STABILITY AND *IN VIVO* ACTIVITY OF TOPICAL CORTICOSTEROID DILUTIONS.

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THESIS SUMMARY

A stability-indicating assay for fluocinolone acetonide and fluocinonide was developed using high-performance liquid chromatography (HPLC). The degradation of fluocinonide resulted in a primary degradation product which was identified as fluocinolone acetonide. This is turn degraded to a series of unidentified products which had similar retention times to those produced when fluocinolone acetonide was used as the original steroid. The disappearance of both steroids followed first-order kinetics.

The HPLC assay method was used to investigate the shelf-life of Synalar® ointment (fluocinolone acetonide 0.025% w/w) and Metosyn® cream (fluocinonide 0.05% w/w) diluted 1 in 4 and 1 in 10 in Lipobase® and Unguentum Merck®. In addition, Metosyn Diluent® was studied for that product. The formulations were stored at 4°C, 25°C and 32°C for a maximum of ten months.

The 1 in 4 formulations of Synalar® showed no loss of steroid in the first four months of storage at 25°C. The 1 in 10 formulations were markedly less stable with shelf-lives of 1-2 months. The 1 in 4 formulations of Metosyn® were all stable for nine months at 25°C. The 1 in 10 formulations were stable over the seven months studied. Storage of the dilutions at 4°C can extend the shelf-life of the products. Elevation of temperature had no significant effect.

The *in vivo* activity of extemporaneous formulations of Synalar® was compared to that of the undiluted steroid, the commercially available diluted product and the bases used by means of a vasoconstrictor assay. The blanching potency of the 1 in 4 dilutions was not significantly different to that of the undiluted steroid, $p<0.05$, and that of the 1 in 10 dilutions was not significantly different to that of the bases alone, $p<0.05$. The two groups were significantly different, $p<0.05$, although there was some overlap between the 1 in 4 dilutions and the 1 in 10 in Lipobase®. There was no linear relationship between dilution and potency, but the choice of base did have an effect on the potency of the formulation.

KEYWORDS: Fluocinolone acetonide, fluocinonide, stability, topical corticosteroids, vasoconstriction.

DEDICATION

I would like to dedicate this thesis to my family, with love and thanks for all their support, guidance and encouragement, but most of all their patience.

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1. INTRODUCTION

1.1 PSORIASIS AND ECZEMA

Topical corticosteroids are widely prescribed in the treatment of the skin conditions psoriasis and eczema.

Psoriasis affects 2% of the population of the United Kingdom, and although it occurs equally in males and females it shows significant racial and ethnic variation. It is a genetically inherited disorder and it is believed that it is the susceptibility to psoriasis that is inherited rather than the condition itself[1]. Precipitating factors stimulating the development or exacerbation of the condition include streptococcal throat infection, hormonal factors (psoriasis often appears at puberty and may be affected by pregnancy), trauma (such as a scar or a cut giving rise to the Kobner phenomenon), drugs (lithium, chloroquine, some beta-blockers, withdrawal of high-dose steroids), and stress.

In normal skin, cells divide in the lower layers of the epidermis and then gradually migrate to the outer layers undergoing flattening and keratinisation. They then die and are slowly shed. This process takes about thirty days but in psoriasis cell turnover takes only five days[2] as the production of new epidermal cells is increased by 30 fold. Live and dead cells occur together at the skin surface producing the characteristic raised red plaques covered with silvery scales. There are several forms of psoriasis; guttate, chronic plaque, flexural, pustular (which may be generalised or localised), palmoplantar pustulosis, psoriasis of the nail, napkin psoriasis and psoriatic arthropathy. The condition occurs most frequently on the scalp, knees and elbows but it may be more widespread[1].

Treatment is by topical therapy except in severe cases where systemic therapy may be administered under hospital supervision. Emollients are used to moisturise the skin and increase the patient's comfort. Tar and dithranol are both effective topical treatments but may be messy to use and the latter also causes burning and staining of the skin. Most patients benefit from exposure to ultraviolet light, although a few patients find this makes their condition

worse. Long-wave ultraviolet light (UVA) is also used in conjunction with 8-methoxypsoralen in the regimen known as PUVA, utilising the photosensitizing properties of the psoralen which is given systemically. Side-effects include a 12-fold increase in the incidence of skin cancer, and a risk of cataract formation. The vitamin A retinoid derivative etretinate is effective alone or in combination with PUVA but its long half-life and teratogenicity means that women patients must not become pregnant while taking the drug, nor for two years after treatment has finished. Other side-effects include calcification of the spinal ligaments which requires monitoring using X-rays. Cytotoxic therapy, for example with methotrexate, is another effective systemic therapy which is limited in its use by its side-effect profile. Methotrexate causes bone marrow toxicity and hepatotoxicity which must both be monitored during treatment.

Due to the hazards of systemic therapy, topical remedies are used in the majority of cases. Topical corticosteroids are frequently prescribed because they are cosmetically acceptable and provide a rapid symptomatic response. However, they only suppress the condition but produce no remission of the disease, so the symptoms return when the steroid is withdrawn. The patient therefore tends to apply the formulation indefinitely in large quantities. The skin also becomes resistant to the anti-inflammatory action of steroids such that a more potent steroid is required to produce an equivalent response. The application of large quantities of potent steroids can result in topical side-effects including skin thinning, skin atrophy and acne. In chronic use, systemic side effects causing suppression of the pituitary-adrenal axis and hypercorticism may result[2]. Topical corticosteroids are, therefore, not the treatment of choice in chronic psoriasis.

Eczema is a term used to describe a wide variety of inflammatory skin conditions. It is derived from the Greek word meaning "to boil over" and is synonymous with the Latin word dermatitis which means "inflammation of the skin"[1].

Eczema may be endogenous where the main cause of the inflammation is internal, or exogenous where the condition is caused by contact with irritants and allergens. However, in practice many eczemas have a multifactorial basis[1].

An eczema is almost always itchy and may show a combination of other features including erythema, exudation, crusting and scaling. Thickening of the skin may result following continued scratching. The terms acute, subacute and chronic are used to describe the severity of the disorder and its duration. Different types of eczema are more common in some age groups than others, such as napkin dermatitis which is caused by occlusive contact of the skin with urine or faeces and is an irritant contact dermatitis. Asteatotic eczema is a drying out of the skin especially seen on the legs of the elderly in the cold weather. Atopy is a tendency to develop hypersensitivity to allergenic substances. Atopic eczema can occur at any time in life, presenting with intense itching of the face and flexures. About 3% of children have the condition and about 25% of the population are atopic and therefore prone to this form of eczema.

The type of eczema determines its management. General measures are appropriate for most types and include avoidance of irritants, use of soap substitutes and emollients, and the use of oral antipruritic drugs such as chlorpheniramine or terfenadine. Topical corticosteroids are indicated in this condition using the lowest potency that is efficacious. Hydrocortisone is the strongest steroid which may be applied to the face. If an infection is present, as may occur in napkin dermatitis or seborrhoeic eczema, a fungicidal preparation is required in addition to the steroid, such as Daktacort® which contains 2% miconazole in addition to 1% hydrocortisone. In severe infections a systemic antibiotic may be required. Erythromycin is usually suitable although *eczema herpeticum* requires treatment with acyclovir. Hand and foot eczema requires potassium permanganate soaks (1:8000) in the acute phase, with potent topical steroids added to therapy as the condition is improving. Topical steroids are not helpful in *pityriasis alba* but coal tar preparations may be beneficial[1].

1.2 POTENCY OF TOPICAL CORTICOSTEROIDS

Topical corticosteroids are classified into groups according to their relative strengths. The British National Formulary[3] uses four categories from class I (mild) through to class IV (very potent). However, this system may not adequately distinguish subtle biological differences which develop over several months of treatment, effecting the efficiency of the control of the pathological process and the length of remissions and occurrence of side effects[4]. In addition there are several problems in classifying corticosteroids into potency groups which may cause confusion amongst clinicians when choosing the most appropriate formulation. Gibson et al[5] summarised these problems as follows;

i) it is not clear which property of the corticosteroid is used to define its potency (e.g. adrenal suppression, clinical efficacy, skin thinning potential),

ii) preparations exist within the same potency group which have significantly different clinical effects. This is not reflected in the potency definitions,

iii) the choice of formulation may be important in different skin conditions.

In the United States of America the classification system is extended to seven groups and potency is evaluated using a vasoconstrictor assay and a clinical efficacy trial of topical corticosteroids in the treatment of cutaneous disease[4,6,7]. Vasoconstriction appears to be a fundamental property of the steroids and it has been shown to correlate well with clinical efficacy[5,8]. The vasoconstriction assay was first described by McKenzie and Stoughton in 1962[9] and has become a widely used method of comparing the potency of topical corticosteroids and their formulations. The assay is easy to perform, permits the simultaneous evaluation of a series of compounds, exposes the individual to only small amounts of the test substance for a short period of time, and is reproducible[8].

An alternative human assay system is based on the ability of corticosteroids to suppress inflammation produced by exposure to ultraviolet irradiation. This was described by Burdick et al[10]. An individual minimum erythema dose (MED) is determined for each subject and the forearm is irradiated with the MED immediately prior to application of the steroids. The study is then performed in the same manner as the vasoconstrictor assay. The authors compared ultra-violet erythema suppression to vasoconstriction and showed a close correlation between the results of the two assays in ranking of corticosteroid preparations.

1.3 REFLECTANCE SPECTROPHOTOMETRY

The traditional method of measuring vasoconstriction was to devise a numerical index to quantify the degree of skin blanching induced by the corticosteroid being studied. Assessment was made by visual, subjective assessment by trained individuals.

Dawson et al[11] developed a theoretical treatment for the optical properties of a layered structure which absorbs and scatters light. This theory predicts that the logarithm to the base 10 of the inverse of reflectance (LIR) of the surface should be a useful parameter for the examination of that structure. This theory was applied to a study of non-pigmented skin *in vivo* where the reflectance (R) of the skin can be expressed as:

$$R = I / Io$$

where I = intensity of light reflected from test surface, and

Io = intensity of light reflected from white surface.

This theoretical model was used to develop a skin spectrophotometer which was used in the clinical situation to measure the light intensity reflected from the visible region of the spectrum (450-760nm).

Changes in skin haemoglobin content were quantified following application of vasoconstricting preparations. Erythema and melanin pigmentation, induced by exposure of the skin to ultraviolet radiation, were also quantified. The accuracy and sensitivity of the instrument was assessed using a series of

pink tiles of known concentration of colour pigment. The ranking correlation coefficient was never less than 0.93 and had an average of 0.97[11]. Subjective assessment by visual inspection gave an average ranking correlation coefficient of 0.48 with individual values ranging from 0.32 to 0.67. The instrumental method of assessment was concluded to be more sensitive, accurate and reproducible than human observation.

Feather et al[12] have developed the Haemelometer, which is a simpler, more portable reflectance instrument for use in the clinical situation, plate 1.1. The instrument has three sections, a power supply, which alters the mains voltage, an electronic drive and signal processing unit, and a skin reflectance measuring head. The head is lined with black perspex and has nine light emitting diodes positioned at 45° to the horizontal to produce uniform illumination of the skin. A silicon photodiode detector is positioned vertically above the illuminated area of skin and detects the light which has been reflected from the skin. Reflectance measurements are made at three wavelengths in order to obtain indices for both haemoglobin and melanin. The skin is illuminated with green (566nm), orange (640nm) and red (670nm) light. The differential absorption of light of wavelengths 640nm and 670nm provides a measure of the gradient of the melanin absorbance curve and therefore of the quantity of cutaneous melanin. The melanin content can therefore be defined as the difference in LIR between 640nm and 670nm. Blood strongly absorbs the green light with a peak wavelength of 566nm compared with the light of wavelengths 640nm and 670nm. Therefore the haemoglobin index is defined as the difference in LIR between 566 nm and 640 nm. The electronic signal processing unit produces a reading in LIR units on a digital display. The instrument can be used on all body sites that are large enough to present a flat surface to the measuring aperture in the head, including those sites subject to movement, giving rapid quantification of cutaneous haemoglobin and melanin content.

Plate 1.1 THE HAEMELOMETER

Reflectance spectrophotometry was used by Feather et al[13] to observe the degree of vasoconstriction induced by topically applied corticosteroids. They compared their results with those obtained by subjective assessment by Barry and Woodford[14]. The average instrumental blanching index correlated well (correlation coefficient 0.97) with the area under the curve indicator used by Barry and Woodford[14]. Feather et al[13] found that the coefficient of variation of the blanching indices was 16.6%, with considerable variation in response to the steroids between subjects.

The authors concluded that the instrumental assessment offered no advantage over the subjective method if an average relative effectiveness was all that was required, but the instrumental method was preferable where information on individual patient response to an applied corticosteroid was required, giving greater sensitivity and reduced reliance on the visual skill of the observer.

1.4 THE INCIDENCE OF DILUTION

Despite the wide range of topical corticosteroids commercially available many physicians prescribe a preparation which requires extemporaneous formulation by the pharmacist. Extemporaneous dilution of proprietary topical corticosteroids is controversial but widespread. Wilcock[15] surveyed the prescribing habits of hospital dermatologists at the Manchester Skin Hospital. One in nine of the medicinal items dispensed required reformulation, proprietary topical corticosteroids being involved in 36% of these preparations. Of those preparations which required substantial dilution, 73% involved the use of a diluent not included in the External Diluent Directory[16]. In the majority of cases no stability data relating to the compounded product were available.

The problem was also highlighted by Smith[17] in 1982 who surveyed prescribing habits in general practice. He reported that 11.2% of the topical corticosteroids prescribed in one month required reformulation by the pharmacist, and 83% of these used an unrecommended diluent. Smith hypothesised that the practice of dilution and compounding of topical corticosteroids is initiated in hospital and extrapolated to general practice.

1.5 REASONS FOR DILUTION

Dermatologists dilute proprietary products to minimise unwanted side effects which arise from prolonged or excessive use, especially in children. These include skin thinning, telangiectasia, and adrenal atrophy. Gibson et al[5] investigated the effect of dilution on clinical efficacy and skin thinning potential. Where dilution of a corticosteroid preparation in its recommended diluent produced a significant reduction in vasoconstrictor potency, this was accompanied by a decreased clinical efficacy and skin thinning potential. Extemporaneous dilution of a proprietary product does not produce a formulation with the same clinical effect but a reduced local side effect potential. Although the diluted preparation may still be potent enough to treat the condition it should not be interpreted as having exactly the same effect. Dilution may be required when a particular steroid is not available in a low strength commercially, as many clinicians argue that steroidal preparations are not interchangeable[18].

1.6 THE DANGERS OF DILUTION

The dangers of dilution were reviewed by Busse[19], and can be categorised into pharmaceutical, biopharmaceutical and bacteriological considerations.

i) *Pharmaceutical*

Pharmaceutical stability is determined by the physical and chemical compatibility of the corticosteroid product and the diluent chosen. Incompatibility may result in obvious physical instability, such as cracking of a base. Less obvious is the problem of stability of the active corticosteroid ingredient. Inappropriate dilution may result in accelerated degradation of the active drug to an inactive form, or a form with a different activity to that which was intended, resulting in formation of a product with an unpredictable pharmaceutical profile. For example, the dilution of betamethasone-17-valerate cream with a neutral to alkaline base, such as emulsifying ointment, can result in the conversion of the 17-ester to the less active 21-ester[20].

ii) *Biopharmaceutical*

The percutaneous absorption of a drug molecule from a topical preparation depends on physiological factors associated with the patient, e.g. site of application, skin hydration and skin temperature, the physicochemical properties of the drug, and the properties of the vehicle[21,22]. Modification of molecular structure, to change the partitioning and solubility characteristics and alter the rate of penetration through the stratum corneum, has been used extensively for corticosteroids.

Busse[19] highlighted the importance of the choice of vehicle. The release of steroid from a topical formulation is dependent on the nature of the vehicle. Dilution with an inappropriate base can lead to unsatisfactory conditions for release. The vehicle provides a means of maintaining the drug at or close to the topical absorption site and controls the drug activity, the rate of diffusion in the vehicle and the partition coefficient between the vehicle and the skin[23]. Dempski et al[22] demonstrated that the *in vitro* release of medicinal agents from topical bases is dependent upon the solubility of that agent in both the base and its surrounding media. They investigated the release of a drug substance into an aqueous environment and used this as a model to assess the release of the drug from a topical formulation into the stratum corneum and the subsequent transfer of that agent into the epidermis. However, the stratum corneum is usually considered to be a lipid matrix but the authors did not discuss this discrepancy.

The percutaneous penetration rate is directly proportional to the concentration of dissolved drug in a formulation[21]. Where the drug has a low solubility in the vehicle and is in suspension within the vehicle, the release rate may be controlled by diffusion within the vehicle. In this case it is directly proportional to the square root of the concentration; therefore to achieve a doubling in the rate the concentration must be increased four-fold. Turi et al[21] stated that, since the release rate of the active ingredient is an important factor affecting topical bioavailability, the development of topical formulations should include the use of theoretical models and appropriate experiments to quantify drug-vehicle interactions and topical bioavailability.

iii) *Bacteriological*

Proprietary topical corticosteroids are manufactured under conditions designed to avoid microbiological contamination. Preservatives are added where necessary to reduce "in-use" contamination to a minimum. Dilution is dangerous from a bacteriological viewpoint because;

a) on dilution the preservative may be diluted out below its effective level, or
b) it may be inactivated due to an incompatibility,
c) extemporaneous dilution in a busy retail pharmacy is unlikely to occur under the aseptic conditions appropriate to produce a microbiologically safe product.

Barrett[24] also reviewed the pitfalls in the extemporaneous formulation of dermatologicals. He suggested that there is probably not a linear relationship between dilution and potency.

This hypothesis has been confirmed by many authors and is discussed in chapter 1.7.

1.7 RELATIONSHIP BETWEEN DILUTION AND POTENCY

Gibson et al[25] investigated a wide range of corticosteroids and their dilutions. They demonstrated that clobetasol propionate 0.05% (Dermovate®), a very potent ointment, diluted one part in ten in white soft paraffin, gave rise to a preparation equipotent to betamethasone valerate 0.1% (Betnovate®), which is classified as a potent topical corticosteroid. Potency was assessed using the vasoconstrictor assay technique to determine the degree of blanching produced by each preparation. There was no significant difference in potency between clobetasone butyrate 0.05% (Eumovate® ointment) a moderately potent preparation, Betnovate® or beclomethasone dipropionate 0.025% (Propaderm® ointment), which are both classified as potent preparations.

Allenby and Sparkes[26] reported that Eumovate® is clinically equal or possibly superior to hydrocortisone 17-butyrate 0.1% (Locoid®), which is classified as a potent preparation.

Ryatt et al[27,28] investigated the effect of serial dilution of Betnovate® ointment in emulsifying ointment and Unguentum Merck® using a modified version of the blanching assay. Betnovate® was diluted 1:4 in emulsifying ointment and the blanching index measured by reflectance spectrophotometry using ten male volunteers[27]. The average blanching index for the undiluted steroid was 0.62 and that for the freshly made dilution was 0.63, which are not significantly different.

The same steroid was diluted four, eight, sixteen and thirty-two-fold in emulsifying ointment and the blanching indices investigated using two male volunteers[27]. The four, eight and sixteen-fold dilutions showed similar potencies as demonstrated by the blanching indices; 0.70, 0.75 and 0.73 respectively, with a rapid loss of effectiveness on diluting thirty-two-fold; blanching index 0.95. However, since only two volunteers were used in the latter study, more data are required before any conclusions can be drawn. Also, the data from the undiluted steroid were not quoted which would have given a complete set of results for this small group.

Betnovate® ointment was diluted four, sixteen and thirty-two-fold in Unguentum Merck® and the blanching potential investigated in ten male volunteers[28]. Statistical analysis showed that the preparations could be split up into two significantly different groups; the Unguentum Merck® base in one group and the preparations containing active steroid in the other. There was no statistical difference between the blanching indices of any pair of diluted preparations demonstrating that the various dilutions of Betnovate® ointment in Unguentum Merck® were equipotent.

A Belgian study[29] investigated the potency of betamethasone-17-valerate (Betnelan-V®) cream, diluted with Beeler's basis and Cold cream®, by measuring skin blanching in eight volunteers. The blanching indices of the steroid, diluted 1:2 and 1:3 in each base, were not significantly different to the blanching produced by the undiluted steroid. Since dilution did not produce the expected reduction in potency, the steroid was subjected to further dilution and the potency investigated in only three volunteers. The 1:5, 1:10

and 1:20 dilutions in Beeler's basis showed no difference in potency to the undiluted Betnelan-V® cream. However, when the steroid was diluted in Cold cream®, the formulations could be divided into two statistically different groups. Undiluted Betnelan-V® cream and the 1:5 diluted formulation were significantly more potent than the 1:10 and 1:20 dilutions; the latter two preparations were equipotent. The blanching potential of each base alone was not investigated in this study.

The clinical efficacy of Betnovate® ointment diluted 1:4 was compared to that of the undiluted steroid by means of a blanching assay using ten volunteers. Gibson et al[30] demonstrated that an extemporaneous dilution, 1:4 in white soft paraffin, had a similar potency to the undiluted steroid but the proprietary 1 in 4 dilution of Betnovate®, Betnovate RD®, was significantly less potent than both the undiluted and the extemporaneous formulation. Again the blanching potential of the base alone was not investigated.

These investigations demonstrate the lack of linearity in the relationship between dilution and resultant potency of topical corticosteroid formulations.

1.8 STABILITY OF DILUTIONS

Extemporaneously diluted corticosteroids are given a maximum shelf-life of 14 days in accordance with the recommendations of the External Diluent Directory[16]. Information on the chemical stability of the more commonly used dilutions would be useful if the shelf-life of such preparations could be extended. It is also important to be aware of dilutions which have a shorter shelf-life than two weeks.

Yip and Li Wan Po[20] studied the stability of betamethasone-17-valerate in Plastibase, white soft paraffin and emulsifying ointment. The latter was studied with and without the addition of 0.005% phosphoric acid. The amount of steroid present in the formulation was analysed by direct densitometry on thin layer chromatographic plates. Betamethasone-17-valerate was shown to degrade by acyl rearrangement to betamethasone-21-valerate, which has 1/15 the activity of the 17-isomer. This was followed by

hydrolysis to form betamethasone. Betamethasone-17-valerate was shown to follow a first-order degradation. The rate of the reaction was investigated using betamethasone-17-valerate diluted 3:1 with emulsifying ointment, and was found to increase with temperature, following the Arrhenius equation.

The stability of three dilutions of betamethasone-17-valerate in emulsifying ointment; 3:1, 2:1 and 1:1 was studied. The higher the amount of emulsifying ointment, the more rapid was the decomposition, as demonstrated by the decrease in half-life of the steroid from 3.14 hours, when diluted 3:1; to 0.686 hours when diluted with an equal ratio of steroid to base. The half-life was also shown to decrease with temperature. The results above were obtained at 22.5°C, but the half-life of the 3:1 formulation was also measured at 20°C and 30°C and was found to be 4.36 hours and 1.25 hours respectively.

Emulsifying ointment, pH 8.9, is more alkaline than betamethasone ointment, pH 5.6, as determined by Yip and Li Wan Po[20]. They added a sample to water, heating to melt, shaking and cooling, and measuring the pH of the aqueous medium. Increasing the ratio of emulsifying ointment raises the pH and accelerates the degradation of the formulation. This indicates that the decomposition is base-catalysed.

Acidification of the extraction solvents using hydrochloric acid prevented decomposition during the extraction procedure. On storage of these acidified extracts, at room temperature, the steroid underwent a slower, acid-catalysed hydrolysis to betamethasone. Phosphoric acid was used to acidify a 3:1 dilution of betamethasone-17-valerate in emulsifying ointment. However, although there was less decomposition, the half-life of the formulation was only 7.5 days which is not practical for clinical use. Degradation was arrested by storage at 4°C. After 48 hours there was no detectable decomposition.

The pH of Plastibase, measured as described above, was found to be 5.8, which is similar to that of betamethasone-17-valerate ointment, pH 5.6. A dilution of betamethasone 3:1 with Plastibase underwent a slow decomposition, the rate of which increased over 150 times when the ratio was

changed to 1:1. It was concluded that Plastibase acts as a catalyst in the reaction and is not a suitable diluent for betamethasone.

White soft paraffin, pH 5.5, mixed 1:1 with betamethasone ointment, pH 5.6, produced a stable product with a half-life of 471 days and is a suitable diluent for this steroid.

Li Wan Po, Irwin and Yip[31] later designed a HPLC assay for the investigation of corticosteroids and their degradation products. This assay was used by Mehta et al[32] to investigate the chemical stability of a 1 in 4 dilution of betamethasone-17-valerate in emulsifying ointment. Betamethasone-17-valerate degraded rapidly; the average half-life was 4.2 hours, and none was detected in any of the diluted preparations after one week. This is comparable to the results obtained by Yip and Li Wan Po[20] of 4.36 hours for a 3:1 dilution. Mehta et al[32] showed that betamethasone-21-valerate was formed by the degradation, reaching a peak level on the third day, and slowly degrading, with a half-life of eight days, to produce betamethasone. The peak level of the free alcohol was reached after three weeks. It has 1/450 the vasoconstrictor potential of betamethasone-17-valerate. The authors recommend that betamethasone-17-valerate should not be diluted with emulsifying ointment.

Ryatt et al[27] studied the pharmacological activity of the dilutions concurrently with the stability work of Mehta et al[32]. Activity was assessed by a vasoconstrictor assay. Reflectance spectrophotometry was used to assess the degree of pallor or erythema produced by the application of the dilutions. There was no significant difference in the blanching indices of betamethasone-17-valerate (Betnovate®) ointment undiluted and the freshly made 1 in 4 dilution of Betnovate® in emulsifying ointment. The vasoconstrictor potential of diluted Betnovate® ointment decreased with time. There was a significant loss of potency after one week. The blanching induced by the three week old formulation was not significantly different to that produced by the emulsifying ointment base alone. Significant differences in blanching were obtained

between the freshly made and one week old diluted Betnovate®, and between the one and three week old diluted preparations.

The findings of Ryatt et al[27] demonstrate the correlation between chemical stability and clinical efficacy. A chemically unstable formulation will not produce the beneficial effects anticipated by the clinician.

Boosaner et al[29] studied the stability of Betnelan-V® cream (pH 5.13) diluted 1:2 and 1:3 in Beeler's basis (pH 6.96) and Cold cream® (pH 3.52), after storage for 30 days at room temperature and at 40°C. The creams were mixed with distilled water, and the pH of the dilution measured. The steroid was stable in all dilutions at room temperature over the time period studied. The stability at the higher temperature depended both on the dilution and the base used. The creams diluted with Beeler's basis had a higher pH than those diluted in Cold cream® but degraded at a slower rate. Within each base, the lower the pH of the dilution the less degradation was detected. The results indicate that the degradation of betamethasone-17-valerate in the cream depends both on the pH of the cream, and other properties inherent to the base used.

The effect of an ambiphilic diluent, Unguentum Merck® (pH 4.2), on the chemical stability of a range of commonly used proprietary topical corticosteroid products was investigated by Ray-Johnson[33].

Unguentum Merck® comprises approximately 60% fat and 40% water. It combines the properties of both an oil-in-water emulsion and a water-in-oil emulsion, and is useful as it can be mixed in any proportions with both creams and ointments.

Six proprietary cream formulations were studied in a 1:1 ratio with Unguentum Merck®. Samples were stored at 4°C, room temperature and 32-°C. The percentage of steroid present was analysed at 16 weeks and 32 weeks by HPLC. The products studied were betamethasone-17-valerate 0.1% (Betnovate®), fluocinolone acetonide 0.025% (Synalar®), flurandrenolone

0.0125% (Haelan®), beclomethasone dipropionate (Propaderm®), clobetasol propionate 0.025% (Dermovate®) and diflucortolone valerate 0.1% (Nerisone®) creams. Loss of active steroid was considered to be small or insignificant for all but Haelan® over the time studied. There was no increase in degradation at the higher temperature. No degradation products could be detected by HPLC, and no physical incompatibilities were demonstrated. The pH of Unguentum Merck® was quoted as 4.2 and that of emulsifying ointment was 7.2 although no method of measurement was described.

The data obtained by Ray-Johnson suggests that Unguentum Merck® would be a suitable diluent for a wide range of topical corticosteroids. However, there are several points to bear in mind before conclusions can be drawn. Ray-Johnson only sampled at 16 and 32 weeks. A greater number of data points are necessary, including a sample at time zero. Full details of the method used are not given, making it impossible to reproduce the work. The method of temperature control is not outlined, which is especially relevant to the storage at room temperature. The dilutions studied used a 1:1 ratio of steroid to Unguentum Merck®. This ratio is not commonly used in clinical practice. A more useful approach would have been to study a range of dilutions including 1 in 4 and 1 in 10, as these are widely used in dermatology. The preliminary work by this author produced encouraging results in favour of Unguentum Merck® and suggests that further investigation would be useful.

Woodford[34] studied the stability and release characteristics of Unguentum Merck® compared to Cetomacrogol Cream (formula A) BPC, a conventional diluent for topical corticosteroids. Five proprietary creams were studied in a 1 in 4 dilution in each base. The creams studied were clobetasol propionate 0.05% (Dermovate®), halcinonide 0.1% (Halciderm®), both very potent steroids, and three potent steroids; betamethasone-17-valerate 0.1% (Betnovate®), hydrocortisone butyrate 0.1% (Locoid®) and fluclorolone acetonide 0.025% (Topilar®).

Stability and efficacy were measured by a vasoconstrictor assay. The dilutions were stored in a cool, dark place and tested when 3 to 4 weeks old for Unguentum Merck® and 1-2 weeks old for Cetomacrogol Cream. The storage temperature was not detailed. The 1 in 4 dilutions were shown to be equipotent in Unguentum Merck® and Cetomacrogol Cream for each steroid. There was no significant difference between the blanching efficacy of the diluted and undiluted proprietary creams. Storage of steroids diluted in Unguentum Merck® had no effect on their potency, under the conditions studied.

The work by Ray-Johnson[33] and Woodford[34] suggests that Unguentum Merck-® is compatible with a range of topical corticosteroids to produce a stable dilution with good release characteristics.

Unguentum Merck® has been shown to comply with the requirements of the British Pharmacopoeia (BP) 1980 for the efficacy of antimicrobial preservatives in pharmaceutical preparations[35]. Unguentum Merck® was used to produce half-strength and one-fifth strength Betnovate® cream, and half-strength Betnovate® ointment. There was more than adequate compliance with the BP recommendations. Addition of sorbic acid, the preservative present in Unguentum Merck®, showed little significant increase in efficacy. After an extended trial to three months no viable organisms were recovered from any of the challenged preparations.

1.9 KINETICS OF DECOMPOSITION

Kinetic studies have been carried out on several corticosteroids.

Prednisolone was studied by Chulski and Forist[36]. Solid buffers were mixed with aqueous solutions of prednisolone. The effect on the A-ring of the steroid was studied by ultraviolet analysis to detect the extent of adsorption and the alpha-ketol side chain was studied by the blue tetrazolium assay to detect side-chain degradation. In the presence of magnesium oxide the suspension was of high pH, and there was extensive degradation of the side-chain, which followed first-order kinetics. This did not occur at lower pH

values. It was concluded that magnesium oxide had produced an alkaline degradation of the side-chain, and corticosteroids similar to prednisolone should not be exposed to materials capable of producing an elevated pH during formulation (fig 1.1).

Guttman and Meister[37] investigated the kinetics of the base-catalysed degradation of prednisolone, in the presence and absence of air. The degradation of prednisolone was first-order with respect to the steroid concentration. Acidic and neutral steroidal products were formed under aerobic and anaerobic conditions (fig 1.2).

In the presence of air prednisolone degraded much more rapidly. The rate constant for the formation of acidic material was markedly decreased by the exclusion of air, while the rate constant for the formation of neutral material remained unchanged. This suggests that two reactions are involved in the formation of steroidal acidic products under aerobic conditions, one pathway is oxygen-dependent and the other occurs in the presence or absence of air.

Base-catalysed degradation of prednisolone was concluded to result from at least three parallel pseudo first-order reactions, two or more oxygen-independent reactions forming neutral and acidic material, and at least one oxygen-dependent reaction forming an additional acidic product. The first order-degradation of the C-17 side-chain has been demonstrated in a formulation by Allen and Das Gupta[38]. Hydrocortisone in polyethylene glycol ointment base underwent decomposition at both the side-chain, and at ring A.

Degradation at the side-chain was first-order, degradation at ring A appeared to be zero-order. A scheme for ring A degradation was described (fig 1.3).

Yip et al[39] also studied hydrocortisone in formulations. The butyrate was investigated in gel and semi-aqueous systems. A carboxypolymethylene polymer gel was used to prepare the former, the latter contained propylene glycol. Analysis by HPLC demonstrated the decomposition pathway as being

Fig 1.1 DEGRADATION OF PREDNISOLONE IN AQUEOUS SUSPENSION, AFTER CHULSKI AND FORIST[36]. I = PREDNISOLONE, II = A KETODIHYDROXY -ETIOCHOLENIC ACID, III = 17- KETOSTEROID.

