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A STABILITY STUDY OF DITHRANOL IN SOLUTION, FORMULATIONS AND IN NORMAL AND PSORIATIC SKIN

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ABSTRACT

For more than 100 years dithranol has been successfully used for the treatment of psoriasis . It is still not fully understood, however, how it exerts its anti-psoriatic effect. From the information available to date it is clear that the actual process of decomposition is central to the therapeutic action of dithranol.Since there is an obvious parallel between the mechanism of decomposition in solution and that of decomposition/metabolism in skin stability studies were carried out in aqueous buffer (pH 5.5). The effect of various factors such as the presence of various metal ions and surfactants on the decomposition pattern (individual rates of dimer and danthron formation) of dithranol in solution were quantified. The effect of, in particular , surfactants on the skin permeation and decomposition/metabolism of dithranol were then investigated.

Because of dithranol's poor water solubility it was necessary to develop an analytical technique capable of the successful quantification of low levels of dithranol and breakdown products in aqueous systems. A previously reported hplc system, which has adequate sensitivity for dithranol and danthron quantification, was used. Improved sensitivity for dimer (3 fold increase), thus allowing the accurate quantification of low levels of dimer in aqueous solutions, was achieved by a modification of the mobile phase. As it was also necessary to establish the amounts of dithranol and breakdown products on and in skin an appropriate extraction method was needed. A procedure whereby skin was first extracted using a mixture of trichloro acetic acid and methanol followed by hplc analysis of the extract was developed.

Surfactant solubilisation of dithranol was used to enhance the water solubility of dithranol. Data from the solubilisation studies shows that in sodium lauryl sulphate and tween 80 (above c.m.c in aqueous buffer pH 5.5) the amount of dithranol in solution is directly proportional to the % of surfactant present. In cetrimide, solubilisation was observed only at low pH e.g pH 0.4. At pH 5.5, because of the interaction that takes place between dithranol and cetrimide causing dithranol to ionise, dithranol ionisation is responsible for enhanced solubility. About 3 fold more dithranol goes into such cetrimide solutions compared to equivalent concentrations of sodium lauryl sulphate and tween 80.

Surfactant presence, however, had implications on dithranol's decomposition pattern. In the sodium lauryl sulphate and tween 80 solutions little change from that seen in buffer pH 5.5 was observed namely ~94% dimer and ~4% danthron were produced following complete decomposition. In the presence of cetrimide (pH 5.5) a marked change was seen with ~84% danthron and ~14% dimer being formed on complete decomposition.

The effect of the inclusion of metal ions i.e Cu^{2+}, Zn^{2+} and Fe^{2+} on the kinetics of the decomposition of dithranol to dimer and danthron were quantified. All had a catalytic effect on the rate of dimer formation , while suppressing

that of danthron.The catalytic coefficients were in the order of Cu^{2+} > Fe^{2+} > Zn^{2+}.

Concentration vs time data generated on placing surfactant solutions of dithranol in contact with animal skin (in vitro) and human skin (in vivo) allowed estimates of the amount of dithranol and breakdown products penetrating into the skin, along with the degree of skin surface decomposition taking place during the permeation process.A pronounced deviation was observed for dithranol decomposition in the formulations on the skin and when not in contact with skin. Skin surface decomposition was found to result in the formation of an , as yet unknown , breakdown product (P_4).

Using the 12-o-tetradecanoylphorbol-13-acetate/hairless mouse psoriasis model it was visually established that the cetrimide-dithranol formulation negated the anti inflammatory effects of dithranol .The tween 80 and sodium lauryl sulphate dithranol formulations reduced the inflammatory response in the psoriasis model to the same degree as an equivalent amount of dithranol delivered in acetone.

A preliminary clinical investigation on the influence of cetrimide on the therapeutic outcome of conventional dithranol therapy was carried out using two patients with psoriasis.Cleansing the skin with a solution of cetrimide before and after treatment with dithranol resulted in both a reduction of side effects and a loss in the therapeutic effectiveness of dithranol.

The data gathered allowed the discussion of the effect of dithranol's decomposition pathway on its therapeutic outcome.

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To Bambala , Somboli , Chipasha and Kangwa

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Introduction.

Psoriasis is a disease whereby the skin exhibits a complex irregularity [Steigleder, (1981)] in the mechanisms which control the epidermal cell division. This study will therefore begin with a review of the structure of the human skin.

The human skin

The skin is a complex, dynamic organ of many cell types and specialized structures serving multiple functions crucial to health and survival. It is one of the largest and most versatile of organs. The average adult has a skin surface area 1.75 m^2 (about 3000 in^2) [McClintic, 1975, Barry , 1983] and comprises 7 - 12% of the body weight [Wood & Bladon, 1985]. The skin provides a number of unique functions :

(1) It protects deeper cells from environmental factors such as chemical and mechanical injury , desiccation , invasion by micro-organisms and the damaging

effects of ultraviolet light.

(2) It regulates and helps maintain body temperature.

(3) It serves as a neuroreceptor organ in monitoring diverse environmental stimuli.

(4) It processes antigenic substances presented to it.

(5) Hairs (specialized keratinized structures) , over the scalp , body , as eyelashes and in the nose provide aesthetic adornment and protect eyes from small particles and filter air breathed into the nose.

The human skin consists of three anatomical layers: (i) the epidermis , which is a thin , dry and tough outer layer, (ii) the dermis , which is essentially the support system containing blood vessels , nerves , hair follicles , sebum and sweat glands , (iii) the subcutaneous fat layer which acts both as an insulator, a shock absorber and reserve depot for calories [Wilson & Washington ,1989, p 109]. Since in psoriasis the epidermis and dermal regions of the skin are involved [van Scott & Flaxman, 1973], this brief review of the structure of the skin , will therefore concentrate on these regions [see Fig 1.1].

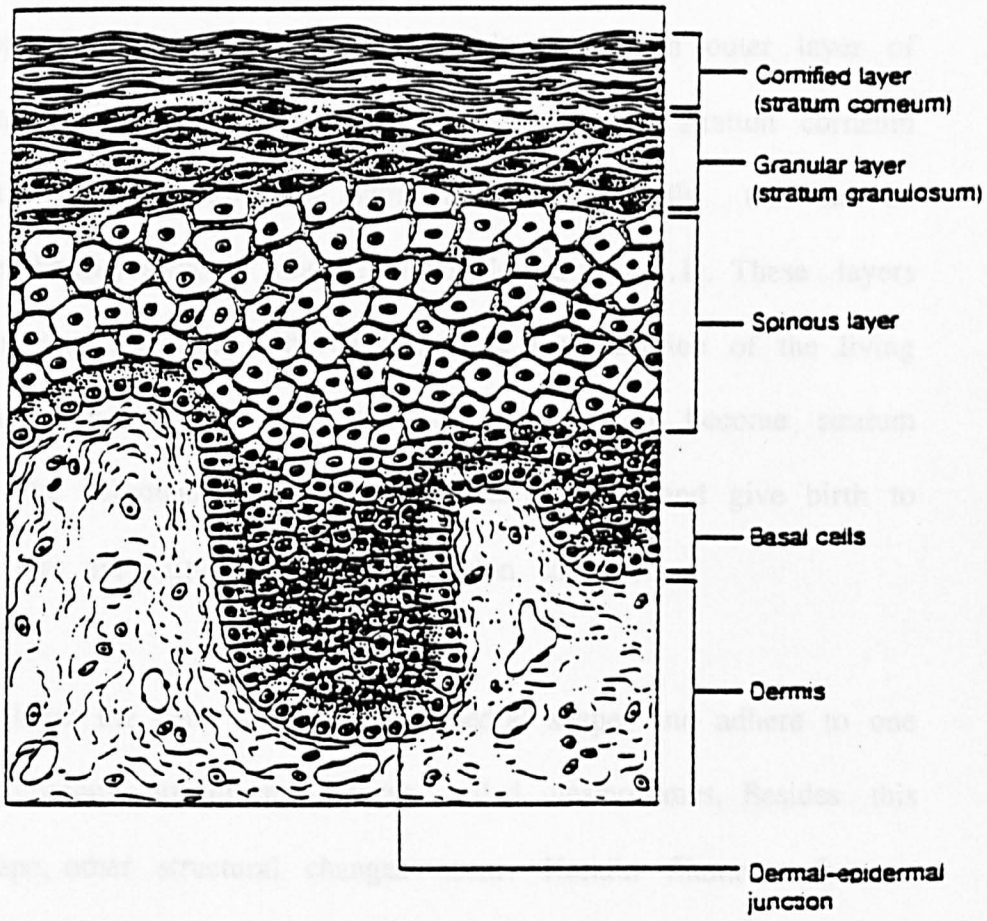


Fig 1.1

Schematic drawing of the epidermis. Three major layers of living cells are distinguished: The columar cells comprising the basal layer, nearest the dermis; the spinous layer, composed of polyhedral cells with many desmosomal attachments on the cell surfaces; and the granular layer, interposed between the spinous layer and the stratum corneum, where cells take on a flattened configuration and contain dense keratohyline granules.

1.1.1 The epidermis

The epidermis is a multilayer consisting of two main parts: an inner region of viable , moist cells termed the Malpighian layer and an outer layer of anucleate, flattened, nonviable , desiccated cells known as the stratum corneum [Orkin et al ,1991]. There are three substrata of living cells that can be recognized: the basal , spinous , and granular layers (Fig 1.1). These layers represent progressive stages of differentiation and keratinization of the living keratinocyte as they move toward the skin surface to become stratum corneum. Thus the columnar basal cells continually divide and give birth to daughter cells that are displaced toward the skin surface.

In the spinous layer the cells take on a polyhedral shape and adhere to one another by numerous attachment devices called desmosomes. Besides this change in shape, other structural changes occur. Keratin filaments form a network within the cytoplasm, and refractile keratohyline granules and submicroscopic lamellar granules appear. The cytoplasmic keratin filaments extend across cells and loop about the perinuclear space of the cells in the spinous layer.

The cells of the granular layer have a flat shape, and the keratohyalin in these cells accrues incrementally among the keratin filaments to form larger and larger masses enclosing the filaments. Histadine-rich basic protein , termed filagrin , has been demonstrated to facilitate the assembly of keratin filaments

into macrofibrils. The keratohyline granules in this layer account for the distinctive granules of the granular layer.

The final stage of keratinization - the abrupt change from the granular layer to the stratum corneum - is attended by a variety of dramatic morphological and biochemical degenerative alterations including the degradation of cellular organelles and nuclei as well as the by the appearance of a thickened cellular envelope of stratum corneum cells. The cornified layer consists of up to 25 layers of anucleate dead cells held tightly together by means of desmosomes.

1.1.2 The dermis

The dermis lies between the epidermis and the subcutaneous adipose tissue. In humans the whole mass of the dermis may constitute 15 - 20% of the total body weight [Orkin et al ,1991].

Grossly , the dermis is a tough , resilient tissue with viscoelastic properties. A 3-dimensional matrix of loose connective tissue is composed of fibrous proteins (collagen and elastin) embedded in an amorphous gel of ground substance (glycosaminoglycans) [Orkin et al , 1991]. The fibrous matrix serves as a scaffolding within which networks of blood vessels, nerves, and lymphatics intertwine. The dermis also contains epidermal appendages such as sweat glands and pilosebaceous units and provides support for these structures.

The dermis can be divided into an upper papillary layer, characterized by interlacing fine collagen fibres and ample interfibrillar spaces, and the deeper reticular dermis recognized by the thicker , aggregated bundles of collagen with lesser amounts of interfibrous space. Elastic fibres are intertwined within the collagenous network [Orkin et al , 1991].

1.2 Psoriasis

1.2.1 Prevalence and clinical features.

Psoriasis is a common scaly erythematous disease of unknown cause showing wide variation in severity and distribution of skin lesions. It usually follows an irregular chronic course marked by remissions and exacerbations of unpredictable onset and duration [Orkin et al , 1989]. Factors that may lead to more lesions include drug reactions, respiratory infections , cold weather, emotional stress, surgery , and viral infections.

Estimates of the prevalence of psoriasis in the general population of the world range from 0.1 to 2.8% [Farber & Nall , 1984].The frequency varies in different geographic regions: 0.5 to 1.5% in the United States [Kraning and Odland , 1979]; 2.8% in Scandinavia [Lomholt, 1963]; 0.4% in the Henan District of the People's Republic of China (Henan Dermatoses Survey Group, 1982); while in a study in the South American Andes no cases were found (Convit, 1963). Psoriasis occurs in males and females with nearly equal frequency. The

average age at onset is in the 20s, ranging from birth to 80 or 90 years. Lesions are commonly observed on the scalp, elbows, trunk, and the lower extremities , but can occur on all body parts including fingernails and toenails. A temporary or permanent remission may occur in the course of the disease. The reason for this is not known.

Psoriatic skin is characterised by the appearance of sharply defined erythematous patches covered with a distinctive crumbling silvery grey scale [Barry , 1983]. When the scales are removed, punctate bleeding points become visible. It's clinical appearance can vary widely and may be classified as:

Psoriasis vulgaris
Guttate psoriasis
Pustular psoriasis
Psoriatic erythroderma
Psoriasis and arthritis

Two categories of psoriatic skin are defined: (i) visible psoriasis , (2) non-visible psoriasis. The latter includes residual psoriasis (clinically non-apparent , but histologically recognizable) , pre-psoriasis (epidermal thickening , increased DNA synthesis , histochemical parakeratosis, infiltration of macrophages, lymphocytes in the dermis, capillary dilatation), and latent psoriasis (absence of any measurable but susceptibility to visible psoriasis after injury - the so called " Koebner reaction")

1.2.2 Skin changes in psoriasis

a) Defects in the epidermis

Psoriatic skin exhibits a complex irregularity [Steigleder, 1981] in the mechanisms that control the epidermal cell division. The characteristic scales which are different to normal stratum corneum and overlie the epidermis are the result of altered keratinization. The abnormality of the keratins causes a decrease in the water holding capacity of the epidermis, thus total epidermal water loss in psoriatic skin is higher than that in normal skin [Serup & Blichmann, 1987 , Marks et al , 1979].

Table 1.1 **Histopathological changes in untreated psoriatic plaques [from Wilborn & Montes, 1974 and Steigleder, 1981]**

Targets	normal	psoriasis
parakertosis	non	appear
cell size	normal	larger
skin thickness	normal	thicker
tonofilaments	numerous	reduced
intercellular space	narrow	large
mitochondria	few, small	increased, enlarged
keratohyalin	numerous	decreased

Histological sections through psoriatic lesions (Table 1.1) show that the granular layer is reduced or absent and there is parakertosis with an increase in the mitotic activity in the basal layer cells [van Scott & Ekel, 1963, van Scott & Flaxman, 1973, Wilborn & Montes,1974, Wood & Bladon, 1985]. The size of psoriatic epidermal cells are larger than normal ones. Marhle (1981) showed that profound alterations of cell membranes and increases in the cell surface area of psoriatic keratinocytes accelerate cell mitosis. The increase in the skin thickness or epidermal hyperplasia is shown to be the result of an increase in cell size [Flaxman & Chopra, 1972]. The type and number of tonofilaments

of psoriatic skin are different from the normal ones and the intercellular spaces in the psoriatic lesions are dilated [Steigleder, 1981]. Table 1.1 also shows that the number and size of mitochondria in psoriatic plaques are different from normal skin.

Examples of other biochemical and immunological deviations from normal skin have been implicated. Hammar (1970) reported a significant rise in the activity of glucose-6-phosphate dehydrogenase in the uninvolved skin and within the psoriatic lesion. An alteration in arachidonic acid metabolism in psoriasis has been reported [Muller, 1988, Kragballe & Voorhees, 1987]. Defects in sterol metabolism, fatty acid metabolism and the esterification process have been demonstrated [Yardley, 1969]. Esterified cholesterol is a vital component in the ordered structure of the keratin fibrils. An incomplete catabolism of phospholipids during keratinisation and a deficiency in a phosphorylation mechanism in psoriasis were shown [Yardley & Godfrey, 1964]. The levels of cAMP [Prunieras ,1979 , Plummer, 1979, Zarchariae, 1979] and folic acid derivatives [Mier & Cotton, 1976] which deviated from normal could indicate the non-clinical presence of psoriasis. The immunological factors IgA and complement are found deposited in the lesion after hyperproliferation [Steigleder, 1981] whilst T-lymphocytes are lower than normal [Zachariae,1979]. Polymorphonuclear leukocytes show an enhanced chemotactic response to various chemo-attractants. This enhancement was shown to be reduced to normal by dithranol [Michaelson, 1980 and Schroder et al , 1985].

b) Defects in the dermis

Histological sections show that there is papillary oedema, dilatation and tortuosity of the papillary capillaries [Ross et al , 1964]. This explains why removal of the scales damages not only the epidermis but also the underlying capillaries, resulting in a characteristic punctate bleeding point [Wood & Bladon, 1985, Barry , 1983]. Inherent vasodilatation and a characteristic psoriatic capillary network have been observed [Ryan, 1979] whilst Kemeny (1989) has shown that the capillary resistance of psoriatic patients is significantly lower than that of healthy individuals.

1.2.3 Animal models

The skin condition is an essential regulating parameter of percutaneous absorption. In most skin diseases an increase in skin permeability is observed which is due to a defective barrier. In dermatological therapy an important question that arises is that of the difference of drug penetration into diseased skin relative to intact skin. To be able to address such a question it is important to establish animal models to mimic diseased skin.

Using normal skin , one of the simplest methods of mimicking the defective permeation barrier of psoriatic skin is by the removal of the stratum corneum by tape stripping (Shroot & Scheafer ,1987). The stratum corneum of normal skin can also be selectively damaged using UV irradiation [Lamaud

& Schalla, 1984]. The use of skin where the stratum corneum is damaged or removed is limited to experiments designed to give information on the permeation characteristics of a particular drug in diseased skin. In order to assess the potential of any particular agent to halt the progress of a given disease of the skin , it is desirable to first produce the disease state in the skin. For psoriasis no perfect model is available to date. An acceptable model is obtained by the treatment of mouse skin with tetradecanoyl phorbol myristate acetate (TPA) [Gupta et al ,1989, Gschwendt et al , 1987]. A number of biological effects are induced including epidermal hyperplasia , activation of protein kinase C , increased release of arachidonic acid and prostaglandins (involved in the inflammatory process) and also induction of ornithine decarboxylase (a participant in regulation of cell growth). The result of the changes induced mimics, to a certain degree , a psoriatic lesion.

1.2.4 Treatment

There is no cure for psoriasis. Several treatments are available , varying from topical to systemic modalities administered singly or in combination, and are all effective to varying degrees in controlling the disease. Remission may occur in the course of the disease.

Topical Therapy - Dithranol, crude coal tar , and corticosteroids are the most frequently used agents for the topical treatment of psoriasis. A detailed discussion of dithranol will follow later in the chapter.

Tars

Crude coal tar is a complex mixture of thousands of hydrocarbon compounds. It affects psoriasis by enzyme inhibition and antimitotic action. Few attempts have been made to assess the pharmacological activity of individual components of purified tar products or of different coal tars themselves . Preparation of 2 to 5% crude coal tar in various bases is effective alone and in combination with other agents such as salicylic acid [Comaish, 1981; Tanenbaum et al 1975, Young , 1970]. One disadvantage with these formulations is the variation in the activity of these products mainly because of the non-standard composition of crude coal tar. Other disadvantages are that the preparations are inconvenient , inconsistent and also usually unacceptable to use. Commercially , coal tar products are available as solutions for baths, paints, ointments , sticks and shampoos.

Corticosteroids

They are available as odourless , non-staining creams, gels and lotions , and patients are willing to use them due their cosmetic appeal. Patients with plaques limited to a few areas respond well to therapy aimed at removing scales and lubricating lesions. Occlusive pliable plastic dressings over topical corticosteroids increase their effectiveness [Watson and Faber, 1977]. Prolonged use of topical corticosteroids , especially fluorinated agents or applications under occlusion, may lead to atrophy of the skin, telangiectasia , straie and

overgrowth of bacterial and fungal infections of the skin. Rapid recurrence of psoriasis (rebound phenomenon) may also occur when topical corticosteroids are used for long periods and then discontinued. Systemic absorption of corticosteroids may be sufficient to cause adrenal gland suppression and Cushing's syndrome , especially with newer "superpotent" topical steroids (eg clobetasol propionate [Temovate]).

Photochemotherapy

Natural sunlight is effective adjunctive therapy for psoriasis , as most patients find who travel to warm, sunny climates. Artificially produced ultraviolet light is an effective method of treating some patients with psoriasis [Farber & Nall, 1984]. High intensity UVA at 360nm is capable of penetrating to the basal cell layer. Patients are given 8-methoxypsoralens systemically and 2 hours later exposed to a controlled dose of UVA. The drug reacts in the skin with UVA to produce a thymidine antagonist.Increasing amounts of ultra-violet are administered each week. Treatment is only available in specialist centres; it has to be carefully regulated, owing to the short-term hazard of severe burning and the long-term hazards of cataract formation [Goldberg et al , 1979], accelerated ageing , and the development of skin cancer [Stern et al, 1979].

Antimitotic drugs

Because epidermal hyperplasia may play a part in the pathogenesis of psoriasis , antimitotic drugs are a logical choice of treatment .Examples include methotrexate and hydroxyurea. These drugs are reserved for use in severe cases or those failing to respond to other treatment [Farber & Nall, 1984]. Methotrexate is more effective, and thus better known, than hydroxyurea [Baker, 1975]. It is used orally or by intramuscular injection with close monitoring of liver, kidney and bone marrow functions.

Occlusive dressing therapy

Previous studies have suggested a potential therapeutic role for occlusion on psoriasis. In 1970 , Fry and colleagues reported the clinical improvements of a significant decrease in mitotic counts, and the reformation of the granular cell layer in psoriatic lesions by covering them with plastic dressings only.

Adverse reactions to occlusion on skin such as potential increase in bacterial infections or discomfort after prolonged applications are likely [Sulzberger & Witten , 1961].

Systemic therapy

Immunosuppressive drugs

Cyclosporin A is an example. It is given orally or by intramuscular injection.

It is found to be effective in severe psoriasis [Griffiths et al , 1989].

Retinoids

Vitamin A has long been recognised as being vital to growth and skin tissue, and synthetic retinoids are demonstrated to be effective in psoriasis [Orfanos & Runne , 1976]. However , the major side effects including skeletal damage and diffuse hair loss, and high severe remission not to mention teratogenicity have limited the use of retinoids in psoriasis.

1.3 Skin permeation studies

In the development of a topical product the kinetics of liberation and subsequent penetration into the skin is vital information. There are two general approaches followed to obtain such information. One approach uses animal or human skin preparations in diffusion cells (in vitro methods). Another approach uses in vivo methods in a variety of animal species , including humans.

1.3.1 In vitro methods

There are various types of diffusion cells used, their design being dependent on the desired objective [Scott,R., 1986]. Franz cells are the most frequently used because of the following advantages: The cells are nonreactive and

nonadditive as they are constructed of glass. The set up is made perfectly water-tight by clamping the two halves together and can be used with whole skin , separated epidermis or even nails. The cells can be modified for a static study or for a dynamic one which better mimics in vivo conditions [Anjo et al , 1980], and is better adapted to non-water soluble substances.

As mentioned above diffusion cells can be used to measure the permeation of a substance through different barriers , human or animal. Whole skin is easy to handle but has a major disadvantage in that most compounds tend to accumulate in the dermis. Animal skin e.g. rat skin has the advantage of being easily standardized (same weight , sex , species) which is important for studies comparing several vehicles for a given drug. To obviate the problem of accumulation in the dermis isolated human epidermis and stratum corneum can be used in these cells. A problem usually encountered with human skin is the difficulty in obtaining hairless mammalian skin , since mammalian skin seems most appropriate. Also the results obtained on separated epidermis are dependent on the thickness of the stratum corneum , and thus the origin of the skin [Schaefer et al ,1982]. When skin from different origins is used a standard is included so that a correction coefficient can be applied. To avoid using separated epidermis with holes in it , tritiated water is allowed to penetrate through it before applying the substance. If the diffusion of the water into the receptor fluid is in the normal range, the epidermis is ready to be used for a penetration study.

1.3.2 In vivo methods

In in vivo studies of permeation the main problems encountered are in quantifying the amount of formulation applied e.g cream , ointment , paste , etc and then sampling the applied formulation to quantify the release of drug over a time period. Such information is essential if release is to be linked to "activity".

One method involves the application of a radioactively labelled (^{14}C and ^{3}H) dithranol formulation , e.g. a cream or ointment , in vivo to human or an animal model then biopsing the treated area. Taking several horizontal sections of this biopsy and analysing for radioactivity allows one to follow the kinetics of penetration of the substance into different cellular layers [Schaefer et al , 1978]. In another approach , once the agent under investigation is applied to the skin, percutaneous absorption is determined by direct analysis of the urine [Wurster & Kramer, 1961]. A major drawback of the above radioactive methods is that information on the decomposition of the parent drug both on and in the skin is not readily attenable.

1.4 Dithranol

1.4.1 History

The natural source of dithranol is araroba or Goa powder. Araroba is extracted

from cavities in the trunk of Andira araroba . Goa powder was first analyzed in London in 1875 when it was found that 85% of the powder was the benzene soluble chrysophanic acid which could be used as a purgative. In 1878 Squire published a book "On the treatment of psoriasis by an ointment of chrysophanic acid" [quoted by Fisher & Maibach, 1975 , Faber ,1983]. The first synthetic antipsoriatic compound (dithranol or anthralin) was produced in 1916 and was named Cignolin. This was after it was shown that the active antipsoriatic component in Goa powder was not chrysophanic acid but a reduced product of it known as chrysarobin (see Fig 1.2).

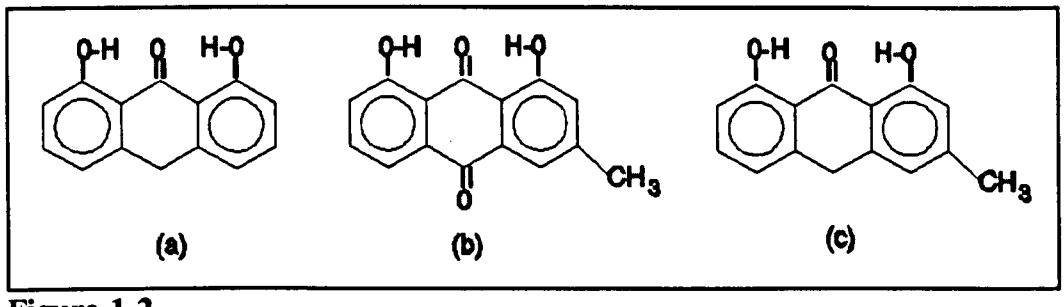


Figure 1.2
The molecular structures of a) dithranol, b)chrysophanic acid and c) chrysarobine

1.4.2 Stability

An appreciation of the chemistry of dithranol is needed in order to understand both the risks and benefits of this drug. The keynote of this chemistry is instability with two focal points: phenolic hydroxyl groups at C1 and C8 , and two reactive hydrogen atoms on C10. The hydrogen bonding

between the phenolic hydrogen atoms and the central carbonyl function account for the relative stability of dithranol in aprotic media such as vaseline ,but in the presence of protic solvents such as water this bonding is disturbed, the keto-enol tautomerism is altered and auto-oxidation centering on C10 can occur (see Fig 1.3).

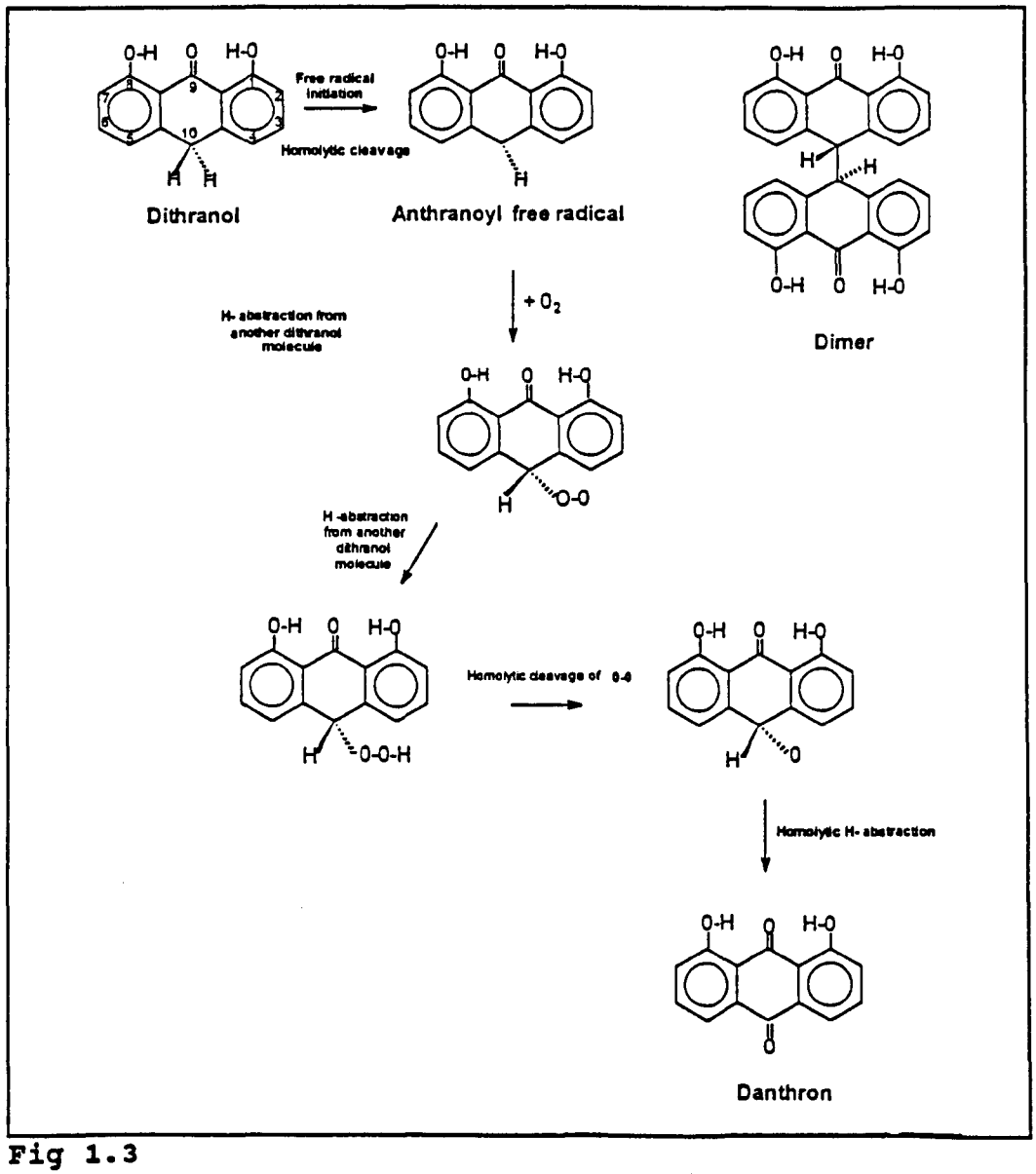


Fig 1.3
The proposed pathway for the free radical degradation of dithranol [from Holder & Upadrashta (1992)].

