacy of the antibiotic in the fibres, but did not address the efficacy of the glucocorticoid. Although the *in-vitro* membrane permeation studies indicate a good release of BV, further tests would be necessary. It would need to be shown, in an animal model for example, that the quantity of steroid released is sufficient to give rise to the desired anti-inflammatory response. This could be performed using a suitably designed vasoconstriction assay. Furthermore, the effects of manufacturing variables, e.g. voltage, needle gauge size, solution flow rate, solution viscosity, surface tension, etc. on the morphology and drug release rates of spun fibers needs to be thoroughly investigated, so that the process is completely characterised. Only then would it be possible to conclude whether the process is reproducible, i.e. whether fibers with a consistent drug content, fibre thickness, etc., can be produced which also release the drug substances at a suitable and reproducible rate. In summary, the process would need to be fully understood and validated before pre-clinical trials could be considered.

7.5 *References*

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8 General Conclusions

The aim of the research performed in this thesis was initially described at the end of chapter 1. The first goal was to develop and validate analytical procedures to enable the stability-indicating analysis of the drug substances and preservatives of interest in formulation candidates. Novel procedures for the analysis of both content and purity were developed and their validity and suitability for the analyses of BV, FA and respective preservatives in cream matrices has been demonstrated.

As planned, the developed procedures were employed to study formulation factors affecting the isomerisation of betamethasone 17-valerate in semi-solid pharmaceutical formulations. It has been demonstrated that both pH and emulsifier concentration are critical with regard to this process. With the help of both microscopy and HPLC experiments it was possible to show that increasing the concentration of emulsifier leads to an increase of the solubility of BV in the aqueous phase of the oil-in-water cream. It was shown that after dissolution the rate of isomerisation is then dependent on the pH of the surrounding matrix. If the pH is low (< 5) then a lower rate of isomerisation is observed than if the pH were higher (>5). It could be shown that the rate of isomerisation can be kept to a minimum by optimising both pH and emulsifier conditions. Maximum stability can be obtained at pH values below 4 and emulsifier concentrations below 1.5 % (w/w). The data gathered through these studies was used to develop a stable cream product which began being marketed in early 2017.

A further goal of the work in this thesis was to investigate the prevalence of polymorphism in commercial sources of FA and to compare the solubilities- and *in-vitro* release rates of any polymorphs observed. This was necessary since the existence of polymorphism may affect the bioavailability of a drug substance from a drug product due to solubility differences between different polymorphic forms. It was demonstrated that Form I and Form III are currently available on the commercial market. Measurements of the intrinsic dissolution rates of both forms indicated that they have comparable solubilities. *In-vitro* membrane permeation studies indicate that both polymorphic forms demonstrate a similar bioavailability from the developed topical cream. It could be concluded that both polymorphic forms are bioequivalent and can be used in semi-solid pharmaceutical preparations.

The final aspect of this research involved investigating the plausibility of incorporating BV and FA into electrospun poly(caprolactone) fibers for use as an

occlusive bandage for the treatment of infected atopic dermatitis. The study results suggest that both drug substances can be incorporated into electrospun fibers in a clinically relevant concentration and that the use of a medicated occlusive bandage could present an alternative to current topical treatments. *In-vitro* release studies have indicated that both BV and FA are released in a short initial burst followed by a longer tailing off phase. It has been demonstrate through agar-diffusion testing that the antibiotic activity of FA released from the electrospun membranes is equivalent to a similar concentration of the pure substance, indicating that FA is released at a sufficient rate from the fibers as to ensure an effective antibacterial activity. Based on the initial experimental results it appears plausible that a drug loaded bandage containing FA and BV for the treatment of atopic dermatitis could be manufactured by electrospinning. As described in Chapter 7, significant further development work is needed before a proper conclusion can be drawn.

9 Publications

The following first author publications arose out of the work in this thesis:

1. **Byrne, J**., Velasco-Torrijos, T. & Reinhardt, R. Development and validation of a novel stability-indicating HPLC method for the simultaneous assay of betamethasone-17-valerate, fusidic acid, potassium sorbate, methylparaben and propylparaben in a topical cream preparation. *J. Pharm. Biomed. Anal.* **96,** (2014).

2. **Byrne, J**., Velasco-Torrijos, T. & Reinhardt, R. An RP-HPLC method for the stability-indicating analysis of impurities of both fusidic acid and betamethasone-17-valerate in a semi-solid pharmaceutical dosage form. *J. Chromatogr. Sci.* **53,** (2015).

3. **Byrne, J**., Wyraz, A., Velasco-Torrijos, T. & Reinhardt, R. Formulation factors affecting the isomerization rate of betamethasone-17-valerate in a developmental hydrophilic cream–a HPLC and microscopy based stability study. *Pharm. Dev. Technol.* **22,** (2017).

4. **Byrne, J**., Reinhardt, R. & Velasco-Torrijos, T. Polymorphism in Commercial Sources of Fusidic Acid: A Comparative Study of the *In Vitro* Release Characteristics of Forms I and III from a Marketed Pharmaceutical Cream. *J. Anal. Methods Chem.* **2017,** 1–7 (2017).