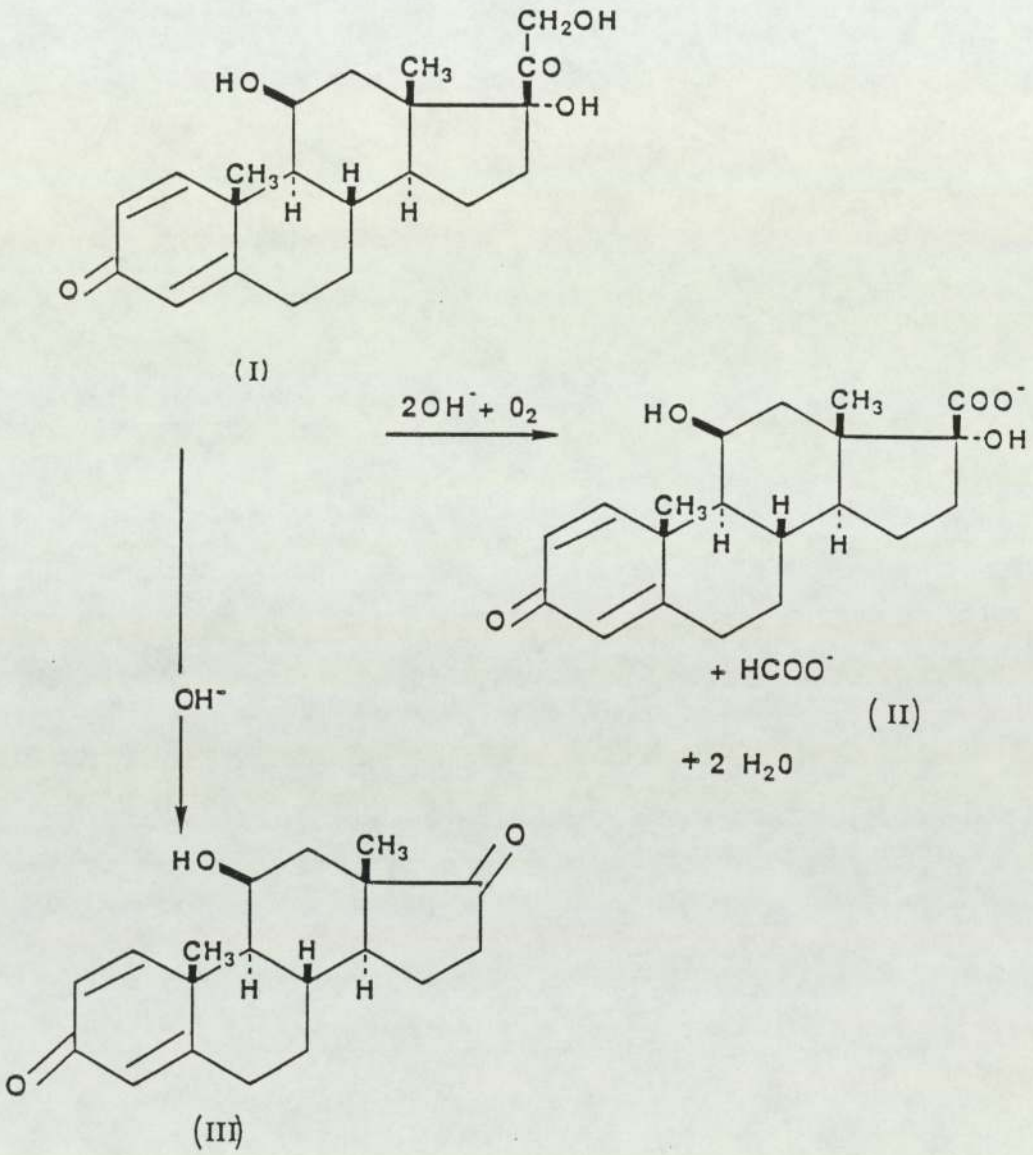
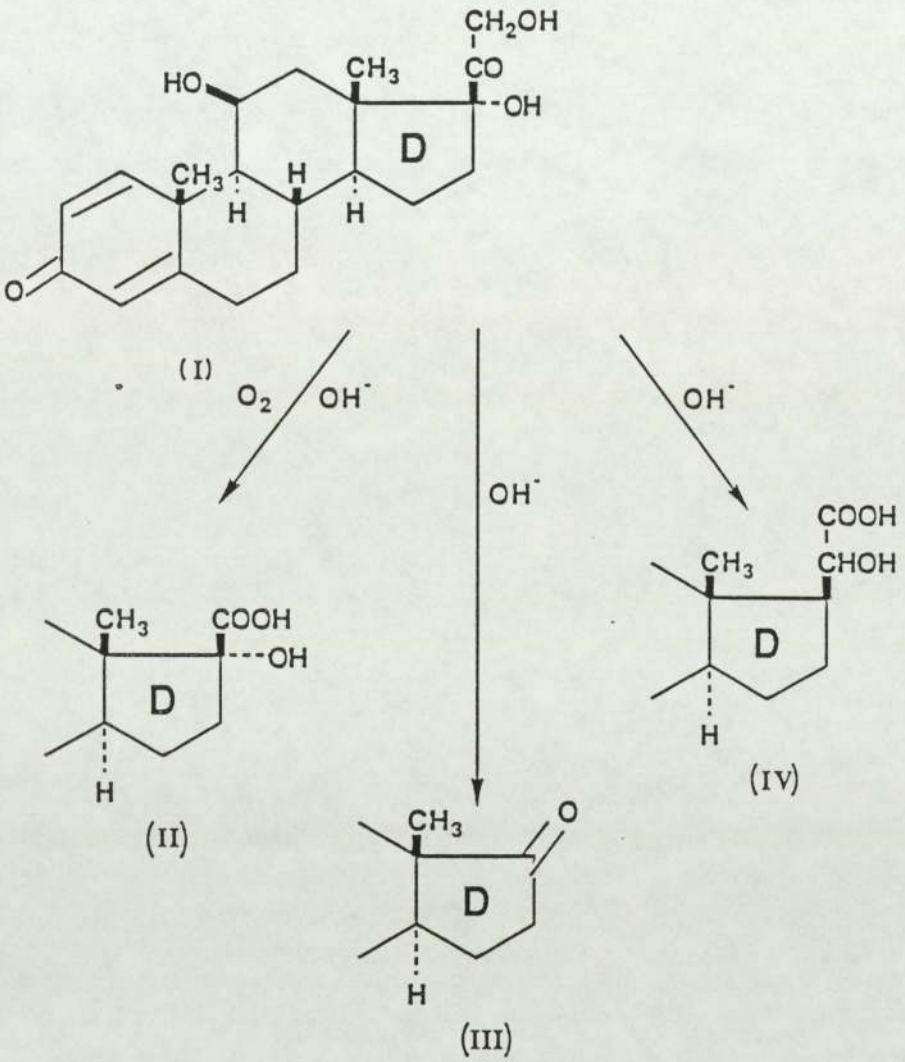


Fig 1.2 DEGRADATION OF PREDNISOLONE UNDER AEROBIC AND ANAEROBIC CONDITIONS, AFTER GUTTMAN AND MEISTER [37]. I = PREDNISOLONE, II = ETIANIC ACID, III = 17- KETOSTEROID, IV = HYDROXYACID.



acyl rearrangement to the C-21 ester of butyric acid, followed by hydrolysis to hydrocortisone (fig 1.4). Kinetic analysis of the gel data showed disappearance of hydrocortisone butyrate followed first-order kinetics. The isomerism to the C-21 ester was base-catalysed and pH dependent. In the absence of the gelling polymer, decomposition was pH dependent, but in the non-buffered system the reaction did not follow first-order kinetics.

Chromatography demonstrated the presence of several degradation products which were not identified but indicate the complexity of the reaction.

The isomerism of hydrocortisone-17-butyrate to the C-21 ester of butyric acid was shown to be reversible, but the hydrolysis is not reversible. Direct conversion of the hydrocortisone-17-butyrate to hydrocortisone is unlikely. The decomposition of hydrocortisone is metal catalysed, and addition of ethylenediaminetetraacetic acid (EDTA) significantly decreased the rate of degradation, changing the profile of the minor decomposition products compared to a control.

The complex decomposition pathways can be satisfied by a series of first-order equations. Das Gupta[40] studied decomposition of triamcinolone acetonide by HPLC. He states that attack on the C-17 side-chain produced acidic products, while attack on the A ring produced neutral products. Degradation followed pseudo first-order kinetics, the rate of which varied with pH. Optimum stability was at approximately pH 3.4 Above pH 5.5 the degradation rate increased rapidly and was directly related to the phosphate buffer concentration used. In the presence of hydrochloric acid buffer solution the major reaction was hydrolysis of triamcinolone acetonide to triamcinolone and acetone. The decomposition decreased with increasing ionic strength when the pH of the solution was greater than 7.

The solutions were stable up to nine times longer at 25°C than at 50°C. Two new peaks corresponding to decomposition products were detected by HPLC, the ratio of the peaks being determined by the composition of the vehicle.

Fig 1.3 SCHEME FOR THE DEGRADATION AT RING A OF HYDROCORTISONE IN POLYETHYLENE GLYCOL OINTMENT, AFTER ALLEN AND DAS GUPTA[38]. I = HYDROCORTISONE, II, III, IV, V = POSSIBLE DEGRADATION PRODUCTS.

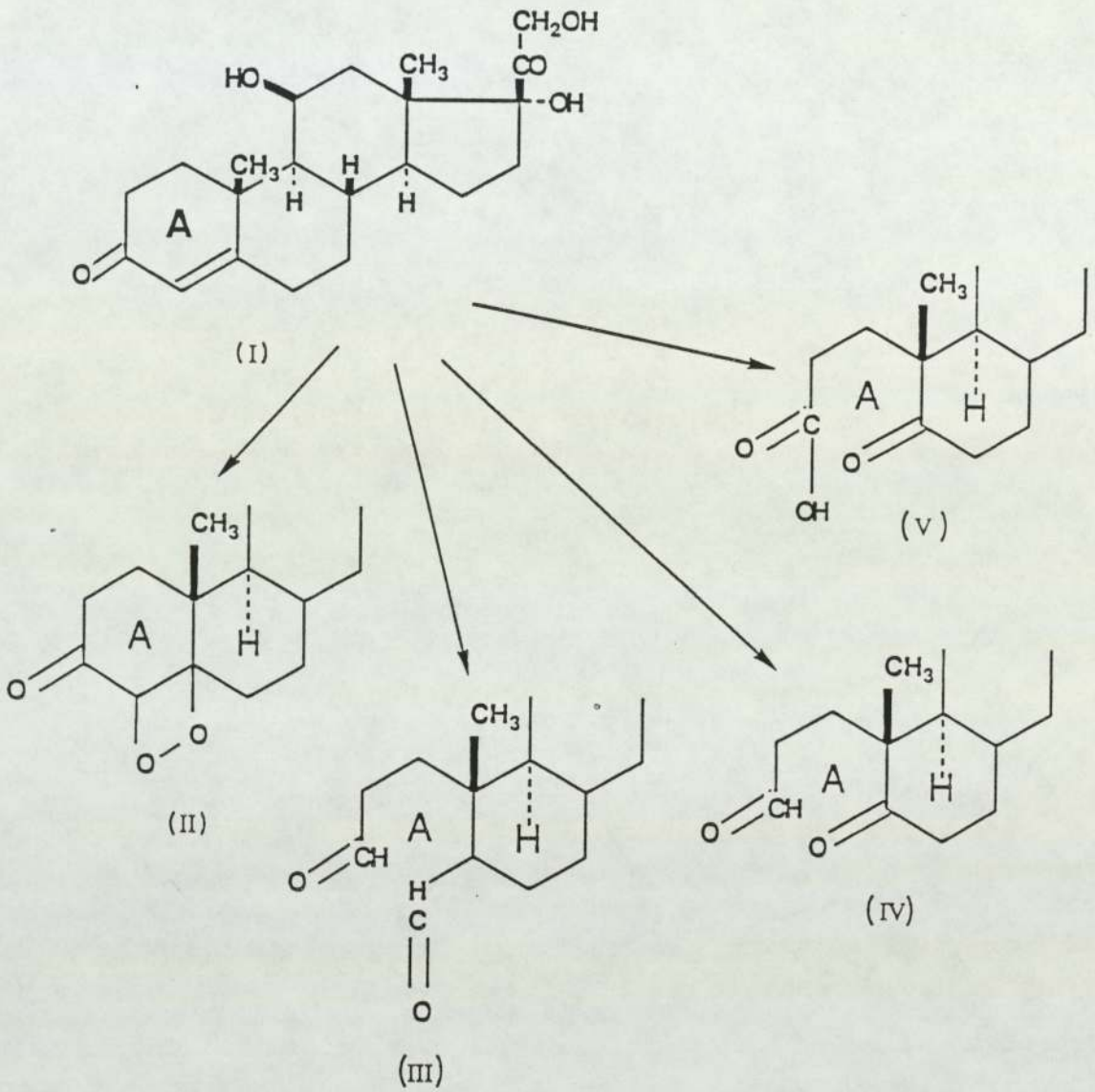
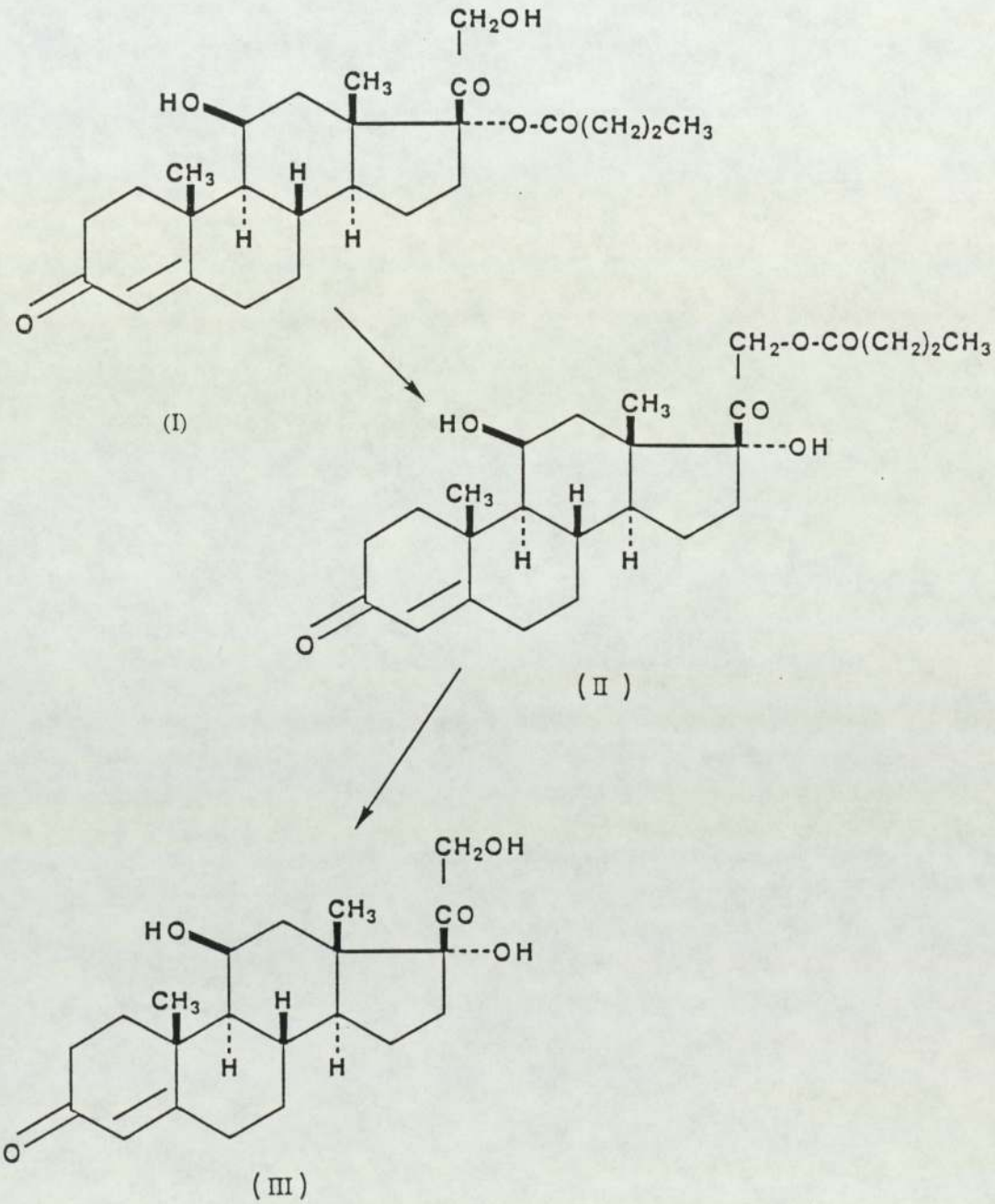


Fig 1.4 DEGRADATION OF HYDROCORTISONE BUTYRATE, AFTER YIP et al [39]. I = HYDROCORTISONE-17-BUTYRATE, II = 21 - ESTER, III = HYDROCORTISONE.



Further investigation of the pH profile of triamcinolone acetonide was carried out by Timmins and Gray[41] (fig 1.5). Specific acid-catalysis is displayed in the region pH 1-3, and specific base-catalysis in the regions 4-7 and 9-12. The pH of maximum stability is approximately pH 4, and a pH-independent region exists at pH 7-9. In the neutral to alkaline region enolization is not a rate-determining step whereas enolization must occur before product formation in the acid region (fig 1.6).

Triamcinolone was also investigated. There was no inflection in the graph of reaction rate versus pH due to enolization and enol ionization as was seen for the acetonide. The pH of maximum stability for triamcinolone is about pH 3.5, and a straight line, slope 1.0, can be drawn through the points above pH 4, implying specific base-catalysis (fig 1.6).

Triamcinolone degrades to the D-homosteroid in basic media, which is not an observed product of triamcinolone acetonide. The acetonide functional group stabilises the steroid ketal in neutral and basic media, preventing hydroxide ion-catalysed decomposition to the D-homosteroid. Below pH 7 the shape of the graph is almost identical to that of the parent steroid.

Temperature and pH dependence of the degradation of fluocinolone acetonide in a topical cream formulation was studied by Kenley et al[42]. Fluocinolone acetonide was incorporated into an oil-in-water cream base. Degradation was studied at pH 2-6, at 23, 40, 50 and 80°C. Fluocinolone acetonide disappearance followed pseudo first-order kinetics. The C-21 aldehyde and C-17 etianic acid were identified by HPLC. Other degradation products appeared close to the solvent front, indicating polar or ionized functional groups. These were not identified.

Fig 1.5 DEGRADATION OF TRIAMCINOLONE ACETONIDE, AFTER TIMMINS AND GRAY[41]. I = TRIAMCINOLONE ACETONIDE, II = TRIAMCINOLONE, III = D - HOMOSTEROID.

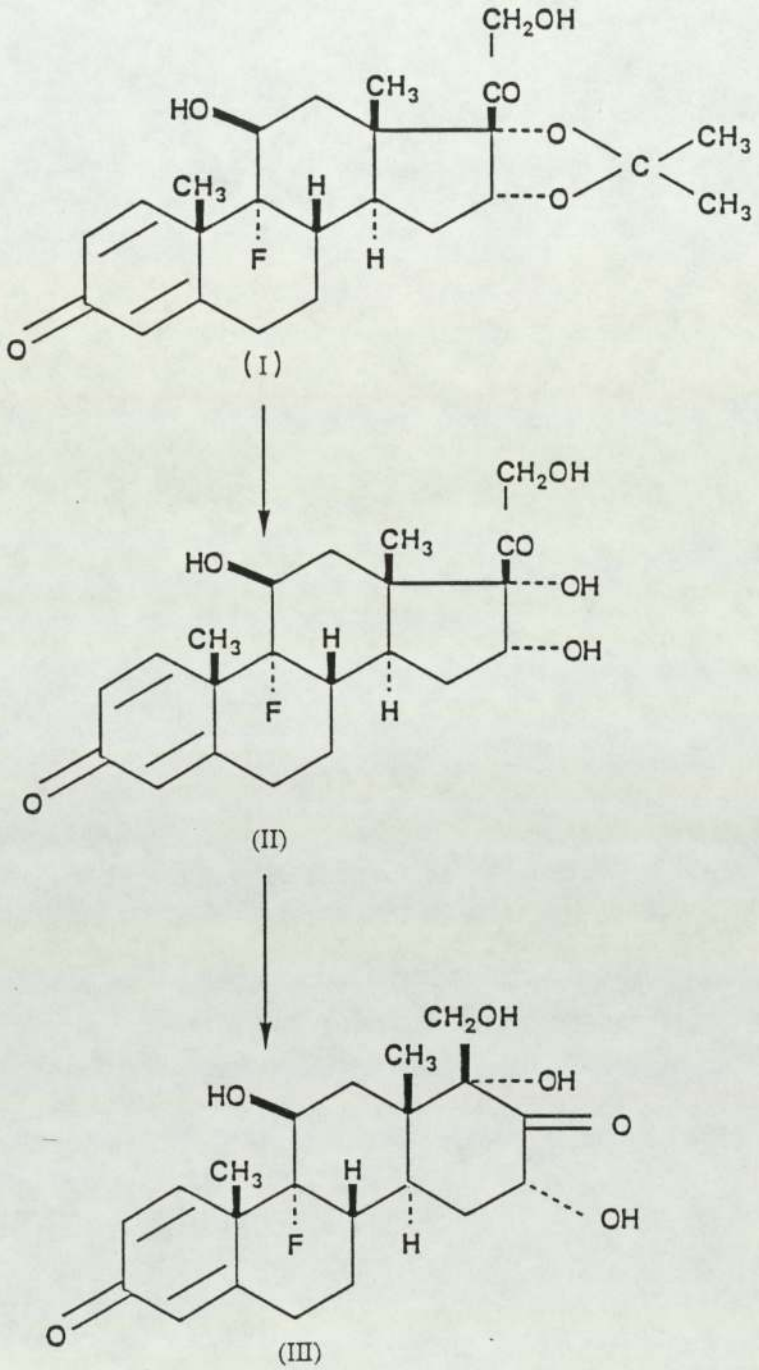
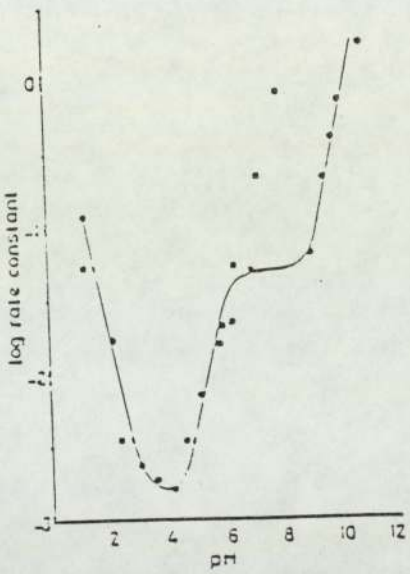


Fig 1.6. pH-RATE PROFILE FOR THE DEGRADATION OF TRIAMCINOLONE ACETONIDE AND TRIAMCINOLONE IN AQUEOUS SOLUTION, AFTER TIMMINS AND GRAY[41] .

EXPERIMENTAL POINTS ● = TRIAMCINOLONE ACETONIDE, ■ = TRIAMCINOLONE.



At all pH values, the fluocinolone acetonide degradation rate constants increased with increasing temperatures. Arrhenius behaviour held for all the creams tested over the entire temperature range of 23-80°C according to the equation:

$$\ln(kobs) = \ln(A) - Ea/RT$$

where:

Ea = Arrhenius activation energy (J/Mol)

R = Gas Constant (J Mol^{-1} K^{-1})

T = Absolute Temperature (K)

A = Preexponential factor ($months^{-1}$)

kobs = observed degradation rate constant ($months^{-1}$)

At all temperatures, the observed degradation rate constant demonstrated a strong dependence on pH, with rate constants increasing at decreasing pH values in the region pH 2.3-3.7, and increasing at increasing pH values in the region pH 4.1-6. There was a rate minimum near pH 4. This is consistent with the mechanism of specific acid-catalysis of fluocinolone acetonide degradation at low pH values, and specific base-catalysis above pH 4.

The pH dependence of fluocinolone acetonide can be described by the equation:

$$kobs = k_O + k_{H+}[H+] + k_{OH^-}[OH-]$$

where:

k_O = pseudo first-order rate constant for spontaneous reaction

k_{H+} & k_{OH^-} = bimolecular rate constants for acid and base-catalysed fluocinolone acetonide degradation.

For each of the three rate constants evaluated, the fit to the Arrhenius equation was good over the temperature range 23-80°C. The fluocinolone acetonide cream shares a similar log(rate)-pH profile over the range pH 2-6 as that demonstrated for triamcinolone acetonide in solution by Timmins and Gray[41] as shown in fig 1.6. In both cases specific acid and specific base-catalysis are evident and rate minima occur near pH 4. The reaction rate

similarities indicate that the fluocinolone acetonide degradation in the cream samples is confined to an aqueous environment that is not affected by the non-aqueous constituents of the cream base.

1.10 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

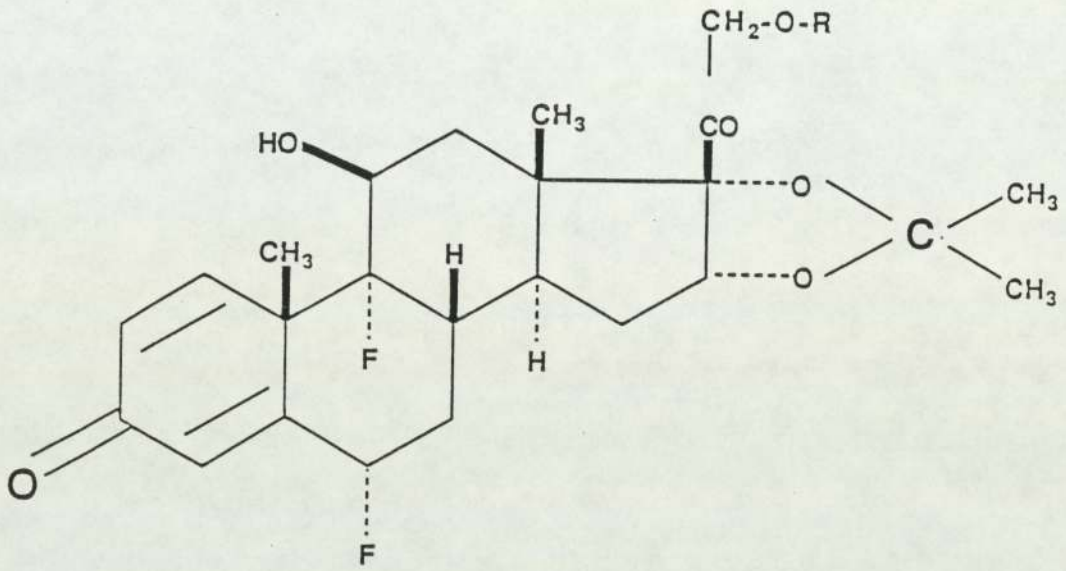
High performance liquid chromatography (HPLC) has become the method of choice in the analysis of corticosteroids over colorimetric procedures. The techniques and applications of HPLC were reviewed by Li Wan Po and Irwin[43] with particular reference to pharmaceutical and clinical sciences, including steroid analysis. The advantages are that rapid and selective quantitation of both standard and formulated steroids can be achieved. The drug can be separated from closely related groups to give a reliable, stability-indicating assay, allowing identification of degradation products and elucidation of kinetic parameters[31,44].

1.11 AIMS

At the Birmingham Skin Hospital the topical corticosteroids fluocinolone acetonide (Synalar®) and fluocinonide (Metosyn®), fig 1.7, are increasingly prescribed as extemporaneous dilutions. The bases used include Unguentum Merck®, Lipobase® and Metosyn Diluent®, but there is little or no information available regarding the stability or clinical efficacy of these formulations.

The aim of this project was to set up a stability-indicating assay using HPLC, which could then be used to investigate the effect of the choice of base and the dilution factor on the shelf-life of each formulation over the temperature range 4°C to 32°C. The clinical efficacy of the formulations was investigated using a blanching assay. The aim was to determine whether there was a relationship between dilution factor and clinical potency and whether the choice of base altered this relationship. Extemporaneously prepared dilutions were compared to a commercially available product of an equivalent dilution factor in order to determine whether there was any difference in the potency of these formulations.

Fig 1.7 STRUCTURE OF FLUOCINOLONE ACETONIDE AND FLUOCINONIDE FLUOCINOLONE ACETONIDE, R = H, FLUOCINONIDE R = -$COCH_3$.



2. METHODS

2.1 EQUIPMENT AND MATERIALS

The following creams, ointments and bases were purchased from the manufacturers:

Synalar® ointment; the 1 in 4 dilution of the ointment and Synalar® lotion (Imperial Chemical Industries PLC); Metosyn® cream and Metosyn® diluent (Stuart Pharmaceuticals Ltd); Betnovate® ointment and scalp application (Glaxo), Lipobase® (Brocades) and Unguentum Merck® (E Merck Ltd).

Fluocinolone acetonide powder was purchased from Sigma Chemical Company and fluocinonide powder was received as a gift from Stuart Pharmaceuticals Ltd.

Hepes buffer was purchased from BDH Chemicals Ltd., as were the acetonitrile (HPLC grade) and hexane (Analar grade). The other reagents used were of laboratory grade. The water used was single distilled water, and the filter paper was from Whatman.

An Orion 811 pH meter with a glass combination electrode was utilised, and all autopipettes were of the air displacement variety. The portable reflectance spectrophotometer used for skin blanching measurements was the Haemelometer Model 4, which is a prototype version of the instrument described in chapter 1.3, from the Birmingham Skin Hospital.

2.2 EXTRACTION AND ANALYSIS

The steroids under investigation were extracted from the proprietary ointment formulations and analysed by HPLC.

2.2.1 EXTRACTION

Extraction was carried out in a 100ml glass separating funnel. To each 1g of formulation 10ml of acetonitrile:water (70:30) and 10ml of hexane were added. After vigorous shaking the funnel was allowed to stand and two layers

separated out, the lower of which contained the extracted steroids and was collected. This was filtered using a coarse filter paper, Whatman number 4, or a finer filter paper, number 6, as required.

2.2.2 ANALYSIS

The extracted steroid was manually injected on to a Hichrom Spherisorb 5 micron ODS 25cm x 4.6mm i.d. column with a guard column packed with pellicular Spherisorb 5 micron ODS. A Rheodyne 7125 valve with an injection loop volume of 20 microlitres was used. The mobile phase consisted of acetonitrile:water (50:50) which was delivered by an Altex 110A pump running at 1ml/minute. A Pye Unicam LC-UV detector, set at 240nm, was connected to both a Tekman chart recorder, running at 300mm/hour, and a Shimadzu C-R 3A Chromatopac integrator to produce peak area ratios of the samples under investigation. The retention times under these conditions were fluocinolone acetonide = 6 minutes, fluocinonide = 10 minutes and butyl 4-hydroxybenzoate = 8 minutes.

2.2.2.1 AMENDMENT TO HPLC METHOD

The HPLC method was changed from manual injection to an automatic procedure by incorporating a Talbot ASI 4 autosampler and a Rheodyne 7010 valve into the system. The injection loop volume was 20 microlitres and the injection time was 22 seconds. A Cecil CE 1100 liquid chromatography pump was used, running at 1ml/minute, to deliver a mobile phase of acetonitrile:water (45:55). The column was changed to a S50ODS 10cm x 4.6mm i.d. with guard column for faster analysis time. The retention times were fluocinolone acetonide = 4 minutes, fluocinonide = 8 minutes and butyl 4-hydroxybenzoate = 6 minutes.

2.2.2.2 CHOICE OF WAVELENGTH FOR ANALYSIS

A sample of Synalar® extract which had been subjected to alkaline degradation in chapter 2.4 was analysed by chromatographing replicate injections over the wavelength range 210-280nm in 10nm increments. The most appropriate wavelength for analysis was found to be 240nm and this was used for all subsequent HPLC investigations.

2.3 POTENTIAL PROBLEMS IN PIPETTING

2.3.1 PRECISION

An automatic pipette was used to measure microlitre volumes of solutions. To assess the precision of this pipette 500 microlitre aliquots of acetonitrile were measured into a glass vial, taking a weight after each addition. The coefficient of variation was calculated.

2.3.2 COMPARISON OF AUTOMATIC AND GLASS PIPETTES

Synalar® ointment was extracted as in chapter 2.2.1. To each of six tubes, 500 microlitres of Synalar® extract and 500 microlitres of acetonitrile:water (70:30) were added using an autopipette. In a further six tubes, a similar reaction mixture was prepared, all measurements being made with 0.5ml class A glass pipettes. The samples were frozen at -20°C. On removal from the freezer 500 microlitres of butyl 4-hydroxybenzoate (1.25×10^{-5}g/ml) were added and the samples analysed by HPLC as in chapter 2.2.2, injecting automatic and glass pipetted samples alternately.

2.4 ACCELERATED DEGRADATION OF STEROIDS

Accelerated degradation of the steroid under investigation was produced by addition of sodium hydroxide to the reaction mixture followed by a buffer, as described below, prior to analysis to prevent damage to the column by the NaOH.

2.4.1 STEROID DEGRADATION IN AQUEOUS SOLUTION

Initial experiments were carried out using aqueous dilutions of Betnovate® scalp application and Synalar® lotion. Degradation was produced by addition of 1-2 ml of molar NaOH dropwise to take the pH to over 12. After 15 minutes the reaction mixture was buffered to a pH of approximately 5 with 0.5-1ml of acetate buffer pH 3.7, added dropwise. The buffered mixture was analysed by HPLC as in chapter 2.2.2. Acetonitrile:water in various proportions was used as the mobile phase in all the experiments.

2.4.2 EFFECT OF pH

Fluocinolone acetonide was extracted from Synalar® ointment as in chapter 2.2.1, and portions of the extract were adjusted to pH 8, 10 and 12 using 0.01M NaOH. The reaction mixtures were left in the dark for 15 minutes, and then buffered to pH 5-6 using acetate buffer pH 3.7 and analysed by HPLC as in chapter 2.2.2.

2.4.3 THE IMPORTANCE OF GLASSWARE

Two similar portions of steroid extract, extracted as in chapter 2.2.2, were stored at 4°C, in a fridge; one in a disposable polystyrene beaker, and one in a 25ml glass beaker. A portion of acetonitrile:water (70:30) i.e. the medium for extraction, without the presence of steroid, was also stored in a fridge, in glassware, as a control. The samples were analysed by HPLC as in chapter 2.2.2.

From the results obtained (chapter 3) all subsequent experiments utilised glassware rather than polystyrene beakers.

2.5 CHOICE OF INTERNAL STANDARD

The sodium salts of the parabens were investigated.

Solutions of ethyl and propyl 4-hydroxybenzoate (1×10^{-4}g/ml) were each prepared using acetonitrile:water (70:30) as the diluent. A 5×10^{-5}g/ml solution of butyl 4-hydroxybenzoate was prepared in a similar manner. The solutions were analysed by HPLC as in chapter 2.2.2 in order to identify a suitable internal standard for the system under investigation. The retention time of butyl 4-hydroxybenzoate was 8 minutes on a 25cm column and 6 minutes on a 10cm, using a mobile phase of acetonitrile:water (45:55). The butyl ester was used as the internal standard in all subsequent experiments.

2.6 CHOICE OF BUFFER

Acetate buffer was used in the preliminary investigations. Acetate buffer B.P.[45] is prepared by dissolving 10g anhydrous sodium acetate in 300ml to 200ml water and adjusting the pH to 3.7 with glacial acetic acid. Water is

added to 1000ml and the pH is readjusted with glacial acetic acid or anhydrous sodium acetate as required. This buffer has been shown to catalyse the degradation of corticosteroids[41] therefore the following alternative buffers were investigated.

2.6.1 BDH BUFFER pH 4

BDH buffer contains 0.05M potassium hydrogen phthalate and this interfered with the chromatogram as discussed in chapter 3.7.

2.6.2 PHOSPHATE BUFFER pH 3.5

A solution of 22.2g of potassium dihydrogen orthophosphate in 100ml of water was adjusted to pH 3.5 with 1M phosphoric acid to produce 1.6M phosphate buffer.

A sample of 5ml of mobile phase (acetonitrile:water (50:50)), 10 microlitres of 0.1M NaOH (to take pH to 12), and 2.5ml of phosphate buffer (to lower pH to 4), was taken as a typical reaction mixture. This was stored overnight in the dark.

2.6.3 CITRATE BUFFER

A 1M solution of citric acid was prepared by dissolving 21g of the monohydrate in approximately 70ml of water and the pH was adjusted to 3.5 with 5-10ml of 20% NaOH. This was made to volume (100ml) with water and the pH adjusted as required.