In the skin [Shroot & Brown, 1986, Cavey et al , 1985] , cell culture [Reichert et al , 1985] , and isolated mitochondria [Fusch et al 1990, Salet et al , 1991] dithranol displays distinct chemical behaviour involving hydrogen atom abstraction and electron transfer to yield the corresponding anthranoyl radical (Fig 1.3). The release of a hydrogen atom from the methylene group at C10 initiates the oxidative decomposition of dithranol and the formation of free radicals [Krebs et al , 1981, Colin et al , 1981] (Fig 1.3). The free radicals produced are biologically active and able to produce further active radicals in a chain reaction [Martinmaa et al , 1981, Whitefield ,1981a, Fusch et al , 1990]. Although these radicals may be responsible for skin irritation and produce oxidation products that cause staining, they are presumably central to the clinical action of the drug [Martinmaa et al ,1981, Misch, et al , 1981] because of the difficulty of separating activity from side effects. Prevention of free radical formation by i) substitution of both hydrogen atoms at C10 , and ii) omission of the methylenic group or replacement by oxygen , produced inactive antipsoriatic compounds: a mono 10-acyl derivative of dithranol is active, but less so than dithranol itself [Schaltegger et al , 1987].

The stability of dithranol will vary according to changes in the environment. Both rate and pattern of decomposition are affected. A number of environmental factors influencing stability are reviewed.

The effect of solvent and pH - Although dithranol is fairly stable in non-polar solvents such as hexane , it is slowly oxidised in relatively more polar ones

such as acetone and methanol [Priprem ,1991] , and also in neutral aqueous solutions where the oxidation rate increases with increasing pH [Upadrastha & Wurster (a), 1988]. The pKa of dithranol in aqueous conditions at 25°C was determined to be 9.06 [Upadrashta & Wurster (a),(1988)]. The autooxidation of the monobasic species is much more rapid than the neutral one and is rate determining in mixtures of both species at equilibrium [Melo et al ,1983]. In basic aqueous solutions dithranol is ionised and oxidation is very fast resulting in the formation of danthron and smaller amounts of dimer [Melo et al, 1983]. In an acidic aqueous solution (pH 3.3) dithranol is non-ionised and was shown to be relatively stable over a 10 hour period [Wang et al , 1987] , the decomposition products formed under these conditions were not mentioned.

Catalysis of dithranol decomposition - Using stable dithranol pastes , Hulsebosch & Ponec-Waelsch (1972) showed that when dithranol was brought into contact with rough solid surfaces such as skin , lint and glass wool, decomposition was catalysed especially in the presence of moisture. Though the catalysis of dithranol decomposition by metal ions such as Zn^{2+} and Fe^{2+} has been reported [Raab & Gmeiner, 1975, Hulsebosch & Ponec-Waelsch, 1972, Priprem ,1991], none of the reports provide information on the effects of the catalytic agents on the individual rates of formation of danthron and dimer. Because of the poor water solubility of dithranol this kind of information for dithranol in aqueous conditions is notably absent in the literature.

The influence of light and temperature - Both of these factors will enhance the decomposition process of dithranol. Raab & Gmeiner (1975) showed that when dithranol was kept in solution (dimethylformamide) the typical absorption spectrum was lost over a period of time. The higher the temperature (80°C as opposed to 30°C) the faster the loss occurred. It was also shown that exposure to ultraviolet light provoked decomposition which occurred at a faster rate than that due to exposure to a temperature of 80°C. In contrast to autooxidation in the dark both the neutral and ionised species of dithranol are readily oxidised when exposed to UV light. The same considerations apply to neutral and basic aqueous solutions although in basic solution photooxidation leads to the formation of the monobasic form of danthron [Melo et al ,1983]. In the studies mentioned above information on the kinetics of decomposition product formation is lacking principally due to the non specific nature of the spectrophotometric methods employed for solution analysis.

1.4.3 Analytical methods

The lack of sensitive and specific analytical procedures for the investigation of dithranol has delayed the answer to such therapeutic parameters of dithranol as effective dose and site of action [Mustakallio , 1981]. Dithranol in the presence of its decomposition products (mainly danthron and dimer) cannot be determined by direct UV spectrophotometry because of overlap in the UV spectra [Caron & Shroot ,1981]. Hplc has improved the situation

because this method in capable of separating and quantitating the amount of dithranol and its degradation products. The use of both normal and reversed phase Hplc has been reported [Cavey et al , 1982 , Burton & Gadde , 1985 , Lee , 1987 ,, Wurster & Upadrashta, 1986, Kneczeke et al , 1989]. Currently the official method used is a normal phase system both in the B.P. (1993) and the U.S.P (XX1). It has been reported however [Burton & Gadde , 1985 , Wurster and Upadrashta , 1986] that separation between dithranol and danthron is better in a reverse phase system.

1.4.4 Skin penetration studies of dithranol

These can be divided into three groups; i) studies aimed at determining the flux properties of the dithranol molecule., ii) the influence of various vehicles on the flux properties of dithranol. , iii) events occurring on and in the skin following application of dithranol to the skin.

Dithranol flux properties - Using both radiolabelling and fluorescence as the detection methods, it has been shown that when dithranol is applied to normal skin in a vaseline base, a high concentration is found in the epidermis , this indicating that the molecule has a rather high flux [Shroot et al ,1981]. When the barrier to permeation into the skin (horny layer) is compromised , dithranol penetration is facilitated. This effect was demonstrated by Schaefer (1980) , who showed that the penetration of dithranol into the epidermis was increased when the horny layer was removed by tape stripping. It was also

observed that penetration of dithranol through the defective skin barrier of the psoriatic lesion is faster than in normal skin [Schalla et al , 1981, Timmerman et al , 1990]. It is on the basis of such observations that the short contact therapy approach using dithranol preparations is advised.

A study using radioactive dithranol [Selim et al , 1981] showed that the penetration in vitro occurs shortly after application and the distribution rapidly became stabilised so that there was little difference between 1 hour and 24 hours of application. The structure of the dithranol , particularly its hydrophobicity [Whitefield , 1981a] contributes greatly to its penetration properties.

Vehicle type - Using vehicles varying in hydrophobicity it was shown that the greater the hydrophobic nature of the vehicle , the greater was the penetration of dithranol into the epidermis (human skin). Penetration into the epidermis from the various bases was in the following order, petrolatum > water in oil cream > oil in water cream > single phase water miscible cream [Kammerau et al ,1975].

Skin surface decomposition - Given the instability of the dithranol molecule , a full account of the skin permeation process is obtained more accurately , if events occurring on the surface as well as in the skin , following the application of dithranol are closely monitored. In the majority of dithranol penetration studies [Kammerau et al , 1975, Schaefer et al , 1981 , Cavey et al

1985] the emphasis is on the events occurring in the skin post application of dithranol dose. Some reports acknowledge the possibility of surface decomposition and the formation of decomposition products other than danthron and dimer, but no skin surface kinetic data is reported [Schalla et al, 1980, Wang et al ,1987]. It would appear that the main reason for the lack of this type of information is the complex nature of the dithranol formulations used in such experiments, leading to sampling difficulties. In the present study, using Hplc and liquid formulations of dithranol the concentration time profiles of dithranol and decomposition products , on the skin surface , during the permeation process was readily monitored.

1.4.5 Mode of action

In a recent review of the mode of action of dithranol [Shroot ,1992] it is suggested that dithranol's effect is not as a result of a direct action on DNA but more likely to be a consequence of a potent effect on cellular respiration and subsequently, energy production. There is strong evidence to suggest that the target organelle for dithranol is the mitochondria [Morliere et al , 1985] and further that the interaction occurs with the electron transport chain on the inner mitochondria membrane resulting finally in a reduction of ATP synthesis. The mechanism by which this process occurs is contested [Fusch et al , 1990 , Salet et al , 1991]. It has also been shown that many enzymes associated with cell proliferation ; Glucose-6-Phosphate-Dehydrogenase; Ornithine Decarboxylase; Lipoxygenase; Protein Kinase C are inhibited by dithranol. In a

earlier comprehensive review [Kemany et al , 1990] these and other targets such as calmodulin and cyclic nucleotides are discussed. However, as these reviewers point out ,dithranol at certain concentrations stimulates epidermal DNA synthesis and hyperplasia thus the antiproliferative effect may not be primordial. When all of the data available is considered, the conclusion that can be drawn is that dithranol's target is not restricted to one cell type, and it is the redox chemistry of the dithranol molecule interfering with cellular metabolism that accounts for the ubiquitous pharmacology of the drug.

Cavey et al (1985) showed that on the application of dithranol to tape stripped hairless mouse skin it was rapidly converted to ether extractable dimer and also to products insoluble in ether, which predominated over the ether soluble fraction. In addition to it's possible relationship to brown oxidation products of dithranol, this insoluble fraction could reflect covalent binding of the drug with e.g. proteins [MeLo et al ,1983 , Kammerau et al , 1975]. The formation in vivo of free radical species from dithranol [Shroot & Brown ,1983] might account for such unspecific interactions , which in turn should have a general effect on cellular metabolism rather than specific effects. This lends support to the idea that active species are rapidily formed when dithranol is applied to diseased skin , and that the mechanism of action does not depend on one chemical entity but on the reactivity of the drug and its transformation products. Based on the presently available information one question that arises is whether or not it would be possible to control this reactivity of the drug and improve the response to it.

1.4.6 Treatment of psoriasis with dithranol preparations

Conventional Regime - Ingram (1953) introduced a combined treatment using tar-ultraviolet light and a dithranol paste. Patients, under the guidance of trained medical staff were given a coal tar bath and exposed to varying increments of UV light to maintain near erythema dosage. The stiff dithranol paste was then applied to the psoriatic plaques, which were powdered with talcum, and the patient was wrapped with a protective stockinette dressing.

The Ingram method has been found to be effective and reliable. The side effects of the dithranol are limited as a result of the stiffness of the paste which prevents spreading to normal uninvolved skin. However, the regimen is not suitable for out-patient care and it relies on homogeneous mixing and the availability of dithranol from the paste [Farber & Nall, 1981].

At present the methods used are various modifications of the original Ingram regimen.

Low Dose - Long Term Application - It has been suggested that a low dose dithranol ointment (0.01 - 0.05%) does not irritate normal skin and would be effective if left in contact with the psoriatic plaque for a long time [Brody,1981]. However , Lowe and Breeding (1981) have shown that 0.05% dithranol o/w cream give equal clinical response to vehicle alone.

High dose-short term application or Short contact therapy - Kammerau et al (1975) used tritiated dithranol to show that the maximal epidermal concentrations of dithranol (from 0.1% in petrolatum) occurred between 60 to 300 minutes after application. Schaefer et al (1980) found enhanced penetration of dithranol into epidermis from which the horny layer had been removed by tape stripping, and that the difference between penetration into normal skin and tape-stripped skin was more noticeable after a short (30 minutes) as opposed to a longer (1000 minutes) period. They concluded that removal of dithranol after 30 minutes should give a relatively higher amount of dithranol penetrating into psoriatic skin, assuming that skin with horny layer removed was a reasonable model for psoriasis. Thus, it was suggested that 1% dithranol (including 1% salicylic acid) in petrolatum be applied for 1 hour and then washed off [Schaefer, 1980]. Runne & Kunze (1982) used 1-3% dithranol ointment for 10 to 20 minutes. Short contact therapy has been shown to be equally effective as conventional dithranol paste and it is suggested as being suitable for out-patient therapy [Marsden et al ,1983].

Adjunct Therapy - The idea of combining dithranol with other therapies has been explored over the years. Reshad et al (1984) studied the combination of dithranol with Ultraviolet A (UV-A) while Morison et al , (1978) combined dithranol with PUVA. The combinations were claimed to be more effective than either of the treatments alone, a claim that was disputed by Grattan et al (1988). The retinoid, etretinate, was also investigated with dithranol therapy [Orfanos & Runne ,1976] and found to reduce clearance time when compared

to dithranol alone.

1.4.7 Formulation

Though effective, the use of dithranol has a number of disadvantages such as its potential irritancy, its staining of skin and clothing, its chemical instability on long term storage in many formulations , and also its use in heavy pastes and ointments requiring bandaging. Ways of improving patient acceptance of, and compliance with , treatment regimens are therefore continually explored.

It has been shown that of the dithranol formulations available aqueous creams are the most popular, mainly because they are the most convenient to apply [Whitefield ,1981 , van Scott & Yu , 1981]. Ointments are less preferred as the greasy base is not well absorbed into the skin and most of the application remains on the skin. The treated areas are therefore covered with protective dressings in order to avoid contamination of the clothing and bed linen which would become extensively stained on washing. An other problem is the softening of ointment bases at skin temperature with the result that the formulation tends to spreads beyond the confines of the psoriatic lesions. High strength ointments would therefore lead to excessive irritation of uninvolved skin. This problem of spreading ointment base is reduced by using a stiffer ointment by incorporation of zinc oxide and starch into the paraffin base [Seville et al ,1979].

Dithranol is rapidly oxidised in the presence of water due to the availability of dissolved oxygen [Upadrashta & Wurster (a), 1988]. To obtain a stable aqueous cream of dithranol, it is dissolved in the oily phase while a water soluble antioxidant is added to the aqueous phase as stabiliser (all dissolved oxygen is removed). In the cream because of dithranol's poor water solubility it partitions in favour of the oily phase and the water soluble antioxidant in favour of the aqueous phase. The stabilised cream therefore consists of dithranol dissolved in oily globules surrounded by an aqueous phase free from dissolved oxygen. The aqueous phase aids hydration of the skin , and also allows the cream to be completely rubbed into the skin.

Recently a new cream formulation in which dithranol is encapsulated in a matrix of semicrystalline monoglycerides [Lindahl , 1992] has been developed and marketed under the trade name Micanol. It is a stable formulation which contains no greasy components and is therefore easy to wash off. Clinical studies reported [Gunnar Volden et al ,1992] have found Micanol to be effective and well suited for both short-and long-contact regimens for in- as well as out-patients. Nils Jorgen Mork et al (1992) report that by carefully instructing the patients to thoroughly rub the cream into the lesions the rate of clinical improvement may be increased further. Christensen & Brolund (1992) conducted studies using a combination of Micanol and UV-B at day centres. They showed that there was no clinical difference between Micanol and other dithranol formulations, however side effects, cosmetic properties, and patient preference were mostly in favour of Micanol.

1.5 Surfactants and dithranol

1.5.1 Surfactants - These are molecules characterized by the possession of both polar and non-polar regions on the same molecule. The polar or hydrophillic region of the molecule may carry a positive or negative charge, giving rise to cationic or anionic surfactants , respectively , or may be composed of a polyoxyethylene chain , as in most of the non-ionic surfactants. The non-polar or hydrophobic portion of the molecule is most commonly a flexible chain hydrocarbon. Because of this dual nature, when surfactant molecule are placed in an excess of water they tend to associate in solution forming particles of colloidal dimensions called micelles. Micelle formation confers unique properties on the solution.The micelles exhibit an interfacial region separating the polar bulk aqueous phase from the hydrocarbon like interior [Tanford , 1988, Israelachvili, 1985]. The interfacial region (also called the Stern layer) has a width equivalent to the surfactant head-group and in the case of ionic surfactants , contains the ionic head- groups , a fraction of the counter ions and water [Tanford , 1980 , Fendler, 1982 , Israelachvili , 1985 ,Bunton & Savelli , 1986]. The Stern layer is very anisotropic causing properties of this region to change abruptly over a distance of a few angstroms. The anisotropy of the micelle renders micellar solutions a special medium in which hydrophobic , amphiphilic or ionic compounds may be solubilized and reagents may be concentrated or separated in aqueous solution [Fendler , 1982 , Bunton & Savelli, 1986].

Typical examples of the main classes of surfactants are:

(1) **Anionic** - The anion is the surface active species eg sodium lauryl sulphate (molecular weight 288.4).

$CH_3.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.\underline{SO_4^-Na^+}$

Hydrophobic Hydrophillic

(2) **Cationic** - The cation of the compound is the surface active species eg Hexadecyl(cetyl)trimethylammonium bromide (cetrimide , molecular weight 365.4).

$CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$

(3) **Non-ionic** - The water soluble moeity of this type can contain hydroxyl groups or a polyoxyethylene chain , eg polyoxyethylene (20) sorbitan mono-oleate (Tween 80 , molecular weight 1309), which is an example of a polysorbate. Commercial polysorbates are a mixture of sorbitol and its mono- and di-anhydrides condensed with an appropriate number of moles of ethylene oxide. They have a complex molecular formula.

1.5.2 Solubilization - To enable the formulation of dithranol in aqueous solution, the problem of it's poor solubility and stability in water needs to be addressed. In this study the solubilization of dithranol in aqueous solutions of surfactants is the approach to be undertaken to improve solubility.

Solubilization by surfactants has been shown to be an effective method for the enhancement of the aqueous solubility of a variety of poorly water soluble drugs [D.E. Guttman et al, 1961, H.B Klevens,1949, Goodhart & Martin , 1962]. The advantage of this approach is that the drug entity can be used without chemical modification and hence toxicological data do not have to be repeated as would be the case when alternative approaches are used to produce more soluble compounds.

1.5.3 Percutaneous absorption

Surfactants can either reduce percutaneous absorption of a compound by reducing its thermodynamic activity in the vehicle or enhance absorption by acting directly on the skin. In a micellar solution of surfactant and penetrant both of these effects will usually operate although which predominates depends on the physicochemical characteristics of the drug, the concentration of the surfactant , and the ability of the surfactant to interact with the skin. Mechanisms by which different surfactants enhance the permeability of the skin are yet to be satisfactorily elucidated.

With the cationic surfactants (eg cetrimide) it seems likely that the main action is on the keratin fibrils of cornified cells and the resulting disruption of the cell/lipid matrix leading to enhanced permeability [Ashton et al , 1986].

Nonionic surfactants (eg Tween 80) cause fluidization of lipid components of

the stratum corneum [Rubin & Ann , 1967] , and their ability to enhance absorption is related to the ease with which they penetrate cholesterol monolayers, thus it would appear they act primarily on lipids.

Most anionic surfactants (eg sodium lauryl sulphate) seem capable of both interacting strongly with keratin and lipids. Sodium alkyl sulphates cause swelling of the human stratum corneum. The swelling is the result of interaction with the keratin filaments in the cornified cells causing an conversion of the filaments. This results in their uncoiling and thus causes swelling. Uncoiling of the keratin filaments means that the stratum corneum presents less of a barrier to percutaneous adsorption [Scheuplein & Ross ,1970]. Anionic surfactants generally accelerate absorption more than cationic or nonionic ones. Whatever the anionic groups , maximum enhancement is obtained by surfactants having a hydrophillic tail consisting of a linear alkyl chain of 12 carbons.

On the whole , there can be little doubt that not only will the surfactants affect the stability of dithranol in solution , and possibly on the skin , but they will also affect dithranol's penetration to a lesser or greater degree and hence its activity.

1.6 Purpose of study

At present dithranol is the main drug used for the treatment of psoriasis. As

indicated in the above introduction problems arise in its use because of its instability, its staining effects and its irritancy particularly on normal skin. Though the molecular mechanism by which it is effective in psoriasis is as yet not fully understood (Fusch et al , 1990) there is evidence to suggest that there is a significant parallel between the mechanism of its decomposition in solution and that of its decomposition/metabolism in skin (Cavey et al , 1985). Both involve free radical reactions the main decomposition products being danthron and dimer. Danthron is considered to be inactive and it may be responsible for the staining and irritancy effect , the dimer may or may not be active [Cavey et al ,1985]. The most likely possibility is that it is the process involving the production of the free radical intermediates (See Fig 1.3) that is responsible for the observed effects. Hence study of the effect of factors governing stability in solution must be pertinent to anti-psoriatic effects. Also it is generally observed that hydration of psoriatic skin aids anti-psoriatic effects e.g. use of occlusive dressings , aqueous creams , and the hydrogel formulation developed by Priprem , (1991). It seems reasonable to suggest that to aid skin hydration and formulation sampling and perhaps the psoriatic activity of dithranol , there is a necessity for a stable , aqueous solution of dithranol.

Very little information is to be found on the stability profile for dithranol in solution particularly with regard to the rate of formation of danthron and dimer. This is particularly true for dithranol in aqueous solution at pH 5.5 - the pH of the outer layer of skin. There is also a lack of information on

how the presence of possible accelerants and inhibitors of dimer and danthron formation , e.g metal ions, antioxidants and surfactants , quantitatively and selectively affect dithranol's stability profile. As the above are of considerable relevance to the proper formulation of dithranol preparations and to its skin "activity", studies involving the production and evaluation of stability profiles for dithranol in solution , particularly aqueous solution at pH 5.5 , and the quantitative effect of the presence of possible accelerants or inhibitors will form a major part of the present project.

An essential part of the above , because of the poor water solubility of dithranol , will be solubilisation studies with various surfactants with the objectives of maximising dithranol's aqueous solubility and stability.

Following on from the above, in vitro metabolism and permeation studies using the developed aqueous formulations will be carried out using in the main hairless mouse skin. The objectives here are two fold, namely , to allow evaluation of the effect of the presence of a) skin on the stability profile of dithranol and b) the presence of the surfactants on the permeation rate and the metabolism of dithranol in the skin. A preliminary comparison will be made with the in vitro results obtained using human psoriatic skin obtained from the Dermatology Department of Aberdeen Royal Infirmary.

Because of the ease of sampling , a preliminary study will be carried out with the objective of establishing the stability/metabolism/permeation profile

seen in vivo following the application of the aqueous surfactant solutions of dithranol to the surface of normal human skin. Particular attention will be paid to any observed relationships between changes in the profiles and corresponding changes in the observed staining and/or irritancy effects of dithranol. The ultimate objective is one of being able to control dithranol stability in skin with a view to controlling and understanding the relationship between stability and therapeutic effect. So , finally an attempt will be made using TPA to produce an animal model to assess the antipsoriatic activity of the dithranol-surfactant aqueous formulations. A brief clinical evaluation of one of these formulations will be made in collaboration with the Dermatology Department of Aberdeen Royal Infirmary.

CHAPTER 2

THE DEVELOPMENT OF ANALYTICAL PROCEDURES

2.1 Introduction

To fulfill the purpose of this study , it was essential to develop procedures to quantify and monitor dithranol and decomposition products in a variety of media. Dithranol , danthron and/or dimer will be required to be quantified in aqueous solutions of surfactants , in whole skin (hairless mouse) and in receptor media (permeation studies).

For analytical work involving dithranol the current method of choice [B.P 1993] is hplc. Priprem (1991) developed a reversed phase hplc technique which has both high sensitivity and good separation between dithranol and danthron. Throughout the study this will be the hplc technique used for dithranol and danthron. Though the dimer peak is well resolved from those of dithranol and danthron , the sensitivity is much poorer compared to that for dithranol and danthron in this system. Modification of the system will be undertaken to maximise the sensitivity for dimer, and also shorten analysis time by reducing retention time. Fluorescence spectrophotometry will be used to study the ionisation behaviour of dithranol in aqueous solutions in the presence and absence of surfactants. To facilitate dithranol permeation studies

using whole skin , the quantification of dithranol , danthron and dimer in skin was required. It was therefore necessary to develop a procedure for the quantification of these compounds in skin.

2.2 Methods

2.2.1 MATERIALS AND EQUIPMENT

Chemicals and reagents

Dithranol and danthron were obtained from Sigma (St.Louis, U.S.A.): and dithranol dimer (dimer), B.P. chemical reference substance was from the B.P.C. Laboratory (Stanmore, U.K.). Dithranol and dimer were stored in a desiccator at room temperature in the dark. Fluorene, used as the internal standard in hplc work, was from Fisons Scientific Equipment (Loughborough, England).

Surfactants - Sodium dodecyl sulphate (**NaLS**) , approx. 99% pure , Hexadecyltrimethylammonium bromide (**CET**) 99% pure , polyoxyethylene sorbitan mono-oleate (**Tw**) , were all obtained from Sigma (St.Louis U.S.A).

Buffer components - Sodium acetate was from Fisons Scientific Equipment (Loughborough, England) ,1M Hydrochloric acid was from May & Baker Laboratory Products (Manchester, England).

Solvents - Methanol , hplc grade , was from Rathburn Chemicals (Walkerburn,

U.K.). Glacial acetic acid (GAA) was from BDH Limited (Poole, England). Hplc water was purified by a millipore Milli-Q system (Harrow, U.K.)

The containers used to store solutions of dithranol and dimer were amber glass unless otherwise specified. Standard and stock solutions of danthron were kept in transparent glassware. All chemicals and reagents were used as received.

Chromatographic system

EQUIPMENT - A Waters Associates (Milford, MA, U.S.A.) Model 6000A solvent delivery pump system was used. Detection was by a Model LC 871 UV-VIS detector operating at 254 nm fitted with a nominal 18 μl flow cell. Injection was by a Rheodyne model 7125 valve (Cotati, C.A., U.S.A.) fitted with a 200 μl fixed volume loop. Recording of chromatograms was accomplished with a BBC recorder SE 120 (BBC Goerz, Austria). Columns were 100 x 2mm ID slurry packed in the laboratory with C-18, 5μm ODS Hypersil, HETP (Macclesfield, U.K.). These were used for all quantitative work. Analysis was performed at room temperature (nominally 20 $\pm$2°C). Equilibration with the mobile phase was complete in about 30 minutes. For all studies , the absorbance range used was 0.005 AUFS.

SOLVENT SYSTEM - The isocratic mobile phase consisted of methanol, hplc water, and glacial acetic acid 60:38:2 % v/v (pH 2.3 $\pm$ 0.02 , at 20°C). The flow rate

was 0.5ml/minute. The combination of mobile phase, temperature and flow rate yielded a back pressure of about 1500 psi. The mobile phase in the reservoir was degassed using a filter prior to use.

Spectrofluorimetry

A Perkin-Elmer Model LS-5B Luminescence Spectrometer was used. The excitation and emission slit widths were set at 2.5 and 10 nm. The calibration scale of the instrument was fixed at 1.00. The scanning speed was 30nm/minute and recorder speed 20 nm/cm. The integration time for reading the fluorescence intensity (FI) at a particular wavelength was 4.2 seconds and for the recording of spectra 16.8 seconds. A 1-cm cuvette was used.

Miscellaneous

The following apparatus was used where and when required:
A Model FS100 ultrasonic bath (Decon, Sussex, England): A Corning pH meter model 10, Corning Limited (Essex, England), standardised with pH 4.00 and 7.00 buffer solutions: A Sartorius Balance (Sartorius Werke AG, Gottingen, Geremany.) for weighing more than 50mg weight of materials and a Mettler Model AE 240 (Mettler Instruments, Buckinghamshire, U.K.) for 5 mg or more. Centrifugation was performed using a model MSE Micro Centaur, Fisons , (England).

2.2.2 STOCK SOLUTIONS

Dithranol

10 ml of a solution of dithranol in GAA of about 400μg/mL, was accurately prepared (monthly) and stored at -21°C. Using such a solution, less concentrated stock solutions in acidified methanol (methanol 98% , GAA 2%) were prepared fresh on a day to day basis. Once prepared solutions in acidified methanol were protected from light and the container kept in ice. Such solutions were not kept longer than 6 hours.

Danthron

An accurately prepared solution of danthron in methanol (~100μg/mL) was used throughout the study.

Dimer

Accurate concentrations of dimer in acetone (about 100 μg/mL) were freshly prepared.

2.2.3 STANDARD SOLUTIONS AND CALIBRATION

Hplc

20 μg/mL fluorene in methanol, stored at room temperature was used as an INTERNAL STANDARD (IS) solution.

DITHRANOL - The GAA stock solution of dithranol was diluted to 100μg/mL using GAA . 0.05ml of the 100μg/mL stock solution of dithranol was transferred to a 10mL volumetric flask. GAA was added to this such that the final volume of GAA was 0.2ml. Methanol was then added to make the volume up to the 10ml mark resulting in a dithranol solution of 0.5 μg/mL in acidified methanol (see above). This procedure was then followed to produce 1, 1.5 and 2 μg/ml solutions of dithranol in acidified methanol. The acidified methanol solutions were diluted 1:10 using mobile phase before injection onto column. 0.05mL of the internal standard stock solution (resulting in 0.1μg/mL of fluorene in the solution to be injected) was added at the stage of dilution with mobile phase. ALL solutions were prepared in triplicate.

DANTHRON - The stock solution was first diluted to 3μg/mL using methanol. 0.1, 0.2, 0.3 and 0.4ml of the 3μg/mL stock solution was transferred to 4 separate 10ml volumetric flasks. 0.05ml of the 20μg/mL fluorene solution was also added to each volumetric flask. The volume was then made up to the 10mL mark using mobile phase. The final concentration of danthron was thus in the range of 0 - 0.12μg/mL. The diluted solutions were injected unto the column. All solutions were prepared in triplicate.

2.2.4 DIMER ANALYSIS - Starting with the mobile phase shown below (Priprem, 1991), modification was made to the amounts of methanol and water (see Table 2.1).

Methanol..... 60 %

Water ..... 38 %

Glacial acetic acid ..... 2 %

Table 2.1

% Methanol	% Water	% Glacial acetic acid
(A) 60	38	2
(B) 75	23	2
(C) 80	18	2

The detection wavelength was fixed at 254nm and using the above mobile phases in turn , dimer 0.20μg/mL was injected onto the column and peak characteristics at various flow rates investigated.

2.2.4.1 Calibration graph for dimer using mobile phase-A and mobile phase-B at a flow rate 0.3 mL/min.

The acetone stock solution was diluted to 50μg/mL. 0.03, 0.06, 0.09, 0.12 and 0.24mL of this solution was transferred to each of 5 different 10ml volumetric flasks. The volume was then made up to the 10mL mark using mobile phase-A. The procedure was then repeated using mobile phase-B diluent.