Metosyn® ointment was extracted as in chapter 2.2.1 and 75 microlitres of 0.1M NaOH were added to 500 microlitres of the extract to raise the pH to 11. Samples were taken at time zero and then at 15 minute intervals for one hour. The pH was lowered to 3.5 immediately after sampling, by the addition of 100 microlitres of citrate buffer. A sample of citric acid buffer in mobile phase was also run with no steroid. The samples were analysed by HPLC as in chapter 2.2.2.

2.6.4 BORATE BUFFER pH 4

Borate buffer was prepared by dissolving 3g of boric acid in approximately 80ml of water and adjusting the pH to 4 using 0.1M NaOH. This was made to volume (100ml) with water and the pH adjusted as required.

Metosyn® ointment was extracted as in chapter 2.2.1 and 50 microlitres of 5M NaOH were added to 5ml of the extract to raise the pH to 12. Samples were taken at time zero and then at 15 minute intervals for one hour. The pH was lowered to 4 immediately after sampling by the addition of 1ml of borate buffer. The sample was analysed by HPLC as in chapter 2.2.2.

2.6.5 HYDROCHLORIC ACID pH 1.5

A 0.08M solution of HCl was used in preliminary experiments to neutralise the NaOH present. To a reaction mixture containing 50 microlitres of 5M NaOH, 500 microlitres of 0.08M HCl were added. This is a ratio of one mole of NaOH to one and a half moles of HCl.

2.6.5.1 EFFICACY OF HCl

Synalar® ointment was extracted as in chapter 2.2.1. The solutions used in this section were cooled by standing in an ice bucket for 15 minutes prior to use. Three replicate samples of cooled Synalar® extract, 0.5ml, were transferred to glass vials using a glass pipette, class A. To each sample 5 microlitres of cooled 5M NaOH were added using an autopipette, followed immediately by 50 microlitres of cold 0.08M HCl using an autopipette. This was intended to immediately quench the reaction. The first vial was analysed immediately, while the two remaining vials stood on the laboratory bench. The second vial stood at room temperature for 20 minutes before analysis and the third vial stood for 40 minutes. 500 microlitres of butyl 4-hydroxybenzoate (1.25×10^{-5}g/ml) were added as an internal standard immediately before analysis. The samples were analysed by HPLC as in chapter 2.2.2.1, except that the mobile phase was acetonitrile:water (45:55). The retention time of fluocinolone acetonide was 4 minutes and that of the internal standard was 6 minutes, using a 10cm column.

2.6.5.2 STORAGE IN A FREEZER

Synalar® ointment was extracted as in chapter 2.2.1 and a 45ml aliquot of the extract was collected, to which 450 microlitres of 5M NaOH were added. A series of 500 microlitre samples were immediately withdrawn and transferred to pre-measured 500 microlitre aliquots of 0.08M HCl. These samples were put into a freezer and analysed after 25.5 hours, 14 days, 31 days and 50 days. Analysis was performed by HPLC as in chapter 2.2.2.1, adding 500 microlitres of butyl 4-hydroxybenzoate (1.25×10^{-5}g/ml) as an internal standard immediately prior to chromatography.

2.6.6 HEPES BUFFER

A 0.01g/ml solution of Hepes buffer (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) was prepared in the following manner. A 1g quantity of Hepes buffer was dissolved in a few ml of water, 70ml of acetonitrile were added, followed by 5ml of 1M HCl. The solution was made up to a volume of 100ml with water and had a pH of 4. The effectiveness of Hepes buffer in the range required (pH 3-5) was demonstrated by the addition of 0.1M HCl. A 100 microlitre quantity of the HCl was added to each of 5ml of water, and 5ml of Hepes buffer (0.01g/ml). The pH was recorded before and after this addition. The efficacy of the buffer was investigated under alkaline conditions by the addition of 50 microlitres of 5M NaOH, in a similar manner.

Hepes buffer was tested for compatibility by adding up to 10ml to samples of 5ml Synalar® extract in the presence of 50 microlitres of 5M NaOH. These were stored in the light (on the laboratory bench) and in a fridge. The Synalar® ointment was extracted as in chapter 2.2.1.

2.6.6.1 EFFICACY OF HEPES BUFFER

Synalar® ointment was extracted as in chapter 2.2.1 and to 5ml of the extract 50 microlitres of 5M NaOH were added followed by 10ml of Hepes buffer (0.01g/ml), pH 4. Samples were withdrawn using a glass pipette at t=0, 15 and 40 minutes, and at 1, 2.5 and 4 hours. The experiment was also carried out with the addition of 100 microlitres of 0.15g/ml sodium sulphite solution.

2.6.6.2 STORAGE IN A FREEZER

An extraction of Synalar® ointment as in chapter 2.2.1 yielded 13.5ml, to which 135 microlitres of 5M NaOH were added, followed by 27ml of Hepes buffer (0.01g/ml), using a glass pipette to give a final pH of 4. Duplicate 1.5ml quantities of sample were removed (equivalent to 0.5ml Synalar® solution and 1ml buffer) and transferred to glass vials. Three vials were analysed immediately; the remaining vials were stored in a freezer at -20°C. Vials were removed after overnight freezing, and after 4 days, 6 days, 29 days and 43 days.

The experiment was repeated, adding 100 microlitres of EDTA (2.5×10^{-3}g/ml of the sodium salt in acetonitrile:water (70:30)) to each vial analysing without freezing, and then after 3 days and 14 days storage in a freezer. An internal standard of 500 microlitres of butyl 4-hydroxybenzoate (1.25×10^{-5}g/ml) was added to each vial immediately before analysis.

A similar experiment was carried out to determine the stability of samples containing steroid extracted from Metosyn® ointment as in chapter 2.2.1, with and without EDTA. To 50ml of the extract 200ml of Hepes buffer (0.01g/ml) were added followed by 500 microlitres of 5M NaOH. The increased proportion of buffer was necessary to quench the reaction and prevent degradation. Aliquots of 2.5ml were withdrawn from the reaction mixture (equivalent to 0.5ml of Metosyn® solution and 2ml buffer) and transferred to glass vials, half of which contained 100 microlitres of 2.5×10^{-3}g/ml EDTA.

The samples were analysed without freezing, and after 3 days and 14 days storage in a freezer. Analysis was by HPLC as in chapter 2.2.2.1, adding 1ml of butyl 4-hydroxybenzoate (1.25×10^{-5}g/ml) as internal standard immediately prior to injection.

The mobile phase used for the stability and efficacy work performed on Hepes buffer was acetonitrile:water (45:55). The retention time of fluocinonide was 8 minutes and that of the internal standard was 6 minutes, using a 10cm column.

2.7 FLUOCINOLONE ACETONIDE AS A STANDARD

Fluocinolone acetonide is the steroid component of Synalar® ointment. The pure compound was dissolved in acetonitrile:water (70:30) to make a 8 x 10^{-6}g/ml solution. This contains the same concentration of fluocinolone acetonide as a 500 microlitre sample of Synalar® extract, assuming 100% extraction of the steroid from the base (see recovery data chapter 3). This solution was subjected to HPLC analysis as in chapter 2.2.2.1 to determine whether any impurities were present which may otherwise be attributed to degradation.

A stock solution of 1.6 x 10^{-5}g/ml fluocinolone acetonide in acetonitrile was used to prepare dilutions in the range 1.2 x 10^{-5}g/ml to 2 x 10^{-6}g/ml in the same diluent. A 1ml sample of each solution was taken and 500 microlitres of butyl 4-hydroxybenzoate (1.25 x 10^{-5}g/ml) added as an internal standard and each solution was analysed by HPLC as in chapter 2.2.2.1. The experiment was repeated with a second set of dilutions, and the peak area ratios of the steroid to the internal standard were used to construct two calibration graphs (chapter 3). The retention time of fluocinolone acetonide was 4 minutes and that of the internal standard was 6 minutes, using a 10cm column and a mobile phase of acetonitrile:water (45:55).

2.8 FLUOCINONIDE AS A STANDARD

Fluocinonide is the steroid component of Metosyn® cream. A stock solution of 2 x 10^{-5}g/ml fluocinonide in acetonitrile was used to prepare dilutions in the range 1.5 x 10^{-5}g/ml to 3 x 10^{-6}g/ml in the same diluent. A 10ml sample of each solution was taken and 0.5ml of butyl 4-hydroxybenzoate (1.25 x 10^{-5}g/ml) was added as an internal standard. Each solution was analysed by HPLC as in chapter 2.2.2.1. The experiment was repeated with a second set of dilutions and the peak area ratios were used to construct two calibration graphs (chapter 3). The retention time of fluocinonide was 8 minutes and that of the internal standard was 6 minutes, using a 10cm column and a mobile phase of acetonitrile:water (45:55).

2.9 KINETIC EXPERIMENTS

2.9.1 DEGRADATION OF FLUOCINOLONE ACETONIDE UNDER ALKALINE CONDITIONS

Initial experiments used 0.08M HCl as a quenching agent which was cooled by standing in an ice bucket for 15 minutes prior to use.

Synalar® ointment was extracted as in chapter 2.2.1. To 45ml of steroid extract, 450 microlitres of 5M NaOH were added. Over a six hour period, 500 microlitre samples were withdrawn from the reaction mixture and transferred to glass vials containing 500 microlitres of cold 0.08M HCl to quench the reaction. The quenched samples were then stored in a freezer at -20°C to await analysis.

Three similar reactions were carried out;

a) in diffuse daylight, at room temperature,

b) in the dark, at room temperature,

c) in the fridge, at 4°C.

On removal from the freezer 500 microlitres of butyl 4-hydroxybenzoate (1.25×10^{-5}g/ml) were added to each sample as an internal standard, and they were then analysed by HPLC as in chapter 2.2.2.1. The retention time of fluocinolone acetonide was 4 minutes and that of the internal standard was 6 minutes, using a 10cm column and a mobile phase of acetonitrile:water (45:55).

The degradation of fluocinolone acetonide in Synalar® ointment under alkaline conditions was repeated using Hepes buffer (1×10^{-2}g/ml), pH 3.6, as a quenching agent. The degradation was investigated under the following conditions;

a) control,

b) in the presence of EDTA,

c) in the presence of sodium sulphite,

d) with both EDTA and sodium sulphite.

Synalar® ointment was extracted as in chapter 2.2.1. The reaction mixtures consisted of 7.5ml of Synalar® extract, and 75 microlitres of 5M NaOH. 100 microlitres of a 3.75 x 10^{-2}g/ml solution of EDTA gave 5 x 10^{-4}g/ml EDTA in the reaction mixture, while 50 microlitres of a 15 x 10^{-2}g/ml solution of sodium sulphite gave 1 x 10^{-3}g/ml in the reaction mixture. The di-sodium salt of EDTA was used, and dilutions were prepared using acetonitrile:water (70:30). The reaction was carried out in a water bath at 30°C, in the dark. Samples of 0.5ml were withdrawn over a 2.5 hour period using class A glass pipettes and added to 1ml aliquots of Hepes buffer (1 x 10^{-2}g/ml) and stored in a freezer at -20°C. On removal from the freezer, 500 microlitres of butyl 4-hydroxybenzoate (1.25 x 10^{-5}g/ml) were added as an internal standard, and the samples were analysed by HPLC as in chapter 2.2.2.1. The retention time of fluocinolone acetonide was 4 minutes and that of the internal standard was 6 minutes using a 10cm column and a mobile phase of acetonitrile:water (45:55).

2.9.2 DEGRADATION OF FLUOCINONIDE UNDER ALKALINE CONDITIONS

The initial experiment studied alkaline degradation under the following conditions;

a) in a fridge, at 4°C,

b) in the dark, at room temperature,

c) in diffuse daylight, at room temperature,

d) in the presence of sodium sulphite,

e) in the presence of EDTA.

d) and e) were also in diffuse daylight, at room temperature.

100 microlitres of 5 x 10^{-2}g/ml of both sodium sulphite and EDTA were added, to give a concentration of 2 x 10^{-4}g/ml in the reaction mixture, using the di-sodium salt of EDTA and using acetonitrile:water (70:30) to prepare dilutions. The reaction mixture consisted of 25ml of Metosyn® extracted from the ointment as in chapter 2.2.1, with the addition of 50 microlitres of 5M NaOH. Aliquots of 500 microlitres of the reaction mixture were added to 1ml quantities of borate buffer (3 x 10^{-2}g/ml) over a five hour time span.

The samples were analysed by HPLC as in chapter 2.2.2.1 adding 500 microlitres of butyl 4-hydroxybenzoate (1.25×10^{-5}g/ml) as an internal standard immediately prior to chromatography. The retention time of fluocinonide was 8 minutes and that of the internal standard was 6 minutes, using a 10cm column and a mobile phase of acetonitrile:water (45:55).

The degradation of fluocinonide in Metosyn® cream was repeated using the same revised method as for Synalar® in chapter 2.9.1, studying the following reaction conditions and quenching with Hepes buffer;

a) control
b) with EDTA
c) with sodium sulphite
d) with both EDTA and sodium sulphite.

The reaction mixture consisted of 7.5ml Metosyn® extract and 75 microlitres of 5M NaOH, to which 100 microlitres of 3.75×10^{-2}g/ml EDTA and 50 microlitre of 1.5×10^{-2}g/ml sodium sulphite were added. The reaction took place in a covered water bath at 30°C, taking 0.5ml samples over a three hour period and quenching with 2ml of Hepes buffer (1×10^{-2}g/ml) pH 3.5. An internal standard of 1ml of butyl 4-hydroxybenzoate (1.25×10^{-5}g/ml) was added prior to analysis by HPLC as in chapter 2.2.2.1. The retention time of fluocinonide was 8 minutes and that of the internal standard was 6 minutes, using a 10cm column and a mobile phase of acetonitrile:water (45:55).

2.9.3 DIRECT COMPARISON OF DEGRADATION OF FLUOCINOLONE ACETONIDE AND FLUOCINONIDE

The experiment discussed in chapter 2.9.1. and 2.9.2 using Hepes buffer as a quenching agent were repeated concurrently in order to make a direct comparison between the data. The conditions studied were;

a) fluocinolone acetonide : control,
b) fluocinolone acetonide + EDTA,
c) fluocinonide : control,
d) fluocinonide + EDTA.

All experimental conditions were as in 2.9.1. and 2.9.2 above. Measurements of pH were taken in the presence and absence of EDTA.

2.10 ACID DEGRADATION

Fluocinolone acetonide and fluocinonide were each subjected to degradation by acid at pH 1.2 and quenched with Hepes buffer (1 x 10^{-2}g/ml). The reaction mixtures consisted of the following:

5ml steroid extract	+ 25 microlitres 2M HCl	+ 2.5ml Hepes buffer (1 x 10^{-2}g/ml)

Measurements of pH were taken in the presence and absence of EDTA. Where EDTA was used, 100 microlitres of a 3.75 x 10^{-2}g/ml solution of the sodium salt in water was added to the reaction mixture.

Each steroid was subjected to acid conditions in an incubator at 30°C. The steroids were extracted as in chapter 2.2.1, and 100 microlitres of 2M HCl were added to 20ml of the extract. Aliquots of 1 ml were withdrawn from the reaction mixture and added to 0.5ml of Hepes buffer (1 x 10^{-2}g/ml), then frozen at -20°C to await analysis by HPLC as in chapter 2.2.2.1. An internal standard of 500 microlitres of butyl 4-hydroxybenzoate (1.25 x 10^{-5}g/ml) was added to each sample immediately prior to chromatography. Samples were taken over a six hour period and also after 24 hours, 30 hours and one week in the incubator.

Samples were initially run without internal standard to ensure that no degradation peaks were masked by the peak of the butyl 4-hydroxybenzoate. The retention time of fluocinolone acetonide was 4 minutes and that of the internal standard was 6 minutes, using a 10cm column and a mobile phase of acetonitrile:water (45:55).

2.11 FORMULATION

Formulation studies were carried out on fluocinolone acetonide using the commercially available Synalar® ointment, and on fluocinonide using Metosyn-® cream.

2.11.1 COMPOUNDING TECHNIQUE

Dilutions of Synalar® ointment and Metosyn® cream were carried out using the tile and spatula method. The tile and spatula method of trituration is carried out on a white porcelain ointment slab. A steel spatula is used to mix the whole of the medicament intimately with a little of the base, and then the remainder of the base is incorporated gradually. The spatula is held flat, and worked with a side to side motion rather than a rotary one[46]. For the stability studies 200g batches were made of steroid:base 1 in 4 and 1 in 10. For recovery experiments 20g batches were prepared. The batches were packed into amber glass jars, the lids of which had been lined with greaseproof paper.

2.11.2 COMPARISON OF EXTRACTION PROCEDURES

2.11.2.1 METHOD 1 - SERIAL EXTRACTION

One gram of the formulation under investigation was weighed directly into a 100ml glass separating funnel. The sample was dispersed in 10ml of hexane and extracted using three aliquots of 2.5ml acetonitrile. After each extraction the funnel was allowed to stand and the acetonitrile layer was collected in a 10ml volumetric flask. After the third extraction 0.5ml of butyl 4-hydroxybenzoate (1.25×10^{-4}g/ml) was added to the 10ml flask as an internal standard and the solution was made to volume with acetonitrile.

The sample was analysed by HPLC as in chapter 2.2.2.1 except that the mobile phase used was acetonitrile:water (40:60). The retention time of fluocinonide was 12 minutes, that of fluocinolone acetonide was 5 minutes, and that of the internal standard was 8 minutes, using a 10cm column.

2.11.2.2 METHOD 2

One gram of the formulation under investigation was weighed directly into a 100ml glass separating funnel. The sample was dispersed in 10ml hexane and extracted using 10ml of acetonitrile as a single addition. After shaking, the funnel was allowed to stand and the acetonitrile layer was collected in a glass vial. A 5ml sample was taken and 0.5ml of butyl 4-hydroxybenzoate (1.25×10^{-4}g/ml) was added as an internal standard.

The sample was analysed by HPLC as in chapter 2.2.2.1 except that the mobile phase used was acetonitrile:water (40:60).

2.11.2.3 METHOD 3

One gram of the formulation under investigation was weighed directly into a 100ml glass separating funnel and dispersed in 10ml of hexane. The internal standard, 0.5ml of butyl 4-hydroxybenzoate (3.75×10^{-4}g/ml), was added to the funnel and the sample was extracted using a single addition of 10ml of acetonitrile. After shaking, the funnel was allowed to stand and the acetonitrile layer was collected in a glass vial.

The sample was analysed by HPLC as in chapter 2.2.2.1 except that the mobile phase used was acetonitrile:water (40:60).

Method 2 was chosen for subsequent investigations.

2.11.3 RECOVERY EXPERIMENTS

2.11.3.1 RECOVERY OF FLUOCINOLONE ACETONIDE FROM 1 IN 4 FORMULATION

In order to calculate the recovery of the steroid from the bases used in formulation, a solution of fluocinolone acetonide is required which contains a quantity of steroid equivalent to that in the formulation. A 1 in 4 formulation of Synalar® ointment in base contains 1g of Synalar® ointment in 4g of diluted product. Synalar® ointment contains 0.025% w/w fluocinolone acetonide, therefore 1.875×10^{-4}g of the acetonide should be added to 3g of base to produce a formulation with an equivalent w/w concentration. A solution of 1.875×10^{-3}g/ml fluocinolone acetonide in acetonitrile was prepared from the powder, 100 microlitres of which contains 1.875×10^{-4}g of the steroid.

2.11.3.1.1 CONTROL SOLUTION

This was prepared by pipetting 15ml of acetonitrile into a glass vial, and adding 50 microlitres of fluocinolone acetonide (1.875×10^{-3}g/ml) using a glass

microsyringe. The vial was shaken and 5ml withdrawn to which 0.5ml of butyl 4-hydroxybenzoate (3.75×10^{-4}g/ml) was added as an internal standard.

2.11.3.1.2 EXTRACTION FROM THE BASE

The bases under investigation are Lipobase® and Unguentum Merck®.

A microsyringe was used to inject 100 microlitres of fluocinolone acetonide solution (1.875×10^{-3}g/ml) into 3g of each base. The steroid was incorporated into the base on an ointment slab using the tile and spatula method. One gram of the resultant formulation was weighed directly into a 100ml glass separating funnel and extracted as in Method 2, chapter 2.11.2.2.

2.11.3.1.3 EXTRACTION FROM HEXANE

A 15ml quantity of hexane was pipetted into a 100ml glass separating funnel and 50 microlitres of fluocinolone acetonide solution (1.875×10^{-3}g/ml) was added using a 100 microlitre microsyringe. The funnel was shaken to mix, then 15ml of acetonitrile were added and the funnel shaken again. The acetonitrile layer was collected and a 5ml sample taken, to which 0.5ml of butyl 4-hydroxybenzoate (1.25×10^{-4}g/ml) was added as an internal standard.

2.11.3.1.4 USE OF DIMETHYLDICHLOROSILANE

In an attempt to improve the recovery of fluocinolone acetonide from a 1 in 4 formulation of Unguentum Merck®, all glassware was treated with 2% w/v dimethyldichlorosilane in 1,1,1-trichloroethane solution. Each item was rinsed with sufficient solution to completely coat the glass surface. Excess solution was allowed to drain away and the glassware was left to dry, then rinsed with distilled water and left to dry again before use. The method described in chapters 2.11.3.1.1. to 2.11.3.1.3 was then followed.

Dimethyldichlorosilane solution was not used in subsequent experiments.

Each sample in chapter 2.11.3.1.1. to 2.11.3.1.4 was analysed by HPLC as in chapter 2.2.2.1 except that the mobile phase used was acetonitrile:water (40:60).

2.11.3.2 RECOVERY OF FLUOCINOLONE ACETONIDE FROM 1 IN 10 FORMULATION

The recovery was calculated as in chapter 2.11.3.1, taking 3g of formulation for analysis, which contained 7.5 x 10^{-5}g of acetonide. This is equivalent to adding 200 microlitres of a 3.75 x 10^{-4}g/ml solution of acetonide to 3g of base.

2.11.3.2.1 CONTROL SOLUTION

This was prepared by adding 200 microlitres of fluocinolone acetonide (3.75 x 10^{-4}g/ml) solution to 10ml of acetonitrile in a glass vial, shaking and removing 5ml, to which 0.5ml butyl 4-hydroxybenzoate (1.25 x 10^{-4}g/ml) was added as an internal standard.

2.11.3.2.2 EXTRACTION FROM BASE

The method used in chapter 2.11.3.1.2 to incorporate the fluocinolone acetonide solution into the base on an ointment tile proved difficult to carry out in a reproducible manner. In the following experiments the base was weighed directly into a 100ml glass beaker and the steroid solution was incorporated into the base in the beaker.

Fluocinolone acetonide (3.75 x 10^{-4}g/ml), 200 microlitres, was incorporated into 3g of each Lipobase® and Unguentum Merck® and dispersed in 5ml of acetonitrile in the beaker. This was transferred to a 100ml glass separating funnel, the beaker was rinsed with 10ml of hexane to disperse the remaining base, and a further 5ml of acetonitrile was used to wash the beaker, adding the washings to the funnel. The funnel was shaken and the acetonitrile layer was collected. A 5ml sample was removed and 0.5ml of butyl 4-hydroxybenzoate (1.25 x 10^{-4}g/ml) was added as an internal standard.

2.11.3.2.3 EXTRACTION FROM HEXANE

Ten ml of hexane were pipetted into a 100ml glass separating funnel and 200 microlitres of fluocinolone acetonide (3.75 x 10^{-4}g/ml) were added using a 500 microlitre microsyringe. The funnel was shaken to mix, then 10ml of acetonitrile were added and the funnel was shaken again. The acetonitrile

layer was collected and 5ml removed, to which 0.5ml of butyl 4-hydroxybenzoate (1.25×10^{-4}g/ml) was added as an internal standard.

Each sample in chapter 2.11.3.2.1 to 2.11.3.2.3 was analysed by HPLC as in chapter 2.2.2.1 except that the mobile phase used was acetonitrile:water (35:65). The retention time of fluocinolone acetonide was 7 minutes and that of the internal standard was 13 minutes, using a 10cm column.

2.11.3.3 RECOVERY OF FLUOCINONIDE FROM 1 IN 4 FORMULATION

The recovery of fluocinonide from a 1 in 4 formulation was calculated as in chapter 2.11.3.1. Metosyn® cream contains 0.05% w/w of fluocinonide, therefore 1.25×10^{-4}g of fluocinonide should be added to 1g of the base to produce a formulation with an equivalent w/w concentration.

A 5×10^{-3}g/ml solution of fluocinonide in acetonitrile was prepared from the powder which contains 1.25×10^{-4}g in 25 microlitres and was measured using a 25 microlitre glass microsyringe.

2.11.3.3.1 CONTROL SOLUTION

This was prepared by adding 25 microlitres of fluocinonide (5×10^{-3}g/ml) to 10ml of acetonitrile in a glass vial, shaking to mix. A 5ml sample was taken to which 0.5ml of butyl 4-hydroxybenzoate (1.25×10^{-4}g/ml) was added as an internal standard.

2.11.3.3.2 EXTRACTION FROM BASE

Fluocinonide (5×10^{-3}g/ml) was incorporated into 1g of each Lipobase®, Unguentum Merck® and Metosyn Diluent® using the method described in chapter 2.11.3.2.2. The only modification was that a 25ml glass beaker was used.

2.11.3.3.3 EXTRACTION FROM HEXANE

Extraction was performed as in chapter 2.11.3.2.3 except that 25 microlitres of fluocinonide were used in place of the fluocinolone acetonide.

Each sample in chapter 2.11.3.3.1 to 2.11.3.3.3 was analysed by HPLC as in chapter 2.2.2.1 except that a mobile phase of acetonitrile:water (40:60) was used.

2.11.3.4 RECOVERY OF FLUOCINONIDE FROM 1 IN 10 FORMULATION

The recovery was calculated as in 2.11.3.1. The 3g of formulation taken for analysis contain 1.5 x 10^{-4}g of fluocinonide and this is equivalent to adding 375 microlitres of a 4 x 10^{-4}g/ml solution of fluocinonide to 3g of base. However, this volume cannot be measured on the 500 microlitre microsyringe so 370 microlitres were used and a correction factor was applied.

2.11.3.4.1 CONTROL SOLUTION

This was prepared by adding 370 microlitres of fluocinonide (4 x 10^{-4}g/ml) solution to 10 ml of acetonitrile in a glass vial, shaking and removing 5ml, to which 0.5ml of butyl 4-hydroxybenzoate (1.25 x 10^{-4}g/ml) was added as an internal standard.

2.11.3.4.2 EXTRACTION FROM BASE

Fluocinonide solution (4 x 10^{-4}g/ml) , 370 microlitres, was incorporated into 3g aliquots of each Lipobase®, Unguentum Merck® and Metosyn Diluent®, using the method described in chapter 2.11.3.2.2.

2.11.3.4.3 EXTRACTION FROM HEXANE

This was carried out as in chapter 2.11.3.3.3, the only modification was that 370 microlitres of fluocinonide solution (4 x 10^{-4}g/ml) was used as the steroid.

Each sample in chapter 2.11.3.4.1 to 2.11.3.4.3 was analysed by HPLC as in chapter 2.2.2.1.

2.11.4 STABILITY OF FORMULATIONS

2.11.4.1 FLUOCINOLONE ACETONIDE (SYNALAR®)

The stability of the commercial preparation Synalar® ointment (fluocinolone acetonide 0.025% w/w) diluted in Lipobase® and Unguentum Merck® was investigated. The following formulations were prepared as 200g batches, using the tile and spatula method described in chapter 2.11.1;

1) Synalar® ointment 1 in 4 in Lipobase®,
2) Synalar® ointment 1 in 4 in Unguentum Merck®,
3) Synalar® ointment 1 in 10 in Lipobase®,
4) Synalar® ointment 1 in 10 in Unguentum Merck®.

Each batch was packed into 1 x 100g and 2 x 50g amber glass jars with tightly fitting screw lids which were lined with greaseproof paper. The 100g sample was stored at 25°C in a cooled incubator and the two 50g samples were stored at 4°C in a refrigerator and 32°C in an incubator. Samples were taken at regular intervals over a nine month period and analysed for steroid content. Three samples were taken on the day of preparation which served as a Day Zero reading and also as a homogeneity test.

2.11.4.1.1 FLUOCINOLONE ACETONIDE : STANDARD SOLUTION

A 1ml quantity of a 9.375 x 10^{-5}g/ml solution of fluocinolone acetonide in acetonitrile contains an equivalent amount of fluocinolone acetonide to 50 microlitres of the standard solution used in chapter 2.11.3.1. This was added to 10ml of hexane and extracted using 10ml of acetonitrile. The acetonitrile layer was collected and 5 ml removed, to which 0.5ml of butyl 4-hydroxybenzoate (1.25 x 10^{-4}g/ml) was added as an internal standard.

2.11.4.1.2 EXTRACTION FROM BASE

Samples of 1g were taken of each 1 in 4 formulation and 3g samples of the 1 in 10 formulations were taken for analysis. Extractions were performed as described in chapter 2.11.2.2. Duplicate samples were taken for each analysis.

All samples in chapter 2.11.4.1.1 and 2.11.4.1.2 were analysed by HPLC as in chapter 2.2.2.1 except that a mobile phase of acetonitrile:water (35:65) was used.

2.11.4.2 FLUOCINONIDE (METOSYN®)

The stability of the commercial preparation Metosyn® cream (fluocinonide 0.05% w/w) diluted in Lipobase®, Unguentum Merck® and Metosyn Diluent® was investigated. The following formulations were prepared as 200g batches, using the tile and spatula method described in chapter 2.11.1;

1) Metosyn® cream 1 in 4 in Lipobase®,
2) Metosyn® cream 1 in 4 in Unguentum Merck®,
3) Metosyn® cream 1 in 4 in Metosyn Diluent®,
4) Metosyn® cream 1 in 10 in Lipobase®,
5) Metosyn® cream 1 in 10 in Unguentum Merck®,
6) Metosyn® cream 1 in 10 in Metosyn Diluent®.

Each batch was packed into 1 x 100g and 2 x 50g amber glass jars with tightly fitting screw lids which were lined with greaseproof paper. The 100g sample was stored at 25°C in a cooled incubator and the two 50g samples were stored at 4°C in a refrigerator and 32°C in an incubator. Samples were taken at regular intervals over an eight month period and analysed for steroid content. Three samples were taken on the day of preparation which served as a Day Zero reading and also as a homogeneity test.

2.11.4.2.1 FLUOCINONIDE : STANDARD SOLUTION

A 1ml quantity of a 1.25 x 10^{-4}g/ml solution of fluocinonide in acetonitrile contains an equivalent quantity of fluocinonide to 25 microlitres of the standard solution of fluocinonide used in chapter 2.11.3.3. This was added to 10ml of hexane and extracted with 10ml of acetonitrile. The acetonitrile layer was collected and 5ml removed, to which 0.5ml of butyl 4-hydroxybenzoate (1.25 x 10^{-4}g/ml) was added as an internal standard.

2.11.4.2.2 EXTRACTION FROM BASE

Samples of 1g were taken of each 1 in 4 formulation and 3g samples of the 1 in 10 formulations were taken for analysis. Extractions were performed as described in chapter 2.11.2.2. Duplicate samples were taken for each analysis.

All samples in chapter 2.11.4.2.1 and 2.11.4.2.2 were analysed by HPLC as in chapter 2.2.2.1 using a mobile phase of acetonitrile:water (45:55). The retention time of fluocinonide was 7 minutes and that of the internal standard was 5 minutes, using a 10cm column.

2.11.5 VOLUNTEER TRIAL

The blanching potential of nine topical preparations was investigated using ten volunteers. The volunteers were young adults (18-33 years), 2 males and 8 females, who had no previous history of skin or other disease and had not used either topical or systemic corticosteroids for at least one month prior to the study. Where extemporaneous formulation (Ex) was required, the tile and spatula method described in chapter 2.11.1 was used, and the products made one week prior to commencement of the trial. The formulations studied were:

A) Synalar® ointment
B) Synalar® ointment 1 in 4 in Lipobase® (Ex)
C) Synalar® ointment 1 in 4 in Unguentum Merck® (Ex)
D) Synalar® ointment 1 in 4 - commercial preparation
E) Synalar® ointment 1 in 10 in Lipobase® (Ex)
F) Synalar® ointment 1 in 10 in Unguentum Merck® (Ex)
G) Lipobase®
H) Unguentum Merck®
I) Control Site (Blank)
J) Betnovate® ointment

A template was cut in Setonplast tape to produce a rectangle with five apertures, each one 3.5cm x 1.5cm in size. The template was applied to the flexor surface of the forearm, avoiding the areas close to the wrist and the

elbow. The sites on the dominant arm were numbered 1-5 and those on the non-dominant arm from 6-10. Readings of the logarithm of the inverse of reflectance (LIR) were measured at each site using a Haemelometer, as described in chapter 1.3. Duplicate readings were taken at each site. Each volunteer was assigned a trial number 1-10 on a random basis by drawing lots. The trial number corresponded to a Latin square arrangement of the formulations as shown in table 2.1.

TABLE 2.1 A LATIN SQUARE OF FORMULATION TO SITE OF APPLICATION FOR TEN VOLUNTEERS

VOLUNTEER	1	2	3	4	5	6	7	8	9	10
SITE										
1	A	B	C	D	E	F	G	H	I	J
2	J	A	B	C	D	E	F	G	H	I
3	I	J	A	B	C	D	E	F	G	H
4	H	I	J	A	B	C	D	E	F	G
5	G	H	I	J	A	B	C	D	E	F
6	F	G	H	I	J	A	B	C	D	E
7	E	F	G	H	I	J	A	B	C	D
8	D	E	F	G	H	I	J	A	B	C
9	C	D	E	F	G	H	I	J	A	B
10	B	C	D	E	F	G	H	I	J	A

A glass insulin syringe was used to apply 0.1ml of each formulation to the corresponding site by a senior pharmacy technician. The sample was spread evenly over the site with a glass rod. The forearm was then occluded with Blenderm tape for three hours, after which the tape was removed and the skin washed with warm water (30°C) and soap and patted dry. The sites were left exposed for half an hour to reduce the effects of trauma due to removal of

the tape, and then readings of the LIR were taken at each site by the observer. The same technician and observer carried out all the applications and readings. Successive readings were taken over several days up to 75 hours after initial application. After the 8 hour reading a surgical skin marker was used to outline each aperture and the Setonplast tape was removed. For the first 24 hours the volunteers were instructed to avoid contact with water on their arms, extremes of temperature, vigorous exercise and consumption of alcohol.

2.11.5.1 PROCEDURE FOR USE OF THE HAEMELOMETER

The instrument has been described in chapter 1.3. The version used in this study was the Haemelometer Model IV, Medical Physics Dept., Leeds Royal Infirmary.