The concentration range of the diluted dimer solutions was thus 0 - 1.2μg/mL. The diluted samples were injected on to the column and the average peak height (dilutions done in triplicate) plotted against concentration of the standard.

Calibration

The calibration lines for the three analytes were checked on a monthly basis with calibration procedure being done in triplicate for each analyte. Linear regression was used for analysis of the ratio of peak height to concentration for dithranol and danthron but not for dimer. The average correlation coefficient, slopes and intercepts are shown in Table 2.2 (dithranol & danthron) and Table 2.4 (dimer).

2.2.4.2 The effect of water on analyte peak height.

Using methanol, the stock solutions described in 2.2.2 were diluted to 20 and 12μg/mL for dithranol and danthron respectively. For dimer acetone was used to dilute the stock solution to 27μg/mL. 6 , 10mL volumetric flasks containing 9.8 , 6 , 4 , 2 , 1 and 0 ml of water were then set up. 0.1 and 0.05mL each of the 20μg/mL dithranol and 20μg/mL fluorene solutions was added to each of the 6 volumetric flasks. Volume was made up to the 10mL mark using mobile phase-A.The solutions were then injected onto the column and the heights of the peaks measured and recorded. The whole procedure was

repeated first using the 12μg/mL solution of danthron (with mobile phase-A as diluent) and then with the 27μg/mL solution of dimer along with mobile phase-B as diluent. With the dimer solutions fluorene was not added and 9.9ml (instead of 9.8) of water was used in one of the volumetric flasks.

2.2.4.3 The effect of surfactant presence on analyte chromatography (retention time and peak height)

Surfactant solutions (NaLS , CET , Tw) of concentrations 0.0 , 0.5 , 1 , 2 , 3 , 4 , 5 % w/v were prepared in 0.2M aqueous acetate buffer pH 5.5. 2mL of each concentration was transferred to a volumetric flask (10mL). 0.1ml of the 20μg/mL dithranol solution (see 2.2.4.2) was added to each volumetric flasks and volume made up to the 10ml mark using mobile phase-A. The solutions were then injected onto the column and peak characteristics recorded.The procedure was repeated using 0.1mL of the 12μg/mL danthron and 20μg/mL fluorene solutions (see 2.2.4.2) with mobile phase-A as diluent.

2.2.5 SAMPLE PRETREATMENT AND ANALYSIS

Hplc

AQUEOUS SOLUTIONS (WITH AND WITHOUT SURFACTANTS) - Samples to be injected onto the column were first diluted with mobile phase. For the quantification of dithranol and its breakdown products dilutions were such that the aqueous phase did not constitute more than 20% by volume of the solutions to be onto the column (see Results/Discussion).

2.2.5.1 HAIRLESS MOUSE SKIN - A mixture of trichloro acetic acid (TCA) and methanol , 20:80 respectively , was used as the extraction solvent. TCA is added because it facilitates the homogenisation process and also enhances stability , especially for dithranol , due to a lowering of the pH. The ability of TCA to precipitate proteins is another reason for it's inclusion. The TCA : Methanol mixture is used cold (4°C) as this aids the settling down of any precipitates in solution . The temperature is kept low during the extraction procedure by surrounding the sample holder in the homogeniser with ice. Methanol is a reasonably good solvent for dithranol , danthron and dimer and therefore aids extraction. Also should the sample require concentration, solvent removal can be achieved at reduced temperatures by blowing off the predominantly methanol solvent.

2.2.5.2 Hairless mouse skin extraction procedure.- Frozen whole skin was used. This was first allowed to thaw by leaving it in a sealed glass container at room temperature. Skin to be extracted ($2cm^2$) is first weighed and then cut into smaller pieces and placed in the glass sample holder of the blender , which is cooled by surrounding it with ice. 3mL of the TCA : Methanol mixture (4°C) is added to the skin which is then homogenised at 13 000 rpm for about 3 minutes. 0.4 mL of the supernatant is diluted to 4 mL with cold mobile phase (4°C). A sample of this solution is then centrifuged (13 000 rpm for 2 minutes) and the clear supernatant injected onto the column (Any further dilution is done using cold mobile phase). A standard mixture of dithranol , danthron and dimer was added to a piece of

chopped up skin in the glass sample holder of the homogeniser and the extraction procedure followed. The percentage recovery of each of the compounds was ascertained.

2.2.6 Calibration graphs for dithranol , danthron and dimer in the presence of skin homogenate.

Using methanol for dithranol and danthron the stock solutions in 2.2.2 were first diluted to 40 and 20μg/mL respectively. The dimer stock solution was diluted to 50μg/mL using acetone. A 2cm^2 piece of hairless mouse skin was then weighed and chopped up into smaller pieces and placed into the glass sample holder of the blender. 0.05mL each of the diluted standards of dithranol and danthron and 0.06mL of the dimer standard were added to the chopped skin. 0.2mL of the 20μg/mL fluorene standard solution was then added. The volume was made up to 4mL using the TCA : Methanol mixture and the extraction procedure as per 2.2.5.2 followed. 0.4mL of the supernatant is diluted to 4mL with cold mobile phase-A (for dithranol and danthron) and cold mobile phase-B for dimer. The diluted solutions are injected on to two separate columns and all peak heights recorded. Using a fresh piece of skin each time more solutions were prepared such that the solutions to be injected on to the columns all contained 0.1μg/mL fluorene and were in the range 0.05 - 0.20μg/mL for dithranol , 0.02 - 0.10μg/mL for danthron and 0.06 - 0.24 for dimer. All solutions were prepared in triplicate. Linear regression was used for analysis of the ratio of the peak height (dithranol and danthron) to internal standard on concentration. For the dimer , the relationship between dimer peak height and concentration of the standard was the one analyzed

by linear regression. The results are shown in Table 2.5. Calibration graphs produced as described above were used for all skin determinations.

2.2.7 Spectrofluorimetry

Using the GAA stock solution of dithranol a standard solution of dithranol (1μg/mL) was prepared in acidified methanol (methanol 98%, GAA 2%). The prepared standard is protected from light (amber coloured container) and kept cold by placing the container in ice. 0.03mL of the standard is added to 2.97mL of the solution under investigation, resulting in a mixture containing dithranol in the region of 10ng/mL which is a concentration within the linear range of response [Priprem, 1991]. The cell is inverted once to ensure mixing of the contents and the fluorescence reading taken quickly to avoid loss of ionised dithranol due to decomposition.

2.3 Results/Discussion

Hplc

The chromatographic system used for dithranol and danthron analysis was the one developed by Priprem (1991). A representative chromatogram of dithranol danthron and fluorene is shown in Fig 2.1

Calibration graphs

The characteristics of the calibration graphs for dithranol and danthron are shown in Table 2.2. The table shows that correlation coefficients ranging from 0.997 to 0.999 were obtained. Linearity of response was obtained throughout the period of the study. It is also seen in the table that the slope for the danthron line is approximately twice that for dithranol, thus indicating that the system has a higher sensitivity for danthron.

For the analysis of dithranol and danthron the hplc system reported by Priprem (1991) is adequate in terms of sensitivity and analysis time. In this system the reported detection limits are 15-20 , and 1.5-2 ng/mL for dithranol and danthron respectively. The analysis for both compounds is done in less than 10 minutes. This system , however, is only of limited use when used for dimer analysis. The major drawbacks are a lengthy retention time of 24 minutes and a broad peak which results in reduced sensitivity. Starting with the mobile phase reported by Priprem (1991) and increasing the methanol content from 60 to 75% , and reducing that of water from 38 to 23% , the retention time was reduced from 24 to 3.5 minutes when the flow rate was set at 0.5mL/minute. With a retention time of 3.5 minutes the dimer peak was not well resolved from the solvent front , reduction in flow rate to 0.3mL/minutes achieved this resolution as the dimer peak has a retention time of 5.8 minutes. These results are shown in Table 2.3. The superior sensitivity of the modified mobile phase is shown when the two mobile phases are used to obtain calibration graphs for dimer (see Fig 2.2). The

characteristics of the calibration lines are tabulated in Table 2.4. Linearity of response was obtained using both mobile phases , comparing the slopes of the two lines shows that there was more than a three fold increase in the slope on modification of the mobile phase. At a signal to noise ratio of 3 the detection limit in the mobile phase with 60% methanol is 55 ng/mL compared to 10 ng/mL with 75% methanol.

There are a number of reports in the literature dealing with the Hplc analysis for dimer. Irene C.L et al (1989) report a reverse phase system where the detection limit for dimer was 10ng/mL and the analysis time was just under 15 minutes. The disadvantage of this system compared to mobile phase-B is the use of a 25 cm column and a flow rate of 2mL/minute leading to increased consumption of mobile phase. In the mobile phase-B system the column length is 10 cm and flow rate 0.3mL/minute. The analysis time of around 6 minutes in mobile phase (B) is much shorter than the 15 minutes in the report by Irene C.L et al. Caron and Shroot (1981) report a system where dimer retention was 8.42 minutes , the disadvantages are the use of a 25 cm column , flow rate 2mL/minute and operation at 60°C which would introduce stability problems. Lee (1987) reports the use of the Caron and Shroot system run at 20°C where dimer retention time was 12 minutes. The same disadvantages of longer column , faster flow rate and longer analysis time are still present when compared to mobile phase-B. The present disadvantages of using mobile phase-B are that a suitable internal standard has not been identified and also that two chromatographic systems need to be

run at the same time to enable the simultaneous determination of dithranol , danthron and dimer. However a fixed volume loop (20μL) was used on the dimer system and repeated injection of dimer 0.10μg/mL gave a peak height of 10.3 $\pm$ 0.2 (n=5). The small standard deviation showing that peak height reproducibility was good. Mobile phase-B will thus be used for all work requiring the hplc analysis for dimer.

Assay validation

For assay validation purposes the effect on the chromatographic analysis of (i) variation in the water content; (ii) the presence of surfactants of different type and concentration ; (iii) the possible presence of interfering substances from the hairless mouse skin extractions , in the injected samples required to be established. Also the % recoveries from the skin for dithranol , danthron , dimer and fluorene and the characteristics of the calibration lines subsequently obtained required to be established.

Effect of water content variation in the injected samples

All samples injected onto the column were first diluted with mobile phase. Because of dithranol's poor aqueous solubility the solutions to be analyzed usually contained low concentrations of dithranol. With such solutions dilutions such as 1: 10 (and greater) are possible at the beginning of experiments when dithranol concentrations are still relatively high. As decomposition proceeds

and dithranol levels decrease less dilute samples need to be injected onto the column. When less dilute samples are used it means that there is an increase in the amount of aqueous phase present in the sample injected. Fig 2.3 shows the influence on analyte peak height of varying amounts of H_2O in samples injected onto the column. For all the analytes the presence of aqueous phase up to 20% v/v did not alter analyte peak height. Above this concentration a general increase in peak height is observed. For dithranol and danthron injection of samples containing > 60% aqueous phase resulted in ~ 100% increase in initial peak height while for dimer and fluorene around 30% increase was observed. Retention time was observed not to be influenced by water content variation. Because of this effect of water, sample dilution (before injection) of less than 1:5 were not made.

The effect of the presence of surfactants of different type and concentration in the injected samples on the chromatographic analysis

The results in Fig 2.4 show that the presence of all three surfactants up to 1% v/v did not alter the chromatography of the analytes. Both retention time and peak height remained constant as surfactant concentration was increased from 0 to 1% v/v. During the course of the study dithranol and its breakdown products was required to be determined in aqueous solutions containing up to 4% surfactants. Since no sample dilutions (using mobile phase) of less than 1:5 were made before injection onto the column , the highest concentration of surfactant present in the sample to be injected was 0.8% v/v (1:5 dilution of a 4% surfactant solution). Determinations in the

surfactant solutions can , therefore , be done without any chromatographic interference from the surfactants.

Hairless mouse skin: the effect of interfering substances on the chromatographic analysis and assay validation.

By following the TCA-Methanol skin extraction procedure developed chromatograms for dithranol , danthron and dimer are obtained that indicate that interference from any substances present in the skin is successfully removed. These chromatograms are shown in Fig 2.5 & 2.6. In Fig 2.7 the results for the recovery of dithranol , danthron , dimer and fluorene when a standard mixture of the four compounds is added to skin which is then put through the extraction procedure , are shown. For all four compounds around 90% recovery was observed. Effects such as binding to protein and the efficiency of the homogenisation of the skin , make 100% recovery almost impossible to achieve. When calibration was done in the presence of skin homogenate Table 2.5 shows that linearity of response was obtained in all cases. The slopes of the three lines were however smaller than those obtained in the absence of skin homogenate , this is most likely linked to the % recoveries possible.

Spectrofluorimetry

The monoanionic species of dithranol is the fluorescent form. It is also very unstable particularly at pH values above it's pKa value of 9.4 [Melo et al (1983)]. In solutions of pH 5.5 dithranol is non ionised and no

fluorescence is observed. Introduction of CET , however , leads to dithranol ionisation , even at this low pH , and the appearance of fluorescence (see chapter 5). When fluorescence characteristics were investigated in solutions of pH > 9.5 isoascorbic acid (0.1% w/v) was added to stabilise the fluorescence signal , which would otherwise decay rapidly (> 90% decay within 5 minutes) in these alkaline conditions. When the fluorescence signal from a 10ng/mL solution of dithranol (0.02M glycine buffer pH 10 + 0.1% isoascorbic acid) was set to read 100% , a standard deviation of ± 11% (n=5) for repeated determination of the same concentration was obtained. This indicates that the fluorescence signal was reasonably reproducible.

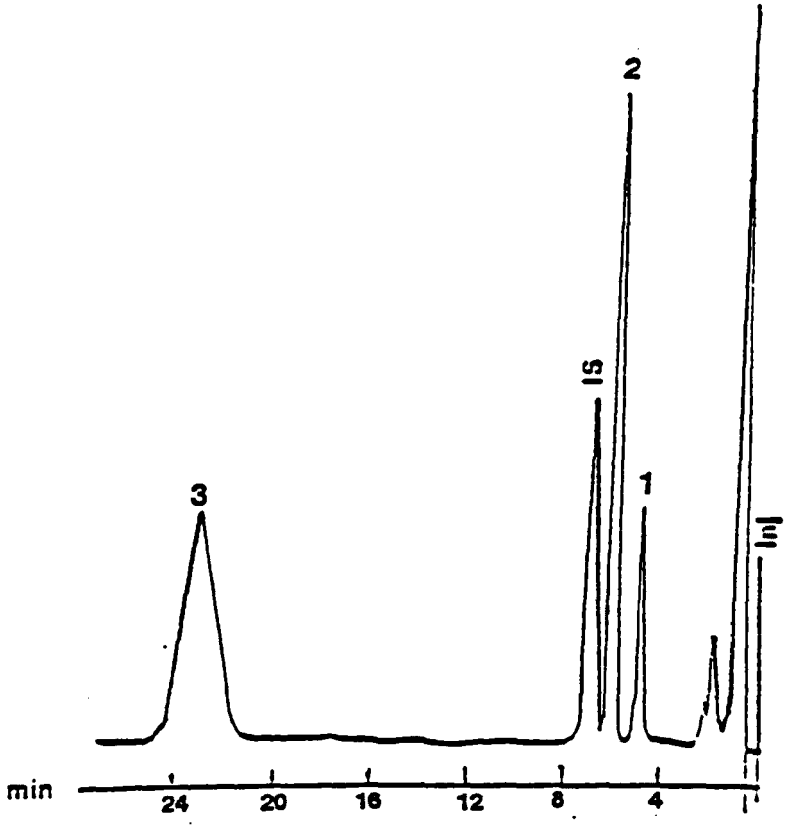


Fig 2.1

Representative chromatogram of dithranol, danthron , dimer and fluorene (internal standard) analysed using mobile phase-A at a flow rate of 0.5mL/minute.

1 = danthron , 2 = dithranol , 3 = dimer , IS = internal standard.

Table 2.2

Calibration line characteristics for dithranol and danthron.

	Slope	C.C	Intercept
Dithranol	6.116 (0.300)	0.997 (0.001)	0.02 (0.01)
Danthron	11.823 (0.441)	0.999 (0.000)	0.01 (0.01)

Results are shown as mean ± SEM (in brackets, n=9). C.C = correlation coefficient.

Table 2.3

The effect of the alteration of the methanol:water ratio and flow rate of the mobile phase on dimer retention.

% MeOH	% H_2O	% GAA	Flow rate mL/min	Retention time (minutes)
60	38	2	0.5	24.0
75	23	2	0.5	3.5
80	18	2	0.5	2.2
75	23	2	0.3	5.8

MeOH = methanol, GAA = glacial acetic acid

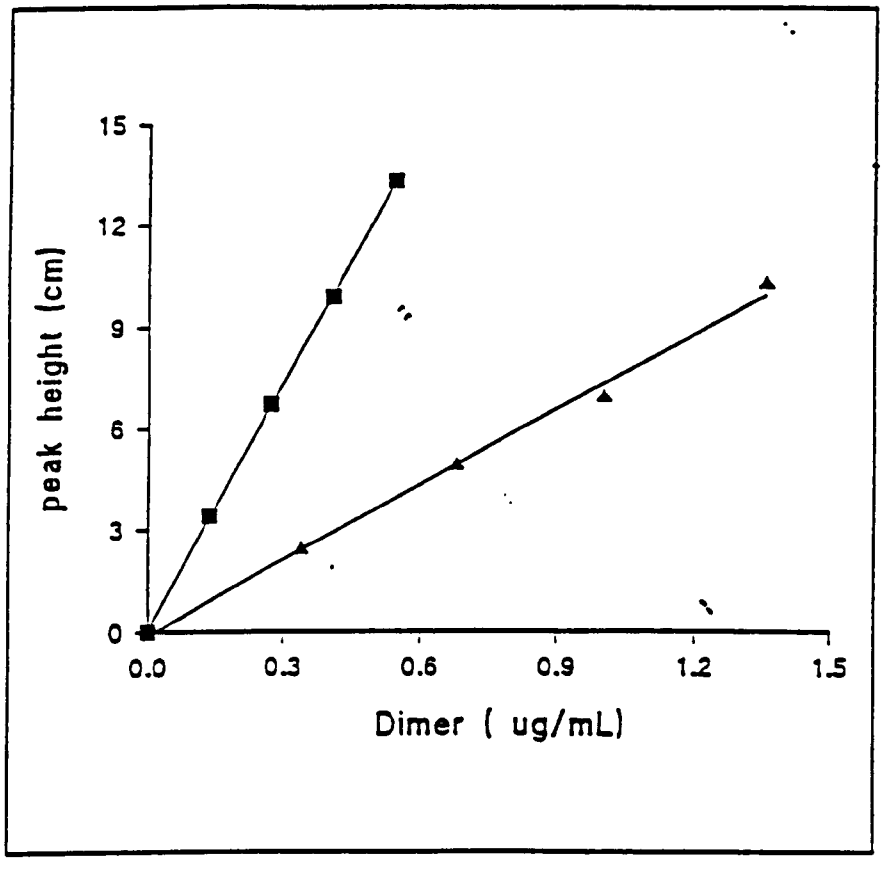


Fig 2.2 Dimer calibration graphs ■ mobile phase-B flow rate 0.3mL/minute, ▲ mobile phase-A flow rate 0.5mL/minute.

Table 2.4

Dimer calibration line characteristics

	Slope	C.C	Intercept
Mobile phase-A	7.4 (0.1)	0.996 (0.001)	-0.15
Mobile phase-B	24.4 (0.2)	0.999 (0.000)	-0.02

Results are shown as mean ± SEM (in brackets,n=9). C.C = correlation coefficient.

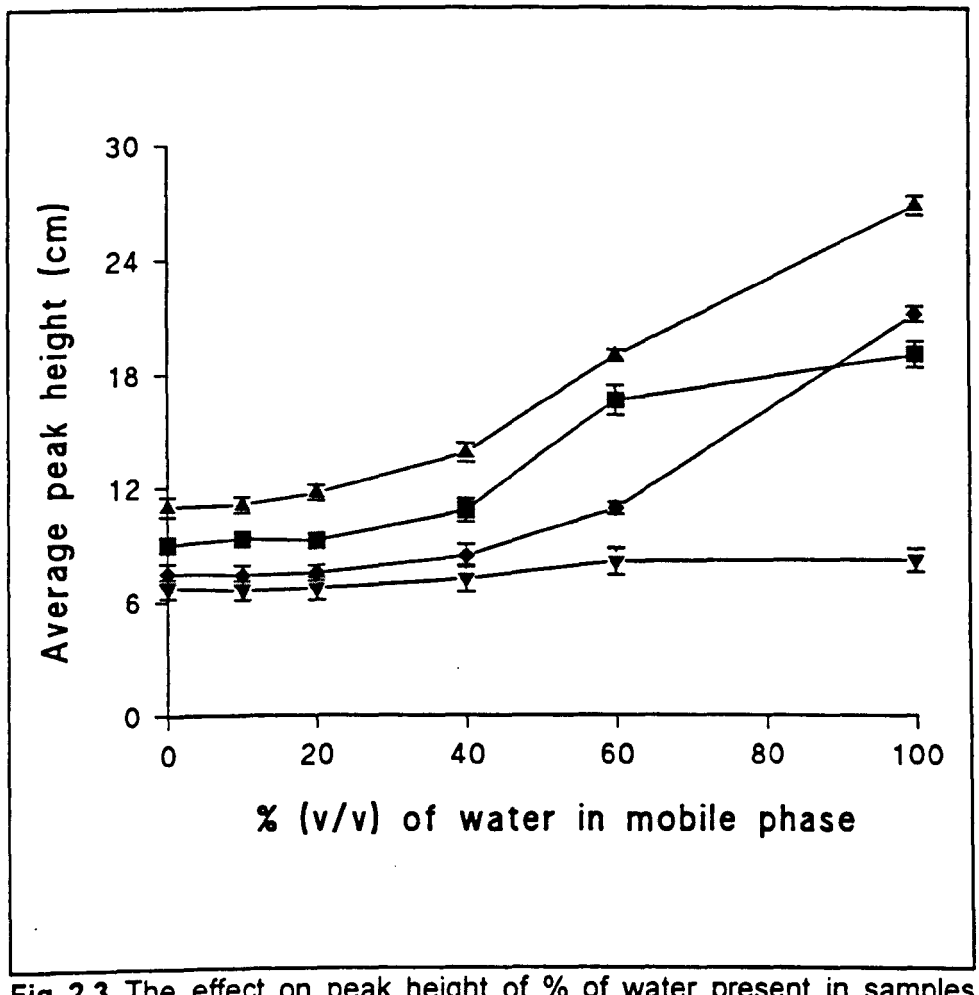
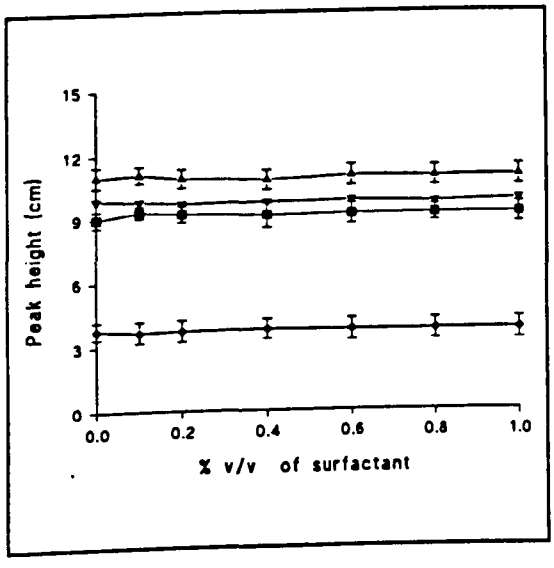


Fig 2.3 The effect on peak height of % of water present in samples injected onto the Hplc column. ■ dithranol, ▲ danthron, ▼ dimer, ♦ fluorene.



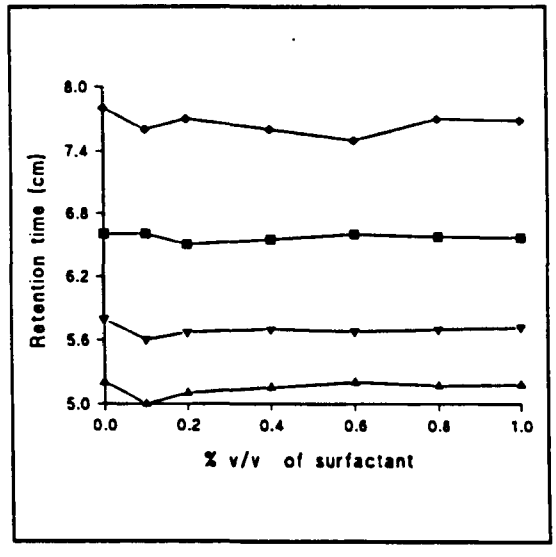


Fig 2.4 (a) **(b)**

The effect on a) peak height and b) retention time , of the amount of surfactant present in samples injected onto the Hplc column. ■ dithranol , ▲ danthron , ▼ dimer , ◆ fluorene.

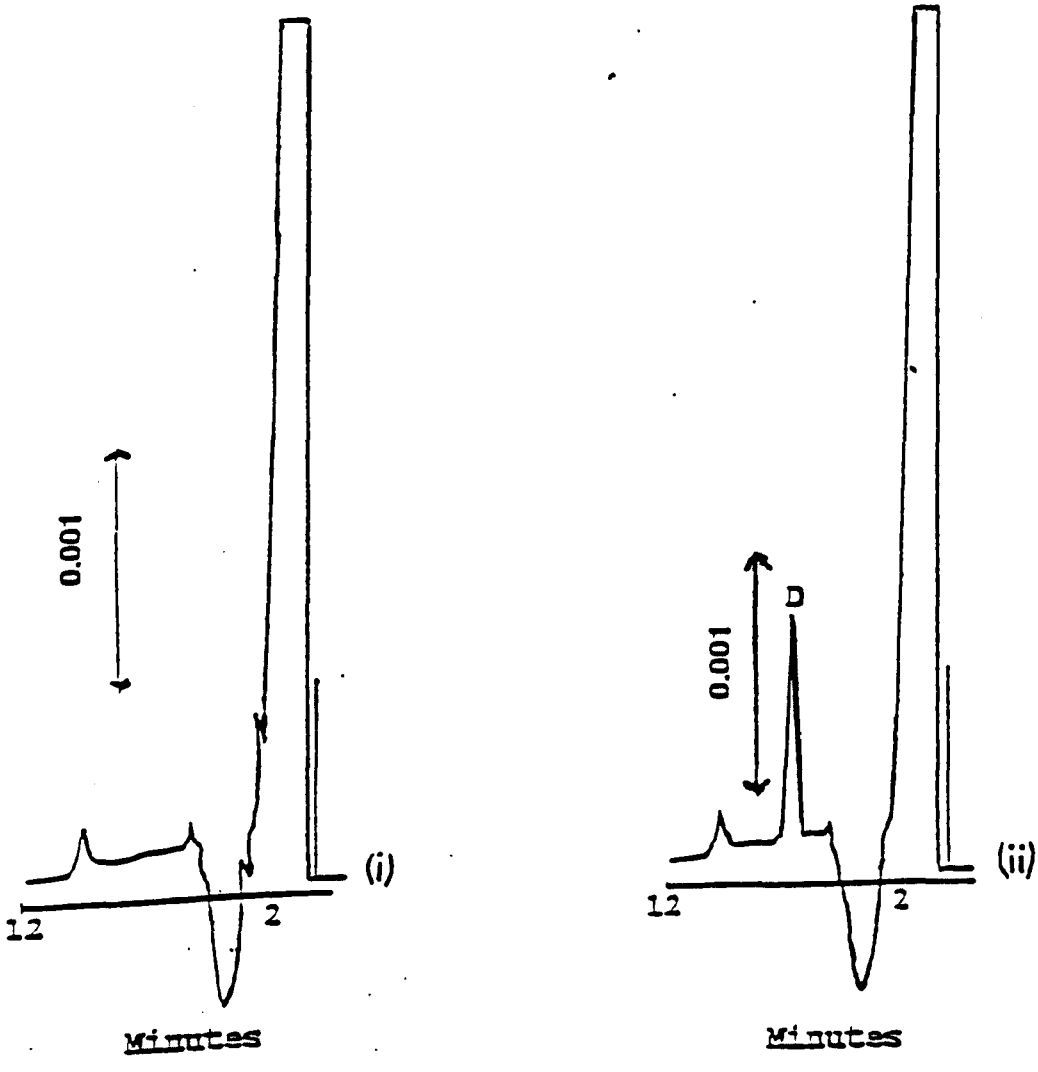


Fig 2.5 chromatograms of the TCA-Methanol extract of (I) hairless mouse skin , (II) hairless mouse skin with dimer added.The chromatograms were obtained using mobile phase-B at a flow rate of 0.3mL/minute. D = dimer.