The measuring head was lightly rested on the skin, without applying pressure, and a reading of the LIR initiated by depressing a footswitch. After 5-10 seconds the reading was displayed on a digital panel meter, and was held on display until the footswitch was depressed again. Three tiles were used to calibrate the instrument, one of each white, light pink and dark pink. A reading was taken on each tile for every reading taken on skin and a normalised blanching index calculated for each preparation.

3. RESULTS AND DISCUSSION

Topical corticosteroids are widely used in the treatment of inflammatory skin conditions. A range of steroid creams and ointments are available commercially of varying potencies, from mild to very potent. However, dermatologists often prescribe a proprietary high potency steroid which is then diluted extemporaneously in an inactive base. Many new bases have been developed in recent years which are becoming increasingly used, but for which there is little or no information available on their stability in the final formulation or on the clinical efficacy of the diluted products as compared to the original steroid preparation.

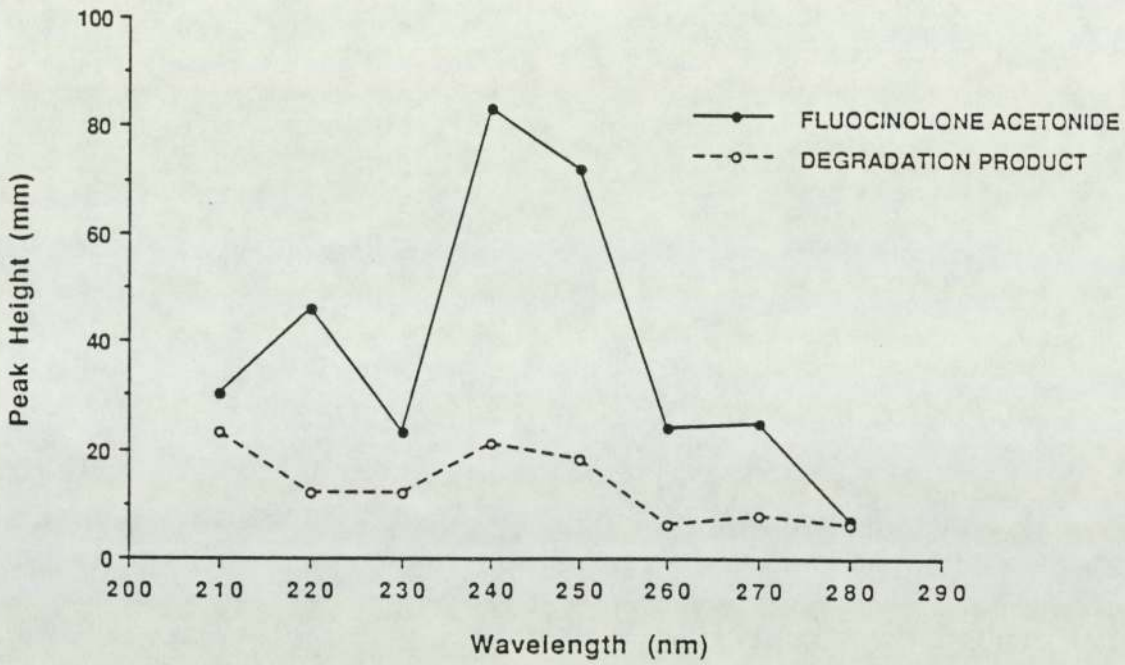
3.1 DEVELOPMENT OF A STABILITY-INDICATING ASSAY

A stability-indicating assay was developed using HPLC because it has been shown to be a fast, accurate and reproducible method of analysis for closely related compounds such as corticosteroids[43]. Initially a Rheodyne 7125 valve was used as described in chapter 2.2.2 which required manual injection. This was later replaced by a Rheodyne 7010 valve in conjunction with an autosampler. The automatic system gave greater flexibility to the operator, was more time efficient and allowed samples to be stored and run overnight.

Fluocinonide and fluocinolone acetonide were extracted from proprietary formulations using the method described in chapter 2.2.1. Ointments are more frequently diluted in clinical practice in the district of West Birmingham than the creams. However, Metosyn Diluent® is produced by the manufacturers for the dilution of Metosyn® cream. This was one of the bases used in the formulation experiments and therefore Metosyn® cream was also included in the stability experiments.

An extract of Synalar® ointment which had been subjected to alkaline degradation was analysed over the wavelength range 210-280nm as described in chapter 2.2.2.2 in order to determine the wavelength at which the maximum absorbance of the parent and the major degradation peak occurred. The sample was repeatedly analysed at 10nm increments and the peak height of

Fig 3.1 GRAPH OF PEAK HEIGHT OF FLUOCINOLONE ACETONIDE AND THE MAJOR DEGRADATION PRODUCT OVER THE WAVELENGTH RANGE 210-280 nm.



the steroid and the major degradation peak (peak A) were measured. A graph of peak height was plotted against wavelength, fig 3.1.

The graph shows a maximum at 240nm, compared with 239nm as stated in the British Pharmacopoeia[47], for both the parent steroid and the major degradation product, therefore 240nm was chosen as the optimum wavelength for analysis. All subsequent HPLC procedures were performed at this wavelength. Addition of 10% phosphoric acid (1 in 10) to the mobile phase to give a pH of 4 did not improve peak resolution and was not incorporated into the method.

3.2 POTENTIAL PROBLEMS IN VOLUMETRIC ACCURACY

During the extraction and analysis procedures microlitre quantities of solutions were required. Initially, these were measured using automatic air-displacement pipettes. However, due to unexpected erratic results the precision of the automatic pipette was investigated. Eight 500 microlitre aliquots of acetonitrile were weighed into a glass vial and the weight recorded after each addition as described in chapter 2.3.1. The weight of each aliquot was calculated and is shown in table 3.1. Acetonitrile has a weight per ml of 0.78g, therefore the samples measured with the autopipette should have weighed 0.39g.

TABLE 3.1 WEIGHTS OF EIGHT 500 MICROLITRE ALIQUOTS OF ACETONITRILE MEASURED WITH AN AUTOMATIC AIR-DISPLACEMENT PIPETTE

ALIQUOT	WEIGHT (G)
1	0.373
2	0.379
3	0.393
4	0.391
5	0.384
6	0.391
7	0.398
8	0.393

The mean of all eight results is 0.388 with a coefficient of variation of 2.16%. However, the first two results were significantly lower than the last six, despite the fact that the pipette tip was rinsed before the first measurement in accordance with the manufacturer's instructions. If the first two results are considered to be part of the wetting process and omitted from the analysis, the mean of the last six results is 0.392 with a coefficient of variation of 1.78%. Even with pre-wetting the precision of the automatic air-displacement pipette was unacceptably low.

The reproducibility of the automatic air-displacement pipette was compared to that of a 0.5ml class A glass pipette to determine whether the problems of volumetric accuracy could be overcome by using a manual technique. Aliquots of a sample reaction mixture were pipetted into glass vials using the automatic pipette and the manual pipette, making three measurements with each as described in chapter 2.3.2. The samples were analysed by HPLC, injecting automatic and manually pipetted aliquots alternately. The first sample analysed has been measured with the automatic pipette and the ratio of the peak area of the steroid present in this sample to the internal standard

was taken as 100%. The mean peak area was calculated for each sample, as shown in table 3.2, and expressed as a percentage of the first aliquot.

TABLE 3.2 INVESTIGATION OF THE VOLUMETRIC ACCURACY OF THE AIR-DISPLACEMENT AUTOMATIC AND MANUAL GLASS PIPETTE

Sample	Mean Peak Area Ratio FA/IS	Mean Peak Area Ratio as a % of automatic 1st measurement
Automatic 1	0.835	100
Automatic 2	1.369	164
Automatic 3	1.392	167
Manual 1	1.403	168
Manual 2	1.416	170
Manual 3	1.4235	171

FA = Fluocinolone acetonide; IS = Internal standard; MEAN PEAK AREA RATIO = Ratio of the peak areas of FA/IS; Automatic 1,2,3 = Three measurements made with air-displacement pipette; Manual 1,2,3 = Three measurements made with glass pipette.

The coefficient of variation of all six readings was large at 17.8%, but if the first measurement taken with the automatic pipette was omitted, the coefficient of variation of the remaining five readings was 1.6%. There was an apparent improved reproducibility using the class A glass pipette due to the low initial measurement made with the automatic pipette. This may be due to poor tip-wetting although the tip was rinsed five times with the test solution to try to overcome this problem, and the result was lower than would have been expected if it had been purely a wetting phenomenon. Alternatively, the steroid may have undergone absorption into the plastic. This was not investigated further. The automatic pipette was used in initial

experiments, but was replaced by glass pipettes and glass microsyringes to avoid these problems.

3.3 ACCELERATED DEGRADATION OF STEROIDS

One aim of this work was to set up a stability-indicating assay which is capable of separating fluocinolone acetonide and fluocinonide from their major degradation products. To test the method, the two steroids were each subjected to an accelerated degradation by the addition of alkali as described in chapter 2.4.1. Prior to degradation each steroid was analysed by HPLC. Initial experiments were performed using a 25cm Spherisorb ODS 1 column and a mobile phase of acetonitrile:water (50:50). The retention time of fluocinonide was 10 minutes, that of fluocinolone acetonide was 6 minutes, and that of the internal standard, butyl 4-hydroxybenzoate, was 8 minutes. The column was changed during the course of the experiments to a 10cm Spherisorb ODS 1 column for a faster analysis time and an improved peak shape. The subsequent retention times were 8 minutes and 4 minutes respectively for the steroids and 6 minutes for the internal standard, using a mobile phase of acetonitrile:water (45:55). The improvement in resolution is shown in fig 3.2 using a sample of fluocinonide degraded in alkali for 3 hours.

On the addition of alkali to the steroid solutions, a rapid degradation was seen. Samples were analysed after 15 minutes contact with the alkaline medium and after one hour. A series of new peaks were seen with shorter retention times than the original steroid. The retention times of these peaks varied between approximately 3 minutes and 6 minutes, for both fluocinolone acetonide and fluocinonide using the 25cm column (fig 3.3 and fig 3.4).

Fluocinonide produced a major degradation peak with the same retention time as fluocinolone acetonide. A number of other peaks were also detected, which had similar retention times and peak shapes for both the degraded fluocinonide and fluocinolone acetonide.

The results indicate that under alkaline conditions fluocinonide degrades to fluocinolone acetonide *via* ester hydrolysis. This is the major degradation

Fig 3.2. CHROMATOGRAPHY OF FLUOCINONIDE SUBJECTED TO BASE-CATALYSED HYDROLYSIS USING a) 25cm b) 10cm SPHERISORB ODS 1 COLUMN. F = FLUOCINONIDE, IS = INTERNAL STANDARD, FA = FLUOCINOLONE ACETONIDE

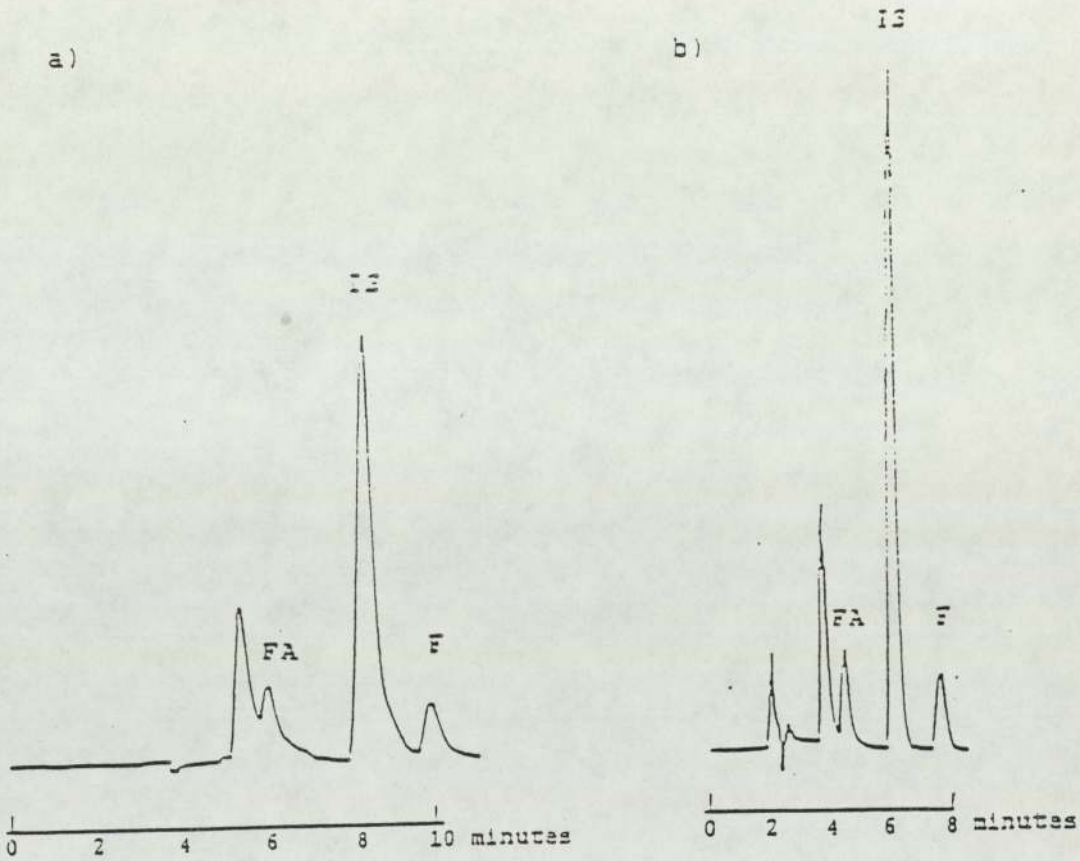


Fig 3.3. CHROMATOGRAPHY OF FLUOCINOLONE ACETONIDE SUBJECTED TO BASE-CATALYSED HYDROLYSIS a) TIME ZERO b) AFTER 15 MINUTES c) AFTER 1 HOUR, USING A 25cm SPHERISORB ODS 1 COLUMN.
FA = FLUOCINOLONE ACETONIDE, A = PRIMARY DEGRADATION PRODUCT, B = SECONDARY DEGRADATION PRODUCT.

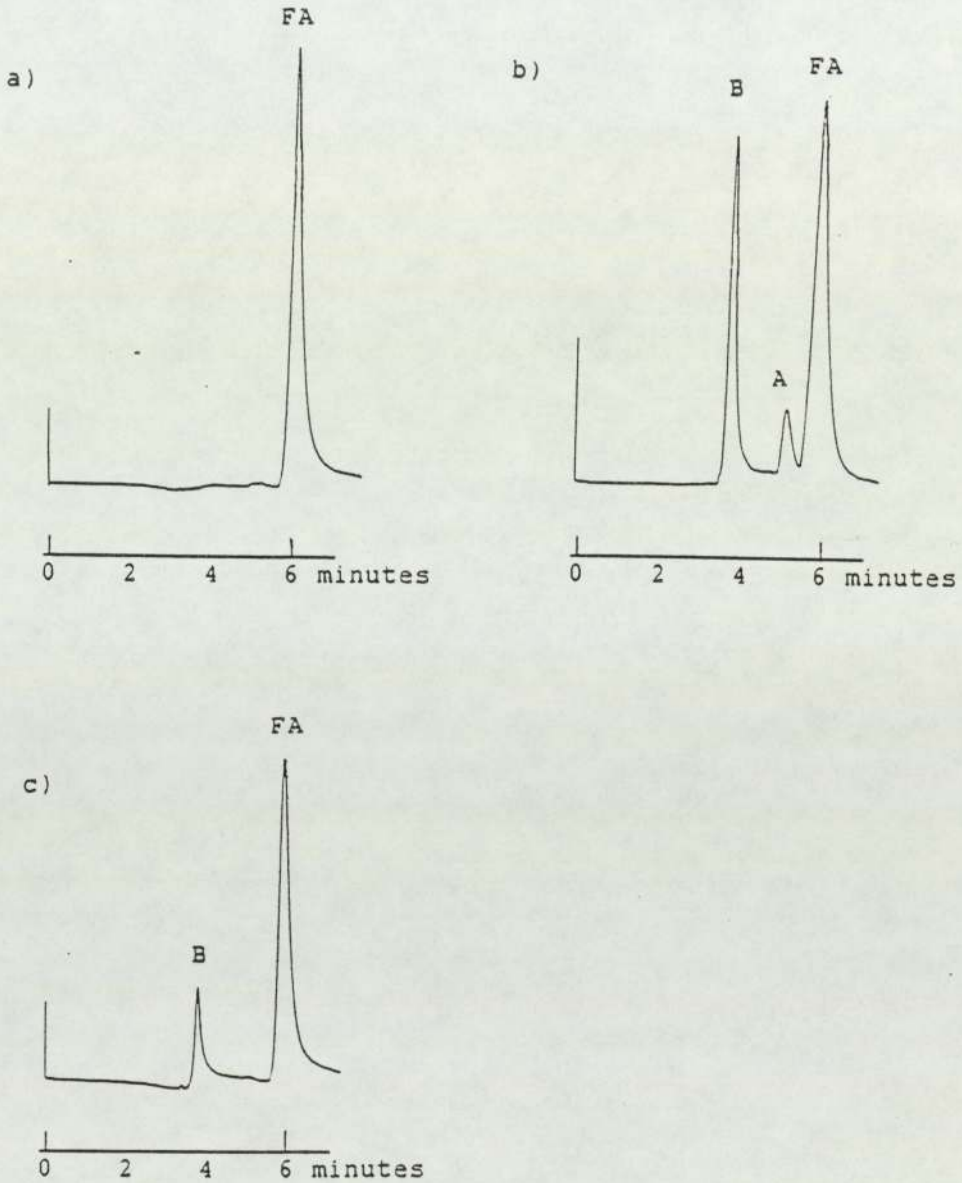
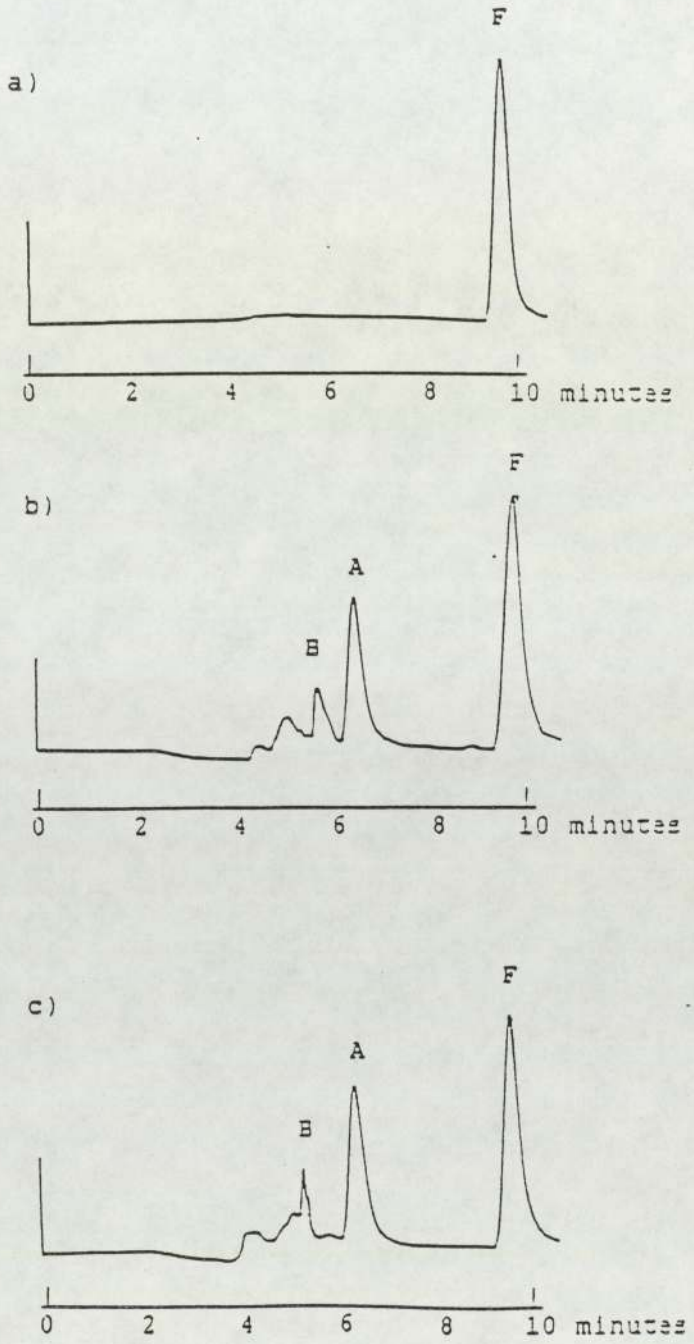


Fig 3.4. CHROMATOGRAPHY OF FLUOCINONIDE SUBJECTED TO BASE-CATALYSED HYDROLYSIS a) TIME ZERO b) AFTER 15 MINUTES c) AFTER 1 HOUR, USING A 25cm SPHERISORB ODS 1 COLUMN. F = FLUOCINONIDE, A = PRIMARY DEGRADATION PRODUCT (FLUOCINOLONE ACETONIDE), B = SECONDARY DEGRADATION PRODUCTS.



product, which in turn degrades to a series of decomposition products. When fluocinolone acetonide is taken as the parent steroid and subjected to alkaline degradation the decomposition profile is similar to that resulting from the degradation of fluocinonide. There was a major degradation product, peak A, and a series of smaller peaks close to the solvent front.

Powell et al[48] investigated the degradation profile of fluocinolone acetonide. They identified four decomposition products, a keto aldehyde, a keto acid, an etianic acid and a tetral compound (fig 3.5). Separation was achieved using a reversed-phase Altex Ultrasphere ODS column and a three-stage linear-gradient delivery of mobile phase. The mobile phase consisted of water and acetonitrile starting with 95% water:5% acetonitrile for five minutes changing to 70% water:30% acetonitrile for ten minutes and finally 50% water:50% acetonitrile for a further 25 minutes.

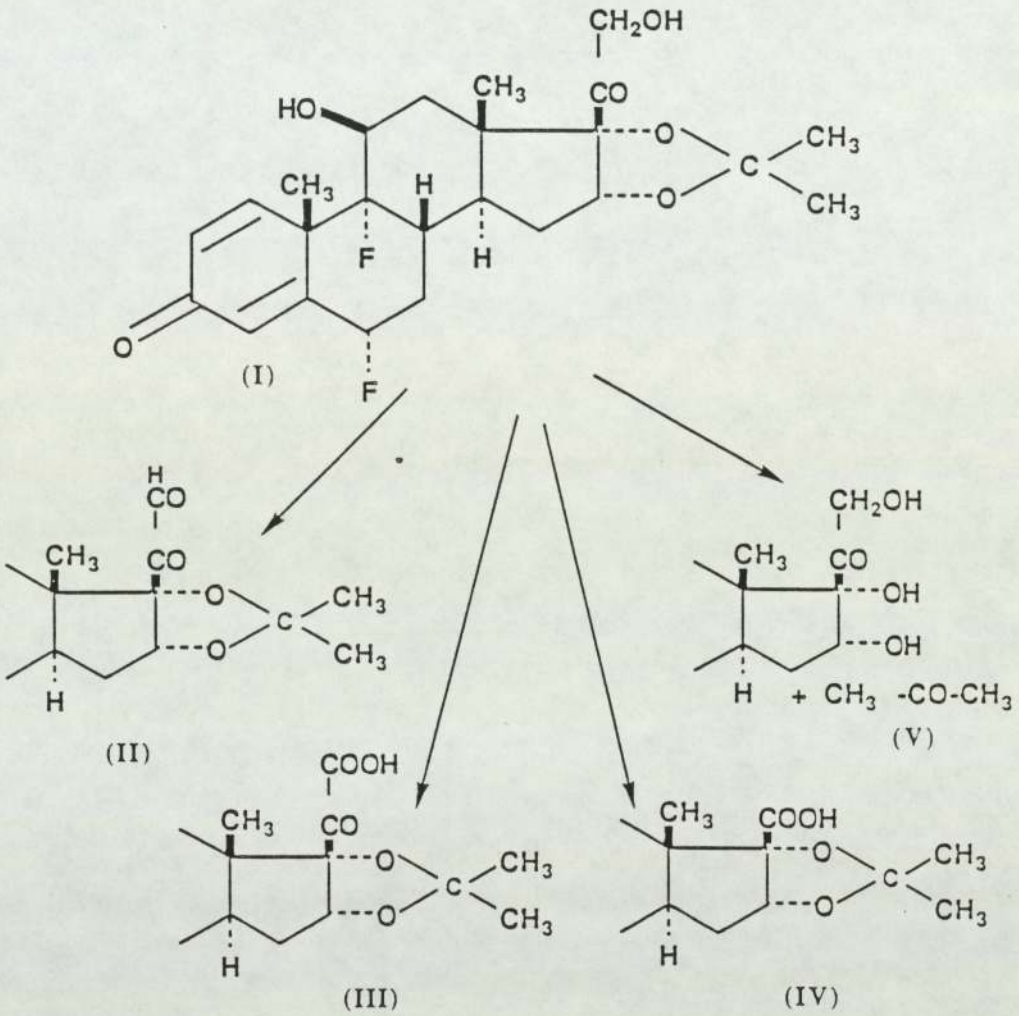
A gradient system was not available for use in this project and using the equipment available the degradation products could not be resolved.

3.4 EFFECT OF pH

Extracts of fluocinolone acetonide were exposed to alkali at pH 8, 10 and 12 as described in chapter 2.4.2 for a period of 15 minutes. The steroid underwent decomposition at each pH value studied to produce a major degradation peak with a retention time of 5.5 minutes on a 25cm column, and a second peak at 4 minutes which correlated to the pattern shown in fig 3.3. The primary degradation peak increased in size with increasing pH.

At pH 12, the mixture showed a degradation peak which had an area of 25% of that of the parent steroid. At pH 10, the degradation peak had a peak area of 12% of that of the parent, and at pH 8 it was only 4% of the peak area of the parent.

Fig 3.5 DEGRADATION PROFILE OF FLUOCINOLONE ACETONIDE, AFTER POWELL et al [48]. I = FLUOCINOLONE ACETONIDE, II = KETOALDEHYDE, III = KETOACID, IV = ETIANIC ACID, V = TETROL.



The pattern of degradation was the same at each pH value, but the more alkaline conditions yielded a more extensive reaction in the same time interval. The results suggest that the hydroxide ion concentration determines the rate of the reaction but not the route taken. However, since all of the degradation products could not be separated this cannot be demonstrated from the degradation profile.

3.5 THE IMPORTANCE OF GLASSWARE

Steroid extracts were stored in disposable polystyrene beakers at 4°C prior to analysis. Samples of Synalar® extract which had been stored in this manner demonstrated a peak on the chromatogram with a retention time of 10.3 minutes in addition to the fluocinolone acetonide peak which had a retention time of 6 minutes, when run on a mobile phase of acetonitrile:water (50:50), using a 25 cm column. The peak at 10.3 minutes had a peak area which was 125% of the area of the steroid peak. Because the unidentified peak had a retention time greater than that of the steroid peak it did not fit in with the degradation profile previously observed. It was thought to be a substance that had leached from the polystyrene beaker. To test this hypothesis a sample of acetonitrile:water (70:30) was stored for 24 hours at 4°C in a similar polystyrene beaker and then analysed by HPLC on a mobile phase of acetonitrile:water (50:50). A peak was seen on the chromatogram which had a retention time of 10.8 minutes, and a similar peak area to that of the unidentified substance eluted from the Synalar® extract. A sample of acetonitrile:water (70:30) was also stored at 4°C in a glass beaker. When analysed under similar conditions no peak was seen on the chromatogram. A sample of Synalar® extract was also stored for 24 hours at 4°C in a glass beaker and analysed by HPLC. Only one peak was observed which had a retention time of 6 minutes and was identified as fluocinolone acetonide.

These results show that the peak produced at 10.3 minutes, after storage in disposable polystyrene containers, was leached from the polystyrene. As the appearance of such peaks may be wrongly attributed to degradation of the steroids, all further work was carried out using glass containers. The use of plastic syringes was also found to be unsuitable as they rapidly disintegrated on contact with acetonitrile.

3.6 CHOICE OF INTERNAL STANDARD

An internal standard was required which was soluble in the solvent mixture used, which had a retention time which would not interfere with the main steroid peak, and which would not mask any peaks produced by degradation products. It should not have a retention time which is comparatively long, slowing down the analytical procedure.

The sodium salts of the paraben esters were investigated using a 10cm column and a mobile phase of acetonitrile:water (45:55) as described in chapter 2.5. Butyl 4-hydroxybenzoate had a retention time of 6 minutes compared to 4 minutes for fluocinolone acetonide and 8 minutes for fluocinonide. The butyl salt did not interfere with the degradation peaks of either steroid and was clearly separated from the steroid peaks on the system described. Butyl 4-hydroxybenzoate fulfilled all the criteria for an internal standard and was used in all subsequent experiments.

3.7 CHOICE OF BUFFERS

An effective buffering system was necessary to maintain the pH of the reaction mixture between 3 and 5 in order (1) to prevent damage to the column, and (2) to quench the acid or base-catalysed degradation. Acetate buffer was used in preliminary experiments as described in chapters 2.4.1 and 2.4.2. However, although this buffer effectively maintained the pH within the range required to quench the reaction, consistent results could not be obtained. An investigation revealed that acetate buffer, which contains sodium acetate in glacial acetic acid and water, has been shown to catalyse the degradation of fluocinonide[41]. A replacement buffer was therefore required which should be:

a) free from catalytic activity,

b) compatible with the acetonitrile present in the extraction media and the mobile phase,

c) capable of neutralising the pH sufficiently to both quench the degradation due to acid or base and prevent damage to the column.

In order to find a buffer which fulfilled these criteria a number of compounds were investigated. BDH buffer pH 4 produced a large peak on the HPLC trace over the time span in which the steroid and its degradation products would be eluted. BDH buffer contains potassium hydrogen phthalate and the peak was attributed to the presence of the phthalate in the buffer, which rendered it unsuitable for use in this investigation. Phosphate buffer was stored in a reaction mixture overnight, during which crystals formed. The temperature in the laboratory had fallen to 20°C. Since the reaction mixture and the buffer were required to be stable over the range of temperatures likely to be encountered in the laboratory this system was judged to be unsuitable, and was therefore abandoned. Citric acid was found to be compatible with the reaction mixture, but produced a broad peak on the HPLC trace, which covered the part of the chromatogram at which the steroid would be eluted. Dilution of the buffer did not reduce the interference caused by this buffer solution, which may have been due to contamination of the citric acid or of the solvent. However, since preparation of a fresh solution did not solve the problem the buffer was not investigated further. Boric acid has a pH range of 3.8 to 4.8 and a 3% solution was investigated as a neutralising agent. Difficulty was encountered in getting the solid into solution. The lowest pH attainable with the 3% solution was pH 4, which lowered the pH of the reaction solution to a minimum of 5 at which the reaction was not effectively quenched. Increasing the ratio of boric acid in the buffer to lower the pH resulted in precipitation of the borate. Erratic and unreliable results were obtained due to the partially quenched reaction continuing at a reduced rate therefore the buffer had to be abandoned.

Hydrochloric acid (0.08M, pH 1.5) was used in preliminary experiments (chapter 2.6.5.1) to quench the base-catalysed degradation of Synalar®

ointment. Three replicate samples of reaction mixture were taken, vials 1, 2 and 3, and alkali added followed by cooled acid, which reduced the pH to 3.5. If the acid was effective at quenching the reaction there should be no loss of steroid from any of the three vials. The first vial was analysed immediately, and at 5 minute intervals thereafter, whilst vials 2 and 3 sat on the bench at room temperature awaiting analysis. The ratio of the area of the fluocinolone acetonide peak to the internal standard was measured for each sample and are shown in table 3.3 as a percentage of the first reading from vial 1.

TABLE 3.3 EFFICACY OF HYDROCHLORIC ACID AT QUENCHING THE BASE-CATALYSED DEGRADATION OF FLUOCINOLONE ACETONIDE (25°C)

Sample	Time after quenching	Peak Area Ratio	Peak Area Ratio as a % of Vial 1 (t=0)
Vial 1	0 mins	2.00	100.0
	5 mins	1.93	96.5
	10 mins	1.75	87.5
	15 mins	1.56	78.0
Vial 2	20 mins	1.79	89.5
	25 mins	1.60	80.0
	30 mins	1.57	78.5
Vial 3	40 mins	1.65	82.5
	45 mins	1.13	56.5

FA = Fluocinolone acetonide; IS = Internal standard; PEAK AREA RATIO = ratio of the peak areas of FA/IS.

As the samples stood awaiting analysis there was a loss of steroid which was apparent both from vial to vial and within each vial itself. Vial 3 had been standing for 40 minutes prior to analysis, after which time there had been a loss of over 17% of the original concentration of fluocinolone acetonide. A one-way analysis of variance of the peak area ratios of FA/IS showed a significant difference between the steroid content in each vial, with an F value of 2.5 and a significance of 0.16, which was low due to the small sample size.

The ideal buffer would also be stable on long-term storage in a freezer at -20-°C. The ability to store samples in a freezer was an important advantage in a laboratory where there were limitations on the availability of the HPLC equipment. It also meant that samples could be stored and run as a batch overnight using the autosampler, which was more efficient in time and solvents than performing short runs of small batches. Freezer stability would also be useful in the design and feasibility of the experiments. Hydrochloric acid was therefore investigated under these conditions. The samples were quenched to pH 3.5 with acid as described in chapter 2.6.5.2. The samples were stored at -20°C and analysed over a 50 day period to determine the change in steroid content with time. The extent of any degradation was also quantified by measuring the peak area of the major degradation product of fluocinolone acetonide as shown in table 3.4. Peak A had a retention time of 2.5 minutes, the acetonide having a retention time of 3 minutes on a 10cm column with a mobile phase of acetonitrile:water (50:50), fig 3.6.

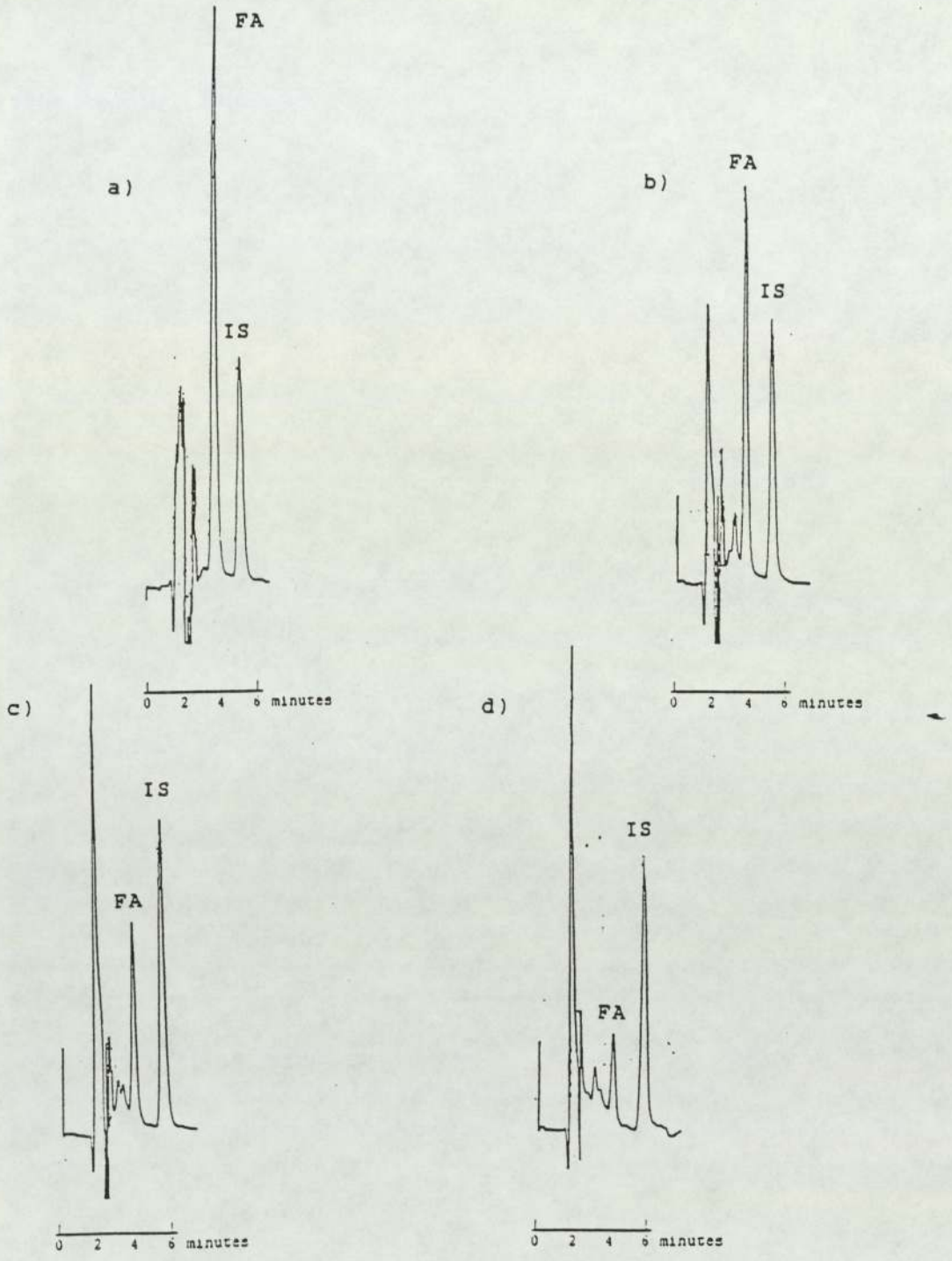
TABLE 3.4 THE QUENCHING EFFICACY OF HYDROCHLORIC ACID ON LONG-TERM STORAGE IN A FREEZER (-20°C)

Storage time	Mean Peak Area Ratio FA/IS (1)	Mean Peak Area Ratio A/IS (2)	MAR (1) as a % of 25.5h	MAR (2) as a % of MAR (1)
25.5hrs	0.3933	0.0181	100	4.6
14 days	0.4223	0.0234	107	6.0
31 days	0.4520	0.0213	115	5.4
50 days	0.4050	0.0315	103	8.0

FA = Fluocinolone acetonide; IS = Internal standard; A = Primary degradation peak; PEAK AREA RATIO = Ratio of the peak areas of FA/IS; MAR = MEAN AREA RATIO.

The amount of steroid present was found to be erratic. The area of peak A increased over the 50 day storage period, suggesting a continuing degradation of fluocinolone acetonide despite the quenching system used.

Fig 3.6. CHROMATOGRAPHY OF FLUOCINOLONE ACETONIDE SUBJECTED TO BASE-CATALYSED HYDROLYSIS a) TIME ZERO b) AFTER 15 MINUTES c) AFTER 45 MINUTES d) AFTER 75 MINUTES, USING A 10cm SPHERISORB ODS 1 COLUMN. FA = FLUOCINOLONE ACETONIDE, IS = INTERNAL STANDARD.



The results of the quenching experiments demonstrated the lack of efficacy and reliability of HCL as a buffer and therefore it was not investigated further.

Hepes buffer, which has a pKa of 7.55, was investigated for efficacy, stability, reproducibility and compatibility in a series of experiments as described in chapter 2.6.6.

The effectiveness of Hepes buffer was assessed by comparing the change in pH brought about by the addition of 100 microlitres of 1M HCl to 5ml of water and to 5ml of buffer. The initial pH of the water was 5.96 and this fell to 2.73 on addition of the acid. When HCl was added to Hepes buffer, pH 4.0, the pH was lowered to 3.5. A similar experiment was performed to assess the buffering capacity under alkaline conditions by adding 50 microlitres of 5M NaOH to 5ml of water and 5ml of Hepes buffer. The pH of the water rose to 10.7 on addition of the alkali and that of the buffer rose from an initial value of 4.0 to 4.9. Hepes buffer was therefore considered to be an effective buffer, maintaining the pH in the range 3.0-5.0 as required for maximum stability of the steroid.

Hepes buffer was physically compatible with a sample reaction mixture stored overnight in the light at room temperature, and in a fridge. Compatibility was judged by visual analysis, looking for precipitation, crystallisation and clarity of solution.

The efficacy of Hepes buffer at quenching the alkaline degradation of fluocinolone acetonide was investigated as described in chapter 2.6.6.1. All of the initial work was done on this steroid and the experiments were then extended to incorporate fluocinonide. Since fluocinonide is more stable than fluocinolone acetonide (see formulation studies chapter 3.11) the initial efficacy experiments were not repeated for the second steroid.

Sodium hydroxide was added to the extract of fluocinolone acetonide, followed by buffer, and samples were withdrawn over a four hour period in

order to follow the extent of the decomposition with time. Two replicate injections were used to calculate the mean peak area ratio of the steroid to the internal standard. This is expressed as a percentage of the value at time zero, after immediate quenching to pH 4.0, as shown in table 3.5.

TABLE 3.5 EFFICACY OF HEPES BUFFER AT QUENCHING THE BASE-CATALYSED DEGRADATION OF FLUOCINOLONE ACETONIDE (25°C): NO ADDITIVES

Sample Time	Mean Peak Area Ratio FA/IS	MAR as a % of value at t=0
0 mins	0.393	100.0
15 mins	0.400	101.8
40 mins	0.406	103.3
1 hr	0.394	100.3
2.5 hrs	0.392	99.8
4 hrs	0.394	100.3

FA = Fluocinolone acetonide; IS = Internal standard; PEAK AREA RATIO = Ratio of the peak areas of FA/IS; MAR = MEAN PEAK AREA RATIO.

A one-way analysis of variance was performed on the individual peak area ratios of fluocinolone acetonide/internal standard and the statistical parameters are shown in table 3.6.

TABLE 3.6 STATISTICAL PARAMETERS FOR EFFICACY OF HEPES BUFFER : NO ADDITIVES (TABLE 3.5)

$F_{0.05\ (1)\ 5,6} = 4.95$ $F = 0.827$	SIGNIFICANCE $p = 0.57$

The F value and the significance value support the chromatographic evidence that there was no degradation of the samples.

The above experiment was repeated with the addition of sodium sulphite to the reaction mixture. Sodium sulphite is an antioxidant which was investigated in the kinetics experiments in chapter 2.9, therefore it was important to determine whether or not the presence of sodium sulphite would effect the efficacy of Hepes buffer. A reaction mixture was prepared as previously except that 100 microlitres of a 0.15g/ml solution of sodium sulphite in water were added prior to quenching to pH 4. Samples were withdrawn over a four hour period, and the results are shown in table 3.7, based on two replicate injections.

TABLE 3.7 EFFICACY OF HEPES BUFFER AT QUENCHING THE BASE-CATALYSED DEGRADATION OF FLUOCINOLONE ACETONIDE (25°C) : SODIUM SULPHITE ADDED

Sample Time	Mean Peak Area Ratio FA/IS	MAR as a % of value at t=0
0 mins	0.407	100.0
15 mins	0.433	106.4
40 mins	0.411	101.0
1 hr	0.399	98.0
2.5 hrs	0.455	111.8
4 hrs	0.395	97.1

FA = Fluocinolone acetonide; IS = Internal standard; PEAK AREA RATIO = Ratio of the peak areas of FA/IS; MAR = MEAN PEAK AREA RATIO.

A one-way analysis of variance was performed on the individual peak area ratios of fluocinolone acetonide/internal standard and the statistical parameters are shown in table 3.8.

TABLE 3.8 STATISTICAL PARAMETERS FOR EFFICACY OF HEPES BUFFER : SODIUM SULPHITE ADDED (TABLE 3.7)

$F_{0.05\ (1)\ 4,5} = 6.26$ $F = 1.424$	SIGNIFICANCE $p = 0.35$

The F value and the significance value support the chromatographic evidence that there was no degradation of the samples compared to undegraded standards, fig 3.3 (a).

There was no evidence of degradation in either the presence or absence of sodium sulphite, either by visual inspection of the chromatograms, or by statistical analysis. Hepes buffer was demonstrated to quench the reaction at room temperature.

The stability of Hepes buffered reaction mixtures was investigated at -20°C, the advantages of freezer stability having been previously discussed.

Synalar® ointment was extracted and sodium hydroxide added to promote degradation, as described in chapter 2.6.6.2. The sample was then quenched with Hepes buffer to pH 4 to arrest the decomposition. A ratio of 1ml buffer: 0.5ml Synalar® extract was used. Samples were taken and analysed immediately, i.e. without freezing, and further samples were transferred to a freezer at -20°C where they were stored for up to 43 days. The samples were analysed by HPLC and the results shown in table 3.9.

TABLE 3.9 STABILITY OF SAMPLES OF FLUOCINOLONE ACETONIDE SUBJECTED TO BASE-CATALYSED DEGRADATION AND QUENCHED WITH HEPES BUFFER, STORED AT -20°C

Time in freezer	Peak Area Ratio FA/IS			MAR as a % of t=0
0 mins	1.342	1.202	1.218	100.0
24 hours	1.249	1.217	1.173	96.7
4 days	1.184	1.186	1.211	95.2
6 days	1.330	1.312		105.3
29 days	1.186	1.155	1.189	93.9
43 days	0.879*	1.130	1.126	90.0

FA = Fluocinolone acetonide; IS = Internal standard; PEAK AREA RATIO = ratio of the peak areas of FA/IS; MAR = MEAN PEAK AREA RATIO.
* = vial showing appearance of new peak

A one-way analysis of variance was performed on the individual peak area ratios of FA/IS and the results are shown in table 3.10 (A). The statistical analysis was only performed on the data collected over the 0-29 day period.

TABLE 3.10 STATISTICAL PARAMETERS FOR STABILITY OF HEPES BUFFER AT -20°C (TABLE 3.9)

$F_{0.05\ (1)\ 5,11} = 4.7$ (A) F = 4.285 $F_{0.05\ (1)\ 1,3} = 216$ (B) F = 0.723	SIGNIFICANCE p = 0.021 p > 0.05

The analysis of variance showed that the means were not homogenous and therefore regression analysis was performed to determine whether the difference was due to random variation or degradation. The results are shown in table 3.10 (B) and show that the slope of the graph was not significantly

different to zero. The variation in the means was due to day to day analytical variation and not degradation.

The chromatograms over the 0-29 day period showed no signs of degradation. After storage at -20°C for 43 days one of the vials, the result of which is marked by an asterisk in table 3.9, demonstrated the appearance of a new peak. This was accompanied by a reduction in steroid but neither of these phenomena were repeated in the other two vials which had been stored concurrently. From the results it is not possible to say whether this loss was due to deterioration on storage because of contamination, or whether this is the limit of effectiveness of the method. It was concluded that storage in a freezer at -20°C was a feasible method of retaining samples for short periods and where it was used a maximum of two weeks' storage time was enforced.

The efficacy of Hepes buffer as a quenching agent for the base-catalysed degradation of fluocinonide was also investigated. The steroid was extracted from Metosyn® ointment as described in chapter 2.6.6.2, sodium hydroxide was added to promote degradation and the sample was then quenched with Hepes buffer to pH 4. A preliminary experiment demonstrated that a ratio of 1ml buffer:0.5ml of Metosyn® extract, as used for the Synalar® samples, did not quench the reaction sufficiently to prevent degradation. Therefore in this experiment 2ml of buffer were used for every 0.5ml of Metosyn® extract. Samples were taken as for the Synalar® and they were stored at -20°C for up to 14 days then analysed by HPLC. The results are shown in table 3.11.

TABLE 3.11 STABILITY OF SAMPLES OF FLUOCINONIDE SUBJECTED TO BASE-CATALYSED DEGRADATION AND QUENCHED WITH HEPES BUFFER, STORED AT -20°C

Time in freezer	Peak Area Ratio FI/IS	MAR as a % of t=0
0 mins	1.000 1.011 0.983	100.0
3 days	1.060 1.051 1.070	106.2
14 days	0.962 0.963	96.5

FI = Fluocinonide; IS = Internal Standard; PEAK AREA RATIO = Ratio of the peak areas of FI/IS; MAR = MEAN PEAK AREA RATIO.

A one-way analysis of variance was not performed on the peak area ratios of FI/IS due to the small data groups and the high values obtained at 3 days. The chromatogram showed no signs of degradation and since the reading at 14 days was greater than 95% of the original steroid concentration it was concluded that samples containing fluocinonide could be stored at -20°C for up to 14 days.

The efficacy of Hepes buffer was investigated in a similar manner with the addition of EDTA to samples of both fluocinolone acetonide and fluocinonide. EDTA is a complexing agent which was investigated in the kinetics experiments described in chapter 2.9. The effect of EDTA on the stability of samples stored at -20°C was therefore investigated over a two week period, in line with the maximum storage time imposed from the results shown in table 3.9. The results for the samples containing fluocinolone acetonide are shown in table 3.12.

TABLE 3.12 STABILITY OF SAMPLES OF FLUOCINOLONE ACETONIDE WITH EDTA SUBJECTED TO BASE-CATALYSED DEGRADATION AND QUENCHED WITH HEPES BUFFER, STORED AT -20°C

Time in freezer	Peak Area Ratio FA/IS		MAR as a % of t=0
0 mins	1.192	1.187	100.0
3 days	1.278	1.298	108.3
14 days	1.155	1.190	98.7

FA = Fluocinolone acetonide; IS = Internal Standard; PEAK AREA RATIO = Ratio of the peak areas of FA/IS; MAR = MEAN PEAK AREA RATIO.

A one-way analysis of variance was not performed on the peak area ratios of FA/IS due to the small data groups and the high values obtained at 3 days. The results for the samples containing fluocinonide are shown in table 3.13.

TABLE 3.13 STABILITY OF SAMPLES OF FLUOCINONIDE WITH EDTA SUBJECTED TO BASE-CATALYSED DEGRADATION AND QUENCHED WITH HEPES BUFFER, STORED AT -20°C.

Time in Freezer	Peak Area Ratio FI/IS			MAR as a % of t=0
0 mins	0.880	0.994	0.992	100.0
3 days	1.040	1.063	1.065	110.6
14 days	0.954			99.9

FI = Fluocinonide; IS = Internal Standard; PEAK AREA RATIO = Ratio of the peak areas of FI/IS; MAR = MEAN PEAK AREA RATIO.

A one-way analysis of variance was not performed on the peak area ratios of FI/IS due to the small data groups and the high values obtained at 3 days.

The results shown in tables 3.11, 3.12 and 3.13 were obtained from concurrent experiments and in each case an increase in steroid concentration was noted at 3 days. This suggests that there was an underlying error responsible for those readings. Since there were no signs of degradation on any of the chromatograms it was concluded that samples containing fluocinolone acetonide and fluocinonide with EDTA could be stored at -20°C for a maximum of 14 days. The presence of EDTA did not alter the stability of the samples on storage.

Storage of each steroid, with or without the presence of EDTA, was shown to be feasible for a period of fourteen days without chromatographic evidence of degradation. Due to the reduction in steroid seen in table 3.9, a longer storage period could not be recommended. Variations of ± 5% of the original steroid content may be due in part to analytical variation, a greater reduction in steroid content must be regarded as a possible degradation.

3.8 FLUOCINOLONE ACETONIDE AND FLUOCINONIDE AS STANDARDS

A solution of fluocinolone acetonide was analysed by HPLC as described in chapter 2.7 and produced a chromatogram with a single peak, the retention time of which was 4 minutes. A solution of fluocinonide was analysed as described in chapter 2.8 and also produced a chromatogram with a single peak, the retention time of which was 8 minutes. These peaks corresponded to fluocinolone acetonide and fluocinonide respectively and the absence of additional peaks shows that no impurities were present that may have interfered with the analysis.

A series of dilutions of fluocinolone acetonide was analysed by HPLC for steroid content, and the mean area ratio of the steroid to the internal standard was plotted against steroid concentration to produce a calibration graph. This was repeated with a second set of dilutions, table 3.14 and fig 3.7.

TABLE 3.14 CALIBRATION CURVE DATA FOR FLUOCINOLONE ACETONIDE

Concentration of Steroid (% w/v)	Mean Peak Area Ratio FA/IS as a % of the undiluted steroid	
	1	2
0.0002	12.4	11.6
0.0004	26.3	24.0
0.0006	38.2	37.9
0.0008	51.8	51.3
0.0012	76.8	75.5
0.0016	100.0	100.0

FA = Fluocinolone acetonide; IS = Internal Standard; PEAK AREA RATIO = Ratio of the peak areas of FA/IS.

Each set of dilutions produced linear calibration graphs. The equations of the lines were $y = 0.0043 + 530x$ ($r = 0.9997$) and $y = 0.0032 + 560x$ ($r = 0.9998$) respectively.

In a similar manner a solution of fluocinonide was subjected to serial dilution and the results are shown in table 3.15 and fig 3.8.

Fig 3.7 CALIBRATION CURVES FOR FLUOCINOLONE ACETONIDE.

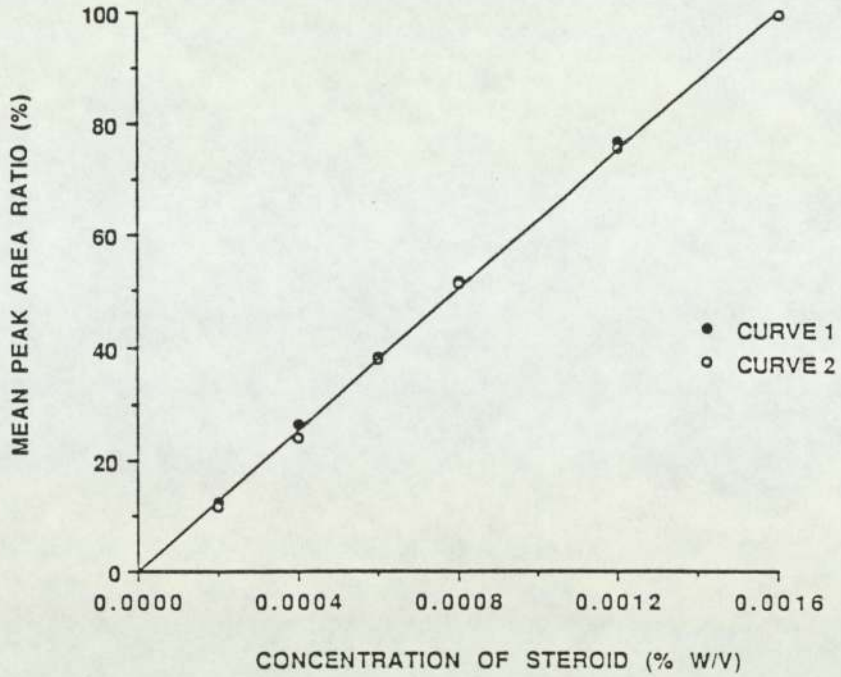


Fig 3.8 CALIBRATION CURVES FOR FLUOCINONIDE.

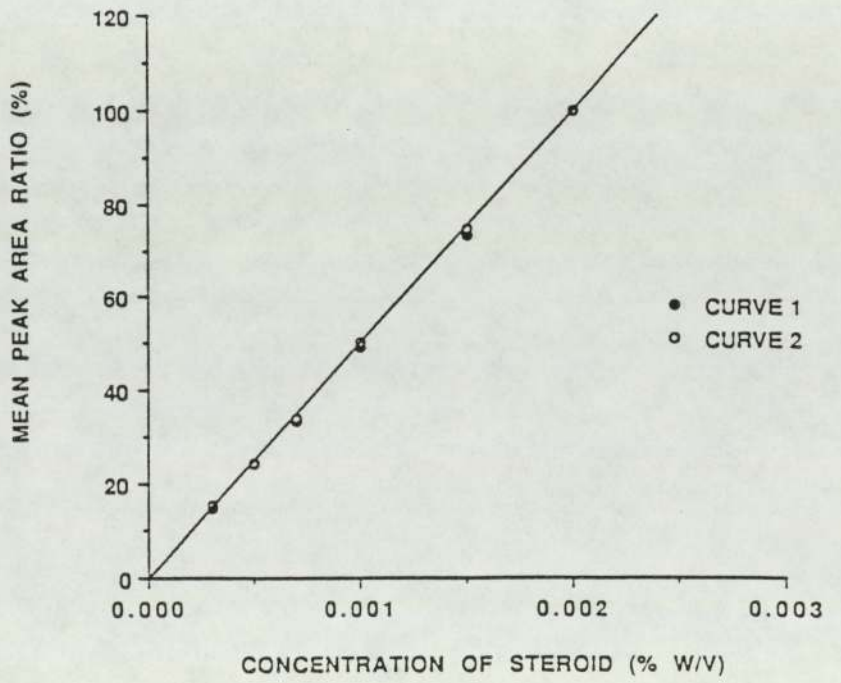


TABLE 3.15 CALIBRATION CURVE DATA FOR FLUOCINONIDE

Concentration of Steroid (% w/v)	Mean Peak Area Ratio FI/IS as a % of the undiluted steroid	
	1	2
0.0003	14.9	15.3
0.0005	24.3	24.5
0.0007	33.7	34.1
0.0010	49.4	50.1
0.0015	73.2	74.7
0.0020	100	100

FI = Fluocinonide; IS = Internal Standard; PEAK AREA RATIO = Ratio of the peak areas of FI/IS.

Each set of dilutions produced linear calibration graphs. The equations of the lines were $y = 0.0047 + 1316x$ ($r = 0.9999$) and $y = 0.0111 + 1337x$ ($r = 0.9998$) respectively.

The HPLC method described in chapter 2.2.2 can be used to determine the steroid content of solutions containing fluocinolone acetonide and fluocinonide. This was developed into a series of kinetic experiments to investigate the effect of storage conditions on the stability of the steroids.

3.9 KINETIC EXPERIMENTS

The experiments described in the previous sections were carried out in order to design and evaluate a method for the extraction and analysis of fluocinolone acetonide and fluocinonide from the commercially available ointment and cream formulations respectively. The method was then used to investigate the effect of storage conditions and additives on the stability of the steroid extracts so that the results could be extrapolated to formulation studies.

3.9.1 DEGRADATION OF FLUOCINOLONE ACETONIDE UNDER ALKALINE CONDITIONS

Preliminary experiments were performed as described in chapter 2.9.1, subjecting fluocinolone acetonide to base-catalysed degradation and quenching with HCl. While the samples were awaiting analysis they were stored in the light at room temperature, in the dark at room temperature, and in a refrigerator at 4°C. Each set of results gave a low reading at time zero, which suggested that the steroid may be coming out of solution, and may be adsorbing on to the glassware. Solubility measurements of fluocinolone acetonide in acetonitrile:water (70:30) in the presence of HCl were not performed.

Although the use of HCl as a quenching system was found to be ineffective, the results obtained demonstrate important points which were relevant to the design of the final method, and so have been discussed for completion.

The samples stored in diffuse daylight at room temperature degraded over five hours, after which no steroid could be detected. The samples stored in the dark and in the refrigerator showed a large degree of variation in the concentration of fluocinolone acetonide present with time. When the temperature in the laboratory fell to below 26°C two immiscible layers were formed, while above that temperature no separation occurred. The solvent system was acetonitrile:water (70:30). The biphasic system may cause precipitation and adsorption, resulting in a variable steroid concentration in solution with time. The concentration of steroid in the sample will also depend on which phase the sample is taken from. A water-bath was used to maintain the temperature of the reaction mixture at 26°C which prevented separation and ensured reproducible results.

The experiment was repeated at 26°C using Hepes buffer as a quenching agent. The degradation was investigated with EDTA added to the reaction mixture, with sodium sulphite added, and with both EDTA and sodium sulphite added to the same reaction mixture. If the addition of a complexing agent or an anti-oxidant could be shown to slow down the degradation of

fluocinolone acetonide then this could be incorporated into formulation studies. The chromatograms produced showed the same degradation profile as fig 3.6 and no difference was detected between those that did or did not have additive present. The concentration of fluocinolone acetonide with time is shown in table 3.16.

TABLE 3.16 CONCENTRATION OF FLUOCINOLONE ACETONIDE PRESENT WHEN SUBJECTED TO BASE-CATALYSED HYDROLYSIS AT 26°C IN THE PRESENCE AND ABSENCE OF EDTA AND SODIUM SULPHITE

Sample (% mM)						
Time (mins)	**0.0**	**15**	**30**	**45**	**60**	**90**
Control	100	51.7	31.02	21.01	12.66	4.38
EDTA	100	53.06	32.32	21.30	10.28	2.96
S. Sulphite	100	49.28	30.14	16.07	6.68	0.00
Both	100	46.85	22.24	8.41	5.52	0.00

The results shown in table 3.16 were plotted as the percentage concentration of fluocinolone acetonide versus time (fig 3.9) and the logarithm (base 10) of the concentration was plotted against time (fig 3.10). The rate constants were calculated using replicate samples and are shown in table 3.17.

FIG 3.9 CONCENTRATION OF FLUOCINOLONE ACETONIDE WHEN SUBJECTED TO BASE-CATALYSED HYDROLYSIS AT 26°C IN THE PRESENCE AND ABSENCE OF EDTA AND SODIUM SULPHITE.

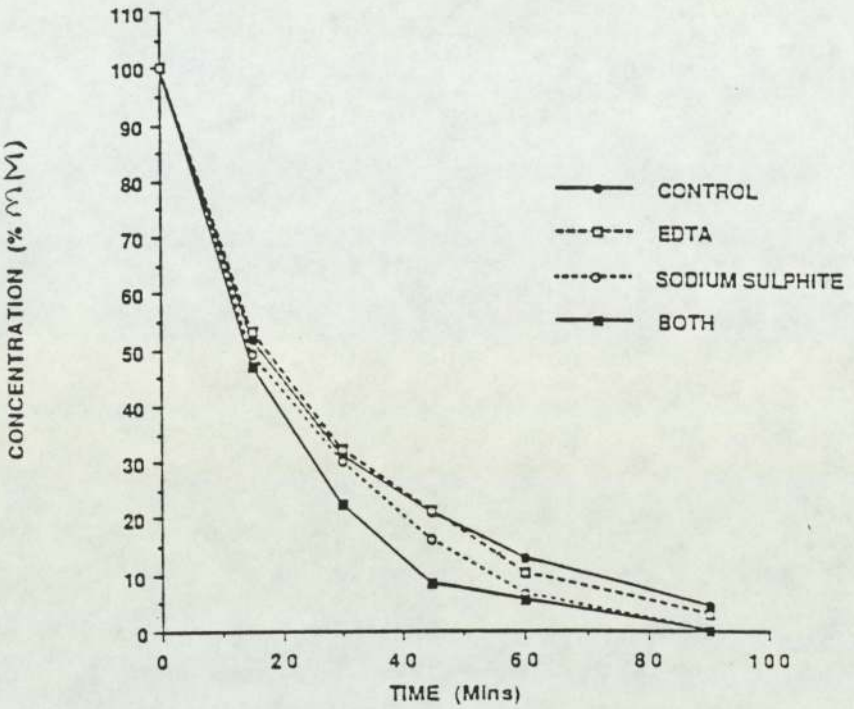


FIG 3.10 LOG CONCENTRATION OF FLUOCINOLONE ACETONIDE WHEN SUBJECTED TO BASE - CATALYSED HYDROLYSIS AT 26°C IN THE PRESENCE AND ABSENCE OF EDTA AND SODIUM SULPHITE.

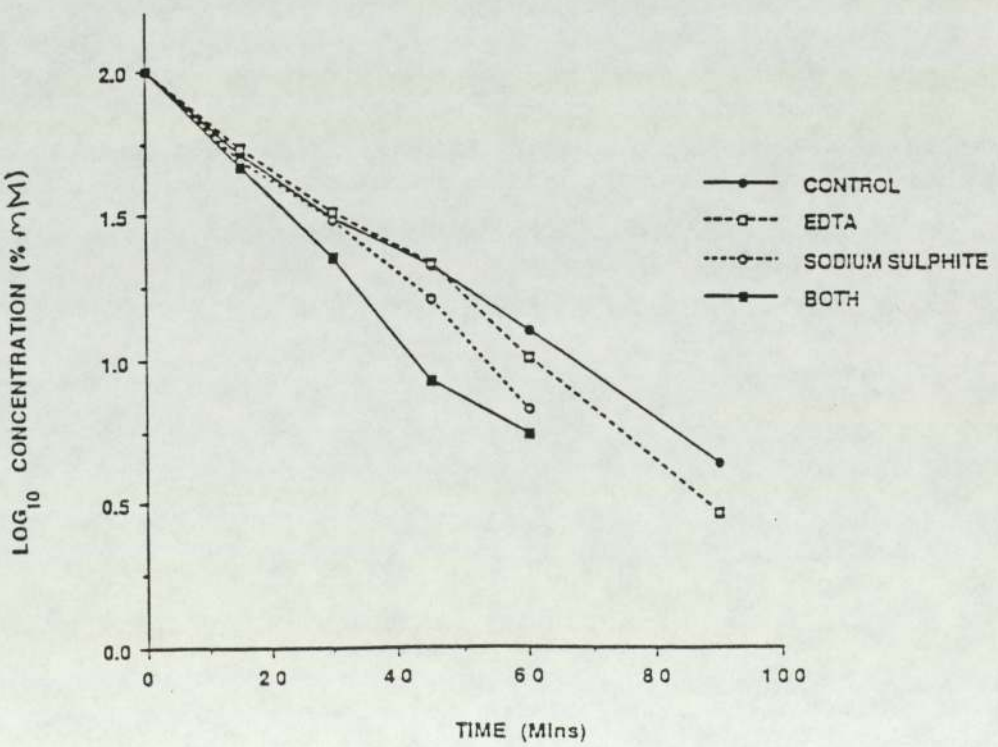


TABLE 3.17 REGRESSION AND RATE DATA FOR THE BASE-CATALYSED DEGRADATION OF FLUOCINOLONE ACETONIDE AT 26°C IN THE PRESENCE AND ABSENCE OF EDTA AND SODIUM SULPHITE

Sample	Rate x 10^{-2} $Mins^{-1}$	Intercept	Correlation Coefficient
Control	3.38	1.97	-0.998
EDTA	3.85	2.01	-0.998
S.Sulphite	4.35	2.01	-0.996
Both	5.02	1.99	-0.995

The addition of EDTA, sodium sulphite, and both EDTA and sodium sulphite together, resulted in an increase in the rate of the degradation reaction and would therefore be of no use in extending the shelf-life of a formulation containing fluocinolone acetonide. The logarithmic plots were linear over 15-90 minutes indicating first-order reaction kinetics for this time period.

The degradation products eluted during these experiments had retention times the same as, or very similar to, that of the solvent front. Various mobile phases were employed in an attempt to separate the degradation products; acetonitrile:water (50:50), (30:70), (20:80), (15:85), (10:90), (5:95) and water alone. Even using a low strength mobile phase the degradation products were not separated, and could not be investigated further at this stage.

The degradation of steroids with a C-17 dihydroxyacetone group occurs primarily at that side chain; both oxidative and hydrolytic reaction pathways are available and transition metals, hydroxide and hydrogen ions catalyse the reactions[42].

The effect of temperature and pH on the degradation of fluocinolone acetonide in an oil-in-water cream base was studied by Kenley et al[42] (temperature and pH dependence were discussed in chapter 1.9). The major degradation products were the C-21 aldehyde and C-17 etianic acid analogues

of fluocinolone acetonide. However, these represented only a minor fraction of the total fluocinolone acetonide lost, as in the experiment described above. Other degradation products appeared with (or shortly after) the void volume indicating polar or ionised functional groups which the authors suggested were secondary degradation products and were not identified, agreeing with the findings of the experiment in this section. The authors concluded that the degradation of fluocinolone acetonide in cream samples occurs in an aqueous phase or compartment that is largely unaffected by the non-aqueous ingredients of the cream formulation.

Shek et al[49] also reviewed the degradation data from reactions of the C-17 side chain of corticosteroids. In aqueous buffer solutions hydrocortisone produces the 17-deoxyglyoxal derivative, the 21-dehydrocortisone, etianic acid and glycolic acid derivatives. Triamcinolone acetonide has been shown to oxidise to the corresponding etianic acid acetonide. Triamcinolone acetonide is a good model for the degradation of fluocinolone acetonide since both molecules are corticosteroids with a dihydroxyacetone functional group at C-17 and cyclic ketal bridging at C-16 and C-17[42]. Shek et al[49] therefore suggested that the ketal side chain of fluocinolone acetonide might degrade in a similar manner to triamcinolone acetonide to produce the glyoxal and etianic acid derivatives, as shown in fig 3.11. Their results will be discussed in chapter 3.9.2.