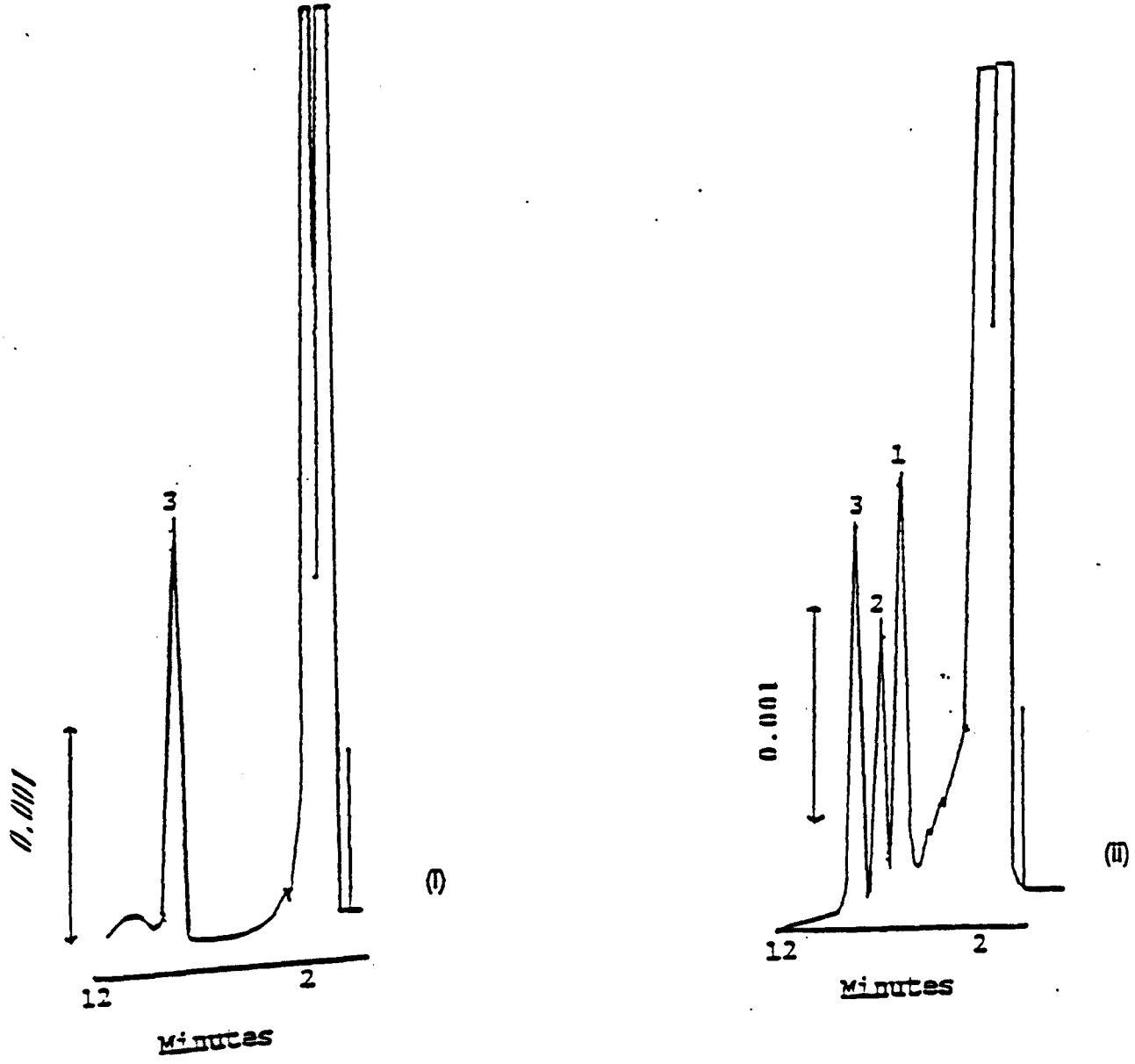


Fig 2.6 Chromatograms of a TCA-Methanol extract of hairless mouse skin with (I) Flourene added, (II) with danthron (1) , dithranol (2), and fluorene (3) added. The chromatograms were obtained using mobile phase-A, at a flow rate of 0.5mL/min.

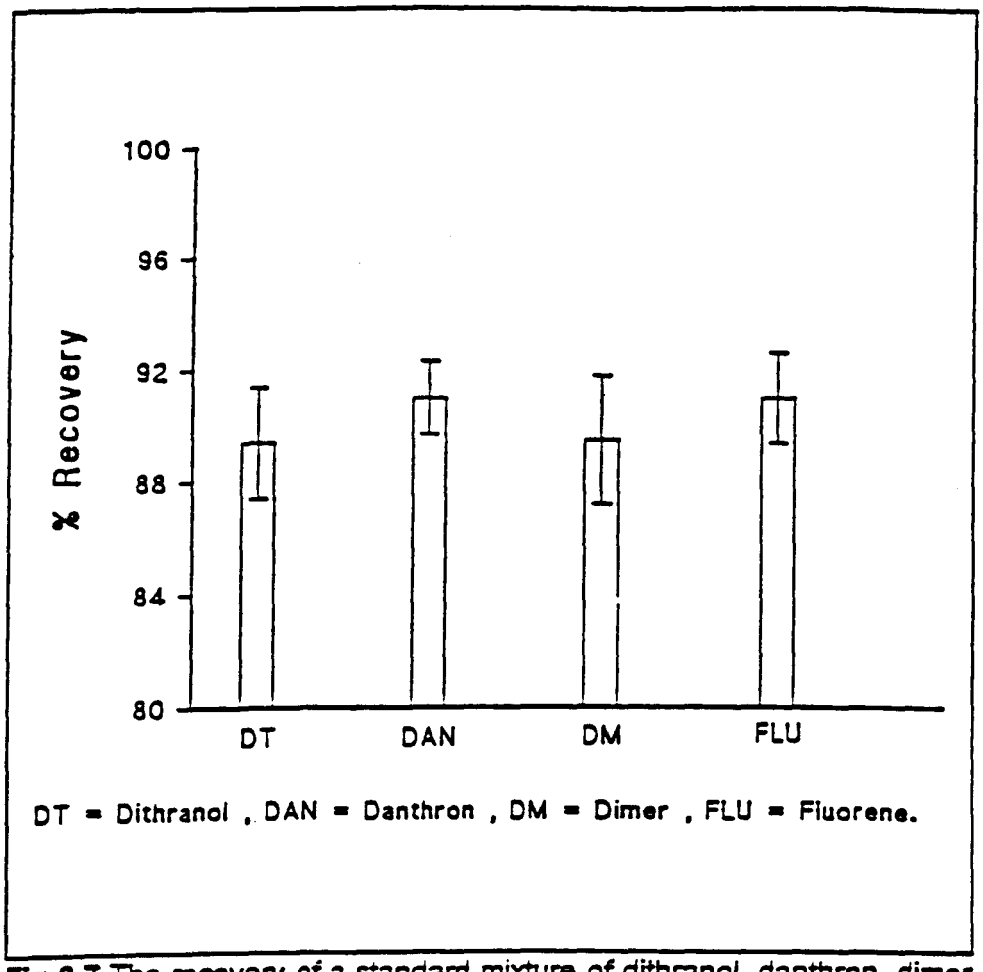


Fig 2.7 The recovery of a standard mixture of dithranol, danthron, dimer and fluorene from hairless mouse skin following the TCA-methanol extraction procedure (n=3).

Table 2.5

Calibration line characteristics for dithranol, danthron and dimer in the presence of skin homogenate

	Slope	C.C	Intercept
Dithranol	5.78 (0.09)	0.996 (0.002)	0.03 (0.02)
Danthron	10.84 (0.67)	0.997 (0.001)	-0.08 (0.01)
Dimer	20.4 (1.1)	0.996 (0.001)	1.35 (0.10)

Results are shown as mean ± SEM (in brackets, n=3). C.C = correlation coefficient. The mobile phase used for dithranol and danthron was (A), and (B) was used for dimer (see Table 2.1 for mobile phase composition).

CHAPTER 3

THE ENHANCEMENT OF THE AQUEOUS SOLUBILITY OF DITHRANOL USING VARIOUS SURFACTANTS.

3.1 Introduction

Dithranol is practically insoluble in water (BP 1988). Determination of the end point for dithranol saturation solubility in aqueous systems is difficult to achieve with great accuracy because of the formation of a colloidal dispersion especially when dithranol is present in excess. This is reflected in the literature where a range of solubilities are quoted - 2μg/mL M.Whitefield (1981) , 5μg/mL Upadrashta and Wurster (1988), 3μg/mL Caron et al (1982),0.1 - 0.4μg/mL M.Kneczke et al (1989). Variations in pH and temperature conditions for the solubility determinations are contributory factors. There are various approaches that can be taken to enhance the solubility of poorly water soluble drugs. The approach that will be concentrated on here is solubilization in surfactant solutions (see 1.5.2). Surfactants have also been shown to influence the percutaneous absorption of drugs [Chowan and Pritchard (1978) , Shahi and Zatz (1978) , Hwang and Danti (1983) , also see 1.5.3]. This property of surfactants could be exploited to improve the effectiveness of delivery of solubilized drug to active sites in the skin.

3.2 Methods

3.2.1 Materials and Equipment

Details of the spectrophotometer used are given in 5.2.1. All other materials and equipment used were as described in 2.2.1

3.2.2 Solubilization

Equilibrium solubility of dithranol in NaLS and Tw 80 was determined by adding an excess of the drug to the test solution containing the assigned quantity of the solubilising agent in acetate buffer (0.2 M sodium acetate adjusted to pH 5.5 using 0.4 M HCl). For CET , solubilization studies were carried out in 1 M HCl (pH 0.4) solutions.To enhance the stability of dithranol iso-ascorbic acid (0.5 %) was added to the Tw, NaLS and CET pH 5.5 solutions. Because of dithranol's good stability properties at low pH (see chapter 4) iso-ascorbic acid was not added to the CET solutions in 1M HCl. The solutions, in 10mL volumetric flasks protected from light with aluminium foil , were left in a water bath at 25°C with periodic shaking. Priprem (1991) showed that the time taken for dithranol to reach equilibrium solubility in aqueous solutions (25°C) was approximately 1 hour. The NaLS , Tw and CET in 1M HCl solutions were therefore analysed for dithranol in solution after 1 hour. Because of CET ionisation effect in the case of the pH 5.5 CET solutions

analysis for dithranol in solution was carried out after 5 - 10 minutes. For all solutions a 1mL sample was removed using a pipette and this was centrifuged at 13000 rpm for 2 minutes. After this an aliquot of the clear supernatant was suitably diluted using the mobile phase , the internal standard added and the diluted sample analysed by hplc.

3.2.3 Ultraviolet absorption spectrophotometry

The effect of surfactant concentration on the UV-absorption spectrum of dithranol in the three surfactants was investigated. The spectra for a fixed concentration of dithranol (4-5μg/mL) under the following conditions were obtained.

(1) Sodium acetate-HCl buffer pH 5.5 at 25°C.

(2) 1M HCl at 25°C.

(3) Chloroform (Analar)

(4) Increasing concentrations (0 - 0.32% w/v) of NaLS and Tw (0 - 0.1% w/v) in buffer pH 5.5, and CET (0 - 0.06% w/v) in 1M HCl , all at 25°C.

3.4 Results/Discussion

Though literature in the field of micellar solubilization is extensive and many monographs and review articles are available [M.E.L M^{c}bain & E.Hutchinson (1955), P.H Elworthy et al (1968), T. Nakagawa (1967) , J.H. Fendler & E. Fendler (1975) , K. Shinoda (1967)] , there is no information that deals specifically with the solubilization of dithranol. Reference to dithranol solubilization without any quantitative or qualitative data presented has , however , been made. C.M Lawrence et al (1987) suggested that the solubilization of dithranol by Teepol (anionic surfactant) facilitated it's physical removal from the skin during short contact therapy. Indeed the general practice in short contact therapy is to remove excess dithranol by washing the treated area with soap. It is somewhat surprising , therefore , that the effectiveness of surfactant solubilization of dithranol has not been investigated.

Surfactants are able to increase the solubility of poorly water soluble drugs by solubilisation because of their unique ability to form micelles in solution (see 1.5.1 & 1.5.2). The nature of solubilisation in micellar solutions and the effects of the solubilized species on the monomer-micelle equilibria depend on the nature of the interactions of the solubilized species with the monomers and the micelles [M.E.L. M^{c}bain and Hutchinson (1955) , P.H. Elworthy et al (1968) , T. Nakagawa (1967)]. Relatively non-polar solubilized species do not interact with the monomers to an appreciable extent. Their solubilization can

be ascribed to the micelles. In solutions of flexible chain surfactants a characteristic feature of such micellar solubilization is the existence of a small range of concentrations below which there is no solubilization and above which the solubility increases approximately linearly with the concentration. Some suitably defined concentration within this narrow range is the critical micellization concentration (c.m.c) of the system containing the solubilized species. This feature reflects the highly cooperative character of the self association of the monomers that leads to the existence of the c.m.c and to the formation of micelles.

On saturating increasing concentrations of NaLS , Tw and CET with dithranol the solubility profiles obtained in the three surfactants was typical of micellar solubilization (see Fig 3.1 , 3.2 & 3.3). Plotting the data in Fig. 3.1, 3.2, & 3.3 as $\Delta S/\Delta C$ v $\bar{C}$ with $\Delta S/\Delta C$ defined as $(S_2-S_1)/(C_2-C_1)$ where C_2 and C_1 are adjacent surfactant concentrations in mol/Litre and S_2 and S_1 are the corresponding solubilities of dithranol in mol/Litre, $\bar{C}$ is the average of C_1 and C_2 , shows that the rate of change of dithranol solubility with concentration of the surfactant increased from a value of nearly zero to a high , nearly constant value , over a narrow range of concentrations around the c.m.c (Fig 3.4, 3.5 & 3.6). Again this is typical of micellar solubilization. If the aqueous surfactant solutions were behaving as cosolvents a plot of $\Delta S/\Delta C$ v $\bar{C}$ would produce monotonically increasing curves [Shinoda et al. 1963]. Fig 3.4 , 3.5 & 3.6 show that no such curves were obtained for dithranol in the surfactant solutions. The possibility of the surfactant solutions behaving as

good cosolvents can thus be ruled out and the enhanced solubility observed ascribed to micellar solubilization.

To gain an understanding of the location and distribution of the solubilised dithranol inside the micelles UV spectroscopic properties were used. The UV spectra of many compounds are sensitive to changes in the environment of the absorbing molecules [Cardinal & Mukerjee, 1978]. Since micelles are characterised by possession of regions of different polarity , an estimate of the location of solubilized material within micelles can be made from UV spectra. On comparison of the UV spectra for dithranol in a polar environment (buffer pH 5.5) and in a non-polar one (chloroform) it is evident (see Fig 3.7) that the spectrum in the polar solvent lacks the spectra detail that it obtained in the non-polar solvent. In the buffer no distinct peaks are identified , whereas in chloroform three peaks , at 258nm , 288nm and 355nm, are evident.

When a fixed concentration of dithranol , was placed in increasing concentrations of NaLS solutions at pH 5.5 it was observed that the spectrum obtained for dithranol became more and more like that obtained in chloroform (see Fig 3.8). The indications are that dithranol molecules are located in an increasingly non-polar environment as NaLS concentration increases. The conclusion from the spectrophotometric evidence gathered is that solubilization is such that the molecules of dithranol are embedded in the hydrocarbon core of the NaLS micelles.

In Tw , the spectrum for solubilized dithranol was very similar in feature to that obtained in buffer alone except for the shoulder at around 388nm and the peak at 258nm both of which are indicative of the presence of the ionised form of dithranol (see Fig 3.9). The similarity in the spectra of dithranol in buffer alone and when solubilised in Tw micelles is an indication that the molecules are in an environment where the polarity is close to that of aqueous buffer - i.e polar. The ionisation peak at 258nm shows that upon solubilisation dithranol molecules enter an environment where the hydrogen bonding which stabilises the keto form of dithranol molecules is disrupted leading to the appearance of the enol form , and thus more of ionised dithranol following the deprotonation of the enol form by water molecules (see scheme 5.1). The micelles that non-ionic surfactants containing polyoxyethylene head groups form in aqueous solutions have a hydrocarbon core surrounded by a mantle , which can be described as a dense solution of polyoxyethylenes. Solubilization in such micelles may involve two broadly defined loci: (a) the hydrocarbon core (non-polar region) along with its interface and, (b) the polyoxyethylene mantle (polar region). From the spectrophotometric evidence obtained in Tw solutions the indications are that the solubilized molecules of dithranol are located in the polar polyoxyethylene mantle of the micelles.

With CET it was observed that the introduction of dithranol into pH 5.5 solutions led to an interaction between the two resulting in the ionization of dithranol (see chapter 5). To obtain solubilization data free from the effect of

this interaction the determinations were done at low pH (1 M HCl) , where the interaction is effectively suppressed. When a fixed concentration of dithranol was placed in increasing concentrations of CET in 1 M HCL the spectra obtained showed a gradual change from the poorly defined spectrum obtained in buffer alone to the well defined type similar to that obtained in chloroform (see Fig 3.10). From this spectrophotometric evidence the indications are that the environment for dithranol molecules became increasingly non-polar as CET concentration rose. It can thus be concluded that the seat of solubilization is the hydrocarbon core of the micelles. Further evidence supporting this conclusion was the lack of any signs of ionisation of dithranol at 258nm. This is an indication that upon solubilisation the molecules of dithranol enter an environment where the hydrogen bonding that stabilises the keto form of the dithranol molecules is not disrupted thus the possibility of the ionisation of dithranol is eliminated.

Following the establishment of the fact that dithranol was successfully solubilised by the three surfactants investigated , the amounts of dithranol going into solution was required to be established. The results of carrying out the solubilization procedure using NaLS (pH 5.5) , Tw (pH 5.5) and CET (pH 0.4) all covering the concentration range 1 - 8% w/v are shown in Fig 3.11. The solubility profiles show that over the concentration range studied the solubility of dithranol was linearly dependent on the per cent of surfactant present. CET solubilised dithranol with greatest efficiency followed by NaLS and then Tw.

With regard to the formulation of solubilised systems of dithranol the solubilisation results indicate that the higher the surfactant concentration the greater the amount of dithranol that goes into solution. As the solutions are intended for direct application to the skin , the limiting factor regarding surfactant concentration is the ability of the skin (diseased and normal) to tolerate the concentration of surfactant used. In the case of CET the ability of the skin to withstand treatment with a formulation with a pH as low as 0.4 also needs to be considered. Another problem is the tendency for NaLS and CET to come out of solution as concentration is increased (> 1%) especially at temperatures below 25°C. This precipitation problem was not observed with Tw.

In CET solutions at pH 5.5 around three fold more dithranol went into solution at any given surfactant concentration , compared to solutions of pH 0.4 (see Fig 3.11). This is because of the dithranol-CET interaction that occurs at pH 5.5 (see chapter 5). While this is ideal for the enhanced solubility of dithranol , the formulation is unstable because of the presence of ionised dithranol. It is therefore necessary to take precautions such as antioxidant addition in order to improve stability and thus the shelf life of the formulation (see chapter 4).

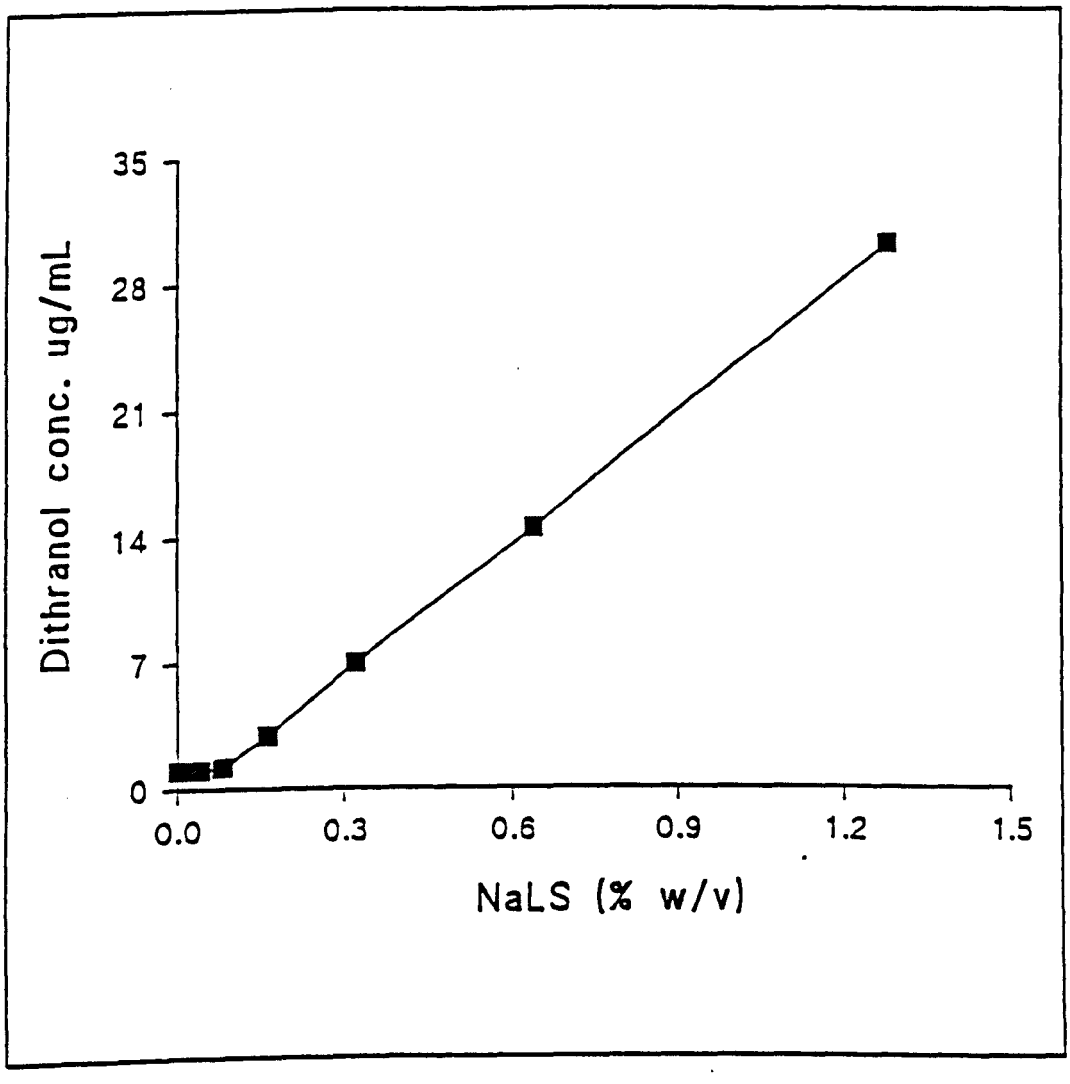


Fig 3.1

The solubility of dithranol in NaLS solutions in buffer pH 5.5 at 25°C.

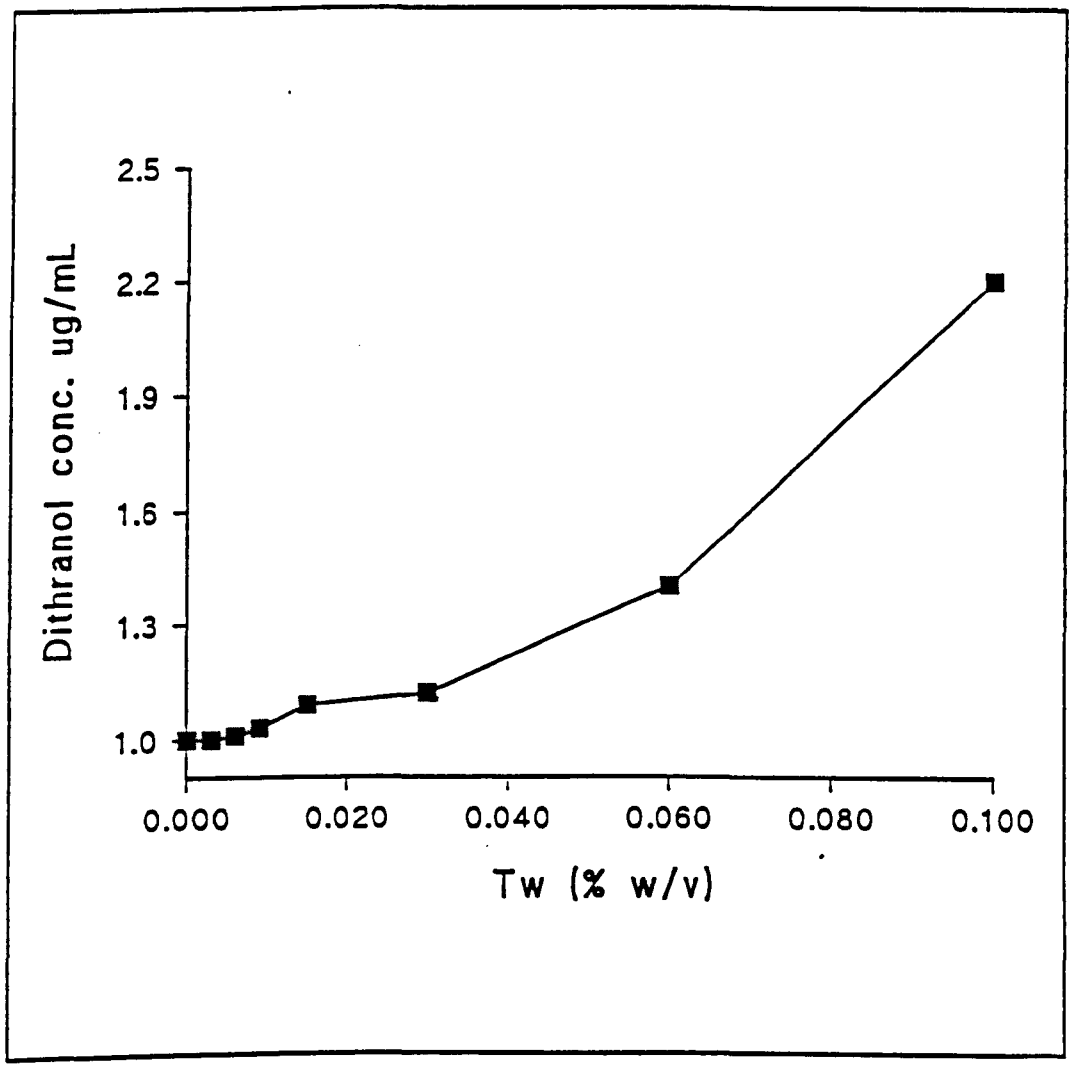


Fig 3.2

The solubility of dithranol in Tw solutions in buffer pH 5.5 at 25°C.

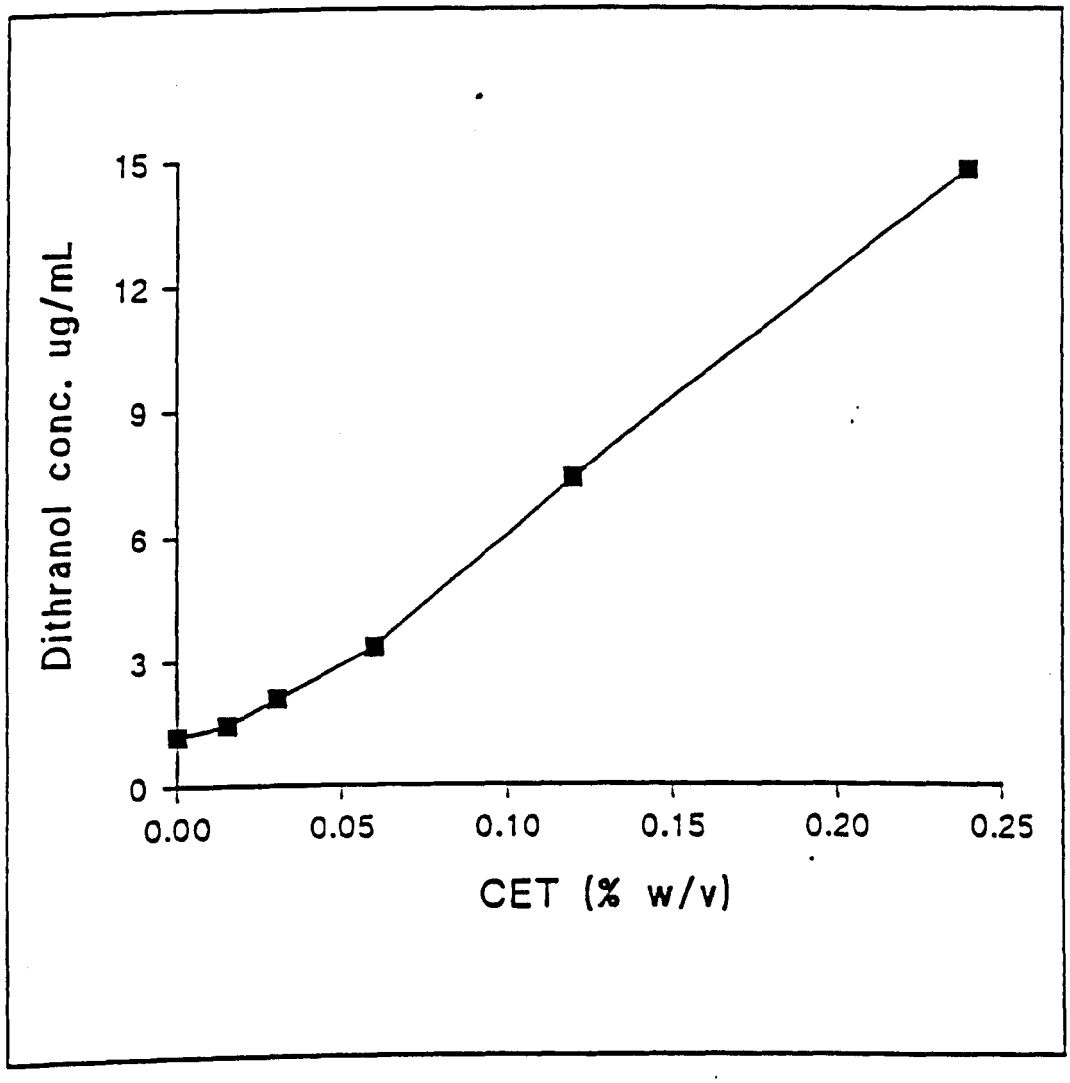


Fig 3.3

The solubility of dithranol in CET solutions at pH 0.4 at 25°C.

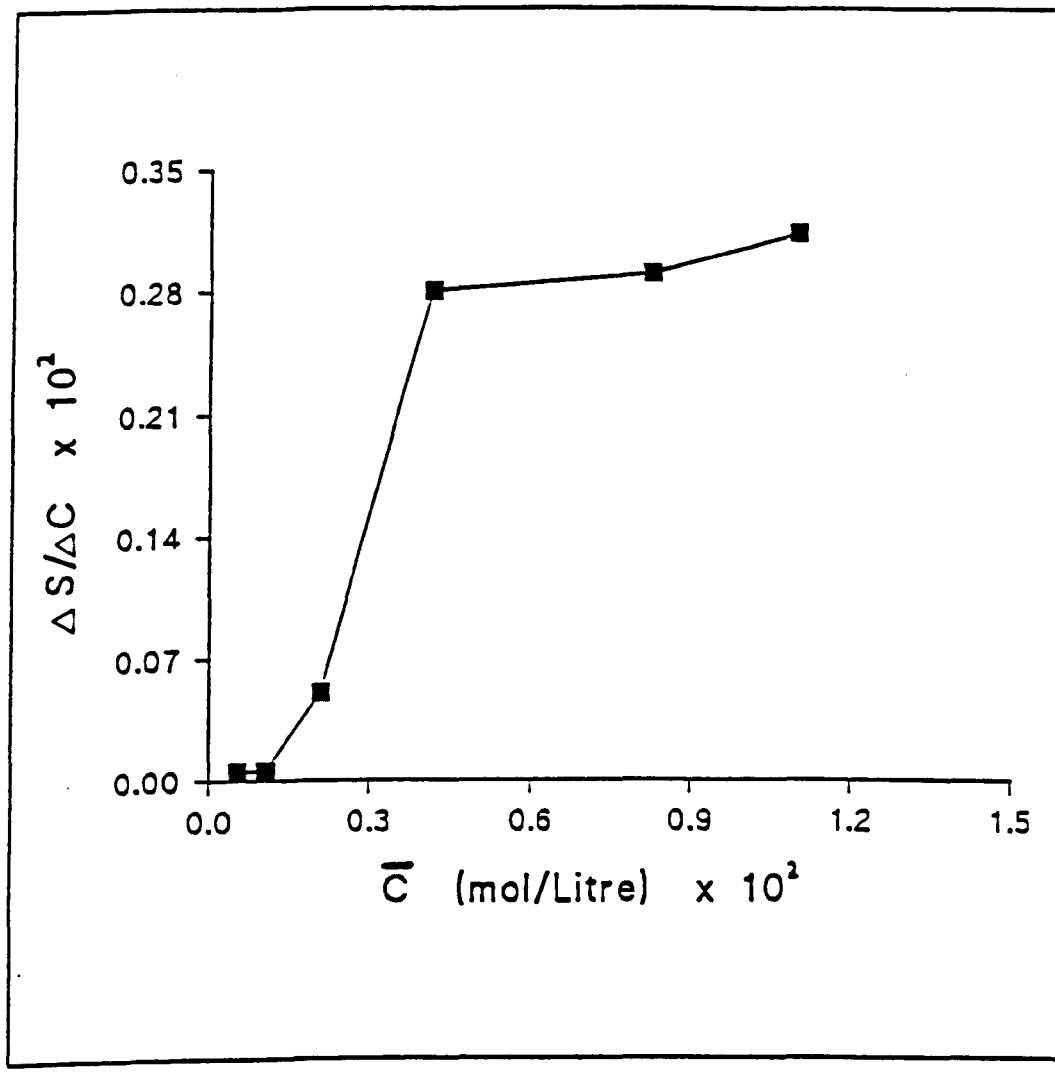


Fig 3.4

The rate of change of dithranol solubility with increasing concentrations of NaLS in buffer pH 5.5 at 25°C.

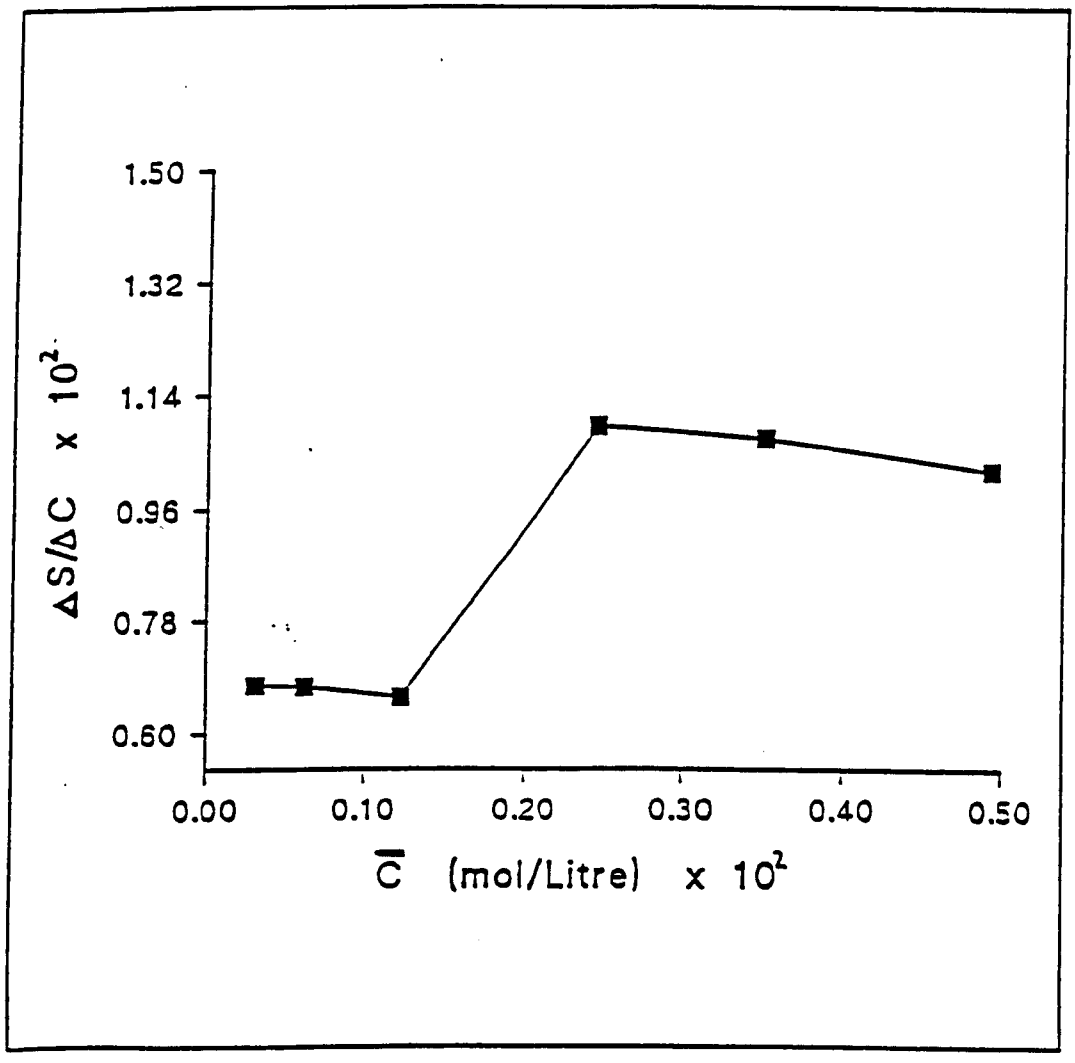


Fig 3.5

The rate of change of dithranol solubility with increasing concentrations of CET at pH 0.4 at 25°C.

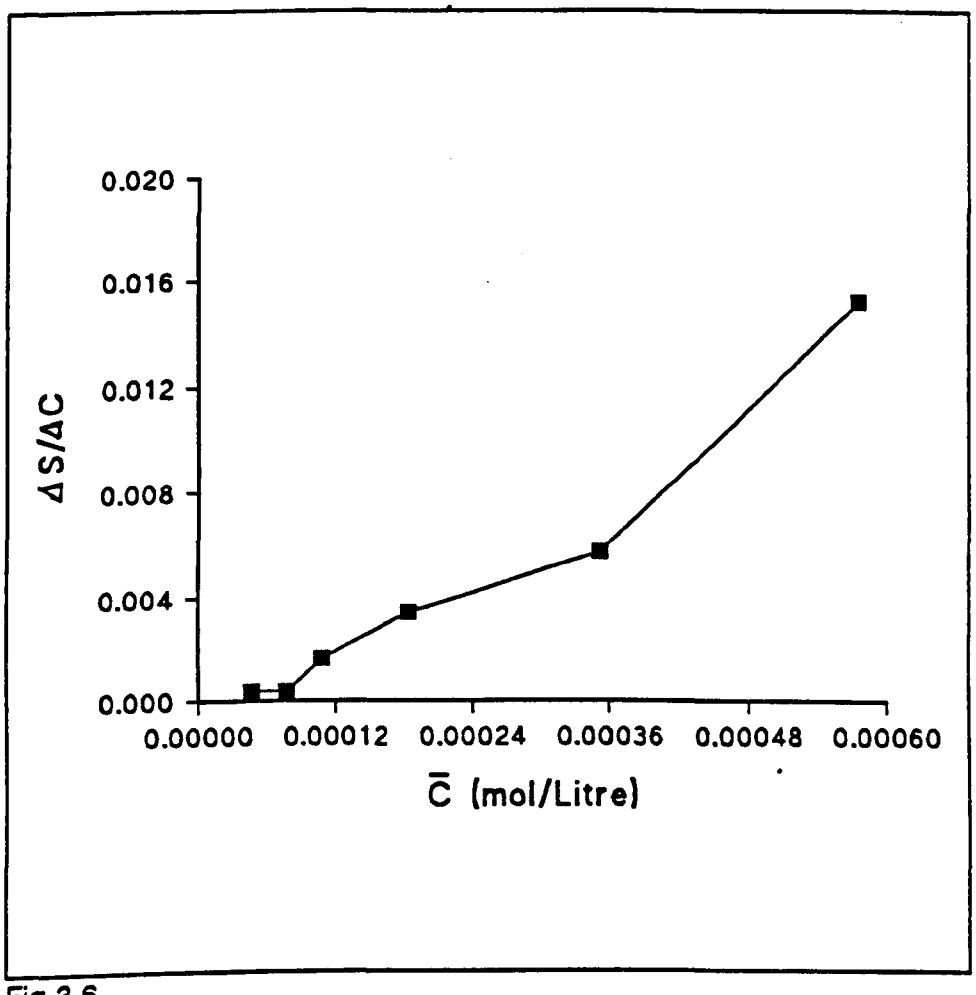


Fig 3.6
The rate of change of dithranol solubility with increasing concentration of Tw in buffer pH 5.5

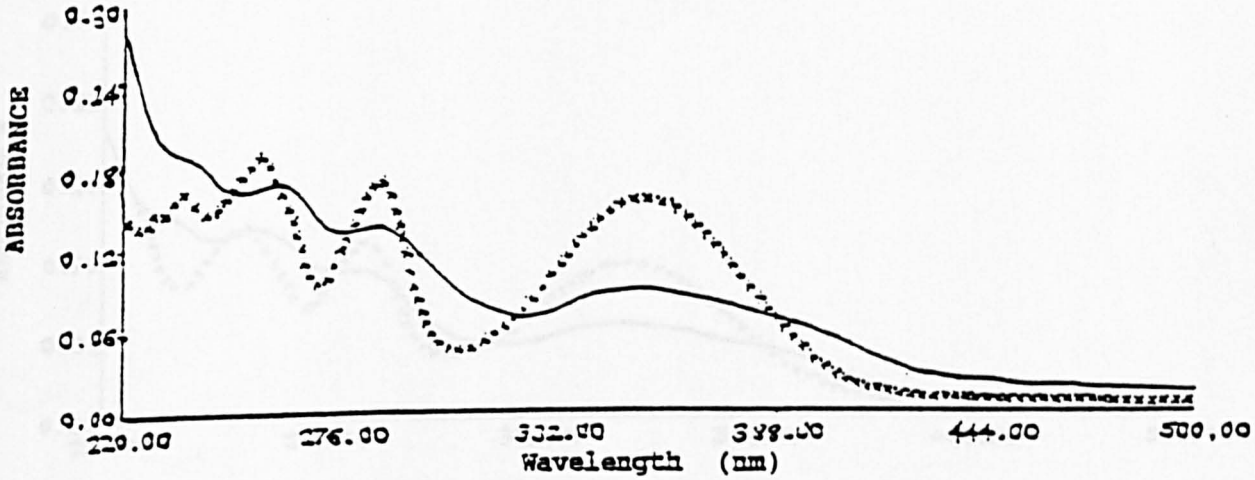


Fig 3.7

The ultraviolet absorption of dithranol (4.5µg/mL) in buffer (———), and chloroform (xxxxx) at 25°C.

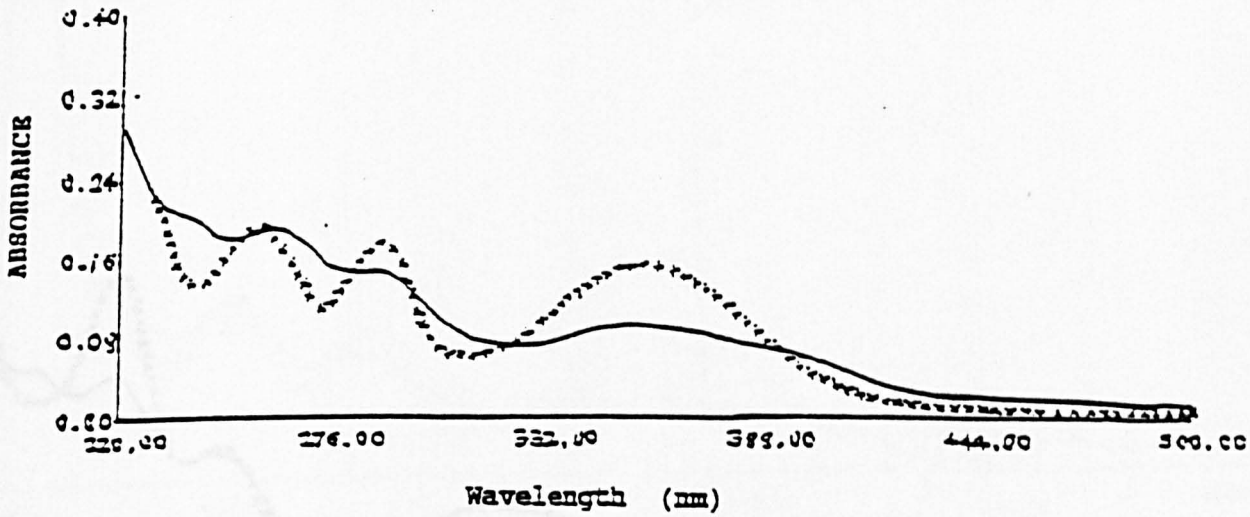


Fig 3.8

The ultraviolet absorption of dithranol (5µg/mL) in 0.16% NaLS pH 5.5 (xxxxx), and in plain buffer pH 5.5 (————).

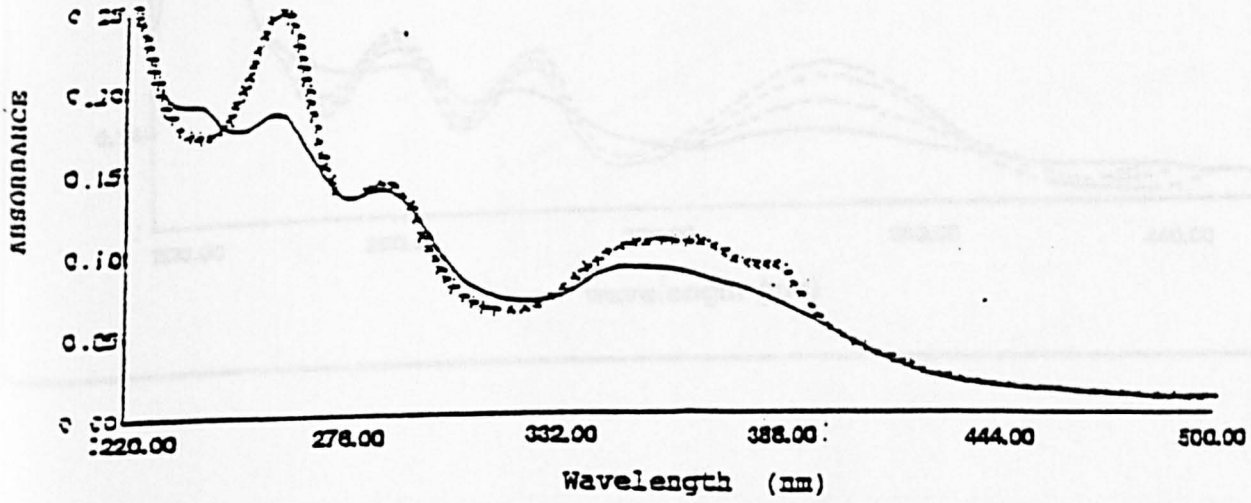


Fig 3.9

The ultraviolet absorption of dithranol (4.2µg/mL) in 0.026% Tw pH 5.5 (xxxxx), and in buffer pH 5.5 (————) at 25°C.

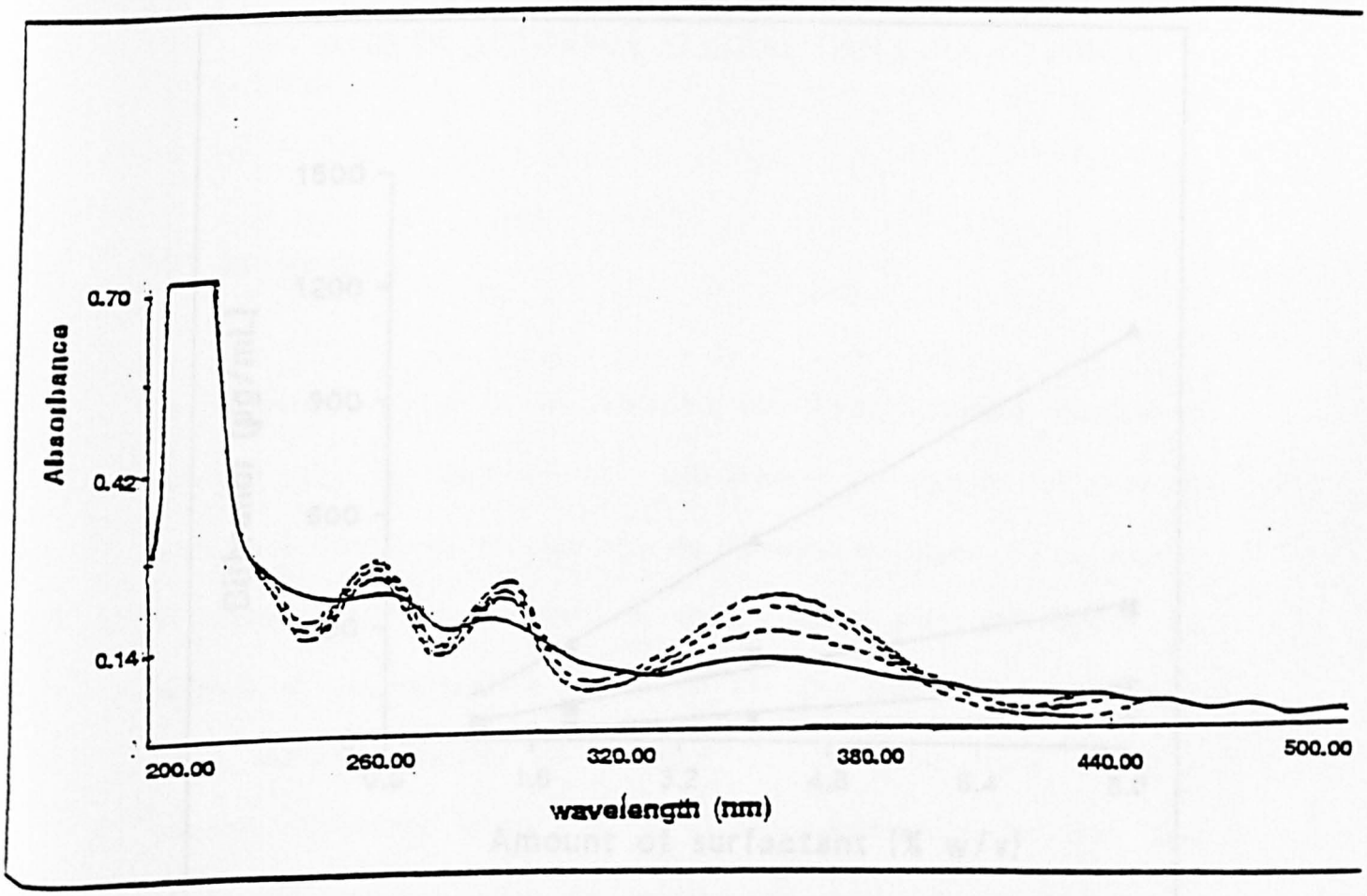


Fig 3.10

The effect of CET concentration on the UV absorbance of dithranol (4.3μg/ml) in 1M HCl at 25°C. —— dithranol in 1M HCl, ---- increasing concentrations (0.04,0.2,0.3,0.5%) of CET.

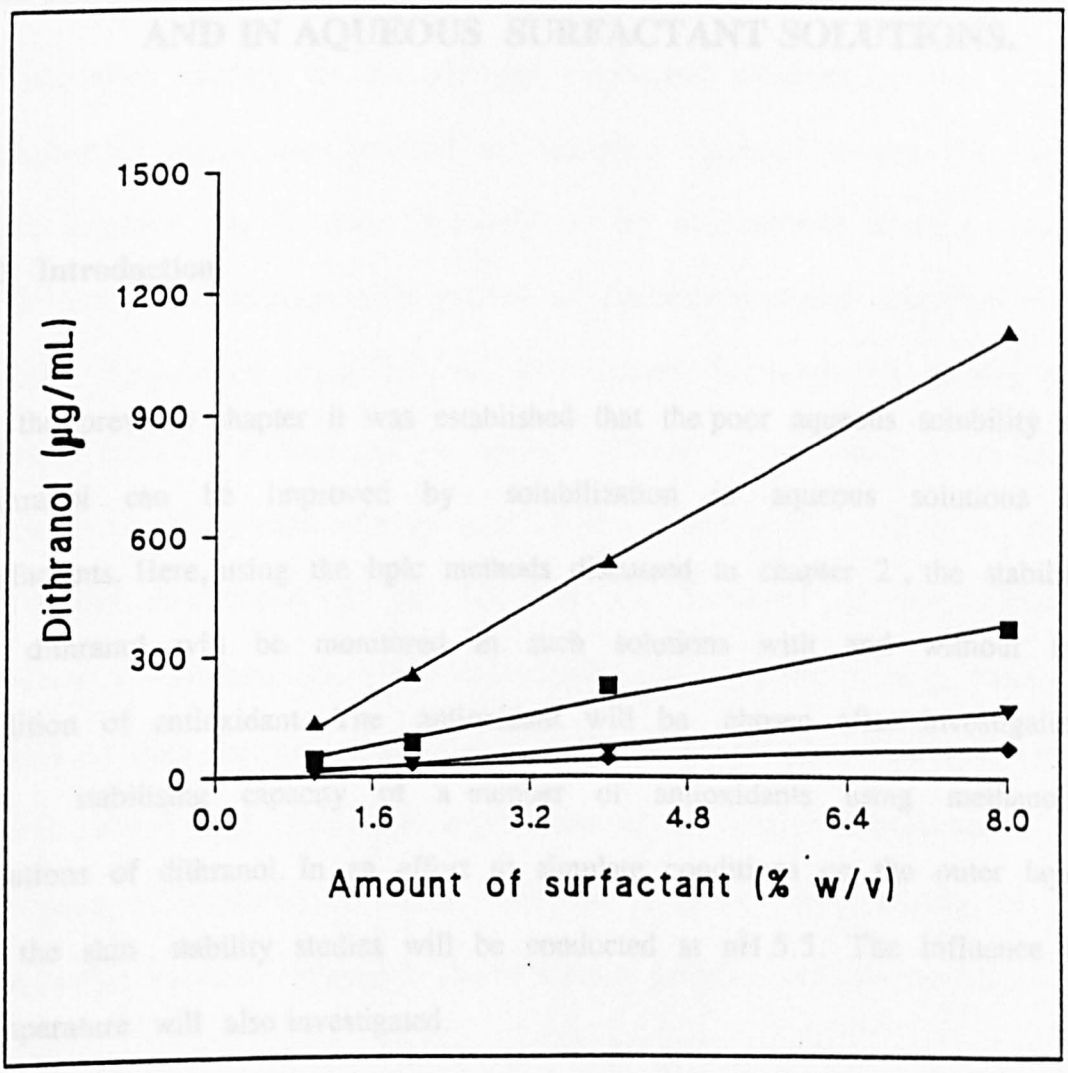


Fig 3.11

The solubility profile of dithranol in aqueous surfactants (1 - 8% w/v) at 25°C.

■ = CET pH 0.4 (y = 47.1717(x) + 3.8837 , CC = 0.989) ; ▼ = NaLS pH 5.5 (y = 22.1804 (x) - 11.6391 , CC = 0.995); ♦ = Tw pH 5.5 (y = 7.9721(x) + 11.6222 , CC = 0.990); ▲ = CET pH 5.5 (y = 139.4696(x) - 20.2609 , CC = 0.999)

CHAPTER 4

THE STABILITY OF DITHRANOL IN METHANOL AND IN AQUEOUS SURFACTANT SOLUTIONS.

4.1 Introduction.

In the previous chapter it was established that the poor aqueous solubility of dithranol can be improved by solubilization in aqueous solutions of surfactants. Here, using the hplc methods discussed in chapter 2 , the stability of dithranol will be monitored in such solutions with and without the addition of antioxidant. The antioxidant will be chosen after investigating the stabilising capacity of a number of antioxidants using methanolic solutions of dithranol. In an effort to simulate conditions on the outer layer of the skin , stability studies will be conducted at pH 5.5. The influence of temperature will also investigated.

Metal ions are known to cause dithranol instability. Also , trace amounts of metal ions are expected to be present in the skin , here, their influence on dithranol decomposition both in the aqueous surfactant solutions and methanolic solutions will be investigated. Methanolic solutions will be used initially to study the stability of dithranol because of its poor water

solubility. Further work will show that the stability patterns in an aqueous environment are similar to those observed in methanolic solutions.

The reasons for studying the above will be , firstly , to identify the conditions of maximum stability for the dithranol - surfactant solutions , so that studies designed to assess the potential of delivering dithranol to the skin using these solutions can be done. Secondly , to try and achieve selective control over dithranol's decomposition pattern to dimer or danthron. Danthron is an inactive antipsoriatic compound , but is implicated in dithranol's staining and irritancy effects.It remains , as yet, unclear whether or not dimer is an active antipsoriatic compound [Cavey et al ,1985 , Krebs et al , 1981]. A more established observation is the fact that the formation of the oxidation products of dithranol (danthron and dimer) , via the free radical route , is central to both the therapeutic and side effects of dithranol [Martinmaa et al , 1981 , Misch et al , 1981]. An appreciation of the relative importance of the formation of danthron or dimer will aid a better understanding of the relationship between dithranol's decomposition pathway and its mechanism of action.

4.2 Methods

4.2.1 Materials

Chemicals

Ferrous sulphate heptahydrate (99% pure) , Zinc sulphate heptahydrate , Cupric sulphate pentahydrate (99% pure), L-ascorbic acid 6-palmitate, D-isoascorbic acid , Citric acid monohydrate , Salicylic acid and L-ascorbic acid were from BDH Chemicals (Poole, U.K).

All other chemicals were as in 2.2.1

4.2.2 Stability of dithranol in methanolic and aqueous solutions

4.2.2.1 In methanolic solutions.

Effect of metal ions - Using concentrated solutions (10 mg/mL) in methanol of Fe^{2+} , Zn^{2+} and Cu^{2+} along with a concentrated solution of dithranol ($\sim$400μg/mL) in acidified methanol (methanol 98% glacial acetic acid 2%), a solution was prepared , in triplicate, containing 40 μg/mL of dithranol and 20μg/mL of Fe^{2+} in a 25mL volumetric flask using acidified methanol to make up the volume. This was repeated , in triplicate , using Zn^{2+} and Cu^{2+} respectively in place of the Fe^{2+} . A blank was also prepared omitting the

metal ion. Once prepared , samples at selected time intervals were withdrawn, appropriately diluted with mobile phase, the internal standard added and analysis done by hplc.

Effect of antioxidants - Stock solutions (1% w/v) of a number of antioxidants, and a stock solution of Fe^{2+} (10mg/mL) were prepared in acidified methanol. Using these , along with a concentrated solution of dithranol prepared as in 4.2.2.1 the following mixtures of dithranol (40μg/mL) , Fe^{2+} (20μg/mL) and an antioxidant (0.5% w/v) were prepared in 25mL volumetric flasks using methanol as solvent.

(i) Dithranol alone

(ii) Dithranol + Fe^{2+}

(iii) Dithranol + Fe^{2+} + Ascorbyl-6-palmitate

(iv) Dithranol + Fe^{2+} + Salicylic acid

(v) Dithranol + Fe^{2+} + Citric acid

(vi) Dithranol + Fe^{2+} + Ascorbic acid

(vii) Dithranol + Fe^{2+} + Isoascorbic acid

Once prepared the solutions were transferred to amber coloured bottles and placed in a water bath at 25°C. At selected time intervals samples were withdrawn , appropriately diluted with mobile phase, the internal standard added and analysis done by hplc.

4.2.2.2 In Aqueous systems

Dithranol stability at pH 5.5 and pH 0.4. - The methanol stock in 4.2.2.1 was diluted to 100μg/mL using methanol. The diluted solution was used to prepare aqueous solutions (10ml) of dithranol (~ 2μg/mL) in 0.2M sodium acetate - HCl buffer , pH 5.5 and in 1M HCl (pH 0.4). The volumetric flasks , protected from light using aluminium foil , were placed in a water bath at 25°C. Solutions were prepared in triplicate. At selected time intervals samples were withdrawn , appropriately diluted with mobile phase and analysed by hplc. To monitor the effect of Cu^{2+} ions on the stability of dithranol in aqueous solution at pH 5.5, about 10μg/mL (Cu^{2+}) was incorporated into the pH 5.5 solutions prepared as described above , and analysed at selected time intervals as per 4.2.2.1

4.2.3 Stability of dithranol in surfactant solutions

The effect of NaLS , CET and Tw - Aqueous solutions of dithranol were prepared as per 4.2.2.2 but incorporating NaLS , Tw and CET respectively to cover the concentration range 0 - 4 % w/v. Samples were withdrawn at selected time intervals, appropriately diluted with mobile phase, internal standard added and analysis done by hplc.

The effect of Cu^{2+} - The following solutions were prepared as per 4.2.2.2 but incorporating Cu^{2+} (10μg/mL).

NaLS (i) 0.02% w/v (below c.m.c)

(ii) 2% w/v (above c.m.c)

Tw (i) 0.003% w/v (below c.m.c)

(i) 2% w/v (above c.m.c)

CET (i) 0.04% w/v (below c.m.c)

(ii) 2% w/v (above c.m.c)

Once prepared samples were withdrawn from the the solutions at selected time intervals appropriately diluted with mobile phase and analysed by hplc.

The effect of antioxidant - 4% w/v solutions of the surfactants containing 40μg/mL of dithranol were prepared with and without the inclusion of 0.5% w/v isoascorbic acid. The solutions were protected from light (amber coloured bottles) and placed in a water bath at 25°C. Once prepared samples from the solutions were withdrawn and then analysed at selected time intervals as per 4.2.2.1

The effect of storage temperature - The isoascorbic acid , stabilised surfactant solutions of dithranol (prepared as outlined above) were left at room temperature (21 $\pm$2°C) and in the fridge (4-5°C). Once prepared samples from the solutions were withdrawn and then analysed at selected time intervals per 4.2.2.1

4.3 Results/Discussion

Decomposition in methanol solutions

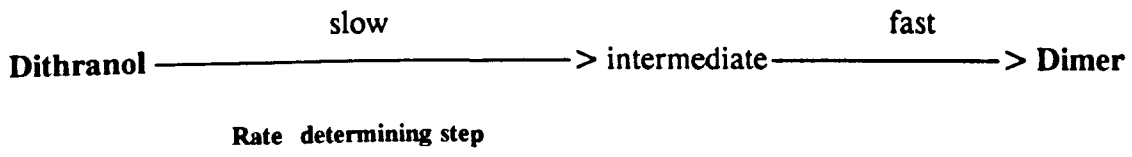
Metal ions such as Zn^{2+} and Fe^{2+} have been shown to cause dithranol instability [Raab and Gmeiner (1975) , Priprem (1991)]. Their presence in trace amounts is expected in skin , it was decided therefore to investigate their effect on dithranol decomposition both in the presence and absence of surfactants. The influence of the presence and absence of metal ions on the rates and amounts of danthron and dimer formed were of particular interest, because the intention was to see whether the decomposition process could be selectively directed towards the formation of either dimer or danthron only. Since dithranol is poorly water soluble preliminary investigations were carried out using methanolic solutions of dithranol ($\sim 40\mu g/mL$).