Powell et al[48] performed long-term stability studies on fluocinolone acetonide using multiply tritiated radiopharmaceuticals. The radiolabelled steroid was incorporated into a cream formulation consisting mainly of propylene glycol, stearyl alcohol and water. HPLC and liquid scintillation counting showed only 3-4% drug degradation after storage at room temperature for 26 months. The products detected were the ketoaldehyde (1.6%), the ketoacid (0.2%), etianic acid (0.6%) and tetrol (0.8%), as shown in fig 3.5. These findings correlate with the hypothesis presented above. If these molecules were present as secondary degradation products in the experiments discussed in this section then they were either present in such small quantities that they could not be

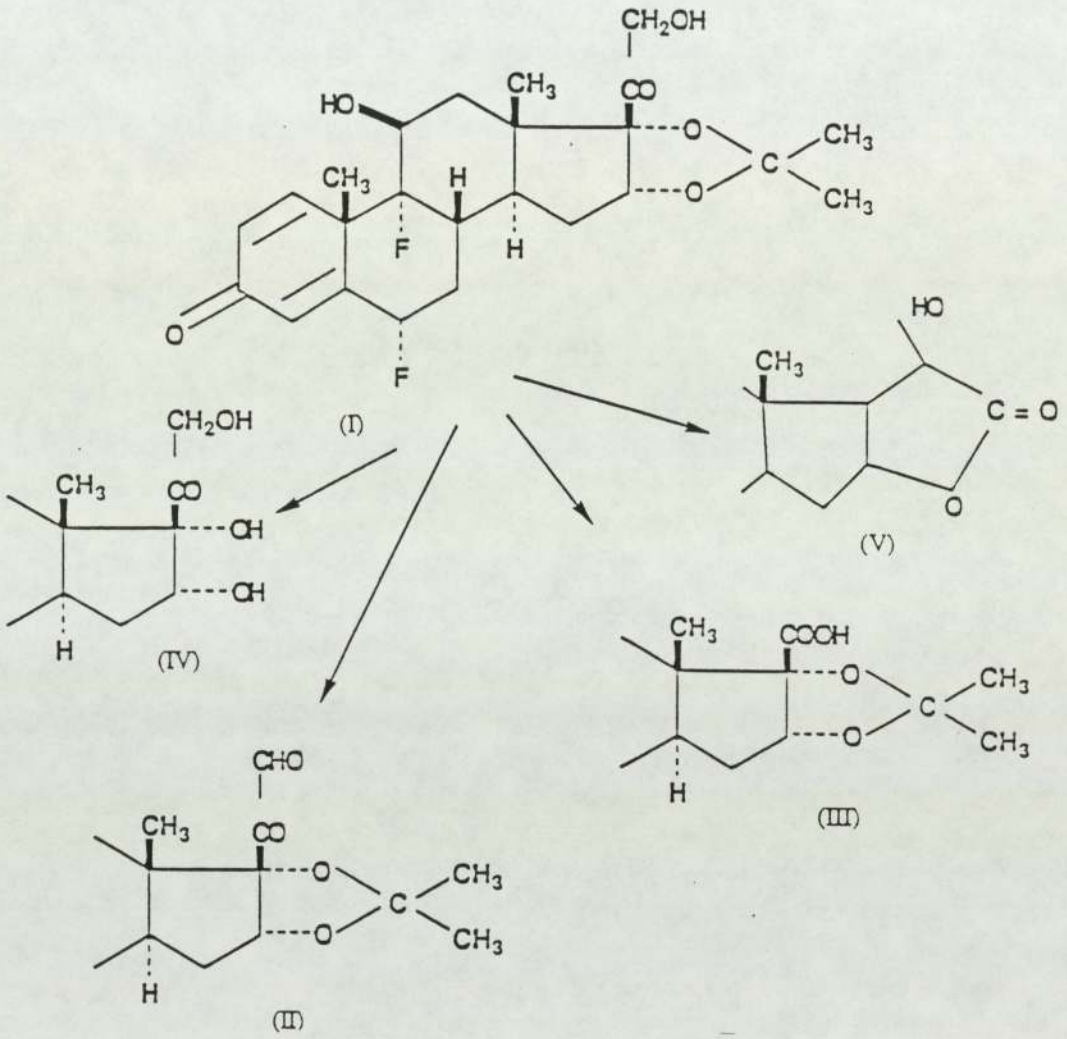
Fig 3.11

FLUOCINOLONE ACETONIDE AND ITS POSSIBLE DEGRADATION PRODUCTS,AFTER SHEK et al [49]

I = FLUOCINOLONE ACETONIDE

II = (GLYOXAL), III (ETIANIC ACID), AND

IV, V = POSSIBLE DEGRADATION PRODUCTS



detected, or they were eluted close to the void volume, again escaping detection.

3.9.2 DEGRADATION OF FLUOCINONIDE UNDER ALKALINE CONDITIONS

The initial work carried out under alkaline conditions used borate as the buffer as described in chapter 2.9.2. The system was not ideal, but demonstrates problems encountered and led to the development of a more appropriate system of analysis.

The reaction was followed in the light and the dark at room temperature, and in the presence of each of EDTA and sodium sulphite, both in the light at room temperature, and at 4°C in a refrigerator.

The samples stored in the light produced two peaks, the primary degradation peak had a retention time corresponding to that of fluocinolone acetonide. Fluocinonide showed rapid degradation over the first hour, falling to 15% of the initial concentration. The appearance of degradation peaks was observed at the first reading after time zero, i.e. after 15 minutes. The new peaks were both less than 10% of the initial fluocinonide concentration and although the concentration of fluocinonide continued to fall, that of the degradation peaks did not increase. The degradation profile was of the same pattern as that demonstrated for the degradation of fluocinolone acetonide in chapter 3.9.1. Further degradation peaks may have been unresolved from the void volume.

Storage in the dark and in the fridge also showed a rapid degradation of similar scale to the light storage; in both cases only one degradation product was observed. This corresponded to the primary degradation peak produced by the reaction in the light, which was fluocinolone acetonide.

In the presence of antioxidant there was an initial fall in fluocinonide concentration to almost zero, followed by a rise after one hour to 40% of the original concentration. A possible explanation is that the steroid may have precipitated out of solution initially, or adsorbed on to the glassware, and then

redissolved into the reaction mixture. The reaction mixture was not subjected to temperature control and therefore temperature fluctuations may have contributed to the results. Only one new peak, that of fluocinolone acetonide, was eluted until the mixture had been standing for 3 hours, when a second degradation product began to appear, corresponding to the secondary degradation product observed under reaction conditions in the light.

When EDTA was added to the reaction mixture, there was a rapid degradation, with only fluocinolone acetonide being eluted. No secondary degradation products were identified.

The degradation of fluocinonide to fluocinolone acetonide has been reported in the literature. Shek et al[49] developed a stability-indicating method for Triple Corticoid Integrated System (TCIS) in a cream formulation. The TCIS consisted of a mixture of three 21-esters of fluocinolone acetonide; fluocinonide, procinonide and ciprocinonide and it was expected that under hydrolytic or solvolytic conditions the major degradation product would be fluocinolone acetonide, and that the cyclic ketal group of the acetonide could then be cleaved to yield fluocinolone and acetone in acidic conditions. The pH of the base was not discussed. As expected, the major degradation product detected by HPLC was fluocinolone acetonide. Extended stability studies showed that the acetonide was stable in the cream base so no further degradation data were obtained. The possible degradation profile of fluocinolone acetonide was discussed in chapter 3.9.1.

The results described demonstrates that the presence or absence of light effects the course of the reaction. The samples stored in the light immediately degraded to two degradation products, compared to only one product from each of the other four samples (i.e. the samples with EDTA or sodium sulphite added, and the samples stored in the dark), indicating that light influences the route of degradation.

The second degradation product, which corresponds to peak A, identified in chapter 3.3, began to appear only after prolonged reaction when sodium

sulphite was present as an antioxidant, suggesting that it may be formed by an oxidative mechanism. The primary degradation product was identified as fluocinolone acetonide.

The effect of air on the degradation of prednisolone[37], as discussed in chapter 1.9, showed that for that steroid there were two or more oxygen-independent reactions and at least one oxygen-dependent reaction involved. The results obtained with fluocinonide suggest that there is at least one oxygen-dependent reaction and at least one oxygen-independent reaction taking place. The reaction products observed represent only about 10% of the original concentration of fluocinonide. The remaining 80-90% loss of steroid could not be accounted for by this chromatographic method. More degradation products may be eluted with the void volume, but these were not separated. The problems encountered with erratic steroid concentrations and fluctuating temperature may also be applicable to degradation products, such that if the temperature falls the product may become less soluble so that it is not detected in the solution.

Fluocinonide was degraded by the addition of alkali at 26°C and quenched with Hepes buffer as described in chapter 2.9.2. The reaction was followed up to 3 hours. The major degradation peak had a retention time corresponding to that of fluocinolone acetonide, and its concentration was also recorded. The presence of fluocinolone acetonide at time zero indicates that this was not a true zero reading as degradation had occurred before quenching.

In the control solution (without additives) the fluocinonide degraded rapidly, with almost complete loss of steroid after only 90 minutes. This was accompanied by the production of fluocinolone acetonide, as the major degradation product. Further degradation products may be eluted close to the void volume and would therefore not be detected by this chromatographic method. The concentration of fluocinolone acetonide then began to decrease as this in turn was degraded but at a slower rate than it was produced. It should be noted that there was a significant amount of fluocinolone acetonide present at time zero. This suggests that the reaction occurs immediately on

adding the alkali, degradation of fluocinonide taking place in the time taken to withdraw the first sample. The results are shown in table 3.18.

TABLE 3.18 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : CONTROL

Time (hours)	Concentration Fluocinonide (% mM)	Concentration F. Acetonide (% mM)
0.00	62.7	37.3
0.25	25.2	90.7
0.50	10.6	119.2
0.75	6.5	91.6
1.00	5.7	87.2
1.50	0.2	75.6
2.00	0.0	64.0
3.00	0.0	48.0

The percentage concentration of steroid was plotted against time (fig 3.12). The logarithm (base 10) (fig 3.13) of the concentration against time showed that the degradation of fluocinonide was linear between 15 minutes and 1.5 hours and the degradation of fluocinolone acetonide was linear between 30 minutes and 3 hours, indicating first-order reaction kinetics over these time periods.

The data shown in table 3.18 was investigated to see if it would fit the parameters of first-order sequential kinetics, as represented by the following equation[50]:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

In a two-stage first-order sequential reaction the initial component, FI, degrades to FA, which in turn is converted to A (FI is fluocinonide, FA is fluocinolone acetonide and A is the secondary degradation product). Statistical analysis gave a rate constant of $3.79s^{-1}$ ($0.0631\ min^{-1}$) for the conversion of FI to FA, and a rate constant of $0.33s^{-1}$ ($0.0055\ min^{-1}$) for the conversion of FA to A. The first rate is much greater than the second, indicating that the degradation of FI to FA is largely complete as the conversion of FA to A proceeds. This is supported by graph 3.12.

The degradation of fluocinonide in alkaline conditions was also investigated with the addition of the complexing agent EDTA to the reaction mixture. The rate and extent of the degradation was markedly reduced by the addition of EDTA compared to the control mixture. The loss of fluocinonide was accompanied by a rapid production of fluocinolone acetonide over the first 15 minutes, which then began to plateau, with only a gradual increase in concentration over the next 2-3 hours. The fluocinolone acetonide showed no signs of degradation, either by a fall in concentration or by inspection of the chromatography traces. The EDTA appears to be slowing down the degradation of fluocinonide and at the same time either slowing down the degradation of fluocinolone acetonide, with its continued production masking its degradation, or completely protecting the acetonide from degradation. The results are shown in table 3.19.

Fig 3.12 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : CONTROL

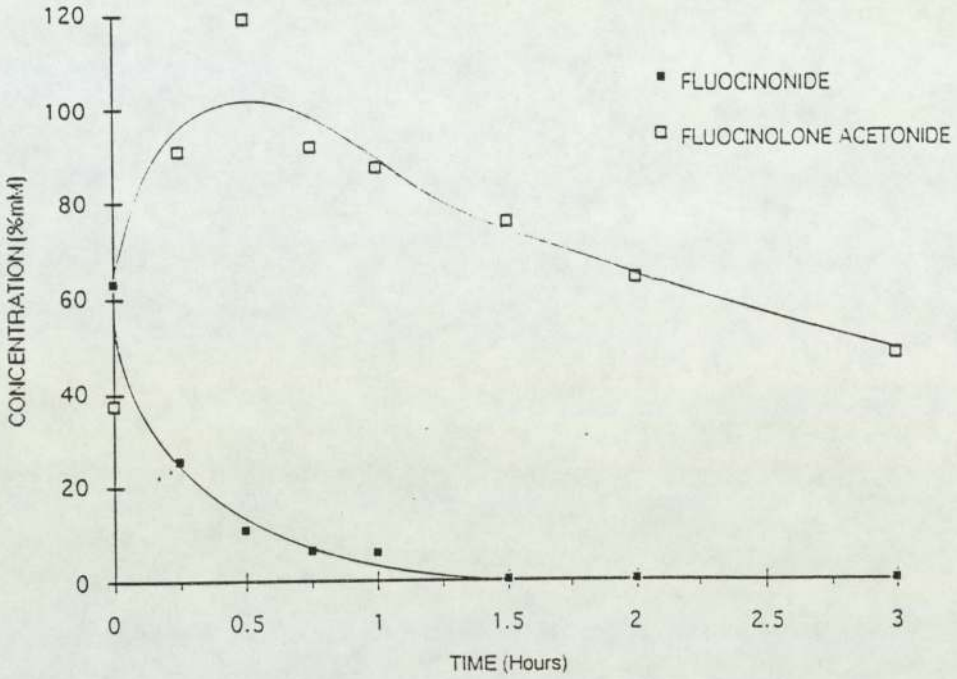


Fig 3.13 LOG CONCENTRATIONS OF FLUOCINONIDE AND FLUOCINOLONE ACETONIDE UNDER BASE-CATALYSED HYDROLYSIS : CONTROL

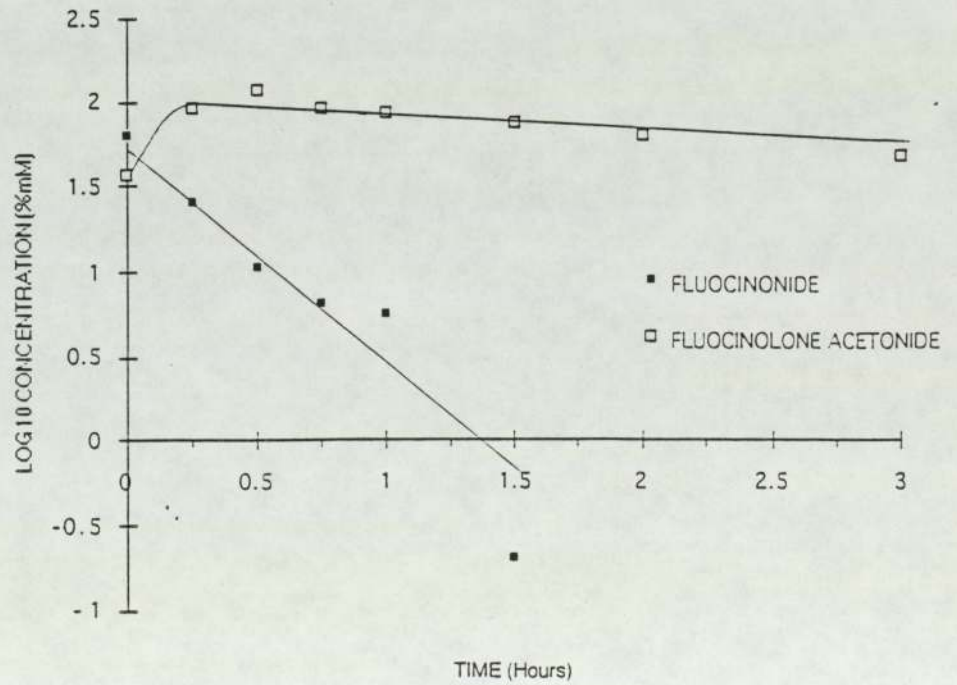


TABLE 3.19 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : EDTA ADDED

Time (hours)	Concentration Fluocinonide (% mM)	Concentration F.Acetonide (% mM)
0.25	27.76	72.4
0.50	21.8	73.7
0.75	19.8	78.0
1.00	18.5	80.2
1.50	15.9	79.5
2.00	11.9	80.9
3.00	4.6	87.4

The percentage concentration of steroid was plotted against time (fig 3.14). The logarithm (base 10) (fig 3.15) of the concentration against time showed that the degradation of fluocinonide was linear between 15 minutes and 3 hours, and that the production of fluocinolone acetonide was also linear between the same time points, indicating first-order kinetics.

The degradation of fluocinonide in alkaline conditions was investigated with the addition of the antioxidant sodium sulphite to the reaction mixture. This resulted in a slightly increased rate of degradation compared to the control mixture, and no fluocinonide could be detected after one hour. There was a rapid production of fluocinolone acetonide over the first 45 minutes. An oxygen-dependent pathway of fluocinonide to an unidentified degradation product may have been blocked by the antioxidant allowing an increased conversion to be acetonide. The acetonide in turn underwent base-catalysed degradation to leave only 23% remaining after 3 hours. The results are shown in table 3.20.

Fig 3.14 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : EDTA ADDED

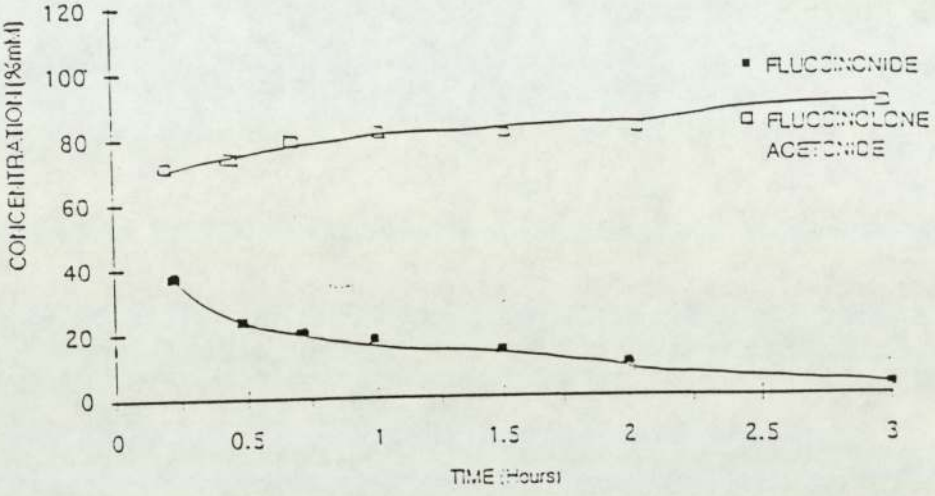


Fig 3.15 LOG CONCENTRATIONS OF FLUOCINONIDE AND FLUOCINOLONE ACETONIDE UNDER BASE-CATALYSED HYDROLYSIS : EDTA ADDED

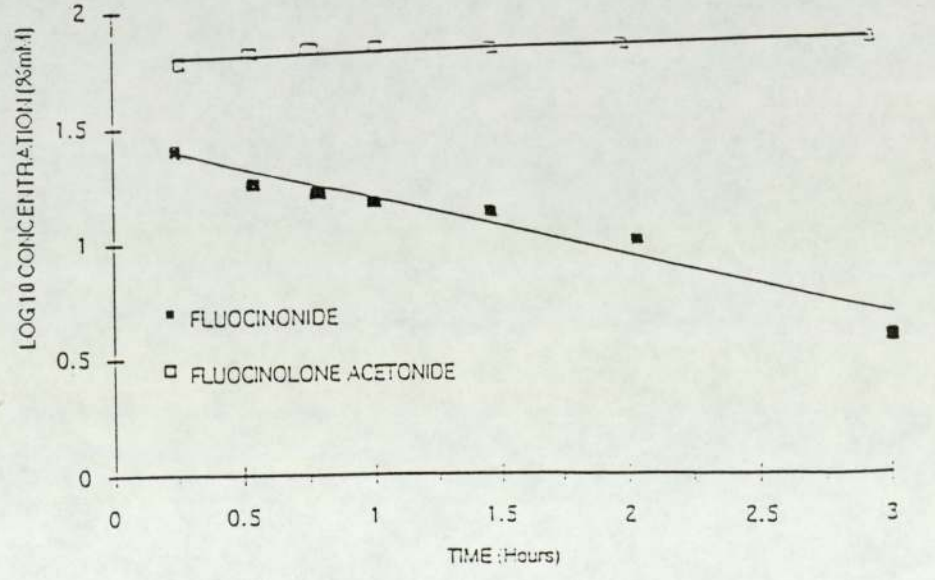


TABLE 3.20 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : SODIUM SULPHITE ADDED

Time (hours)	Concentration Fluocinonide (% mM)	Concentration F. Acetonide (% mM)
0.25	35.1	64.9
0.50	12.8	97.4
0.75	4.7	131.3
1.00	0.0	84.8
1.50	0.0	48.7
2.00	0.0	51.6
3.00	0.0	14.8

The percentage concentration of steroid was plotted against time (fig 3.16). The logarithm (base 10) (fig 3.17) of the concentration against time showed that the degradation of fluocinonide was linear between 15 and 45 minutes, and the degradation of fluocinolone acetonide was linear between 45 minutes and 3 hours, indicating first-order kinetics.

Fig 3.16 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS . SODIUM SULPHITE ADDED

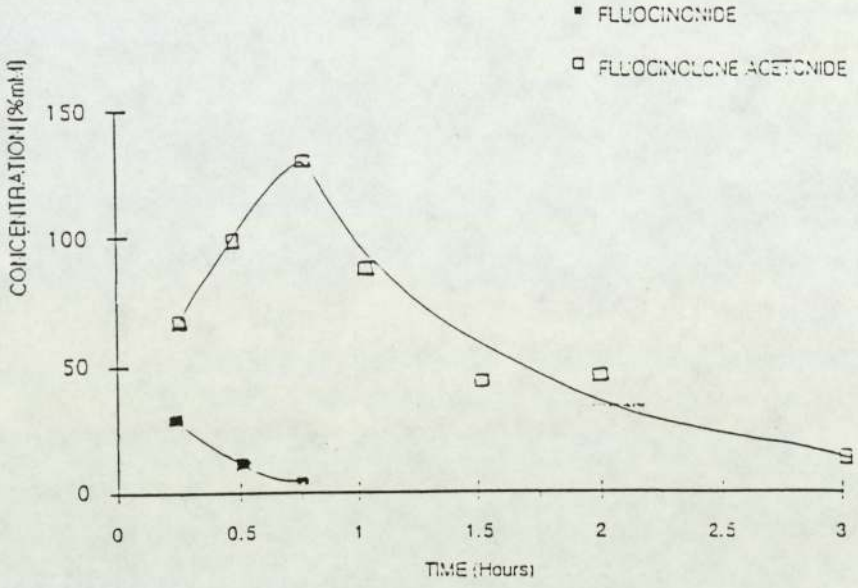
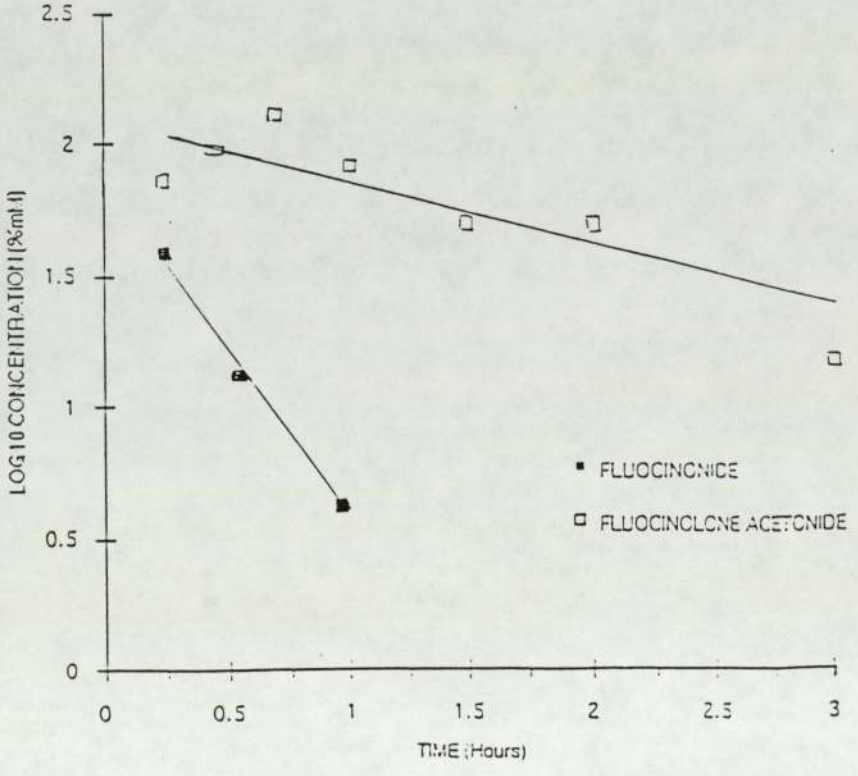


Fig 3.17 LOG CONCENTRATIONS OF FLUOCINONIDE AND FLUOCINOLONE ACETONIDE UNDER BASE-CATALYSED HYDROLYSIS . SODIUM SULPHITE ADDED



The degradation of fluocinonide by base-catalysed hydrolysis was investigated with the addition of both EDTA and sodium sulphite. The results were erratic. Individually, sodium sulphite has been shown to increase or sustain the rate of the degradation whilst EDTA slows down or protects the steroid from degradation. When present together the fluocinonide underwent base-catalysed degradation but after 3 hours there was 9.6% of the fluocinonide remaining compared to 8.4% with EDTA alone, whilst with sodium sulphite alone and with no additives present no residual fluocinonide was detected. A graph of the percentage concentration of fluocinonide present against time (fig 3.18) revealed several points which did not fit closely to the curve. The degradation of fluocinonide gave rise to the production of fluocinolone acetonide. A graph of the percentage concentration of acetonide against time did not give a good fit to the curve (fig 3.18). It was concluded that there was no benefit from the concurrent use of both additives to the stability of the steroid. The results are shown in table 3.21.

TABLE 3.21 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : BOTH EDTA AND SODIUM SULPHITE ADDED

Time (hours)	Concentration Fluocinonide (% mM)	Concentration F. Acetonide (% mM)
0.00	74.9	25.2
0.25	47.0	92.3
0.50	32.6	107.0
0.75	31.7	102.8
1.00	20.2	77.6
1.50	25.0	134.3
2.00	16.3	121.7
3.00	9.6	117.5

The percentage concentration of steroid was plotted against time (fig 3.18). The logarithm (base 10) (fig 3.19) of the percentage concentration against time showed that the degradation of fluocinonide was linear between 15 minutes and 3 hours. The plot for fluocinolone acetonide was also linear for the production of steroid for the same time interval, indicating first-order kinetics over the 3 hour time period. However, for each steroid several points did not give good fit to the straight line due to the scatter of results discussed previously.

The logarithmic plots (figs 3.13, 3.15, 3.17 and 3.19) for the degradation of fluocinonide without additives, with EDTA, with sodium sulphite, and with both EDTA and sodium sulphite were subjected to regression analysis using the mean of the data collected from replicate injections. The regression data are shown in table 3.22.

Fig 3.18 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : BOTH EDTA AND SODIUM SULPHITE ADDED

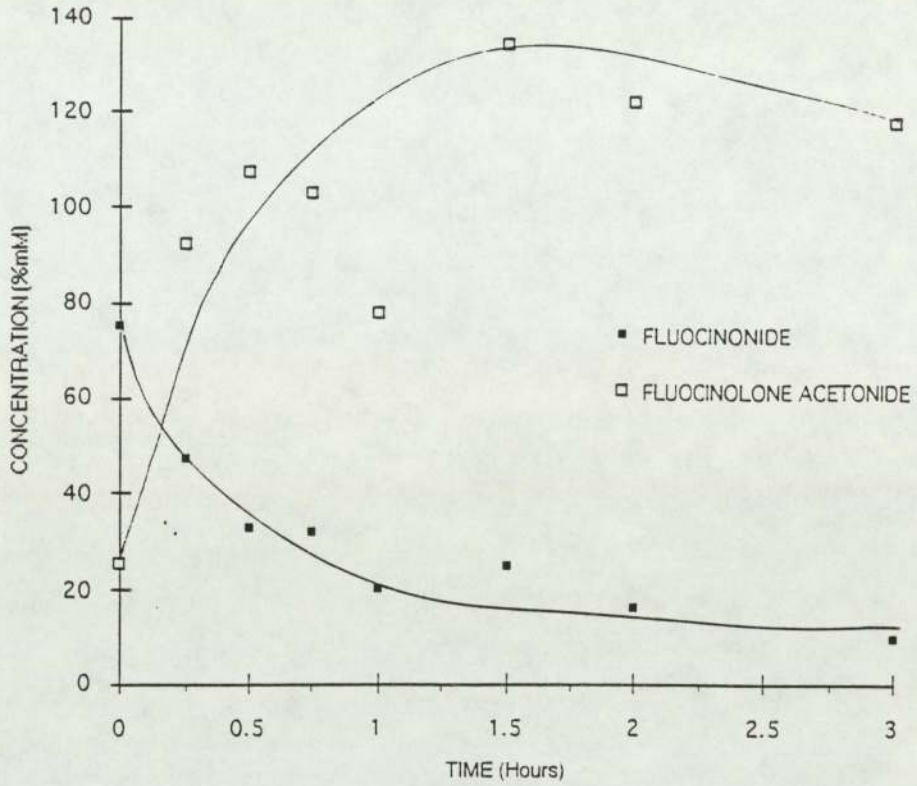


Fig 3.19 LOG CONCENTRATIONS OF FLUOCINONIDE AND FLUOCINOLONE ACETONIDE UNDER BASE-CATALYSED HYDROLYSIS : BOTH EDTA AND SODIUM SULPHITE ADDED

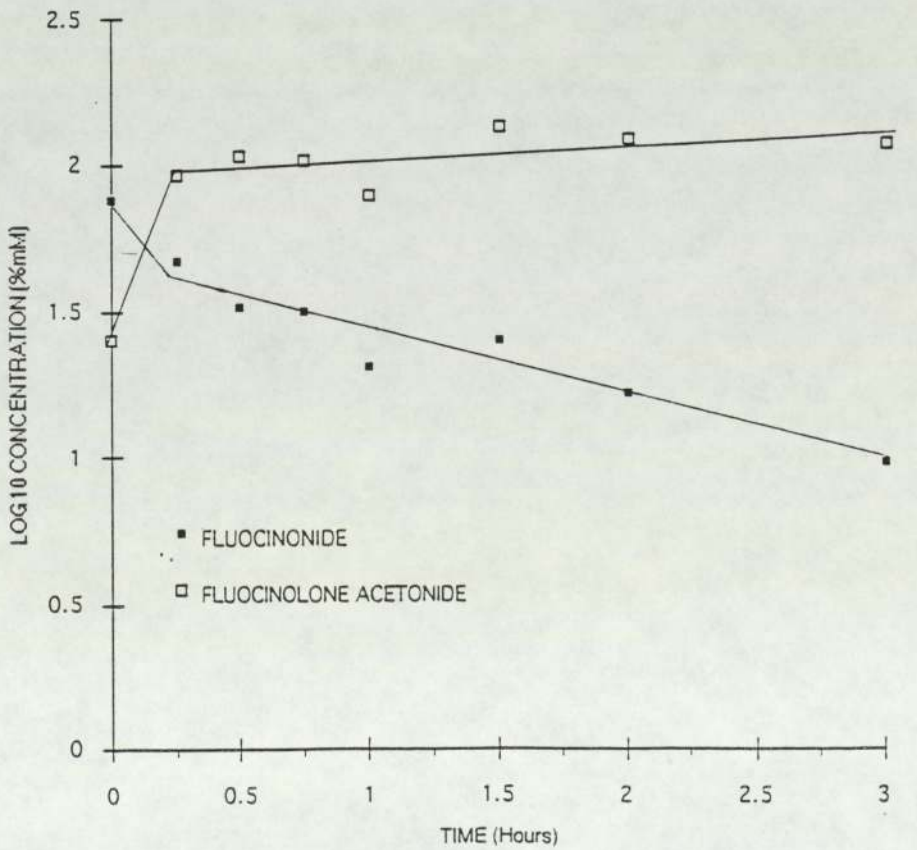


TABLE 3.22 REGRESSION AND RATE DATA FOR THE BASE-CATALYSED DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE IN THE PRESENCE AND ABSENCE OF EDTA AND SODIUM SULPHITE

Sample	Correlation Coefficient	Rate x 10^{-2} $Mins^{-1}$	Intercept
Control:			
Fluocinonide	-0.956	6.31	2.107
F. Acetonide	-0.978	0.55	1.876
EDTA:			
Fluocinonide	-0.987	0.67	1.774
F.Acetonide	0.915	-0.12	1.771
S.Sulphite:			
Fluocinonide	-0.999	6.77	2.296
F. Acetonide	-0.970	1.52	2.255
Both:			
Fluocinonide	-0.938	1.01	1.835
F. Acetonide	0.523	-0.16	1.655

The logarithmic plots indicate that there are possibly two rates of reaction, an initial rapid decomposition between time zero and 15 minutes, followed by a second phase. However, since no data were collected between 0 and 15 minutes it is not possible to determine if this is an experimental artefact or an actual change in rate. It was not feasible within the bounds of this project to investigate this effect further.

The following conclusions were drawn from this section of work. The degradation of fluocinonide was accelerated by the addition of sodium sulphite. The degradation of fluocinolone acetonide was similarly accelerated by the antioxidant. The addition of EDTA slowed down the degradation of the fluocinonide significantly, and the fluocinolone acetonide showed no signs of degradation. The concentration of the acetonide was still increasing over the time period studied. The addition of both additives to the reaction media

produced an erratic graph, as seen by the poor correlation data for the acetonide. The effect was less pronounced on the parent steroid.

3.9.3 DIRECT COMPARISON OF THE BASE-CATALYSED DEGRADATION OF FLUOCINOLONE ACETONIDE AND FLUOCINONIDE

The results discussed in 3.9.1 and 3.9.2 showed that in the presence of alkali fluocinonide degraded to fluocinolone acetonide, which in turn degraded to a series of secondary degradation products which were not identified. The addition of alkali to fluocinolone acetonide produced the same degradation profile as that described for the acetonide when it was investigated as a primary degradation product of fluocinonide, as far as the limitations of the HPLC method would allow. The addition of sodium sulphite to the reaction mixture increased the rate of degradation of both fluocinolone acetonide and fluocinonide. The presence of EDTA increased the rate of degradation of the acetonide in 3.9.1, but slowed down or prevented the degradation of the acetonide in 3.9.2 where it was a primary degradation product.

To confirm the effect of EDTA, the base-catalysed degradation of fluocinonide and fluocinolone acetonide were performed concurrently as described in chapter 2.9.3. The addition of EDTA to fluocinolone acetonide slowed down the degradation compared to the control but did not prevent the reaction occurring. The results are shown in table 3.23.

TABLE 3.23 DEGRADATION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : CONTROL AND WITH EDTA

Concentration Fluocinolone Acetonide (% mM)		
Time (hrs)	Control	With EDTA
0.00	100	100
0.25	40.52	60.52
0.50	19.55	29.00
0.75	11.30	17.87
1.00	6.84	12.55
1.50	0.00	4.16
2.00	0.00	1.20
3.00	0.00	0.00

The concentration of steroid was plotted as a percentage of the concentration of fluocinolone acetonide present at time zero (fig 3.20). A logarithmic plot (base 10) (fig 3.21) was linear between 15 minutes and one hour for the control, and between 15 minutes and 2 hours for the sample with EDTA, indicating first-order reaction kinetics over these time periods.

The addition of EDTA markedly reduced the extent and the rate of degradation of fluocinonide compared to a control solution without additives. As in chapter 3.9.2, fluocinolone acetonide was rapidly produced from the fluocinonide in the control solution, reaching a peak concentration after only 30 minutes. This in turn degraded to a series of secondary degradation products which were not identified. In the presence of EDTA the concentration of fluocinolone acetonide gradually increased over the 4 hour period studied after an initial rapid production. This confirms the results discussed in chapter 3.9.2. The results are shown in table 3.24.

Fig 3.20 DIRECT COMPARISON EXPERIMENT : DEGRADATION OF FLUOCINOLONE ACETONIDE BY BASE - CATALYSED HYDROLYSIS : CONTROL AND WITH EDTA.

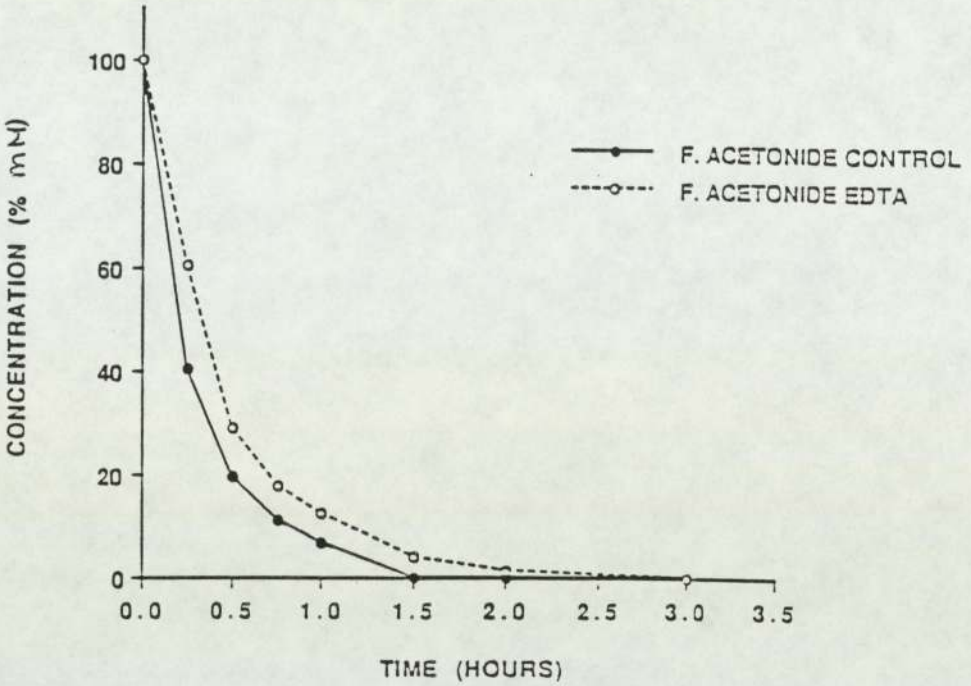


FIG 3.21 DIRECT COMPARISON EXPERIMENT : LOG CONCENTRATION OF FLUOCINOLONE ACETONIDE UNDER BASE - CATALYSED HYDROLYSIS : CONTROL AND WITH EDTA.

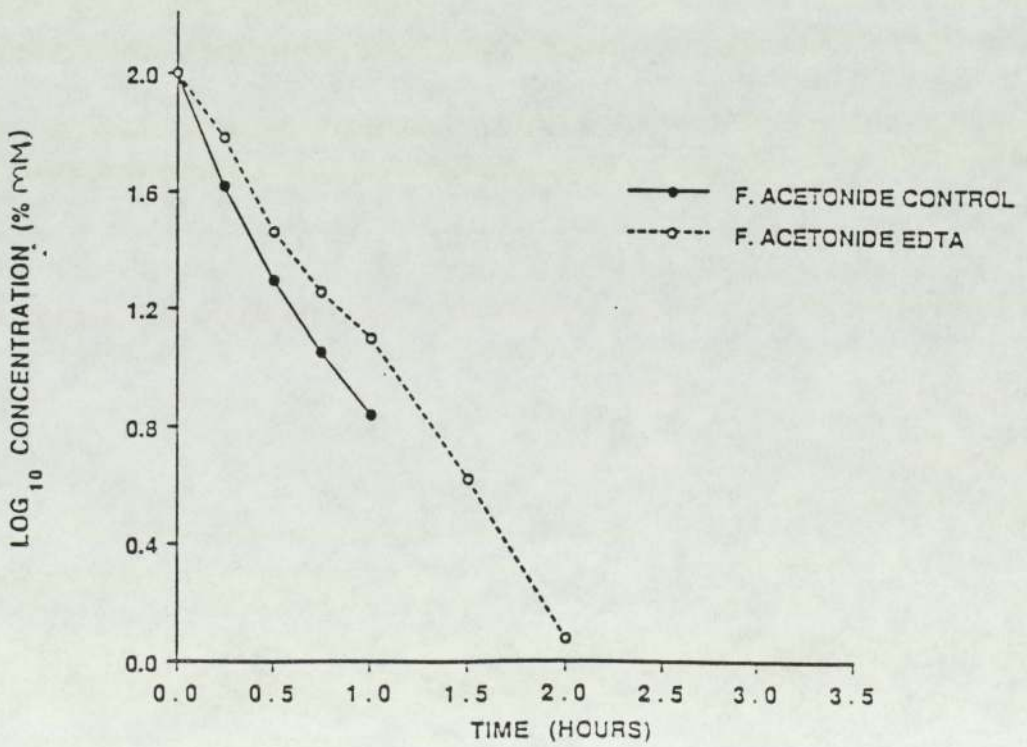


TABLE 3.24 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : CONTROL AND WITH EDTA

Time (hours)	Concentration Fluocinonide (% mM)		Concentration F. Acetonide (% mM)	
	Control	With EDTA	Control	With EDTA
0.00	66.0	67.5	34.0	32.5
0.25	32.1	45.6	122.2	88.9
0.50	19.2	42.7	144.2	93.3
0.75	11.9	39.7	136.2	95.4
1.00	7.3	38.7	124.2	98.7
1.50	3.7	33.7	120.2	112.8
2.00	-	30.8	112.2	117.1
3.00	-	26.8	78.1	124.7
4.00	-	22.8	61.1	128.0

The percentage concentration of steroid was plotted against time (fig 3.22). Logarithmic graphs (base 10) (Fig 3.23) of percentage concentration versus time were also produced. The plot for fluocinonide was linear between 15 minutes and 1.5 hours for the control, and linear between 15 minutes and 4 hours for the samples with EDTA. This indicates that fluocinonide degraded by first-order kinetics over these time periods. The plot of the degradation of fluocinolone acetonide (control) was linear between 15 minutes and 1.5 hours, and the production of the acetonide

Fig 3.22 DIRECT COMPARISON EXPERIMENT : DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE BY BASE-CATALYSED HYDROLYSIS : CONTROL AND WITH EDTA

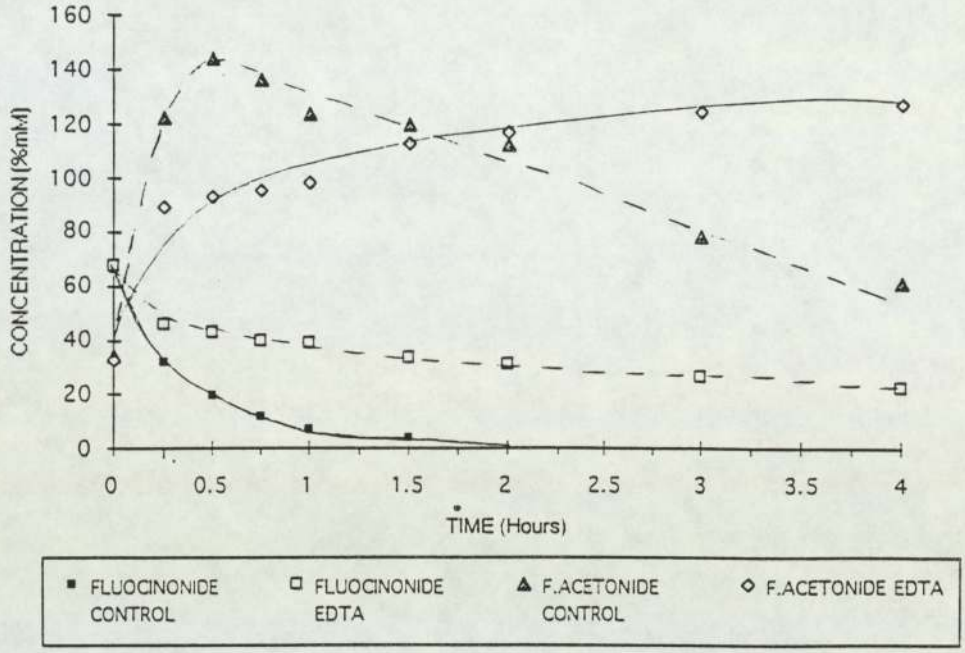
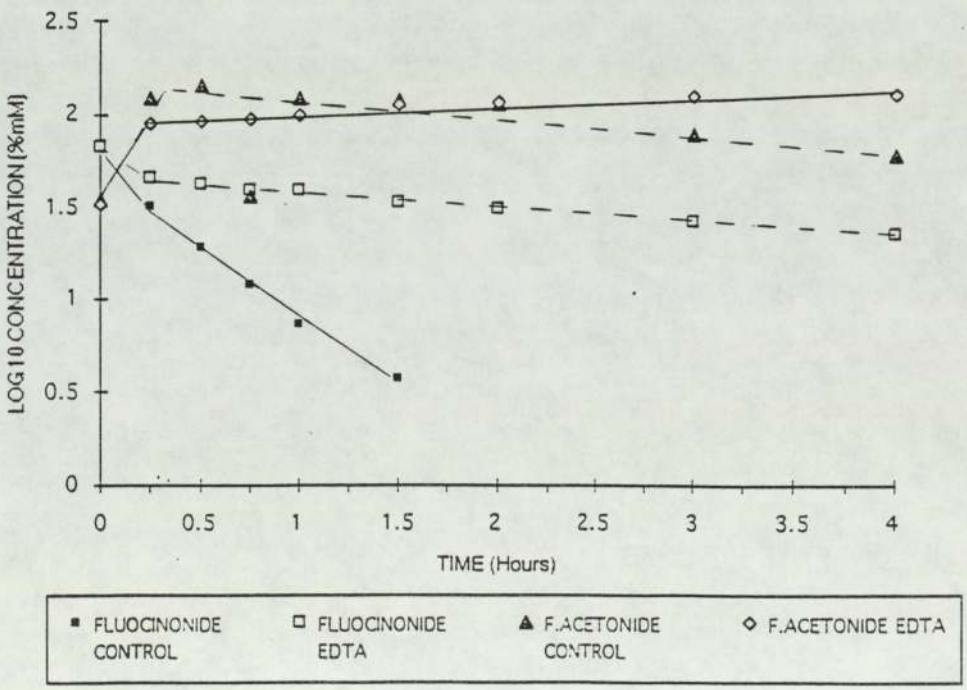


Fig 3.23 DIRECT COMPARISON EXPERIMENT : LOG CONCENTRATION OF FLUOCINONIDE AND FLUOCINOLONE ACETONIDE UNDER BASE-CATALYSED HYDROLYSIS : CONTROL AND WITH EDTA



from fluocinonide in the presence of EDTA was also linear between 15 minutes and 4 hours, indicating that both the degradation and the production of fluocinolone acetonide followed first-order kinetics. The results were subjected to regression analysis using replicate samples.

Fluocinolone acetonide degraded at a rate of 0.0442 $mins^{-1}$ with no additives, and 0.0362 $mins^{-1}$ in the presence of EDTA. This was compared to a degradation rate for fluocinonide of 0.0327 $mins^{-1}$ and 0.003 $mins^{-1}$ for the control solution and with EDTA respectively. The rate of degradation of the acetonide was 0.0042 $mins^{-1}$ in the control but with the addition of EDTA the acetonide was being produced at a rate of 0.0016 $mins^{-1}$.

The regression data for fluocinolone acetonide can be compared to that in table 3.17. It can be seen that the rate of degradation of the acetonide was slightly increased in the presence of EDTA in chapter 3.9.1, but was slightly decreased under similar conditions in chapter 3.9.3. Chromatograms showed that EDTA does not confer any benefit to the stability of the system.

The regression data for fluocinonide can be compared to that in table 3.22. There was poor correlation between the data, but in each experiment the addition of EDTA markedly reduced the rate of degradation of fluocinonide. The primary degradation product was fluocinolone acetonide which increased in concentration over the 3-4 hours of the investigations, compared to a rapid degradation to secondary degradation products as seen in the control solutions.

Yip et al[39] investigated the effect of EDTA on the decomposition of hydrocortisone-17-butyrate which undergoes acyl rearrangement to the 21-ester and then hydrolysis to hydrocortisone. The decomposition of hydrocortisone is metal-catalysed and the rate of this degradation is significantly decreased by the addition of EDTA. Yip et al[39] found that the addition of EDTA to hydrocortisone 17-butyrate had a stabilising effect compared to control systems. The chromatograms produced by HPLC showed that the profiles for hydrocortisone and the minor degradation products were

markedly different when EDTA was added to the system compared to the control. However, the rate constants for isomerization and hydrolysis were not significantly altered. The minor degradation products were not identified but were thought to be due to oxidation of the side chain of hydrocortisone leading to the formation of steroid glyoxals, 17-oxosteroids and glycolic acids.

In the experiments described in chapters 3.9.1, 3.9.2 and 3.9.3 EDTA had a stabilising effect on fluocinonide. A similar effect was conferred upon fluocinolone acetonide, but only when it was a primary degradation product of fluocinonide and not when it was the original steroid. One possible explanation of this anomaly is that whilst the fluocinonide was degrading the concentration of fluocinolone acetonide was still increasing at a faster rate than it was being degraded, so that there was an overall increase in the concentration of fluocinolone acetonide, but an underlying degradation was still occurring. However, this was not supported by the chromatograms.

A second explanation would be that the addition of EDTA lowered the pH of the fluocinonide reaction media, but not that of the fluocinolone acetonide. The pH of the control solutions and the solutions containing EDTA, used in chapter 2.9.3, was measured for each steroid. The fluocinolone acetonide + sodium hydroxide solution had a pH of 13.0 in the absence of EDTA, and a pH of 12.8 with EDTA added. The fluocinonide + sodium hydroxide solution had a pH of 12.7 in the absence of EDTA, and pH of 12.8 with EDTA added. The addition of EDTA did not significantly alter the pH of either reaction mixture and therefore could not explain the results described above.

There was some variation in the rate data obtained in the duplicate experiments. An attempt was made to repeat the investigation in order to obtain consistent kinetic data, but although the pattern of degradation remained unchanged reproducible kinetic data could not be achieved. The temperature of the water-bath was recorded at 15 minute intervals and was found to be constant over the four hour period of the investigation. The samples were left to stand in the water-bath for 30 minutes before the addition of alkali to allow them to attain equilibrium. All solutions used were

prepared from stock solutions which were shown to be stable over the period of the investigation. The same glass pipettes were used for each set of dilutions. Every attempt was made to keep conditions constant. The reason for the variations in data was not determined.

The assay method described allows the degradation of each steroid to be followed, and reproducible chromatographic, but not kinetic, data to be collected. A stability-indicating assay was required to allow the investigation of the effect of formulation on steroid stability. However, the stability tests were detecting small changes occurring over weeks or even months and therefore did not encounter the same problems as were faced in the kinetic experiments, where large changes in concentration were occurring over a rapid time scale. The reproducibility of the assay method and the degradation pattern elucidated were of more relevance to the formulation studies than the establishment of rates of reactions, therefore it was not feasible, within the bounds of this project, to investigate the kinetic data further.

From the results discussed in chapter 3.9 it can be concluded that fluocinolone acetonide degrades to a series of minor degradation products which are eluted close to the void volume and which were not identified. The addition of sodium sulphite and EDTA had no beneficial effects on the stability of the steroid in alkaline solutions.

Fluocinonide was shown to degrade to fluocinolone acetonide in alkali and the acetonide then degraded to a series of minor degradation products which were not identified. The addition of EDTA slowed down the degradation of fluocinonide and the concentration of fluocinolone acetonide continued to increase over the time period studied with no signs of degradation. The addition of EDTA to an unstable formulation, for example when diluted in an alkaline base such as emulsifying ointment, may have a stabilising effect on that formulation and increase the shelf-life of the product. The addition of sodium sulphite was not beneficial.

3.10 ACID DEGRADATION

Fluocinonide and fluocinolone acetonide were subjected to acid degradation at pH 1.2 as described in chapter 2.10 under the same conditions and temperature as the alkaline degradation described in chapters 2.9.1 and 2.9.2. The samples were firstly run without internal standard to make sure that the butyl 4-hydroxybenzoate did not mask any degradation peaks. This internal standard was found to be suitable for use in the investigation.

The pH of the buffered solution was 3.8. This was not affected by the presence of EDTA. EDTA was not investigated further under these conditions.

Fluocinolone acetonide showed no signs of degradation over the first 90 minutes after the addition of acid. The fluctuations in the results as shown in table 3.25 suggest that temperature equilibration may not have been achieved at time zero, despite standing the samples in a water-bath for 30 minutes before the addition of the acid. The low result at 30 hours may be due to loss of steroid or a fluctuation in the data since a similar result was obtained at 5 hours without any signs of degradation. The results are shown in table 3.25 and are expressed as the percentage of the concentration of fluocinolone acetonide at time zero.

TABLE 3.25 DEGRADATION OF FLUOCINOLONE ACETONIDE IN THE PRESENCE OF ACID (pH 1.2)

Time (hours)	Mean Peak Area Ratio Fluocinolone Acetonide (%)
0.00	100.00
0.25	95.45
0.50	98.69
0.75	97.71
1.00	98.36
1.50	100.18
2.00	93.53
3.00	83.19
4.00	85.05
5.00	74.25
6.00	85.23
24.00	86.94
30.00	73.95

% Mean peak area ratio = ratio of the peak areas of steroid/internal standard expressed as a percentage of the concentration of fluocinolone acetonide at time zero.

Fluocinonide was shown to degrade to fluocinolone acetonide in acidic conditions but the reaction occurred much more slowly than under alkaline conditions. The fluctuations in the results shown in table 3.26 again suggest that temperature equilibration may not have been achieved at time zero. After one week there was a significant concentration of fluocinolone acetonide produced and a corresponding loss of fluocinonide.

TABLE 3.26 DEGRADATION OF FLUOCINONIDE AND PRODUCTION OF FLUOCINOLONE ACETONIDE IN THE PRESENCE OF ACID (pH 1.2)

Time (hours)	Mean Peak Area Ratio Fluocinonide (%)	Mean Peak Area Ratio F. Acetonide (%)
0.00	100.00	1.80
0.25	121.66	2.61
0.50	122.33	2.50
0.75	118.29	3.52
1.00	121.42	2.61
1.50	124.11	2.50
2.00	123.94	2.56
3.00	125.77	2.64
4.00	123.76	2.72
5.00	125.72	2.72
6.00	121.90	3.07
24.00	123.07	5.13
30.00	118.73	3.96
168.00	100.22	10.33

% Mean peak area ratio = ratio of the peak areas of steroid/internal standard expressed as a percentage of the concentration of fluocinonide at time zero.

The results from tables 3.25 and 3.26 were subjected to regression analysis using replicate samples. The fluocinolone acetonide degradation rate was 7.06 x 10^{-3} hrs^{-1}. The fluocinonide degraded more slowly at a rate of 1.07 x 10^{-3} hrs^{-1}. Extended stability studies were not performed.

3.11 FORMULATION

3.11.1. COMPARISON OF EXTRACTION PROCEDURES

In preliminary work, a mixture of acetonitrile:water (70:30) was used in the extraction procedure instead of pure acetonitrile. Separation under these conditions was incomplete and the lower layer remained cloudy on standing. Separation was not improved by either filtering or centrifugation. Replacing the acetonitrile:water mixture with pure acetonitrile produced a rapid separation resulting in a clear lower layer.

Three methods of extracting fluocinolone acetonide from base were investigated. In the first method (method 1), as described in chapter 2.11.2.1, extraction was carried out using three aliquots of 2.5ml acetonitrile and the concentration of fluocinolone acetonide was determined after extraction with each aliquot. The results in table 3.27 show that complete extraction of the steroid had been achieved with the first two aliquots and additional extractions did not therefore increase the yield.

TABLE 3.27 EXTRACTION OF FLUOCINOLONE ACETONIDE FROM BASE (METHOD 1)

Aliquots of Acetonitrile	Mean Peak Area Ratio (as % of total)
1	91.1
2	8.9
3	0.0

Mean Peak Area = Ratio of the peak areas of steroid/internal standard.

The recovery of fluocinolone acetonide using method 1 was calculated as 85% compared to a control solution (not extracted). The recovery of the steroid using method 2 and method 3 as described in chapters 2.11.2.2 and 2.11.2.3 was 83% and 84% respectively. There was no advantage in using either of the three methods. Serial extractions were more lengthy than methods 2 and 3 but did not significantly increase the recovery of steroid, therefore a single

extraction method was preferred. Methods 2 and 3 were both feasible extraction procedures; however, some samples were to be stored in a freezer while awaiting analysis and the presence of the internal standard may have affected the stability of the samples on storage, therefore method 2 was chosen for subsequent investigations.

Eight replicate extractions were carried out using this method and each sample was injected on to the column three times to give the mean peak area ratio for that sample. The peak area ratios for the eight samples are shown in table 3.28.

TABLE 3.28 REPLICATE EXTRACTIONS OF FLUOCINOLONE ACETONIDE (METHOD 2)

Peak Area Ratio				Mean	Coefficient of Variation
0.251	0.253	0.245	0.253	0.250	1.64%
0.250	0.245	0.255	0.245		

Peak area ratio = Ratio of the peak areas of steroid/internal standard.

The results in table 3.28 showed that method 2 was reproducible and suitable for use in the formulation studies.

3.11.2 RECOVERY EXPERIMENTS

All glassware used in the recovery experiments was treated with dimethyldichlorosilane solution in an attempt to suppress the adsorption of steroid on to the glass and thereby improve the recovery. The treatment procedure was lengthy and had no effect on the recovery, so untreated glassware was used throughout.

The following equations were used to calculate the data:

Peak area ratio (PAR) = Ratio of the peak areas of steroid/internal standard.

Recovery from base = (PAR from base/PAR from control) x 100.

Recovery from hexane = (PAR from hexane/PAR from control) x 100.

Recovery = Recovery from base/Recovery from hexane) x 100.

In chapter 2.11.3.1, fluocinolone acetonide was extracted from the base using 10ml of acetonitrile compared to using 15ml of acetonitrile to make the control solution. A correction factor was applied to take account of this difference. The acetonide was incorporated into the base under investigation by injection of the steroid into the base on a tile. This method proved acceptable for investigation of Unguentum Merck®, but Lipobase® gave erratic results and reproducible recovery data could not be obtained. To overcome this, the recovery of fluocinolone acetonide from Lipobase® was compared to the recovery of fluocinolone acetonide from Synalar® ointment.

Fluocinolone acetonide was extracted from 1 in 4 formulations in Lipobase® and Unguentum Merck® as described in chapter 2.11.3.1. Each sample was injected three times and the mean peak area ratio for that sample was used to calculate the recovery. The results in table 3.29 show that 97% recovery of fluocinolone acetonide could be achieved from Unguentum Merck® and 96% from Lipobase®. Although the latter was lower than expected, six replicate extractions gave a coefficient of variation of 1.19% which shows that the method is reproducible.

TABLE 3.29 RECOVERY OF FLUOCINOLONE ACETONIDE FROM 1 IN 4 FORMULATION IN LIPOBASE® AND UNGUENTUM MERCK®

Formulation F. Acetonide	Recovery (%)		Mean Recovery (%)	Coefficient of variation (%)
1 in 4 in Unguentum Merck®	98.07 96.67		97.37	-
1 in 4 in Lipobase®	95.41 97.52 94.18	95.00 95.53 96.98	95.77	1.19

The recovery of fluocinolone acetonide from the same two bases was investigated for 1 in 10 formulations as described in chapter 2.11.3.2. Instead of incorporating the steroid into the base on a tile, the steroid was injected into the base in a glass beaker. This improved the recovery by avoiding loss of steroid solution, since the beaker was washed out with the extraction media to ensure complete transfer. Each sample was injected three times and the mean peak area ratio for that sample was used to calculate the recovery. The results in table 3.30 show that approximately 100% recovery of fluocinolone acetonide was achieved using this method.

TABLE 3.30 RECOVERY OF FLUOCINOLONE ACETONIDE FROM 1 IN 10 FORMULATION IN LIPOBASE® AND UNGUENTUM MERCK®

Formulation F. Acetonide	Recovery (%)	Mean Recovery (%)
1 in 10 in Lipobase®	108.4 100.2 100.0	102.9
1 in 10 in Unguentum Merck®	100.3 101.1 100.5	100.6

The beaker method was used to investigate the recovery of fluocinonide from 1 in 4 and 1 in 10 formulations in Lipobase®, Unguentum Merck® and Metosyn Diluent®. Each sample was injected three times and the mean peak area ratio for that sample was used to calculate the recovery. The results in table 3.31 show that the extraction procedure, as described in chapter 2.11.3.3, achieved over 95% recovery for the extraction of fluocinonide from 1 in 4 formulations in each base.

TABLE 3.31 RECOVERY OF FLUOCINONIDE FROM 1 IN 4 FORMULATION IN LIPOBASE®, UNGUENTUM MERCK® AND METOSYN DILUENT®

Formulation Fluocinonide	Recovery (%)	Mean Recovery (%)
1 in 4 in Lipobase®	100.68 99.07	99.88
1 in 4 in Unguentum Merck®	99.77 100.23	100.00
1 in 4 in Metosyn Diluent®	96.00 95.5	95.75

Fluocinonide was extracted from 1 in 10 formulations in each base as described in chapter 2.11.3.4. Each sample was injected three times and the mean peak area ratio for that sample used to calculate the recovery. The results in table 3.32 show that the recovery of fluocinonide from a 1 in 10 formulation in Metosyn Diluent® was lower than an acceptable limit of 95%. Six replicate extractions gave a coefficient of variation of 2.9% showing that although the recovery was low it was reproducible and was therefore considered to be acceptable.

TABLE 3.32 RECOVERY OF FLUOCINONIDE FROM 1 IN 10 FORMULATION IN LIPOBASE®, UNGUENTUM MERCK® AND METOSYN DILUENT®

Formulation Fluocinonide	Recovery (%)		Mean Recovery (%)	Coefficient of Variation (%)
1 in 10 in Lipobase®	98.2 106.2 103.9	98.2 106.0 103.9	102.7	3.6
1 in 10 in Unguentum Merck®	96.7 97.4 100.7	96.8 96.9 100.7	98.2	1.99
1 in 10 in Metosyn Diluent®	88.5 93.7 89.6	90.3 86.8 86.7	89.3	2.9

The recovery data discussed above demonstrates that the extraction procedure outlined in method 2, chapter 2.11.2.2, is a feasible method of analysis of steroid content of formulations and was used in the following stability investigations.

3.11.3 STABILITY OF FORMULATIONS

The stability of Synalar® ointment diluted 1 in 4 and 1 in 10 in Lipobase® and Unguentum Merck® was investigated as described in chapter 2.11.4.1. The ointment was chosen because it is more commonly diluted than the cream at the Birmingham Skin Hospital. Dilutions were stored at 4°C, 25°C and 32-°C, and samples were taken over ten months to determine the steroid content with respect to time and temperature. The results from the stability studies at room temperature are shown in table 3.33 for 1 in 4 formulation in Lipobase® and table 3.34 for the 1 in 4 formulation in Unguentum Merck®. They show that there was no loss of steroid over the first 112 days (4 months) of storage of Synalar® ointment diluted 1 in 4 in Lipobase® or 1 in 4 in Unguentum Merck® at 25°C. This was followed by a fall in steroid content over the subsequent six months. It was concluded that the standard 14 day expiry recommended by the External Diluent Directory[16] is unrealistic for the

formulations described above, which could be given a shelf-life of at least four months at room temperature.

Unguentum Merck® has been shown to comply with the B.P. 1980 test for efficacy of antimicrobial preservatives in pharmaceutical products by Tanner and Woodford[35]. Data were shown for samples of Unguentum Merck® challenged over a three month period. No viable organisms were detected. The base was also used to produce half-strength Betnovate® ointment. No viable organisms were detected when the product was challenged over a three-month period. Therefore, the authors concluded that Unguentum Merck® conferred microbial stability on the products in excess of the 14 day shelf-life currently used. Further investigation of the effect of microbiological contamination on diluted products is required, especially "in-use" contamination where organisms are introduced into the product by the patient. This may influence both the stability of the formulation, and its suitability for use on skin lesions.

TABLE 3.33 STABILITY OF SYNALAR® OINTMENT DILUTED 1 IN 4 IN LIPOBASE® : STORED AT 25°C

Storage Time (days)	F. Acetonide content as % of steroid at time zero			Mean
zero	100	99.5		100
14	102	98		100
28	85	85.5		85
42	101	98		99.5
56	100	101	99	100
84	101	101		101
112	113	98	99	103
147	91	92	91	91
175	92	95	94	94
203	89	89		89
280	84	86		85

TABLE 3.34 STABILITY OF SYNALAR® OINTMENT DILUTED 1 IN 4 IN UNGUENTUM MERCK® : STORED AT 25°C

Storage Time (days)	F. Acetonide content as % of steroid at time zero			Mean
zero	99.8	100.3		100
14	101	100	101	101
28	88	92	98	93
42	101	100		101
56	98	99	102	100
84	98	100	103	100
112	98	98	103	100
147	89	93	106	96
175	90	92	89	90
203	88	89	89	89
280	84	83		84

Synalar® ointment was also diluted 1 in 10 in Lipobase® and Unguentum Merck® and the results from the stability studies at room temperature are shown in tables 3.35 and 3.36 respectively. Fluocinolone acetonide was more stable in Lipobase® than in Unguentum Merck®, when diluted one part in ten with base. Based on these results, a two month expiry could be given to the formulation in Lipobase® and a one month expiry to that in Unguentum Merck® rather than the 14 day limit used at present. The 1 in 10 formulations were markedly less stable than the 1 in 4 formulations probably due to diluting out the original properties of the commercial preparation.

TABLE 3.35 STABILITY OF SYNALAR® OINTMENT DILUTED 1 IN 10 IN LIPOBASE® : STORED AT 25°C

Storage Time (days)	F. Acetonide content as % of steroid at time zero			Mean
zero	102	100	97	100
28	108	97		103
56	100	103	102	102
77	91	100		95

TABLE 3.36 STABILITY OF SYNALAR® OINTMENT DILUTED 1 IN 10 IN UNGUENTUM MERCK® : STORED AT 25°C

Storage Time (days)	F. Acetonide content as % of steroid at time zero			Mean
zero	99	100	101	100
28	99	98	93	97
56	91	95	93	93
77	95	90		93

Stability data were collected for the 1 in 4 and 1 in 10 dilutions in Lipobase® and Unguentum Merck® at 4°C. Samples were taken after 8 and 9 months. On storage in a refrigerator the 1 in 4 dilutions retained at least 95% of the steroid content after nine months. The 1 in 10 dilution in Lipobase® was stable under these conditions for eight months with some loss of activity during the ninth month of storage. The 1 in 10 formulation in Unguentum Merck® showed 20% loss of steroid content when analysed after eight months, and a further 5% loss during the ninth month.

TABLE 3.37 STABILITY OF SYNALAR® OINTMENT DILUTED 1 IN 4 AND 1 IN 10 WITH LIPOBASE® AND UNGUENTUM MERCK®, STORED AT 4°C : CONTENT OF FLUOCINOLONE ACETONIDE EXPRESSED AS A PERCENTAGE OF STEROID PRESENT AT TIME ZERO

Synalar® in base	Content of F. Acetonide after storage at 4°C (Mean in brackets)		
	Time Zero	224 days	252 days
1 in 4 Lipobase®	100 99.5 (100)	116 111 (114)	102 90 (96)
1 in 4 U. Merck®	99.8 100.3 (100)	101 97 (99)	100 94 (97)
1 in 10 Lipobase®	102 100 97 (100)	110 100 (105)	96 86 (90)
1 in 10 U. Merck®	100 99 101 (100)	80 80 (80)	74 76 (75)

Extemporaneously prepared formulations of Synalar® ointment in Lipobase® and Unguentum Merck® at a dilution of 1 in 4 retained a concentration of greater than 95% over the nine month storage period at 4°C. The formulations could therefore be given a shelf-life of nine months based on the stability data. A 1 in 10 dilution of Synalar® ointment in Lipobase® may be stored in a similar manner for up to eight months, although in light of the degradation during the ninth month, a shorter shelf-life of 6 months may be more appropriate. Prolonged storage of 1 in 10 dilutions of Synalar® ointment in Unguentum Merck® cannot be recommended on the data obtained from this study, although storage for less than eight months at this temperature was not investigated.

Stability data were similarly collected for the 1 in 4 and 1 in 10 dilutions in Lipobase® and Unguentum Merck® stored at 32°C. Samples were again taken at 8 and 9 months.

Storage of the 1 in 4 dilutions in Lipobase® and Unguentum Merck® at the elevated temperature of 32°C did not result in loss of steroid after nine months. There was a 20% loss of steroid after eight months for the 1 in 10 dilutions in each base, as shown in table 3.38.

TABLE 3.38 STABILITY OF SYNALAR® OINTMENT DILUTED 1 IN 4 AND 1 IN 10 IN LIPOBASE® AND UNGUENTUM MERCK® STORED AT 32°C: CONTENT OF FLUOCINOLONE ACETONIDE EXPRESSED AS A PERCENTAGE OF STEROID PRESENT AT TIME ZERO

Synalar® in base	Content of F. Acetonide after storage at 32°C (Mean in brackets)		
	Time Zero	224 days	252 days
1 in 4 Lipobase®	100 99.5 (100)	120 114 (117)	105 110 (108)
1 in 4 U. Merck®	99.8 100.3 (100)	99 99 (99)	100 99 (100)
1 in 10 Lipobase®	102 100 97 (100)	80 80 (80)	88 84 (86)
1 in 10 U. Merck®	100 99 101 (100)	78 82 (80)	78 76 (77)

Synalar® ointment diluted 1 in 4 in Lipobase® and Unguentum Merck® was shown to be stable at 4°C and 32°C. The formulations appeared to have an increased stability at these temperatures than at 25°C. It is possible that this is because the formulation stored at 25°C was sampled more frequently, thus introducing a larger air space into the jar (to replace the ointment removed) which resulted in more oxygen available for degradation, or contaminants may

have been introduced on sampling which may have increased the rate of degradation. The shelf-life of such formulations should be restricted to four months, as determined at 25°C. The 1 in 10 formulations both showed significant loss of steroid at 4°C and 32°C and again the shelf-life should be restricted to that determined at 25°C, two months for the dilution in Lipobase® and one month for that in Unguentum Merck®.

The stability of Metosyn® cream diluted 1 in 4 and 1 in 10 in Lipobase®, Unguentum Merck® and Metosyn Diluent® was investigated as described in chapter 2.11.4.2. The cream was chosen for analysis because the commercially available Metosyn Diluent® was under investigation, and this is not suitable for diluting the ointment. Dilutions were stored at 4°C, 25°C and 32°C and samples were taken over nine months to determine the steroid content with respect to time and temperature.