The influence on dithranol decomposition of Zn^{2+}, Fe^{2+} and Cu^{2+} (all $20\mu g/mL$) is shown in Fig 4.1. The decomposition of dithranol followed first order kinetics. In methanol the decomposition rate was 0.043 hr^{-1}. In the presence of $20\mu g/mL$ of Cu^{2+},Zn^{2+}, and Fe^{2+} the rate was increased to 0.257, 0.047, and 0.074 hr^{-1} respectively. Decomposition in the absence of any metal ions led to the appearance of danthron and dimer in solution . When introduced , the presence of all three types of metal ion caused an enhancement in the rate of dimer formation while danthron formation was markedly decreased. In the concentration time profile for dimer formation in methanol (see Fig 4.2)

it is seen that , in any given time period , e.g. in the first 5 hours , more dimer was formed in the solution containing Fe^{2+} compared to that without. In the case of danthron (see Fig 4.3) Fe^{2+} greatly reduced its rate of formation (similar profiles were obtained when either Zn^{2+} or Cu^{2+} were present).

Using the data in Fig 4.1 for dithranol decomposition in methanol in the absence of any metal ions, Table 4.1 shows that on comparing the amount of dithranol decomposed to the amount of dimer formed it is clear that the dimer concentration is half that of the dithranol reacted this indicating that dimer is being immediately formed. The table also shows that danthron is formed but the amount formed is very small relative to the dimer. Fig 4.4 shows the direct relationship between amount of dithranol decomposed and dimer formed. The gradient of 2.22 shows that for every two moles of dithranol decomposed one mole of dimer is formed.

Dithranol decomposition resulting in the formation of dimer and danthron is proposed to proceed via a free radical intermediate (see chapter 1 , Fig 1.3). Here, based on present results it is proposed that the mechanism for dimer formation is a consecutive one as shown below. The first step is the slow , rate determining first order step whereby the free radical intermediate is formed from dithranol. The second step is the rapid conversion of the intermediate to dimer.



The relationship between the amount of dithranol decomposed and amount of dimer and danthron appearing in solution both in the presence and absence of metal ions (see Table 4.2 & 4.3) is evidence which supports dimer formation in solution is as shown above.

Fig 4.1 shows that in the presence of the metal ions first order kinetics still apply, but the rates are increased . The rate is dependent on the type and concentration of metal ion used. Reducing the concentration of Fe^{2+} resulted in a slower rate of dimer formation (see Fig 4.2), showing that with varying Fe^{2+} the reaction followed pseudo first order kinetics. The results show that metal ion presence had a catalytic effect on the decomposition reaction. This is supported by the fact that the final ratio of dimer : danthron (when the reaction is complete) was the same with and without the metal cations.

For first order kinetics the general catalysis equation given below, can be applied to find the individual catalytic coefficient values for any catalytic agent(s) present.

$$k_{exp} = k_0 + k_{M^{2+}}.[M^{2+}] \qquad \text{Eq. 4.1}$$

where k_{exp} = the overall first order rate constant , k_0 = the specific reaction rate in methanol alone , $k_{M^{2+}}$ = specific reaction rate associated with catalytic species (catalytic coefficient) , $[M^{2+}]$ = concentration of catalytic species (metal cations).

At a concentration of 20μg/mL for all three metal ions the mean catalytic coefficients (n=3) were $1.22 \pm 0.14 \times 10^3$ M hr^{-1} for Cu^{2+} which was 7.5 times greater than that found for Zn^{2+} ($1.63 \pm 0.21 \times 10^2$ M hr^{-1}) , while the catalytic coefficient for Fe^{2+} $2.61 \pm 0.27 \times 10^2$ M hr^{-1} was 1.6 times greater than that for Zn^{2+}.

The overall conclusions that can be drawn from the results using methanolic solutions are that metal ion presence was selectively enhancing the rate of dimer formation while markedly reducing that of danthron. The increase in rate of dimer formation is also dependent on metal ion type. Metal ion presence can therefore be used to control the rate of formation of these two degradation products.

For the purpose of stabilisation of the final preparation an antioxidant was required. A variety of compounds have been suggested as an antioxidant for dithranol, e.g salicylic and ascorbic acid (Lee , 1987) various alpha hydroxy acids [Van Scott and Yu , (1985)], ascorboyl-6-palmitate Weller et al (1990).The stabilising capacity of these compounds was assessed using methanolic solutions of dithranol. Antioxidant assessment was done in the presence of Fe^{2+} so as

to eliminate those antioxidants that gave only a small degree of protection to dithranol. The results are shown in Fig 4.5 where it can be seen that isosascorbic acid gave the greatest protection, prolonging the half-life of the dithranol - Fe^{2+} mixture by approximately 5.7 fold from 5.9 to 32.3 hours. In the presence of ascorbic acid and ascorboyl-6-palmitate the half-life was prolonged to 26.6 and 25.2 hours respectively. Protection was much less however with salicylic and citric acids where half-life was prolonged to 12.5 and 11.5 hours respectively.

Decomposition in aqueous solutions

There is only a limited amount of reported work on the decomposition characteristics of dithranol in aqueous solutions. There is , however , considerable variation in the results, in the few reports available. Cavey et al (1982) investigated the stability of dithranol in an aqueous buffer (1% acetone - Ringer buffer , pH 7.5) at 37°C , reporting that dithranol underwent complete decomposition within 4 hours, with the resultant formation of dimer (40%) and no detectable danthron. The results reported by Priprem (1991) differ from these. Priprem found that using phosphate buffer pH 7 at 20°C , dithranol showed reasonably good stability ($t_{1/2}$ = 7 days) , and following complete decomposition , danthron (4%) was detected in solution while no dimer was found. For the above , variation in experimental conditions most likely accounts for the discrepancies observed . Two different buffer systems were used and there is also a 17°C difference in temperature. There were also

differences in the co-solvent used in the preparation of the aqueous solutions of dithranol. Cavey et al (1982) dissolved dithranol in acetone which was then added to the Ringer buffer whereas Priprem (1991) dissolved dithranol in glacial acetic acid and then this was added to the aqueous medium. Apart from the difference in temperature and buffers used the co-solvents used also would affect the intramolecular hydrogen bonding , responsible for dithranol stabilisation, leading to variation in observed results. In the presence of glacial acetic acid decomposition is delayed possibly due to less interference with dithranol's intramolecular hydrogen bonding whereas in the presence of acetone the interference is greater leading to less stable preparations.

The results reported by Upadrashta and Wurster (1988) are in closer agreement with those of Cavey et al (1982). They showed that at pH 7.4 (Tris-HCl buffer) at 25°C dithranol decomposed following first order kinetics with a half-life of 43.9 minutes. Cavey at al reported complete decomposition within 4 hours (1% acetone - Ringer buffer) at 35°C. Using the time taken for complete decomposition (240 minutes) to occur an estimate of a half-life of 24 minutes is obtained if decomposition is said to be complete after 10 half-lives. When the 10°C difference in temperature is considered the half-life estimate is seen to be in good agreement with the results reported by Upadrashta and Wurster (1988). As mentioned above Upadrashta and Wurster (1988) showed that dithranol decomposition in aqueous solutions followed first order kinetics. This is in agreement with the findings of Melo

et al (1983) who used aqueous buffer pH 10.4 . Using acetate buffer pH 5.5 and 1M HCl (pH 0.4) present results also show that dithranol decomposition followed first order kinetics in these aqueous conditions (see Fig 4.6). At pH 5.5 the decomposition rate constant was 0.636 hr^{-1} (correlation coefficient 0.998) giving a half-life of 1.09 hours ; in 1M HCl (pH 0.4) the decomposition rate constant was 0.037 hr^{-1} (correlation coefficient 0.995) and half-life 18.8 hours. The difference in stability of dithranol at the two pH values is in general agreement with the trend observed by of Upadrashta and Wurster, (1988) who showed that in aqueous solutions dithranol stability was enhanced with a reduction in pH.

Following decomposition in aqueous solutions present results show that dimer and danthron were the main breakdown products detected , ~94% dimer and ~4% danthron being formed on complete decomposition (see Table 4.4). Both in the presence and absence of Cu^{2+} ions the ratio of dithranol decomposed to dimer formed approachs the expected value of 2 only towards the end of the period over which the experiment was run (see Table 4.5 & 4.6). When the amount of danthron formed in solution is taken into account (very small relative to dimer amounts) , it is seen that for any given time period , especially in the initial stages , more dithranol has decomposed than the amount of dimer appearing in the solution. These results indicate that dimer was not being formed as soon as dithranol started decomposing. This suggests the formation of an intermediate which builds up then goes on to form the dimer. This is different from the observations in methanol where

the results suggest dimer was formed as soon as dithranol started decomposing.

Qualitatively the patterns observed in methanol were also observed in the aqueous solutions (similar mechanism of degradation). The main difference was the indication of intermediate build up observed in the aqueous conditions which was absent in methanol. It could well be that the situation is closer to that observed in methanol, but because of the practical problems linked to working in aqueous conditions using much reduced concentrations (16 times less concentrated), a deviation was observed. Also , in the presence of Cu^{2+} the reaction rate was extremely rapid , this factor making it difficult to obtain many data points. In retrospect a reduction in the concentration of Cu^{2+} (perhaps 10 fold) would have slowed the reaction down allowing more data points to be obtained. In methanol solutions higher concentrations ($\sim 40\mu g/mL$) of dithranol were used , and also methanol provides a less problematic analytical environment.

Kinetically the presence of Cu^{2+} ions in the aqueous dithranol solutions , at pH 5.5, resulted in an increase in dimer formation (danthron formation remaining very small in comparison to dimer) due to the catalytic effect of the ions (see Fig 4.7). Using equation 4.1 and data such as that shown in Fig 4.8 the mean catalytic coefficient (n=3) of Cu^{2+} ($10\mu g/mL$) was calculated as $6.58 \pm 0.31 \times 10^{4}$ M hr^{-1}. The catalytic coefficent (bearing in mind the difference in dithranol and metal ion concentration) is around 54 times greater

in aqueous conditions compared to the situation in methanol. The fact that there was no statistically significant difference ($p < 0.05$) between the amounts of danthron and dimer formed following complete decomposition (see Fig 4.9) in the presence and absence of Cu^{2+} , provides supportive evidence that metal ion presence had a catalytic effect.

Present results for the amount of danthron formed , about 4% , following dithranol decomposition at pH 5.5 are in agreement the findings of Priprem (1991) who found a similar amount of danthron at pH 5. Present findings , however , differ from those of Cavey et al (1982) who reported no detectable danthron at pH 7.5. The results for dimer formation also show discrepancies. Present results show that at pH 5.5 94% dimer was formed. In complete contrast to these findings Priprem found no detectable dimer at pH 5, while Cavey et al found 40% dimer at pH 7.5 .

The hplc method used by Priprem (1991) is capable of very adequately seperating dithranol , danthron and dimer, but suffers a lack of sensitivity for dimer (see chapter 2). Dithranol concentrations of about 4μg/mL were used in the investigations carried out by Priprem therefore only low dimer levels are expected in solution , thus the finding of no dimer in solution was most probably due to the poor sensitivity of the system. The method used by Priprem has been modified and sensitivity for dimer increased (see chapter 2) . Using this improved method for dimer analysis shows that at pH 5.5 there was 94% dimer formation. The finding of 40% dimer formation by Cavey

et al is consistent with dithranol decomposition at the higher pH value of 7.5 compared to pH 5.5. As pH is increased the fraction of ionised dithranol in solution also increases (see chapter 5) resulting in less dimer and more danthron production. It is not clear , however , why Cavey et al did not detect any danthron in solution.

Decomposition in surfactant solutions

Below c.m.c - Table 4.4 shows that in NaLS (0.02%) and Tw (0.003%) both in the presence and absence of Cu^{2+} the percentages of dimer and danthron formed was not altered relative to the situation in buffer alone. In CET (0.04%) dithranol decomposition resulted in a large increase (from ~4% to ~84%) in the amount of danthron formed , while dimer formation decreased from ~94% to ~14% . On the addition of Cu^{2+} the effect observed was similar to that seen in aqueous solutions in the absence of surfactant (see Fig 4.7 - 4.9). Using surface tension measurements [Moody & Lubwika ,(1992) , also see chapter 5] evidence was obtained for the possibility of a surface interaction between dithranol and cetrimide , particularly at pH 5.5. Formation of a dithranol-CET complex with dithranol present in the ionised form along with decomposition at the surface , where 0_2 is abundant would account for the large amount of danthron formed in the CET solution.

With regard to the kinetics , dithranol decomposition still followed first order kinetics both in the presence and absence of Cu^{2+} (see Figs 4.10 - 4.12). In

the absence of Cu^{2+} , decomposition rate constant for dithranol in 0.02% NaLS was 0.986 hr^{-1} (correlation coefficient 0.994) giving a half-life of 0.702 hours. In 0.003% Tw the rate was 1.08 hr^{-1} (correlation coefficient 0.996) giving a half-life of 0.642 hours , and in 0.04% CET the rate was 0.299 min^{-1} (correlation coefficient 0.989) giving a half-life of 2.31 minutes. On addition of Cu^{2+} the decomposition rate constant in NaLS was increased to 6.726 hr^{-1} (correlation coefficient 0.995) giving a half-life of 0.103 hours. In Tw the rate constant was increased to 8.668 hr^{-1} (correlation coefficient 0.998) giving a half-life of 0.080 hours. In CET it was not possible to follow the rate of dithranol decomposition with time , as dithranol decomposition was almost instantaneous on the addition of the Cu^{2+} ions.

Above c.m.c - Surfactant presence above the c.m.c was observed not to alter the percentages of dimer and danthron formed following complete decomposition of dithranol , this being similar to the observations below the c.m.c. Using unbuffered aqueous solutions of NaLS , Tw and CET at 25°C the following patterns regarding decomposition products were observed . In CET the ratio of dimer : danthron formed following complete decomposition of dithranol was 3:2 , while in both NaLS and Tw the ratio was 4:1 [Moody & Lubwika , (1992)] . The ratios of decomposition products formed in the unbuffered solutions cannot be directly compared to those obtained at pH 5.5 (present results) because of the pH influences on the molecular species of dithranol present (see chapter 5) , which in turn influences decomposition products formed.

The effect of Cu^{2+} on dithranol decomposition rate in surfactant solutions above their c.m.c is shown in Table 4.7. The results show that in 2% NaLS solutions Cu^{2+} addition resulted in dithranol decomposition rate being increased from 0.383 to 0.468 hr^{-1}. This increase in decomposition rate is smaller than that seen below the c.m.c where decomposition rate was increased by a factor of 6.82 compared to 1.22 above the c.m.c. The indications are that Cu^{2+} is less efficient as a catalyst of dithranol decomposition when dithranol is located in the non-polar interior of the NaLS micelles (see chapter 3). This situation bears some similarity to the observations made in methanol and aqueous buffer where it was seen that the catalytic coefficient of Cu^{2+} was greater for dithranol decomposition in the more polar aqueous buffer , compared to the relatively less polar methanol solutions.

In Tw solutions the addition of Cu^{2+} resulted in an increase in dithranol's decomposition rate by a factor of 3.86 which was about half the effect seen below the c.m.c where decomposition rate was increased by a factor of 8.02. Because of the similarity of the catalytic efficiency of Cu^{2+} above and below the c.m.c , the indications are that dithranol is in a relatively polar environment when solubilised in the micelles of Tw. This is in agreement (see Fig 4.15) with the observation that in the micellar environment of Tw the rate of dithranol decomposition was close to that observed in buffer alone. It also agrees with the assessment that solubilised molecules of dithranol in Tw micelles were located in the polyoxyethylene mantle (see chapter 3).

In CET solutions at concentrations below c.m.c (pH 5.5) it was observed that introduction of Cu^{2+} resulted in an extremely rapid decomposition of dithranol (reaction more than 90% complete inside a minute). Above the c.m.c the effect of Cu^{2+} was less dramatic. The decomposition rate of dithranol being increased by a factor of 4.73. Again the results indicate that the solubilization of dithranol , here in the micelles of CET , results in dithranol entering an environment where Cu^{2+} is less efficient a catalyst of dithranol decomposition.

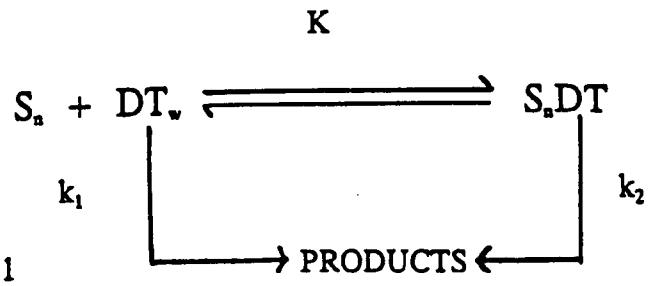
Investigating the effect of surfactant concentration on dithranol decomposition rates yielded the following results. For dithranol ($\sim$2.5μg /mL) in NaLS solutions when surfactant concentration was greater than 0.18% w/v the rate of decomposition of dithranol was observed to reduce with increasing surfactant concentration , while below this concentration increase in surfactant concentration resulted in an enhanced rate of decomposition (see Table 4.8 & Fig 4.13). A similar pattern was observed in CET solutions (pH 0.4) below and above 0.079% w/v (see Table 4.9 & Fig 4.14). In both surfactant solutions the concentration at which the change in the stability pattern of dithranol was observed is in close agreement with the literature c.m.c values quoted for these surfactants - NaLS (0.24% w/v) , CET (0.09% w/v). The results for Tw are shown in Table 4.10 and Fig 4.15. The plot of k_{obsd} vs Tw concentration is bell shaped , similar to what was obtained in NaLS (pH 5.5) and CET (pH 0.4). Unlike the case in NaLS and CET, there is no point on the graph where dithranol decomposed at a slower rate than that observed

in buffer alone The results show that in the presence of the micelles of Tw (Literature c.m.c value 0.014% w/v) dithranol decomposition was actually enhanced compared to buffer alone. It was also observed that above 0.5% w/v there was little further change in the observed rate of decomposition , with increasing surfactant concentration.

The ability of surfactants present in aqueous solutions to influence reaction rates was established many years ago. Fendler and Fendler (1975) give a comprehensive compilation of various aspects of micellar catalysis and inhibition covering a number of decades. A more recent review of this subject is given by De Oliveira and Chaimovich (1993). In accounting for the influences of surfactants on micellar reaction kinetics ,micellization and micellar structure have been discussed in terms of various models [Fisher and Oakenfull (1977)]. For kinetic work it is convenient to use the pseudophase model i.e to assume that a micelle can be regarded as a submicroscopic aggregate which behaves as a seperate reaction medium with its own properties [Shinoda et al (1963) , Schick (1967)]. Reaction can then be regarded as ocurring either in the aqueous or micellar pseudophases, with reactants being rapidly equilibrated beween the two pseudophases [Bunton et al (1968), Bunton and Robinson (1968), Menger and Portney (1967) , Fendler and Fendler 1970]. In the present studies decomposition of dithranol in the aqueous solutions investigated was observed to follow first order kinetics. Such kinetics are a characteristic of unimolecular reactions i.e those reactions where the rate determining step (RDS) involves only one molecule [Morrison and Boyd

(1983)]. In the case of dithranol the RDS is the formation of the anthranoyl free radical (see chapter 1, Fig 1.3). There are examples in the literature of a number of unimolecular reactions whose rates are changed by incorporation into a micelle [Fendler and Fendler (1975) , Bunton (1977)]. Since concentration of substrate in a micelle does not affect the first order rate constant for the reaction, the micellar rate effect upon unimolecular reactions must arise from the properties of the micelle as a submicroscopic reaction medium [Bunton (1977)]. For unimolecular reactions incorporation of substrate into micelles results in the substrate entering an environment which may favour or inhibit the reaction. In the case of dithranol , incorporation in the micelles of NaLS and CET resulted in prolonged half-lives showing that the micellar environment favoured dithranol stability , while in Tw the micellar environment caused an enhancement in rate of dithranol decomposition relative to that in water.

Use of the pseudophase model is convenient for the consideration of the influence of surfactants on micellar reaction kinetics. The relationship between the reaction rate constant and surfactant concentration can be treated very simply by considering the distribution of substrate between the aqueous and micellar pseudophases (scheme 4.1).



Scheme 4.1

In scheme 4.1 S_n = micellar surfactant , DT_w = dithranol in the aqueous phase , S_nDT = dithranol associated with the micelles.

The rate equation for scheme 4.1 is given by ;

$$\frac{-d(DT_w+S_nDT)}{dt} = \frac{d[DT]}{dt} = \frac{d[P]}{dt} \qquad \text{Eq. 4.2}$$

where $[DT_t]$ is the stoichiometric concentration of dithranol at time t. Starting with Eq.4.2 an equation , which allows the calculation of the rate of decomposition in the micelles and also the association constant for the binding of solubilised agent with the surfactant micelles relation can be derived [Fendler & Fendler,1975].

$$\frac{1}{k_1-k_{obsd}} = \frac{1}{k_1-k_2} + \frac{1}{k_1-k_2} \cdot \frac{N}{K(C_D-c.m.c)} \qquad \text{Eq. 4.3}$$

In Eq.4.3 k_1 = first order decomposition rate in absence of surfactant , k_2 = first order decomposition rate for solubilised dithranol, K = micelle association constant , C_D = total concentration of surfactant ($molL^{-1}$) , c.m.c = critical micelle concentration, N = aggregation number.

A plot of the left hand side of Eq. 4.3 vs 1 / (C_D - c.m.c) allows the calculation of k_2 and K. Eq. 4.3 has been used in the literature to obtain binding constants between micelles and a variety of drugs , mono-p-nitrophenyl

dodecanedioate [Menger and Portnoy (1967)] , p-substituted phenyl acetates [Bruice et al (1968)] , p-nitrophenyl diphenyl phosphate [Bunton and Robinson (1969a)] also 2,4-dinitrophenyl sulphate [Fendler et al (1970)]. Because of the fit of experimental data to equations such as Eq. 4.3 , its use is justified. One must be aware , however , of the approximations used in its derivations [Colter et al (1964)]. In deriving Eq. 4.3 it has been assumed that the substrate does not complex with monomeric surfactant , that the association has 1:1 stoichiometry , that the substrate does not significantly alter the micellization and the c.m.c . In NaLS (see Fig 4.16) the micellar decomposition rate constant was 0.157 hr^{-1} and K was 29.8 x N. In CET (pH 0.4 , see Fig 4.17) the micellar decomposition rate was 0.002 hr^{-1} and K was 60.80 x N. The values obtained for the association constants suggest that dithranol's association with NaLS micelles is less than with CET micelles.

The kinetic data for dithranol decomposition in Tw solutions (see Fig 4.15) shows that there was little change in dithranol's decomposition rate at concentrations above 0.5% .Eq. 4.3 , therefore , can not be applied in this case. The good fit , however , of the data for NaLS and CET to Eq. 4.3 means such equations can be used to predict the rate of decomposition of micellized dithranol in these surfactants at a given surfactant concentration , this being of some importance for formulation purposes. This ability to predict stability was tested by placing a known amount of dithranol in a solution of known micellar concentration and allowing decomposition to proceed for a selected time period . The amount of dithranol remaining is

assayed after this time period and compared to the calculated amount expected using Eq.4.3. Using NaLS of micellar concentration 0.38% (dithranol 5μg/mL) Eq.4.3 predicts a decomposition rate constant of 0.50 hr^{-1} giving a half-life of 1.39 hours. Allowing decomposition to proceed for 3.84 hours should have resulted in about 15% of initial dithranol remaining in solution. When done experimentally 13 $\pm$ 2% was found. In CET , 0.2% micellar concentration , 50% of initial dithranol was predicted would be present after 21.34 hours. Experimentally 46 $\pm$1.7% was found. In both surfactants stability predictions were reasonably accurate , experimental findings being within 5% of predicted amounts. In CET solutions when the pH is raised to 5.5 Eq 4.2 can not be used for stability predictions because of the interaction between the two at this pH. In using Eq.4.3 it is assumed that no interaction between surfactant monomers and the solubilizate occurs.

The dithranol stability modifications observed can be explained by considering the site of solubilization of dithranol (see chapter 3). Incorporation of dithranol into the hydrocarbon core of micelles is expected to result in a reduced rate of decomposition compared to that in water as dithranol is more stable in a non-polar environment. This is the observed effect with both NaLS and CET (at pH 0.4) and is in agreement with the evidence in chapter 3 for the site of solubilisation of dithranol in these two surfactants. In Tw it was shown that dithranol is located in a polar environment upon solubilisation (see chapter 3) . Since dithranol is unstable in polar environments the enhanced rate of decomposition observed following solubilisation is expected

, and is in agreement with the assessment of dithranol's environment in Tw micelles (see chapter 3).

After identifying isoascorbic acid as the most suitable antioxidant of the ones investigated , its effect on the stability of dithranol in the surfactant solutions was assessed. The results are shown in Table 4.11. The table shows that IAA had an effective stabilising influence in all three surfactant solutions. The half-life for dithranol decomposition was prolonged by factors of 30 , 19 and 4 in NaLS , Tw and CET respectively. Antioxidants such as isoascorbic acid act by providing electrons and easily avialable hydrogen atoms that are then accepted by free radicals (see proposed decomposition pathway) , this process stops the chain reaction [Martin et al , p385]. Inclusion of isoascorbic acid in the dithranol - surfactant solutions will therefore serve to maximise stability.

Using the stability data presented in Table 4.11 the shelf-life (taken as the time taken for 10% decomposition of initial dithranol to occur) for the dithranol - surfactant solutions was calculated as shown in the table. In a clinical setting the NaLS solutions would be preferred because of the longer shelf-life, the prepared solutions being useful for up to 12 hours after which they should be discarded. For the Tw and CET solutions the shorter shelf-lives would require that freshly prepared solutions were made each time the dithranol - surfactant solutions were needed.

For Tw solutions stored in the fridge (4 - 5°C) the shelf-life was prolonged

from 1.4 to 15 hours. At such temperatures NaLS and CET were observed to precipitate from solution , thus fridge storage would be disadvantageous.

Taken together the information gathered indicates that for the purpose of delivery of dithranol to the skin in aqueous solutions of surfactants , there can be no doubt that choice of surfactant and concentration will markedly affect the stability of dithranol. Based on the observed results the recommendations are:

(1) As high a surfactant concentration as possible be used both for stability and solubility reasons (see chapter3), the final concentrations used being kept below those where skin irritation is observed.

(2) Isoascorbic acid be added in order to maximise stability.

(3) For CET and Tw solutions stored at room temperature, freshly prepared solutions should be used. NaLS solutions should be used within 12 hours once prepared. Tw solutions stored in the fridge should be used within 15 hours. NaLS and CET solutions should not be stored in the fridge.

(4) With regard to pH of the solution , it should be as low as can be tolerated by the skin ,in particular with CET solutions , as this will prolong the shelf-life of the formulation. In CET solutions , though more dithranol will go into solution if the pH is increased , there is a problem with the stability

of such solutions and precautions such as antioxidant addition are necessary . Here , the reason for using solutions of pH 5.5 was because the intention was to simulate stability on the surface of the skin.

(5) The presence of metal ions and choice of surfactant , by changing the kinetics of the decomposition of dithranol , may well alter the antipsoriatic effect observed.

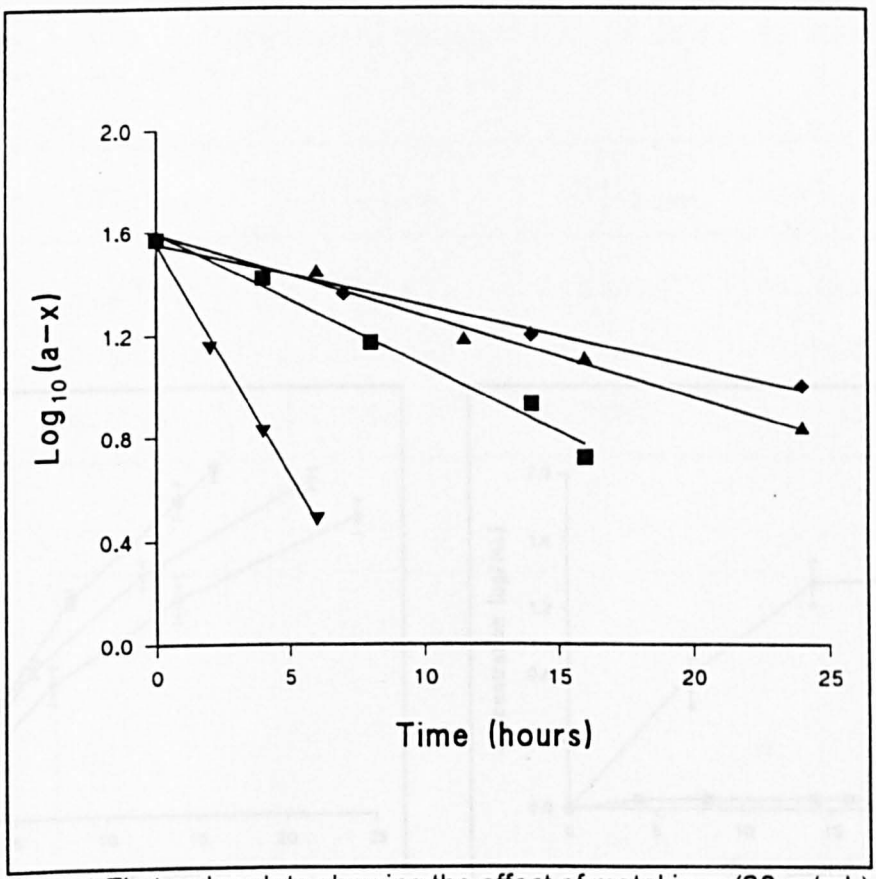


Fig 4.1 First order plots showing the effect of metal ions (20μg/mL) on the decomposition of dithranol (~40μg/mL) in methanol at 25°C. ♦ Metal ions absent, ▲ Zn^{2+} ,■ Fe^{2+} , ▼ Cu^{2+} .

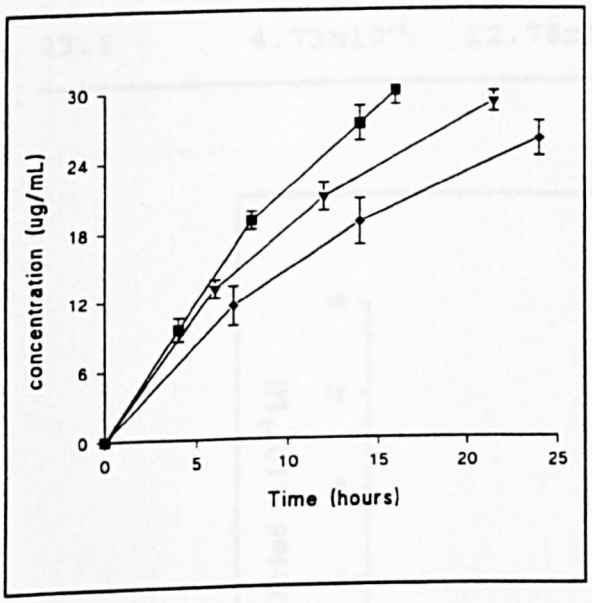


Fig. 4.2 Dimer formation following dithranol decomposition (~40μg/mL) in methanol at 25°C. ♦ Fe^{2+} absent, ■ Fe^{2+} 20μg/mL , ▼ Fe^{2+} 10μg/mL.

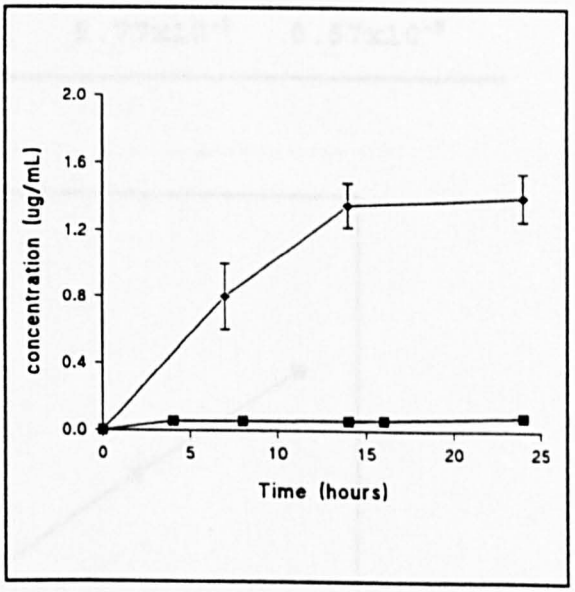


Fig 4.3 Danthron formation following dithranol decomposition (~40μg/ml) in methanol at 25°C.
♦ Fe^{2+} absent, ■ Fe^{2+} 10 & 20μg/mL

Table 4.1

Table showing the amount of dimer and danthron formed at different times following dithranol (1.76×10^{-4} M) decomposition in methanol at 25°C.

Time (hr)	$[DT]_{left}$	$[DT]_{reacted}$	$[DM]_{formed}$	$[DAN]_{formed}$
6.9	1.20×10^{-4}	5.60×10^{-5}	2.51×10^{-5}	0.15×10^{-5}
13.8	8.22×10^{-5}	9.38x10-5	4.14×10^{-5}	0.55×10^{-5}
23.8	4.73×10^{-5}	12.78×10^{-5}	5.77×10^{-5}	0.57×10^{-5}

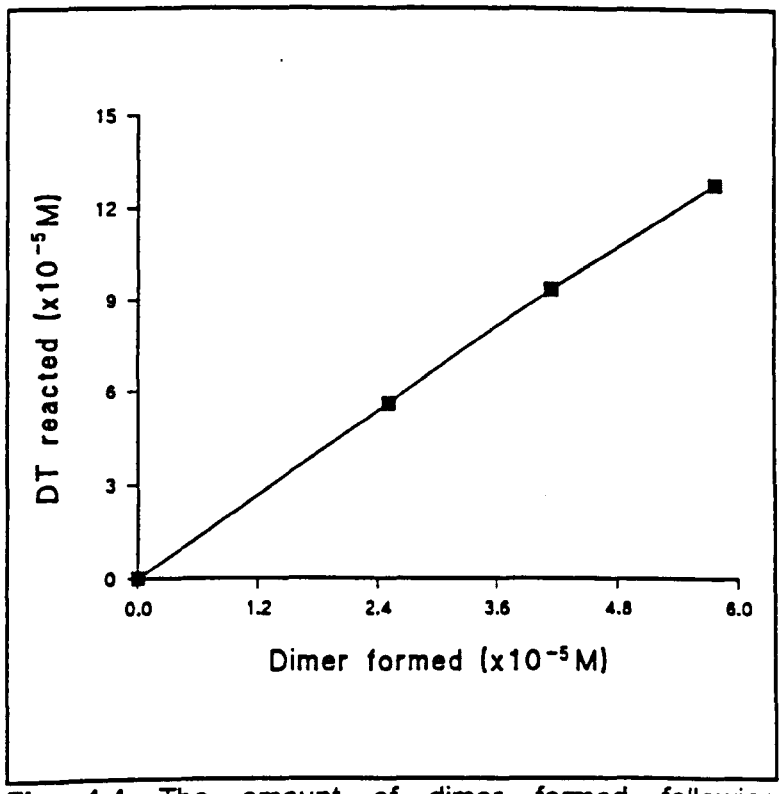


Fig 4.4 The amount of dimer formed following decomposition of dithranol(1.76×10^{-4} M)in methanol at 25°C.
DT = dithranol. **y = 2.2256(x) + 0.0296 (cc = 0.999)**

Table 4.2

Table showing the amount of dimer formed at different times following dithranol (1.76×10^{-4} M) decomposition in methanol at 25°C in the absence of any metal ions.

Time (hr)	$[DT]_{reacted} - [DAN]$	$[DM]_{formed}$	Ratio
6.9	5.45×10^{-5}	2.51×10^{-5}	2.2
13.8	8.88×10^{-5}	4.14×10^{-5}	2.1
23.8	12.30×10^{-5}	5.77×10^{-5}	2.1

Table 4.3

Table showing the amount of dimer formed at different times following dithranol (1.76×10^{-4} M) decomposition in methanol at 25°C in the presence of Fe^{3+} (3.58×10^{-4} M).

Time (hr)	$[DT]_{reacted}$	$[DM]_{formed}$	Ratio
4.1	6.70×10^{-5}	2.07×10^{-5}	3.2
8.2	10.86×10^{-5}	4.14×10^{-5}	2.6
14.4	14.33×10^{-5}	6.07×10^{-5}	2.4
16.3	14.99×10^{-5}	6.66×10^{-5}	2.3

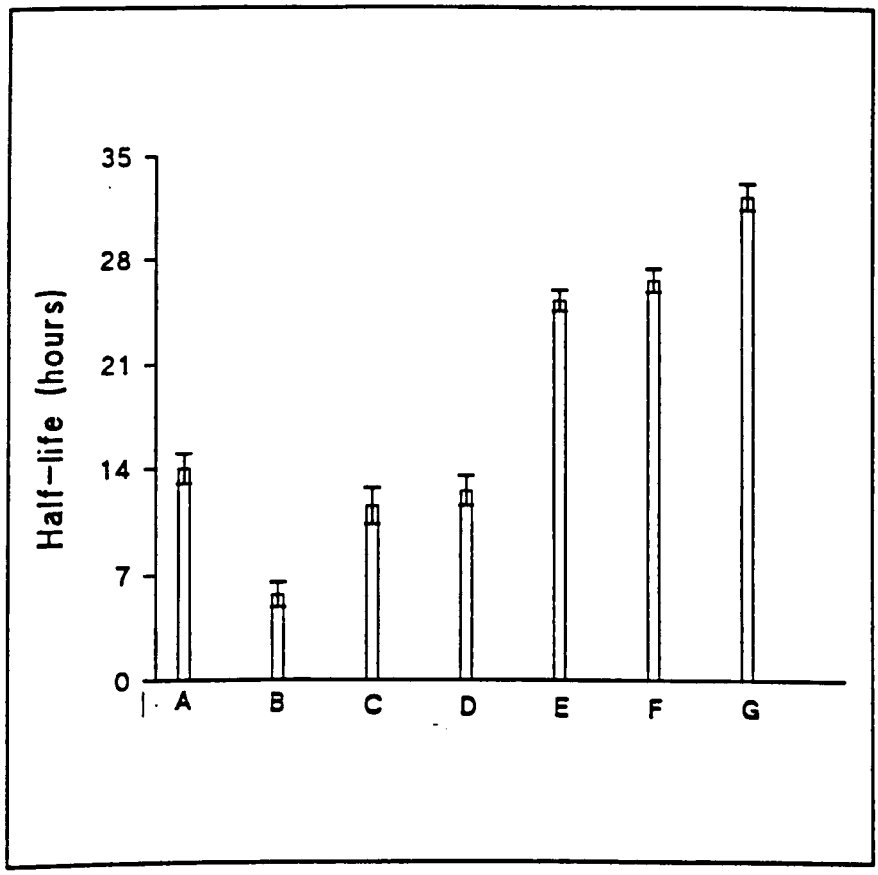


Fig 4.5
The effect of various antioxidants (0.5% w/v) on the half-life for the decomposition of dithranol (~40μg/mL) in the presence of Fe^{2+} (20μg/mL) in methanol at 25°C. Results are shown as mean ± SEM (n=3).

A = DT alone , B = DT + Fe^{2+} , C = citric acid , D = salicylic acid , E = ascorboyl-6-palmitate , F = ascorbic acid , G = isoascorbic acid.

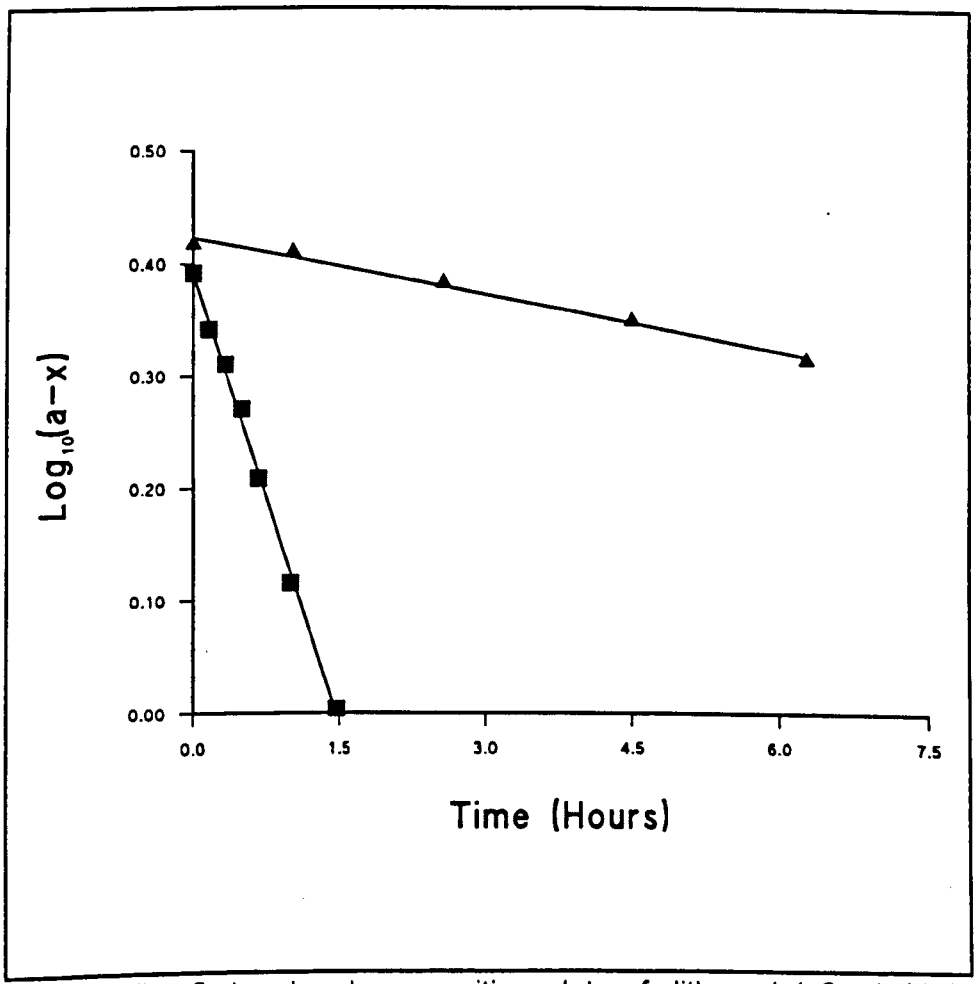


Fig 4.6 The first order decomposition plots of dithranol (~2μg/mL) in aqueous solutions at 25°C.
▲ 1M HCL (pH 0.4) , ■ buffer pH 5.5.

Table 4.4

The amounts of danthron and dimer formed from dithranol (2.5μg/mL) following complete decomposition in aqueous surfactant

	Cu2+ absent		Cu2+ present	
	Dimer	Dan	Dimer	Dan
Buffer pH 5.5	~94%	~4%	~94%	~4%
0.02% NaLS	~94%	~4%	~94	~4%
0.04% CET	~14%	~84%	~14%	~84%
0.003% Tw	~93%	~2.6%	~94%	~3%

Table 4.5

Table showing the amount of dimer formed following the decomposition of dithranol (~2.5μg/mL) in aqueous buffer pH 5.5 at 25°C.

Time (mins)	$[DT_{reacted}]$	$[Dimer_{formed}]$	Ratio
10	1.11×10^{-6}	0.67×10^{-6}	1.6
20	2.12×10^{-6}	1.02×10^{-6}	2.1
30	3.02×10^{-6}	1.09×10^{-6}	2.8
40	3.83×10^{-6}	1.42×10^{-6}	2.7
60	5.21×10^{-6}	1.82×10^{-6}	2.9
90	6.80×10^{-6}	2.51×10^{-6}	2.7

Table 4.6

Table showing the amount of dimer formed following the decomposition of dithranol (~2.5μg/mL) in aqueous buffer pH 5.5 at 25°C in the presence of Cu^{2+} (10μg/mL).

Time (mins)	$[DT_{reacted}]$	$[Dimer_{formed}]$	Ratio
3.6	5.7×10^{-6}	1.35×10^{-6}	4.2
7.2	8.11×10^{-6}	1.89×10^{-6}	4.3
13.5	10.13×10^{-6}	4.95×10^{-6}	2.0

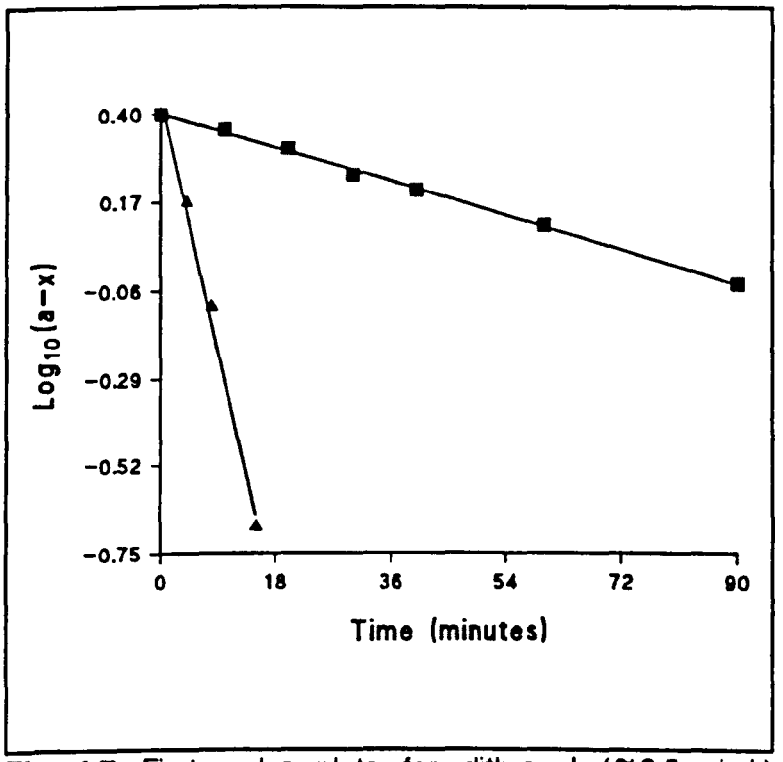


Fig 4.7 First order plots for dithranol (~2.5μg/mL) decomposition in aqueous buffer pH 5.5, at 25°C. ■ Cu^{2+} absent, ▲ Cu^{2+} present.

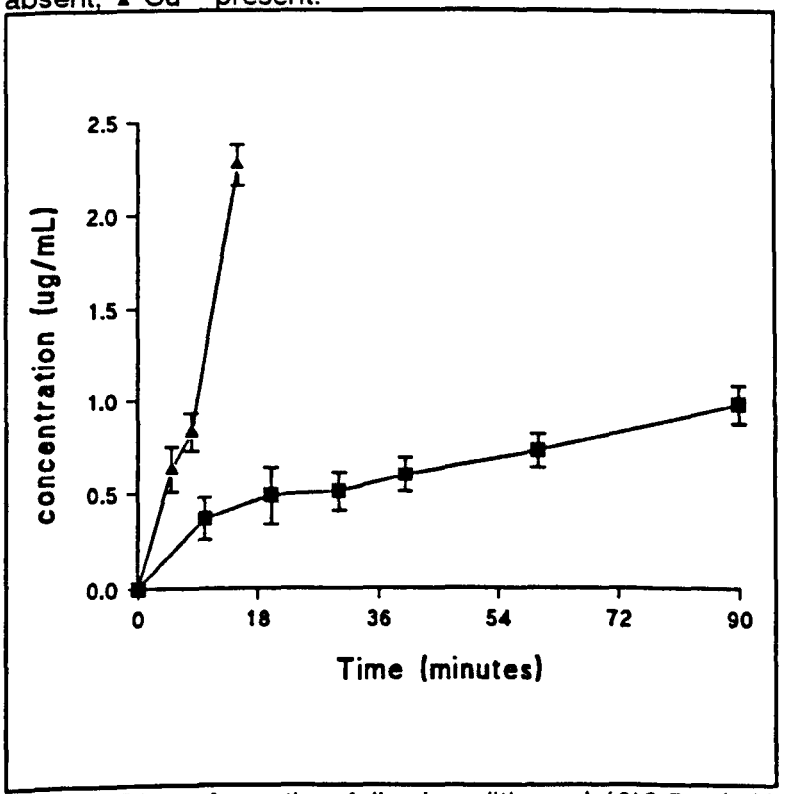


Fig 4.8 Dimer formation following dithranol (~2.5μg/mL) decomposition in aqueous buffer pH 5.5, at 25°C. Results are shown as mean ± SEM (n=3). ■ Cu^{2+} absent, ▲ Cu^{2+} present.

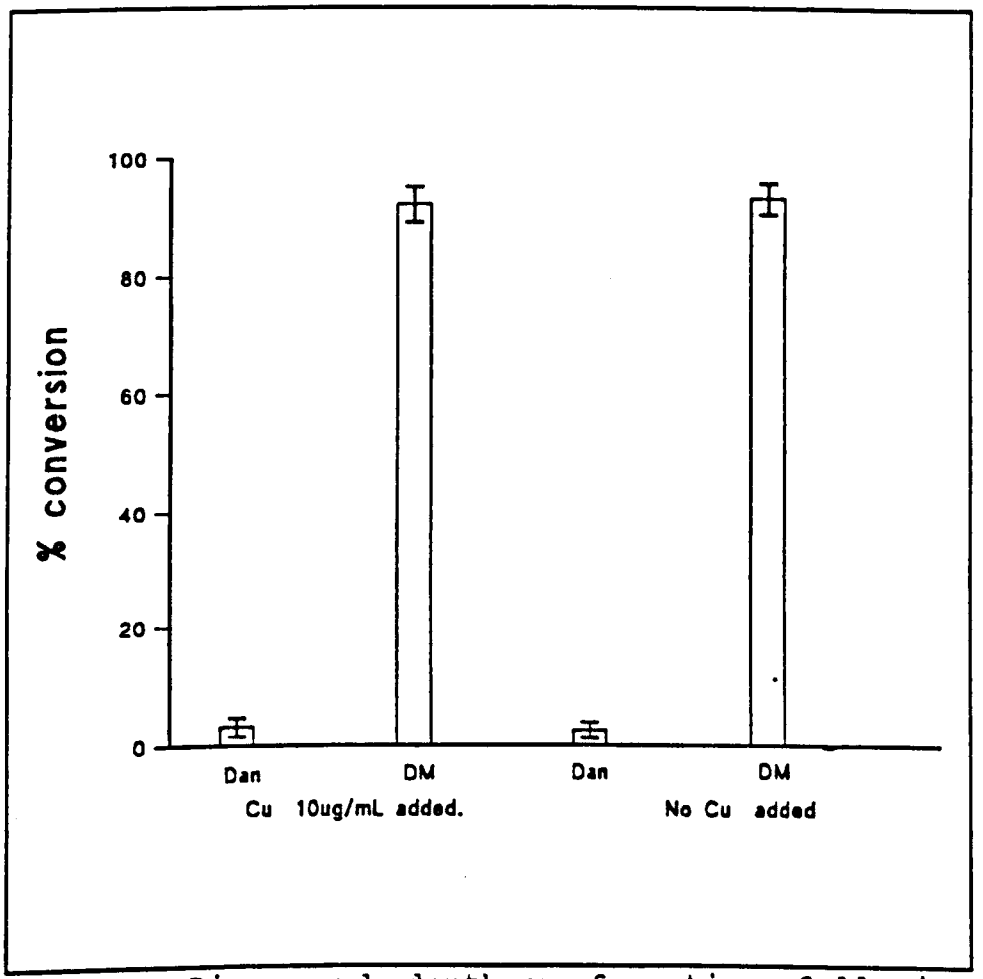


Fig 4.9 Dimer and danthron formation following complete decomposition of dithranol(~2.5μg/mL) in aqueous buffer pH 5.5 at 25°C. Results are shown as mean ± SEM (n=3). Dan =danthron, DM =dimer

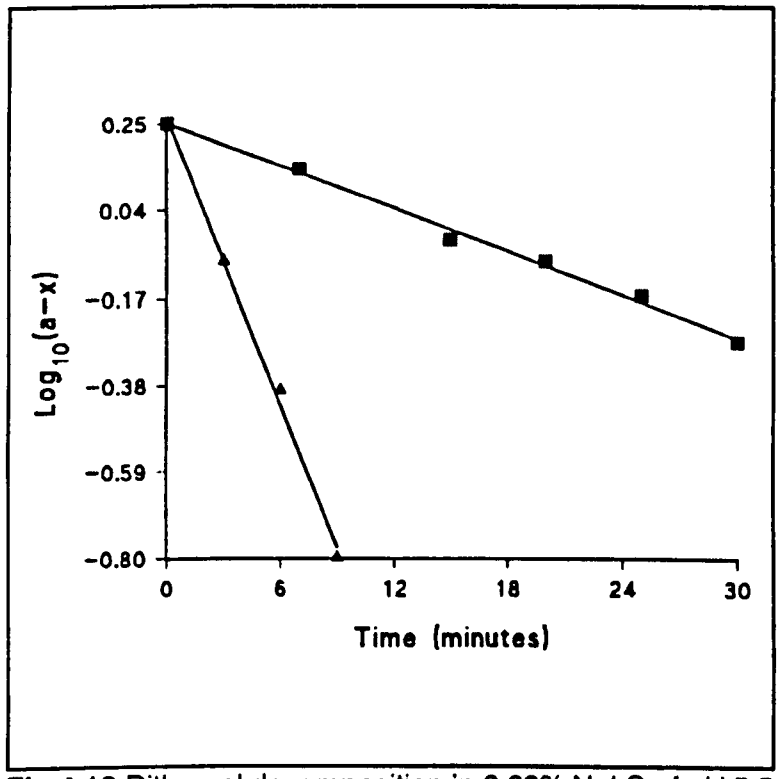


Fig 4.10 Dithranol decomposition in 0.02% NaLS of pH 5.5 at 25°C, ■ Cu^{2+} absent, ▲Cu^{2+} present.

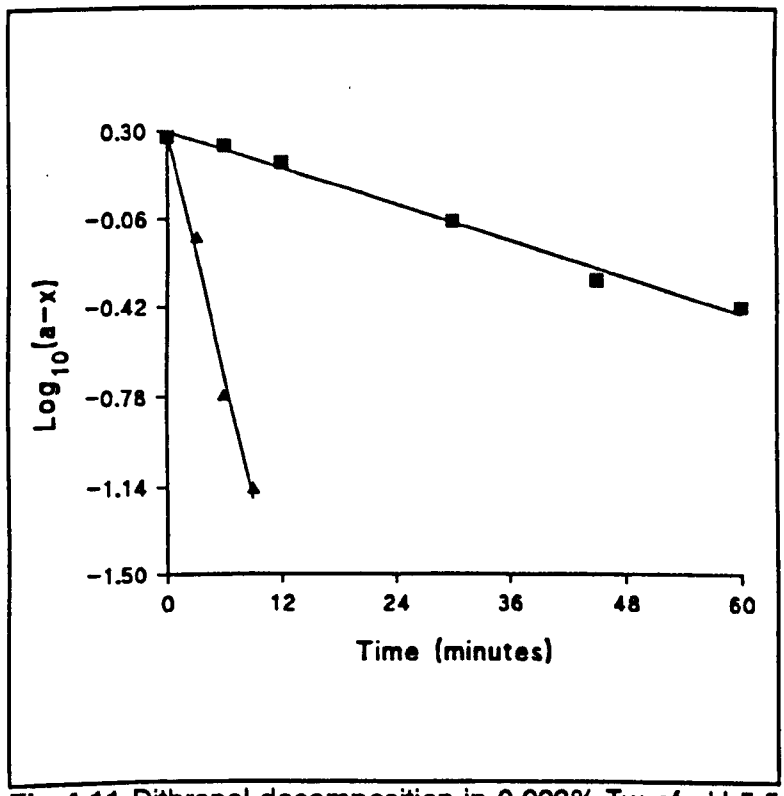


Fig 4.11 Dithranol decomposition in 0.003% Tw of pH 5.5 at 25°C, ■ Cu^{2+} absent, ▲Cu^{2+} present.

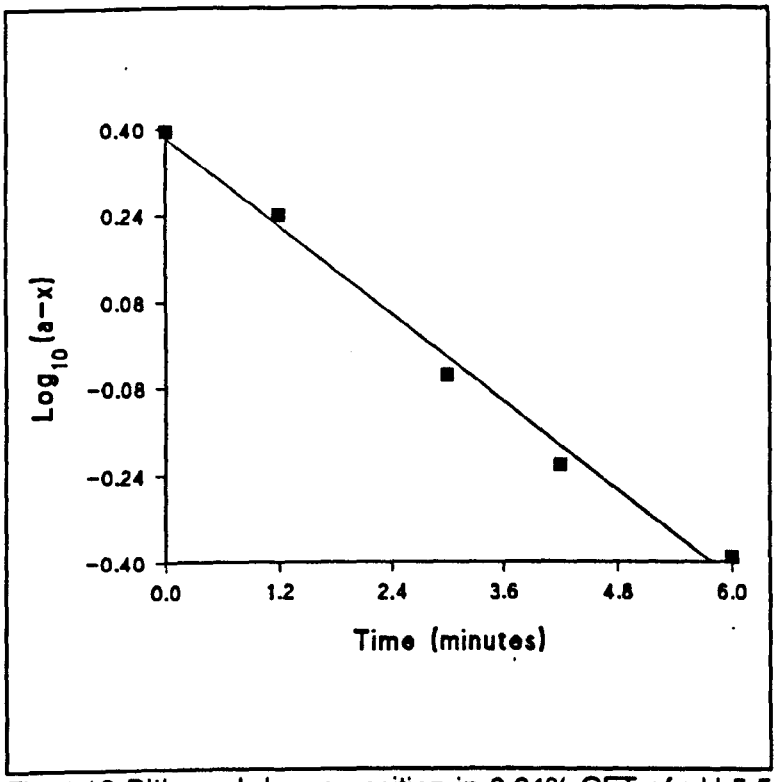


Fig 4.12 Dithranol decomposition in 0.04% CET of pH 5.5 at 25°C, with Cu^{2+} absent.

Table 4.7

The effect of Cu^{2+} on the rate of decomposition of dithranol (~25μg/ml) in 2% solutions (pH 5.5) at 25°C.

Surfactant	Half life (hr) No Cu^{2+}	Half life (hr) With Cu^{2+}
2% NaLS	2.01	1.48
2% CET	0.90	0.19
2% Tw	0.50	0.13

Table 4.8

The first order rate constants for dithranol decomposition in NaLS solutions pH 5.5 at 25°C

NaLS (%w/v)	K_{obsd} hours^{-1}
0.00	0.635
0.02	0.986
0.04	1.202
0.10	1.262
0.18	0.624
0.50	0.533
1.00	0.430

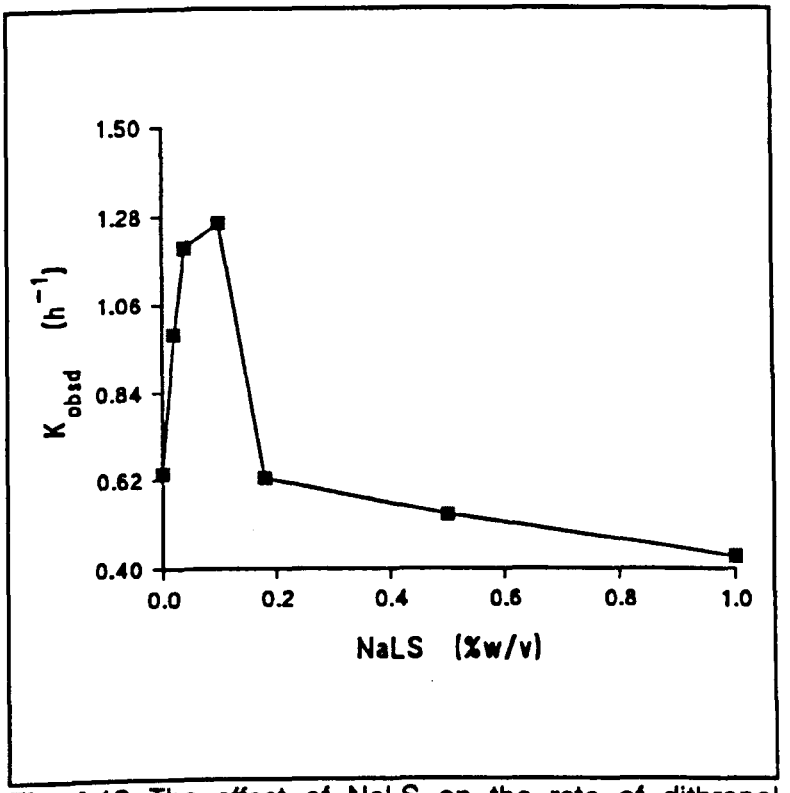


Fig 4.13 The effect of NaLS on the rate of dithranol decomposition in aqueous solutions of pH 5.5 at 25°C

Table 4.9

The first order rate constants for dithranol decomposition in CET solutions pH 0.4 at 25°C.