The results of the stability data at room temperature are shown in table 3.39 for the 1 in 4 formulation in Lipobase®, table 3.40 for the 1 in 4 formulation in Unguentum Merck®, and table 3.41 for the 1 in 4 formulation in Metosyn Diluent®. There was no loss of steroid from any of the formulations over the nine month period studied.

TABLE 3.39 STABILITY OF METOSYN® CREAM DILUTED 1 IN 4 IN LIPOBASE® : STORED AT 25°C

Storage Time (days)	Fluocinonide content as % of steroid at time zero			Mean
zero	101	98		100
14	103	105	105	104
28	107	107	107	107
42	101	104	100	102
58	103	109	107	106
84	109	110	108	109
119	105	108	107	107
147	101	104	106	104
175	104	107	104	105
252	103	106		105

TABLE 3.40 STABILITY OF METOSYN® CREAM DILUTED 1 IN 4 IN UNGUENTUM MERCK® : STORED AT 25°C

Storage Time (days)	Fluocinonide content as % of steroid at time zero			Mean
zero	100	99		100
14	99	97	94	97
28	104	104	92	100
42	97	82	95	91
58	97	103	99	100
84	94	101	107	101
119	98	97		98
147	99	98	100	99
175	102	102	97	100
252	100	98		99

TABLE 3.41 STABILITY OF METOSYN® CREAM DILUTED 1 IN 4 IN METOSYN DILUENT® : STORED AT 25°C

Storage Time (days)	Fluocinonide content as % of steroid at time zero			Mean
zero	100	101	100	100
14	103	99	99	100
28	97	98	99	98
42	96	91	98	95
58	98	101	101	100
84	100	100	103	101
119	98	102		100
147	105	104	104	104
175	98	98	96	97
252	100	99		100

The low results at 42 days in tables 3.39-3.41 were attributed to sampling error, since the results from subsequent samples were consistently higher. It was concluded that Metosyn® cream was stable for at least nine months at 25°C when diluted 1 in 4 in Lipobase®, Unguentum Merck® and Metosyn Diluent®.

Metosyn® cream was also diluted 1 in 10 in each base and stability studies were performed at room temperature over a three month period. These results are shown in tables 3.42 to 3.44. Metosyn® cream was stable for two months when diluted 1 in 10 in each base and stored at 25°C. There was no loss of steroid after 56 days using either Lipobase® or Unguentum Merck® as the diluent, and with the latter base there was no significant loss of potency after 77 days storage. Dilution using Metosyn Diluent® sustained an acceptable level of potency (95%) of the steroid for 56 days, although this was lower than that seen with the other bases. However, the use of Metosyn Diluent® does enable the original characteristics of the product to be maintained on dilution which may be advantageous, since the properties of the

vehicle are as important as the choice of active constituent, as discussed in Chapter 1.

TABLE 3.42 STABILITY OF METOSYN® CREAM DILUTED 1 IN 10 IN LIPOBASE® : STORED AT 25°C

Storage Time (days)	Fluocinonide content as % of time zero						Mean
zero	101	102	100	99	98	99	100
28	96		97		97		97
42	99		98		95		97
56	102		103		102		102
77	91		93				92

TABLE 3.43 STABILITY OF METOSYN® CREAM DILUTED 1 IN 10 IN UNGUENTUM MERCK® : STORED AT 25°C

Storage Time (days)	Fluocinonide content as % of time zero						Mean
zero	101	102	106	97	98	98	100
28	93		95		95		94
42	96		98				97
56	102		102		99		101
77	98		95				97

TABLE 3.44 STABILITY OF METOSYN® CREAM DILUTED 1 IN 10 IN METOSYN DILUENT® : STORED AT 25°C

Storage Time (days)	Fluocinonide content as % of time zero				Mean
zero	100	102	97	99	100
28	90		89	88	89
42	94		94	96	95
56	95		98	92	95
77	92		90		91

Stability data were collected for 1 in 4 and 1 in 10 dilutions in each base (Lipobase®, Unguentum Merck® and Metosyn Diluent®) at 4°C. Samples were taken after 7 and 8 months and the results are shown in table 3.45. There was no significant loss of steroid except from the 1 in 10 dilution in Metosyn Diluent® where the steroid content fell to 93% after 8 months.

TABLE 3.45 STABILITY OF METOSYN® CREAM DILUTED 1 IN 4 AND 1 IN 10 IN LIPOBASE®, UNGUENTUM MERCK® AND METOSYN DILUENT®, STORED AT 4°C: CONTENT OF FLUOCINONIDE AS A PERCENTAGE OF STEROID AT TIME ZERO

Metosyn® in base	Content of Fluocinonide after storage at 4°C (Mean)		
	Time Zero	196 days	224 days
1 in 4 Lipobase®	101 98 (100)	110 106 (108)	106 105 (106)
1 in 4 U. Merck®	100 99 (100)	97 99 (98)	101 97 (99)
1 in 4 M. Diluent®	100 101 100 (100)	100 102 (101)	100 97 (99)
1 in 10 Lipobase®	101 99 (100)	104 104 (104)	102 98 (100)
1 in 10 U. Merck®	103 97 (100)	100 109 (105)	98 100 (99)
1 in 10 M. Diluent®	100 100 (100)	98 95 (97)	93 93 (93)

Stability data were similarly collected for 1 in 4 and 1 in 10 dilutions in each base stored at 32°C. Samples were again taken at 7 and 8 months. The results in table 3.46 show that there was no significant degradation except in the 1 in 10 dilution in Metosyn Diluent® where the steroid content fell to 93% after 8 months. Since this fall in steroid content also occurred in the sample stored at 4°C it was concluded that 7 months is the maximum shelf-life of this dilution.

TABLE 3.46 STABILITY OF METOSYN® CREAM DILUTED 1 IN 4 AND 1 IN 10 IN LIPOBASE®, UNGUENTUM MERCK® AND METOSYN DILUENT®, STORED AT 32°C: CONTENT OF FLUOCINONIDE AS A PERCENTAGE OF STEROID AT TIME ZERO

Metosyn® in base	Content of Fluocinonide after storage at 32°C (Mean)		
	Time Zero	196 days	224 days
1 in 4 Lipobase®	101 98 (100)	111 112 (112)	111 109 (110)
1 in 4 U. Merck®	100 99 (100)	103 103 (103)	98 96 (97)
1 in 4 M. Diluent®	100 101 100 (100)	103 103 (103)	97 98 (98)
1 in 10 Lipobase®	101 99 (100)	109 111 (110)	104 104 (104)
1 in 10 U. Merck®	103 97 (100)	107 107 (107)	100 100 (100)
1 in 10 M. Diluent®	100 100 (100)	98 98 (98)	93 90 (93)

Metosyn® cream diluted 1 in 4 in Lipobase®, Unguentum Merck® and Metosyn Diluent® were stable for at least 9 months at 4°C and 25°C, and at least 7 months at 32°C and therefore the latter would be a suitable shelf-life for the dilutions. The 1 in 10 formulations were also stable for 7-9 months at 4°C and 32°C. However, similarly to dilutions of Synalar® ointment, the stability was greatly reduced at 25°C, therefore a maximum shelf-life of 2 months must be given to these formulations.

Fluocinonide in Metosyn® formulations was shown to be more stable than in the pre-formulation studies. This may be because in the formulation studies, the product is stored in glass jars with minimal air space, which limited the

oxidative degradation pathway. In the pre-formulation studies oxidative and non-oxidative pathways were available. Fluocinonide was also more stable than fluocinolone acetonide in formulation. Degradation of fluocinolone acetonide proceeds by oxidation of the C-17 side chain and by loss of the acetonide group. Substitution with an acetate group at the C-21 hydroxyl group as in fluocinonide appears to protect one or both of these sites from degradation, This correlates with the results of Barnes et al[51] who investigated the stability of steroid ointments diluted with Compound Zinc Paste B.P. and found similar relative stabilities of fluocinonide and fluocinolone acetonide.

3.12 VOLUNTEER TRIAL

The blanching potential of nine topical preparations was investigated as described in chapter 2.11.5 using a portable reflectance spectrophotometer. The formulations studied are shown in table 3.47.

TABLE 3.47 FORMULATIONS USED FOR VASOCONSTRICTOR ASSAY

Synalar® ointment (fluocinolone acetonide 0.025% w/w)	= (S)
Betnovate® ointment (betamethasone 0.1% w/w)	= (B)
Synalar® ointment 1 in 4 - commercial preparation	= (SC4)
Synalar® ointment 1 in 4 in Lipobase®	= (SL4)
Synalar® ointment 1 in 4 in Unguentum Merck®	= (SM4)
Synalar® ointment 1 in 10 in Lipobase®	= (SL10)
Synalar® ointment 1 in 10 in Unguentum Merck®	= (SM10)
Lipobase®	= (L)
Unguentum Merck®	= (UM)

The machine used was a prototype, the Haemelometer model IV. This machine did not produce absolute reflectance measurements but the readings could be calibrated to give a reproducible ratio by means of two glazed ceramic tiles. For each LIR reading taken on the skin, readings were also taken on a white tile and a pink tile. The blanching index (BI) was then calculated as a ratio of these readings:

$$BI = \frac{skin - white\ tile}{pink - white\ tile}$$

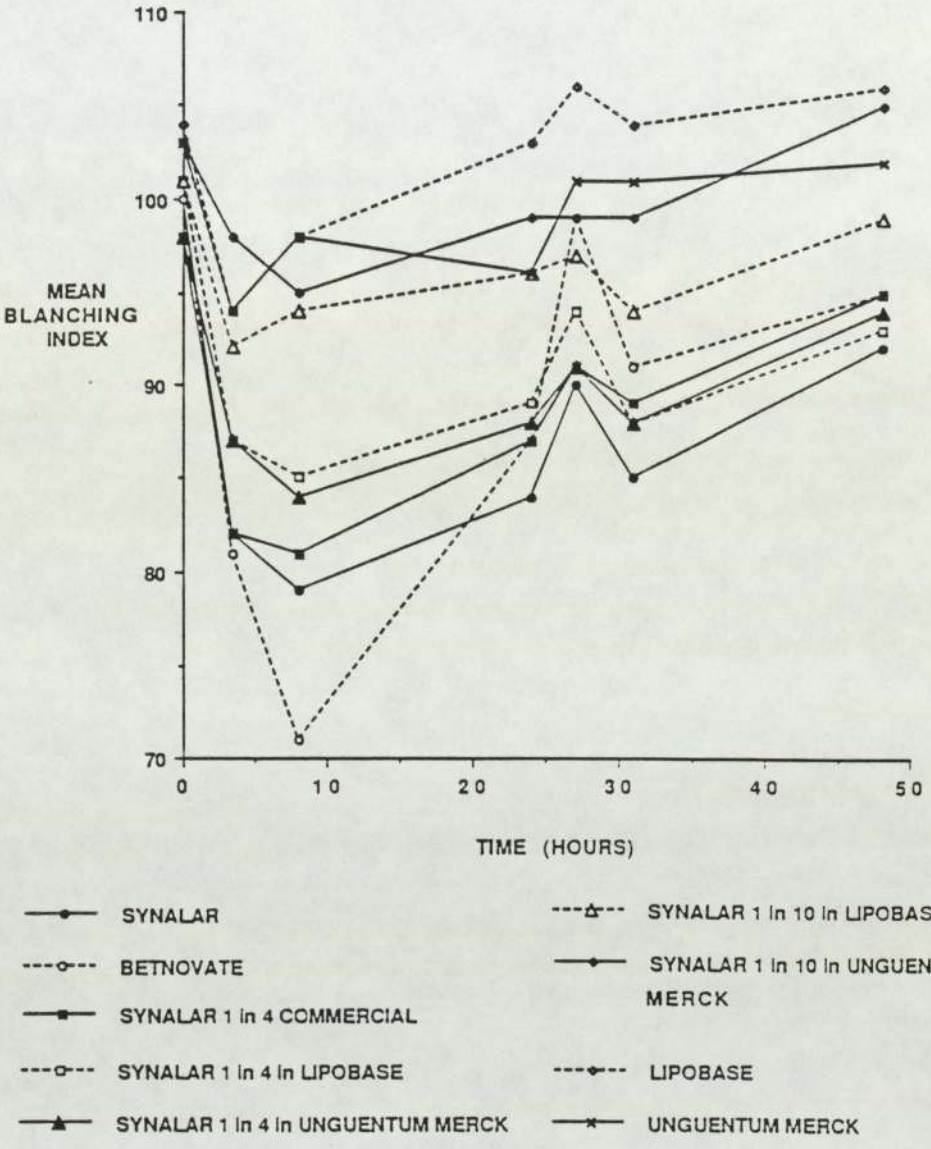
Although the actual LIR readings varied with time, the BI showed good precision. The calibration was tested by taking fifty readings at the same site on the forearm of one volunteer. The blanching index was calculated as above. The mean was 0.825 with a coefficient of variation of 3.1%.

There were ten sites available per volunteer, five on each forearm, and the formulations were applied to these according to a Latin square design (table 2.1) to overcome any possible variation due to position differences. One site was assigned as a control site to which no ointment was applied, to take into account fluctuations in body temperature and exercise which would have altered the blood flow through the forearm and may have caused variations in response which were not due to the formulations. The blanching indices were compared using the following ratio:

$$Relative\ BI = \frac{BI\ at\ time\ t\ of\ site}{BI\ at\ time\ t\ of\ control}$$

Gibson et al[5] studied the relationship between the 7 hour blanching reading, the peak reading and the area under the curve (AUC) value of the vasoconstrictor assay. They concluded that the AUC value is the most accurate predictor of relative differences in clinical efficacy between

FIG 3.24 GRAPH OF THE MEAN BLANCHING INDICES OF NINE TOPICAL PREPARATIONS AGAINST TIME.



corticosteroid preparations and it allows reasonably accurate predictions to be made regarding likely differences in local clinical effects between these preparations. It has been shown by Barry and Woodford[52] that Synalar® ointment exerts its maximum blanching activity later than many other corticosteroids and its AUC assessment predicts greater potency than its blanching value at 7 hours, which is a value often used to compare other steroids. Therefore, a series of readings of the blanching index was taken over 48 hours and used to construct an area under the curve representation of the corticosteroid blanching activity. Data were collected for up to 75 hours post application but were only assessed up to 48 hours. The data from the 10 volunteers was used to calculate the mean blanching index (MBI) which was plotted against time, in hours, for each formulation (fig 3.24). The AUC was determined to 24 hours (AUC 24), table 3.48, and also to 48 hours (AUC 48) as shown in table 3.49.

TABLE 3.48 AREA UNDER THE CURVE TO 24 HOURS FOR TEN VOLUNTEERS

Patient No.	1	2	3	4	5	6	7	8	9	10
Product										
Synalar	123	490	389	535	450	764	525	179	291	509
Betnovate	325	398	413	582	114	817	530	277	461	566
Commercial 1 in 4	514	526	156	320	491	795	358	-159	373	358
Lipobase 1 in 4	282	295	215	255	451	468	546	200	242	99
U.Merck 1 in 4	245	193	316	421	241	721	282	84	382	305
Lipobase 1 in 10	163	201	182	-216	72	729	56	-167	-33	260
U.Merck 1 in 10	-228	107	208	-258	74	500	-13	-49	58	214
Lipobase	-52	72	123	-2	21	-20	-188	-63	122	-56
U.Merck	208	17	42	-12	130	250	54	-213	307	-105

TABLE 3.49 AREA UNDER THE CURVE TO 48 HOURS FOR TEN VOLUNTEERS

Patient No.	1	2	3	4	5	6	7	8	9	10
Product										
Synalar	-143	1214	585	601	730	1058	1108	328	552	1106
Betnovate	152	667	786	550	-3	1049	1098	385	999	811
Commercial 1 in 4	467	940	17	683	913	939	741	-231	485	823
Lipobase 1 in 4	160	619	358	273	933	592	967	330	383	493
U.Merck 1 in 4	174	523	390	539	656	865	599	183	748	753
Lipobase 1 in 10	60	412	175	-367	404	884	112	-185	-112	672
U.Merck 1 in 10	-904	317	221	-316	189	380	-5	-132	87	463
Lipobase	-196	186	37	-32	292	-462	-711	-138	179	-261
U.Merck	210	-108	-174	-41	432	108	65	-446	626	-153

The AUC data were subjected to statistical analysis using the Newman-Keuls multiple range test, a non-parametric test which has been used to compare blanching potencies in similar studies[5,25,30]. This test was performed on the AUC 24 and AUC 48. The AUC 24 data, table 3.50, was divided into two main sets. The first group contained the undiluted steroids, of which Betnovate® ointment produced the highest degree of blanching, and these were not significantly different in potency to the 1 in 4 formulations. The second group contained the 1 in 10 formulations which were not significantly different in potency to the bases alone. These two groups were significantly different, $p<0.05$, although there was some overlap between the extemporaneous 1 in 4 and the 1 in 10 Lipobase® formulations. This is because although the mean AUC values were quite different (SL4 305.3, SM4 319.0 and SL10 124.7) there was a large degree of variability in patient response to each formulation and this is reflected in the lack of significant differences between some of the data groups.

TABLE 3.50 RELATIVE POTENCIES OF OINTMENTS EXPRESSED AS AREA UNDER THE CURVE (0-24 HRS) (MEAN OF TEN VOLUNTEERS)

Underlined figures indicate groups of treatments that were not significantly different from each other, $p<0.05$. For abbreviations see table 3.47.

B	S	SC4	SM4	SL4	SL10	UM	SM10	L
448.3	425.5	373.2	319.0	305.3	124.7	67.8	61.3	-4.3

The test shows that undiluted Synalar® ointment and the 1 in 4 formulations studied are equipotent. The lack of linearity between potency and the dilution factor may lead to unsafe medical practice, because a doctor may anticipate that dilution 1 in 4 will significantly reduce the potency. He may therefore prescribe a diluted formulation for more liberal application than its undiluted form, or he may prescribe it for use in children, where a reduced potency preparation may be desirable. Conversely, the 1 in 10 formulations have no significantly greater potency than the bases alone and therefore the presence of the steroids may not confer clinical efficacy on those preparations. Alternatively, the assay method may not be sensitive enough to detect low levels of blanching activity. Use of such potentially ineffective formulations is dangerous for the patient since a flare-up of the disease may occur, and the practice of extemporaneous dilution also carries a risk of introducing contamination into the product, which effects the stability of the formulations and may cause infection in the patient if applied to broken skin. This practice should therefore be avoided where there is no evidence that the 1 in 10 formulations confer any clinical efficacy.

It is accepted that the choice of base can be as important as the choice of active ingredient, because different bases have properties which may affect the state of the skin, for example; emollient, cooling, astringent, hydrating or occlusive properties. The bases Unguentum Merck® and Lipobase® both produced clinical responses in this study. The area under the curve values would have been zero if no response had been observed. The Lipobase® produced a minimal response up to 24 hours, but a greater response up to 48 hours. Unguentum Merck® caused an increase in skin colour. The bases may change the physical state of the skin, e.g. by hydration or occlusion which may account for these observations.

The AUC 48 was also investigated; table 3.51. The order of activity was similar to that for the AUC 24, table 3.50, except that the prolonged blanching data showed Synalar® ointment to be the more potent over Betnovate® ointment, although this was not a significant difference. This confirms the work done by Barry and Woodford[52] in which Synalar® took longer to produce its blanching effect compared to most other corticosteroids.

There was no significant difference between the Synalar® dilutions 1 in 10 in Lipobase® and the 1 in 4 extemporaneous and commercial preparations.

TABLE 3.51 RELATIVE POTENCIES OF OINTMENTS EXPRESSED AS AREA UNDER THE CURVE (0-48 HRS) (MEAN OF TEN VOLUNTEERS)

Underlined figures indicate groups of treatments that were not significantly different from each other, $p<0.05$. For abbreviations see table 3.47.

S	B	SC4	SM4	SL4	SL10	UM	SM10	L
713.9	649.4	577.7	543.0	510.8	205.5	51.9	30.0	-110.6

The area under the curve data to 24 hours was assessed retrospectively to determine the number of volunteers which would have been required to detect any differences between the treatment, as described by Pocock[53].

To detect a difference between the area under the curve of Synalar® ointment (treated as the control group[53]) and the 1 in 4 dilution in Lipobase®, 64 volunteers would have been required. To detect a difference between the Synalar® ointment (treated as the control group[53]) and the 1 in 10 dilution in Lipobase®, 10 volunteers would have been required, and 109 volunteers to detect a difference between in the 1 in 10 in Lipobase® (treated as the control group[53]) and Lipobase® alone. These dilutions were chosen as they were at the extremes of the significance groups as determined by the Newman-Keuls test.

The above results are compatible with the Newman-Keuls analysis in that there was a significant difference detected between the Synalar® ointment and the 1 in 10 in Lipobase® with 10 volunteers, but no significant difference was

detected between the Synalar® ointment and the 1 in 4 in Lipobase® or between the 1 in 10 in Lipobase® and the Lipobase® alone.

This work confirms the findings of other authors that;

1. there is no linear relationship between dilution and potency,
2. the base used is an important factor in determining the potency of the final formulation.

Ryatt et al[27,28] showed that Betnovate® ointment diluted 1 in 4 in emulsifying ointment was equipotent to the undiluted steroid, and that dilutions of Betnovate® ointment in Unguentum Merck® diluted 4, 16 and 32-fold were not significantly different in potency to the undiluted steroid. Although my studies support the results regarding the 1 in 4 studies, my work did show a reduction in potency on diluting to 1 in 10 for at least some of the formulations. However, this highlights the fact that the choice of base has an important effect on the clinical efficacy of the final formulation and potency cannot be assumed or estimated without the appropriate clinical evidence to support that choice of formulation. The results of other authors is discussed more widely in chapter 1.7.

In my investigations, the commercially available 1 in 4 formulation did not have a significantly lower potency than the undiluted steroid. It is possible that the assay was not sensitive enough to accurately predict the potency of the formulations, or that any differences in potency were masked by the large interpatient variation in response. Alternatively, in line with the work of other authors who have shown no decrease in potency by diluting Betnovate® ointment 1 in 4 in a number of bases; emulsifying ointment[27], Unguentum Merck®[28] and white soft paraffin[30], a 1 in 4 dilution of a steroid may not be sufficient to significantly reduce its potency and therefore the place of such a preparation in clinical practice, even a commercially prepared formulation, must be questioned. It may be more appropriate to choose a steroid from a lower potency group.

It is interesting to note that peak blanching occurred in the first 8 hours, followed by a trend towards baseline as the effect of the dose diminished. At 31 hours a second peak in blanching activity was observed, followed by a trend towards baseline. This pattern was seen in most of the individual patient data as well as in the mean data and was therefore not thought to be an artefact. The skin may act as a reservoir for the steroid, such that when the molecule of steroid occupying a receptor site is metabolised, it is replaced from the reservoir by another steroid molecule, thereby reactivating the receptor site and prolonging the effect of a single application of topical formulation. Further work needs to be done in this area, but this could have an impact on the dosing schedules employed.

The standard deviation of the mean blanching indices is shown in table 3.52. There was a large degree of interpatient variation but this is an accepted phenomenon and is one of the reasons why there is such a large range of corticosteroid formulations on the market.

TABLE 3.52 STANDARD DEVIATION (n-1) OF THE MEAN BLANCHING INDICES

Time (hrs)	0	3.5	8	24	27	31	48
Product							
Synalar	11	8	12	12	17	16	14
Betnovate®	11	13	13	11	21	14	11
Commercial 1 in 4	13	15	15	12	14	12	9
Lipobase 1 in 4	14	9	8	7	17	10	12
U. Merck 1 in 4	16	13	11	6	12	12	11
Lipobase 1 in 10	13	16	13	12	13	16	14
U. Merck 1 in 10	16	15	11	13	16	14	9
Lipobase	12	9	5	11	12	13	12
U. Merck	8	12	9	10	7	11	9

The interpatient variation can be shown by looking at the data for some of the individual patients. The graph for volunteer number 4 (fig 3.25) to 24 hours shows how little or no blanching was achieved with either the 1 in 10 formulations or the bases alone. The two undiluted steroids, Synalar® and Betnovate® ointments, produced marked blanching with a peak at 8 hours, and the 1 in 4 formulations showed intermediate activity. The onset of action of the commercial preparation appeared to be slower than that of the other formulations in this volunteer. The data obtained from volunteer number 3 (fig 3.26) did not fit into the expected blanching pattern. There was no, or minimal, blanching detected. The skin colour either did not change from the time zero reading (for the undiluted steroids) or became more pigmented than the base-line reading. Two possible explanations are offered for these uncharacteristic results. Firstly, the control site was in position number 5, which is the site closest to the wrist area. The regions of the forearm

FIG 3.25 GRAPH OF THE BLANCHING INDEX AGAINST TIME: VOLUNTEER NUMBER 4.

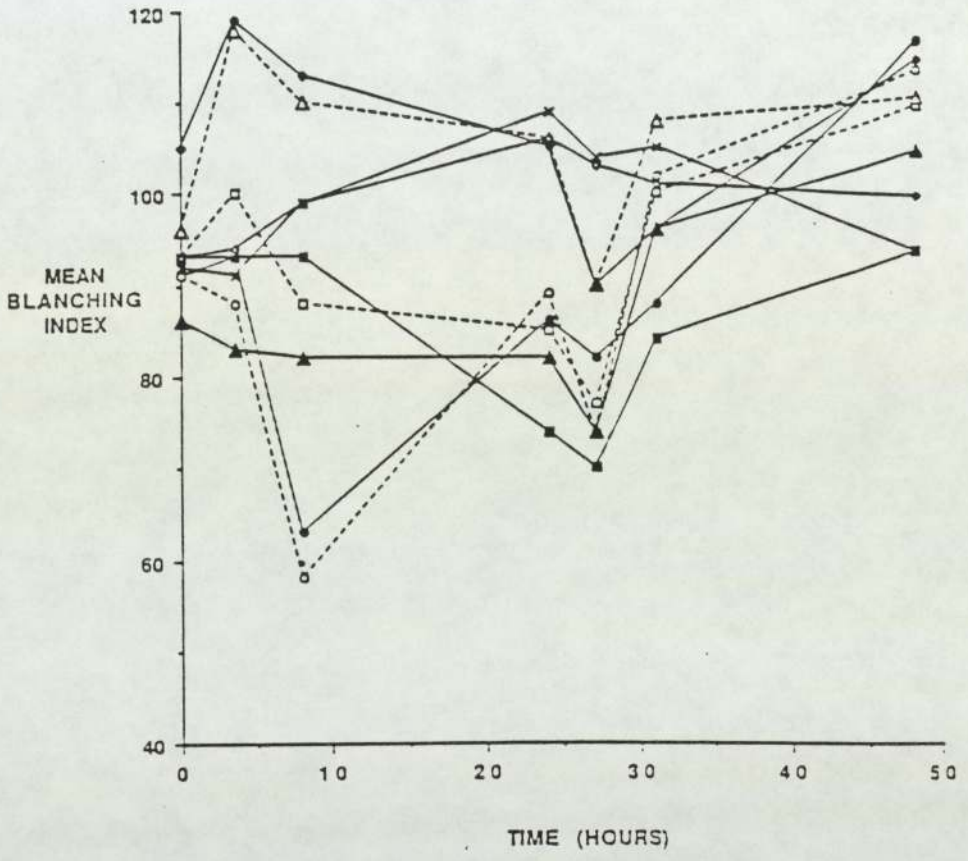
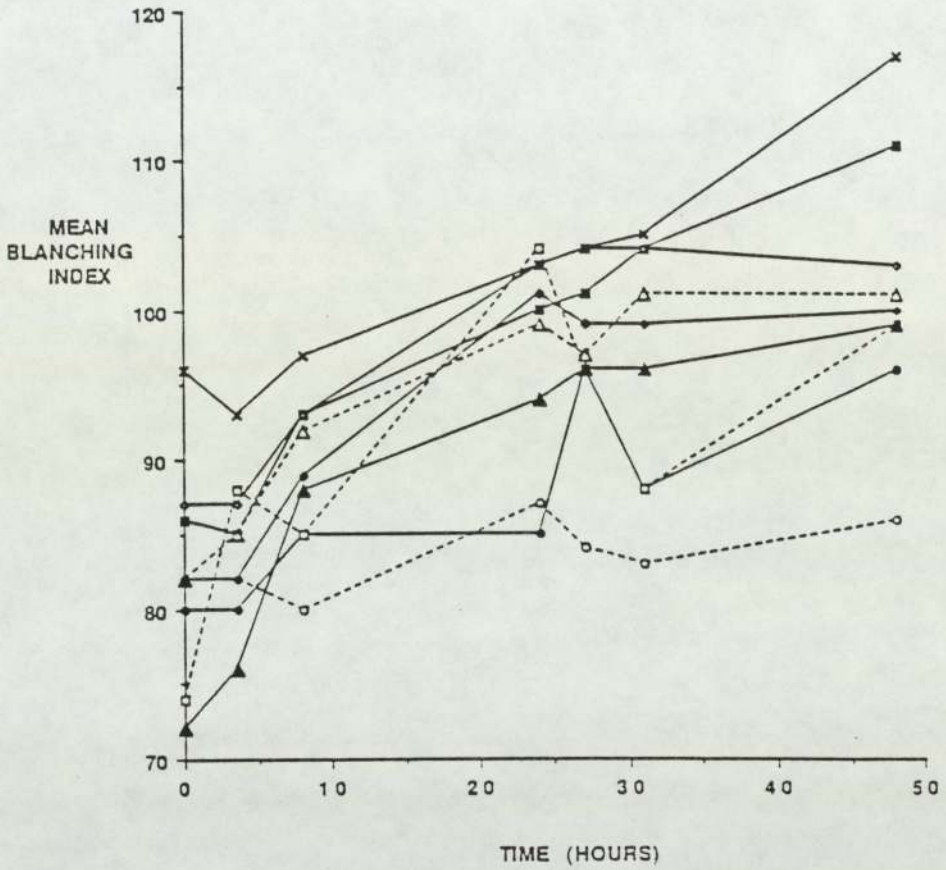


FIG 3.26 GRAPH OF THE BLANCHING INDEX AGAINST TIME : VOLUNTEER NUMBER 3.



immediately adjacent to the wrist and elbow are avoided because the skin in the flexion areas tends to react differently to the rest of the body surface. Although in this experiment every attempt was made to ensure that the template was attached to the centre of the forearm, it is possible that site 5 was positioned too close to the wrist affecting the control site readings. Since all the measurements are expressed as a ratio of the control site, any abnormal readings will distort the remaining results.

Secondly, the volunteer may have exhibited a hypersensitivity reaction to the topical formulations and the increase in skin pigmentation may have been an allergic response. However, the volunteer had no previous history of hypersensitivity to topical formulations and it would therefore seem unlikely that the volunteer was allergic to all of the formulations studied, including the bases.

I believe that it is more probable that the control site readings led to a distortion of the results and masked any blanching which may have occurred,

Ideally, the trial would have been extended to involve a larger population and to determine the importance of other factors such as application to the dominant or non-dominant arm. A similar trial also needs to be carried out for Metosyn® and its dilutions. Unfortunately, due to time constraints these investigations were not incorporated into this project.

4. CONCLUSIONS

A stability-indicating HPLC assay method was developed to investigate the degradation profile of fluocinolone acetonide and fluocinonide under alkaline conditions. Fluocinonide degraded to produce a major degradation peak which had a similar retention time to that of fluocinolone acetonide. This underwent further degradation, following first-order sequential reaction kinetics, to a series of secondary products which were not identified. Fluocinolone acetonide itself degraded by first-order kinetics to yield a series of products which had similar retention times and peak profiles to those shown by the secondary degradation products of fluocinonide. It was therefore concluded that fluocinonide degrades to fluocinolone acetonide, which then degrades in the same way as if the acetonide had been the original steroid.

The effect of the complexing agent EDTA on the stability of solutions of fluocinonide and fluocinolone acetonide was investigated. EDTA did not increase the stability of fluocinolone acetonide in alkaline solutions. The addition of EDTA to alkaline solutions of fluocinonide markedly reduced the rate of degradation of fluocinonide to fluocinolone acetonide, and also appeared to slow down or prevent the degradation of the acetonide to further degradation products. The EDTA appears to increase the stability of fluocinonide and fluocinolone acetonide, but to the latter only when it is a degradation product and not when it is the parent steroid.

The addition of the antioxidant sodium sulphite was also investigated. The rate of degradation of both steroids was increased.

The stability-indicating assay was used to investigate the shelf-life of diluted topical steroid preparations. Synalar® ointment diluted 1 in 4 in Lipobase® and Unguentum Merck® showed no loss of steroid content on storage for 4 months at 25°C. The preparations were also shown to be stable at 4°C and 32°C for at least 8 months. The same steroid was also diluted 1 in 10 in the same bases and these preparations were found to be markedly less stable than

the 1 in 4 formulations, with shelf-lives of between 1 and 2 months at 25°C. Reducing the storage temperature to 4°C increased the stability of the Lipobase® formulation only.

Metosyn® cream was diluted in Metosyn Diluent® in addition to the above bases. The 1 in 4 formulations were all stable for 9 months at 25°C and the 1 in 10 dilutions were stable for a minimum of two months. The preparations were also shown to be stable at 4°C and 32°C for at least 7 months at both levels of dilution.

It was concluded that Synalar® ointment diluted 1 in 4 in the bases studied could be given a shelf-life in excess of that currently recommended by the External Diluent Directory of two weeks, which is an arbitrary figure given to all extemporaneous formulations. The stability work carried out in this project indicates that an expiry date of four months after dilution would be appropriate. The shelf-life of the 1 in 10 formulations could be extended to one month. It was also concluded that the shelf-life of the various Metosyn® dilutions is grossly inappropriate and this could be extended to 7 months after the date of dilution for the 1 in 4 formulations, and 2 months for the 1 in 10 dilutions. However, the effect of microbial contamination was not investigated.

The clinical efficacy of Synalar® ointment and its dilutions, commercial and extemporaneous, was assessed using a vasoconstrictor assay. The blanching potency, which correlates with the clinical potency, was not significantly different for the 1 in 4 dilutions compared to the undiluted steroid, $p<0.05$. The potency of the 1 in 10 formulations was not significantly different to that of the bases alone, $p<0.05$. There was some overlap between the 1 in 4 dilutions and the 1 in 10 in Lipobase®. It was concluded that since the 1 in 4 formulations are equipotent with Synalar® ointment there is little place for this dilution in clinical practice, and a steroid from a lower potency group should be employed. Furthermore, since the 1 in 10 dilutions had no detectable clinical benefit over the bases alone, there seems to be little place for the 1 in 10 formulations. There is no linear relationship between dilution

and potency, and this can lead to incorrect assumptions or estimates about clinical potency. The practice of diluting topical corticosteroids should be discouraged.

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