CET (% w/v)	$k_{obsd.}$ hours^{-1}
0.00	0.018
0.01	0.021
0.02	0.026
0.04	0.032
0.08	0.040
0.12	0.018
0.24	0.015
0.50	0.012

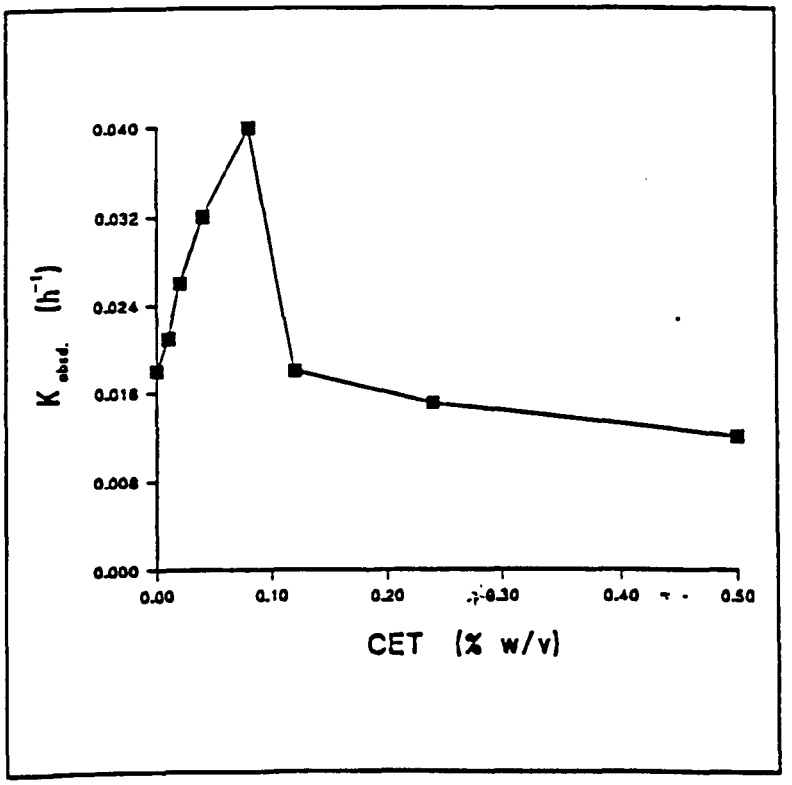


Fig 4.14 The effect of CET concentration on the rate of dithranol decompostion in aqueous solutions of pH 0.4 at 25°C.

Table 4.10

The first order rate constants for the decomposition of dithranol in Tw solutions pH 5.5 at 25°C.

Tw (% w/v)	$k_{obsd.}$ hours^{-1}
0.00	0.635
0.003	1.08
0.015	3.24
0.05	3.35
0.15	2.57
0.50	1.36
1.00	1.37

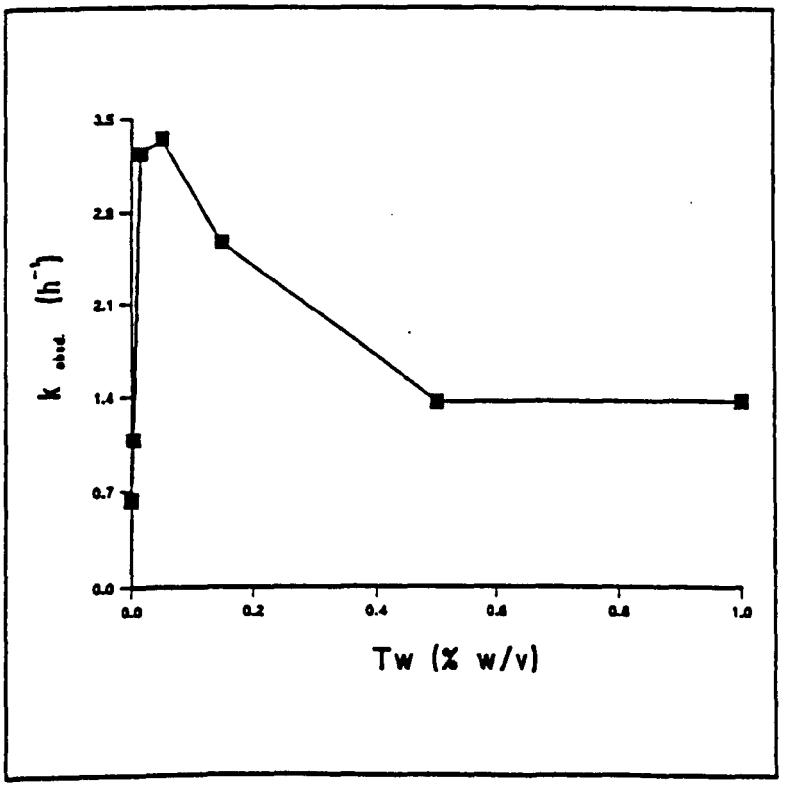


Fig 4.15 The effect of Tw on the rate of dithranol decomposition in aqueous solutions of pH 5.5 at 25°C

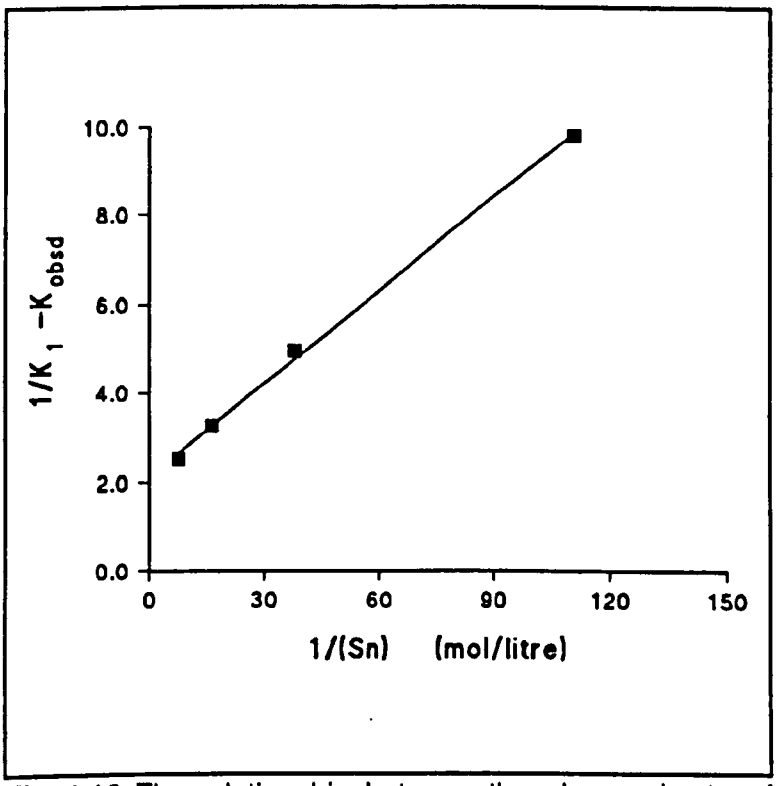


Fig 4.16 The relationship between the observed rate of dithranol decomposition and NaLS concentration (above c.m.c.) in aqueous solutions of pH 5.5 at 25 °C.

Correlation coefficient (r^2) = 0.998. See Eq4.3 and accompanying text.

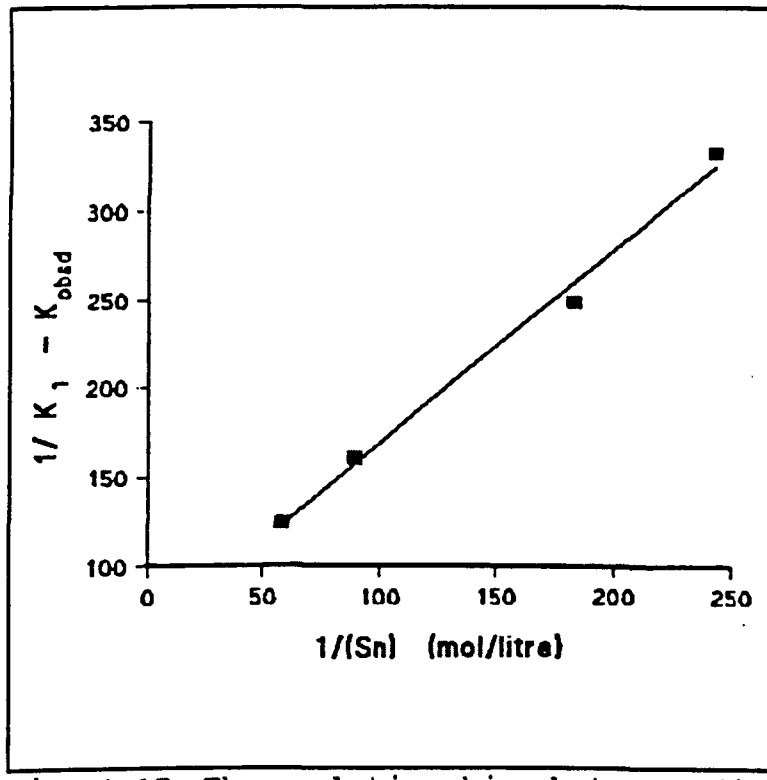


Fig 4.17 The relationship between the observed rate of dithranol decomposition and CET concentration (above c.m.c) in aqueous solutions of pH0.4 at 25°C.

Correlation coefficient (r2) = 0.992. See Eq4.3 and accompanying text.

Table 4.11 The decomposition rate characteristics of dithranol in surfactant solutions of pH 5.5 at 25°C.

	$T_{0.5}$(hours) IAA absent	$T_{0.5}$(hours) 0.5% IAA present	k (hr-1) 0.5% IAA	SL(hours) 0.5% IAA
4% NaLS	2.9 ± 0.5	86 ± 0.7	0.008	12.5
2% CET	0.9 ± 0.2	4.1 ± 0.3	0.171	0.6
4% Tw	0.5 ± 0.1	9.6 ± 0.3	0.072	1.4

The results are presented as mean ± SEM (n=3). IAA = isoascorbic acid. SL = Shelf life , which is taken as the time for 10% decomposition of initial dithranol to occur.

CHAPTER 5

THE EFFECT OF SURFACTANTS ON THE IONISATION BEHAVIOUR OF DITHRANOL IN AQUEOUS SOLUTIONS.

5.1 Introduction

Dithranol is a weak acid. It's ionic equilibrium in solution is influenced by factors such as pH changes and the presence of other ionic species. The UV absorption spectra for the ionised and non-ionised form of dithranol are markedly different [Upadrashta and Wurster (1988)]. This property of the molecule can be used to monitor the relative amounts of ionised and non-ionised dithranol in solution. This can also be achieved by fluorescence measurements , by exploitation of the fact that of the two forms only the ionised species is fluorescent [Melo et al (1983)].

Upadrashta and Wurster (1988) studied the ionisation behaviour of dithranol in aqueous buffers varying in pH from 5.10 to 11.74 . Using absorbance measurements at 258nm they determined the pKa to be 9.06 at 25°C. Despite the presence of three ionisable phenol groups no spectroscopic evidence for the second and third ionisation reactions were observed.

In this study surfactants will be used as a means of enhancing the water solubility of dithranol (see chapter 3). It is therefore desirable to investigate any effect that the surfactants to be used will have on the ionic equilibrium of dithranol in aqueous solutions. This will be done using both absorbance and fluorescence measurements. Surface tension measurements will be made to ascertain any changes in surface activity caused by possible intermolecular reactions between the surfactants and dithranol.

5.2 Methods

5.2.1 MATERIALS and EQUIPMENT

Chemicals and reagents

Glycine , (Fisons Scientific Equipment) Sodium hydroxide 1M (May & Baker Laboratory Products). All other chemicals as in 2.2.1

Equipment

UV absorbance measurements were made using a Perkin Elmer Lambda 15 UV/Visible Spectrophotometer. All other equipment is as described in 2.2.1

5.2.2 Ionic equilibrium of dithranol in aqueous solutions

5.2.2.1 pKa determination using absorbance measurements

Dithranol solutions (2.73μg/mL) were prepared in 0.02 M glycine -NaOH buffer of varying pH (8.1 - 9.4), and the absorption spectra, with appropriate blanks placed in the reference beam , obtained. 1cm cells thermostatted at 25°C , were used. Maximum absorbance for the concentration of dithranol used was obtained using buffer pH 11. Minimum absorbance (i.e absorbance of dithranol when fully non-ionised) was obtained using pH 1.

5.2.2.2 pKa determination using fluorescence measurements

The method of Moody & Lubwika , 1993 was followed. 3mL of 0.02M glycine - NaOH buffer (pH 6.9 - 9.5) were pipetted into 1cm fluorescence cells thermostatted at 25°C . Using a 100 μL adjustable pipette , 30μL of buffer was removed and replaced with 30μL of a 1μg/mL solution of dithranol in acidified methanol. The final concentration of dithranol was therefore in the region of 10ng/mL. Solution preparation was done in the fluorescence cell , to enable a fluorescence reading to be taken as soon as a solution was prepared. The excitation and emission wavelengths were set at 390nm and 518nm respectively. The maximum fluorescence for the concentration of dithranol used was obtained using glycine - NaOH buffer pH 10.5. 0.1% isoascorbic was added to stabilise the signal.

5.2.3 The effect of surfactants on the dissociation of dithranol in aqueous solutions.

5.2.3.1 Cetrimide.

The effect of pH and CET concentration on the absorbance at 258nm.

3mL of 0.2M sodium acetate - HCl buffer pH 5.5 were transferred to 1cm UV absorbance cells. Gradually increasing concentrations of CET (0 - 0.5 % w/v) were prepared directly in the cells. Dithranol was added to these solutions by removing 30μL of buffer from the absorbance cell and replacing

it with 30μL of a 430μg/mL solution of dithranol in acidified methanol. Thus the final concentration of dithranol in each CET solution was 4.3μg/mL. Using appropriate blanks absorption spectra were then obtained. This procedure was then repeated in the range 0 - 0.5% w/v CET at pH 4.6 and in 1M HCl (pH 0.4).

The effect of change in the dithranol concentration on the absorption spectrum and the absorbance at 258nm in the presence of a fixed excess of CET (0.2% w/v).

2mL of 0.2% CET in 0.2M acetate buffer pH 5.5 were transferred to a 0.5 cm UV absorbance cell. Gradually increasing amounts of dithranol were then added directly to the cell. The dithranol was added by removing a volume of the 0.2% CET solution, using a graduated pipette , and replacing this with the same volume of a 500μg/mL solution of dithranol in methanol. The solutions were such that the final concentration of dithranol was 5.0 , 7.5 , 10.0 ,12.5, and 15.0μg/mL. The absorption spectra were then recorded.

The effect of pH and CET concentration on the measured fluorescence intensity using an excitation wavelength of 390nm and emission wavelength of 518nm

For fluorescence measurements the CET concentration range used was 0 - 0.16 % w/v. Using a 1μg/mL dithranol in methanol solution, dithranol was added to each CET solution as per the procedure described above for absorbance investigations to give a final concentration of 10ng/mL dithranol in each solution. Fluorescence measurements were done at pH 5.5 and pH 4.6.

5.2.3.2 Tw and NaLS

The effect of pH and CET concentration on the absorption spectra of dithranol in Tw and NaLS.

Tw solutions covering the concentration range 0 - 0.3% were prepared in 0.2M acetate buffer pH 5.5 and in 0.02M glycine buffer pH 8 and 10. Dithranol was added to each of the Tw solutions as described for the absorption measurements for CET the final concentration of dithranol in each solution being 4.3μg/mL. The absorbance spectra of these solutions was then recorded. The procedure was repeated using NaLS (0 - 0.5%) in glycine buffer pH 10.

5.2.3.3 The dissociation constant determination for dithranol in the presence of an excess of CET

Using UV absorbance measurements.

0.2M acetate buffer solutions pH 4.55 - 5.50 were prepared. Each buffer solution contained 0.2% w/v CET. 3mL of each buffer prepared was transferred to a 1cm UV absorbance cell and dithranol added to the absorbance cell. Dithranol (4.3μg/mL) was added as per 5.2.3.1 (the effect of pH and CET concentration on the absorbance at 258nm). The absorbance spectra of the solutions were then recorded. Maximum absorbance was determined using pH 11.

Using fluorescence measurements

0.2M acetate buffer solutions (pH 4.55 -5.50) at 25°C were prepared as described above. After transfer of 3ml of each of the buffer solutions into a 1cm fluorescence cell, 60μL was removed and replaced with 30μL of a 4% CET solution in water, the solution was mixed by inversion of the contents in the cell after which 30μL of a 1μg/mL solution of dithranol in acidified methanol was added and the fluorescence reading obtained. The final concentration of CET , therefore , was 0.04%. Maximum fluorescence was determined using buffer pH 5.7 containing 0.3% CET with 0.1% isoascorbic acid added to stabilise the fluorescence signal.

5.2.4 Surface tension measurements at pH 5.5 and pH 0.4

These were performed using a White torsion balance. The value recorded was the mean of three replicate samples. Measurements were done at room temperature (21 $\pm$1°C)

CET solutions (10mL) covering the concentration range 0 - 0.02% were prepared in 0.2M acetate buffer pH 5.5 . The surface tension of these solutions was recorded. Using an accurately prepared concentrated solution of dithranol ($\sim$860μg/mL) in acetone 50μL was added to each of the CET solutions to give a final concentration of dithranol in each of about 4.3μg/mL.

For the measurements at pH 0.4 the procedure described above was repeated

using 1M HCl (pH 0.4).

5.3 Results/Discussion

Dithranol is a weak organic acid. It's pKa in aqueous conditions at 25°C was determined as 9.06 by Upadrashta and Wurster (1988), who used UV absorbance measurements at 258nm of dithranol in aqueous buffers. Using the data presented in Table 5.1 and the equations outlined by Upadrashta and Wurster (1988), graphical determination of pKa (see Fig 5.1) gave a value of 9.12 ± 0.10 (n=3) , which is in good agreement with the value obtained by Upadrashta and Wurster.

As with absorbance measurements the relative amounts of ionised and non-ionised dithranol at a particular pH can be determined by measuring the fluorescence at the given pH. The pKa can be determined using the appropriate form of the Henderson - Hasselbach equation

$$pH = pKa + Log\frac{F_{obsd}}{F_{max} - F_{obsd}} \quad (constant\ temperature) \qquad \text{Eq. 5.1}$$

where F_{obsd} = % fluorescence intensity at a given pH

F_{max} = % fluoresence intensity of fully ionised dithranol.

Eq. 5.1 shows that a plot of pH v Log $F_{obsd} / F_{max} - F_{obsd}$ is a straight line. This indicates a direct relationship between the pH of the solution and the ratio of ionised to non-ionised dithranol. The equation also shows that when the Log of the ratio is zero , pH = pKa , this, therefore , allows the graphical determination of pKa. Using a fixed concentration of dithranol (10ng/mL) the data presented in Table 5.2 was obtained. Using this data and equation 5.1 graphical determination of the pKa (see Fig 5.2) gave a value of 8.64 $\pm$ 0.26 (n=3). Though measurement of fluoresence has the advantage of being specific for the ionised form only and is about 100 times more sensitive than UV absorbance measurement , there is the problem of signal instability , especially at high pH. Since this method depends on the acurracy of the F_{max} value, any error in it will lead to inaccuracies in the pKa determination. This would contribute to the statistically significant difference ($p < 0.05$) in the pKa values obtained using the two methods. The pKa determined using absorbance is more reliable because the absorbance of ionised dithranol is relatively more stable than the fluoresence arising from it.

The effect of surfactants on the ionic equilibrium of aqueous dithranol

In Fig 5.3 the effect of CET concentration (0 - 0.02% w/v) on the absorbance spectrum of dithranol (~4.3μg/mL) at pH 5.5 is shown. On the addition of CET up till 0.02% w/v a linear relationship is observed between the absorbance at 258nm and 388nm and CET concentration. When CET is increased beyond 0.02% loss of linearity is seen with a gradual increase to

a plateau region. The effect observed was pH dependent , the lower the pH the less the absorbance seen (see Fig 5.4). By comparing the gradients of the linear portions of the absorbance vs CET concentration plots (see Fig 5.5) it can be seen that the absorption capacity of the ionised dithranol at 388nm is about half that seen at 258nm. Hence for quantitative determination of the ionised form measurement at 258nm would be preferred. The UV spectra obtained when the above procedure was carried out in 1M HCl are shown in chapter 3 (see Fig 3.10). Unlike the results seen at pH 5.5 , there was no increase in the peak at 258nm with increasing CET concentration. This is a clear indication that the ionisation reaction was effectively suppressed. What was observed instead was a gradual change in the spectrum from one lacking in spectral detail to the type obtained in chloroform .The change being associated with the solubilisation of dithranol (see chapter 3).

On monitoring the effect of CET using fluorescence a similar pattern to that obtained using absorbance measurements was observed. Gradually increasing the concentration of CET while keeping pH and dithranol concentration constant resulted in fluorescence gradually increasing to a plateau region (see Fig 5.6).

Fixing the pH at pH 5.5 , and the CET concentration at 0.2% w/v (a concentration in the plateau region of the absorbance v CET concentration plot) and adding increasing amounts of dithranol to such solutions resulted in the absorbance values shown in Table 5.3. A plot of the absorbance (258nm)

v dithranol concentration shows that the relationship between the two was linear (correlation coefficient 0.998). The gradient of this plot gives the Beer Lambert Law constant which was calculated to be 4.68×10^4 mol^{-1} dm^3 cm^{-1}. Using the data in Table 5.1 (i.e A = 0.642, for dithranol 2.73μg/mL at pH 11) the Beer Lambert Law constant for fully ionised dithranol was calculated as 5.3×10^4 mol^{-1} dm^3 cm^{-1}. Comparing the two values of the Beer lambert law constant shows that in 0.2% CET at pH 5.5 dithranol is ~88% ionised. According to Eq.5.7 if pH is fixed , then pK′ and the log ratio will also be fixed. Since dithranol is ~88% ionised a value of 4.63 is obtained for pK′. This value agrees well with 4.68 found when a fixed concentration of dithranol was placed in 0.2% CET solutions of varying pH (see later).

Both absorbance and fluorescence measurements showed that dithranol was being made to ionise at pH values much lower than pH 9 when CET was introduced into solution. The indications are that there is an interaction between the two molecules which results in an increase in the acid strength of dithranol. The shape of both the absorbance (see Fig 5.4) and fluorescence (see Fig 5.6) profiles shows that the interaction between the two molecules rises to a maximum before reaching a pH dependent plateau region, where the reaction becomes independent of the amount of CET present. This is an indication that the reaction is at equilibrium for that pH. Since the acid strength of the complex is greater than that for dithranol, as the pH rises so the complex ionisation will increase. This is the reason why the plateau regions with both the absorbance and fluorescence measurements increase as

pH is increased (see Fig 5.4 and Fig 5.6).

The dissociation of dithranol as shown above is influenced by CET presence. By carrying out pKa determination of dithranol in the presence of an excess of CET i.e a CET concentration where the dithranol-CET interaction is independent of CET concentration, the pK′ value can be obtained. Using 0.2% w/v CET and a fixed concentration of dithranol (4.4μg/mL) the data presented in Table 5.4 was obtained. Using such data a pK′ value of 4.68 $\pm$ 0.09 (n=3) was determined. Fluorescence measurements were also used for pK′ determination. Using 0.04% w/v CET and dithranol 10ng/mL the data in Table 5.5 was obtained. Using such data pK′ was determined as 5.01 $\pm$ 0.13 (n=3). The two methods give rise to two significantly different ($p > 0.05$) pK′ values. As previously outlined the reliability of the fluorescence method depends on the accuracy of the F_{max} determination, which itself is prone to error because of fluorescence signal instability. Using absorbance measurements an almost identical pK′ value was obtained despite two different approaches being undertaken. Keeping pH and CET concentration constant and varying that of dithranol lead to a pK′ estimate of 4.63 and keeping CET and dithranol concentration constant and varying pH lead to a pK′ estimate of 4.68.

Based on these results it is postulated that the relationship between dithranol and CET may be represented as;

$$[DT] + [CET] \underset{\text{(constant temperature)}}{\rightleftharpoons} [CET\text{-}DT] + [H+] \quad \text{Eq.5.2}$$

$$K = \frac{[CET\text{-}DT]\,[H^+]}{[CET]\,[DT]} \; (constant temperature) \quad \text{Eq. 5.3}$$

The equilibrium equations 5.2 and 5.3 show that increasing either dithranol or CET concentration will drive the equilibrium to the right i.e promote complexation and ionisation of dithranol. The equations also show that increasing H^+ will drive the equilibrium to the left i.e suppress complexation and ionisation . Reducing H^+, it is predicted will promote complexation formation and of ionisation of dithranol. In the presence of a fixed concentration of CET the equilibrium equation 5.3 can modified to that shown in Eq.5.4 which can be further modified , through the steps indicated, to the equation shown in Eq 5.7.

at constant [CET] ,

$$K' = K[CET] = \frac{[CET\text{-}DT]\,[H^+]}{[DT]} \quad \text{Eq. 5.4}$$

$$[H^+] = \frac{K'[DT]}{[CET\text{-}DT]} \quad \text{Eq. 5.5}$$

$$Log[H^+] = LogK' + \frac{Log[DT]}{[CET\text{-}DT]} \quad \text{Eq. 5.6}$$

$$pH = pK' + \frac{Log[CET-DT]}{[DT]} \qquad \text{Eq. 5.7}$$

[CET-DT] = ionised dithranol (complexed)

[DT] = non-ionised dithranol

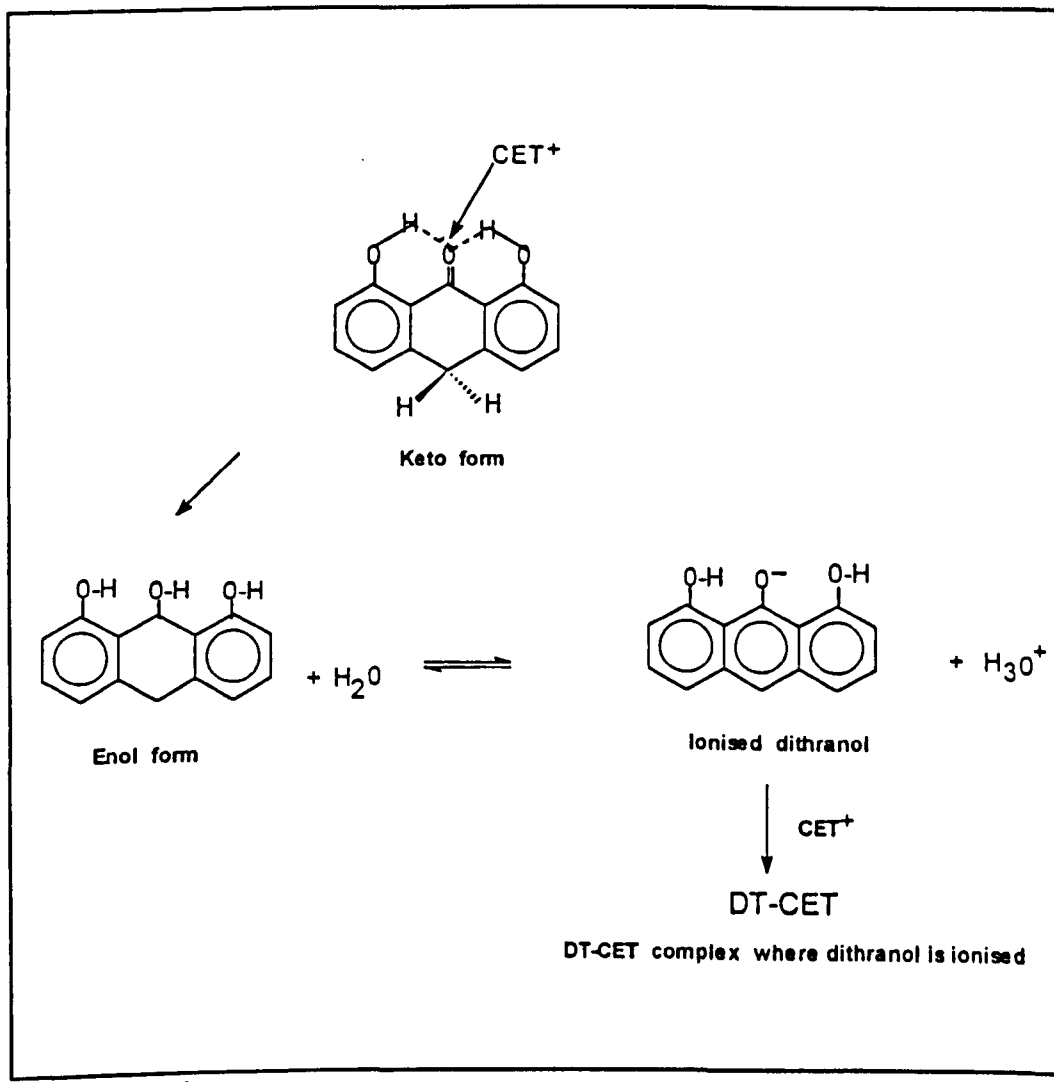
pK′ = apparent dissociation constant

Eq. 5.7 shows that a plot of pH v Log [CET-DT] /[DT] should be a straight line, which allows the graphical determination of pK′ (cf pKa determination using Eq.5.1). The pK′ value obtained in an excess of CET was 4.68 and 5.01 using absorbance and fluorescence respectively. The pK′ value obtained using absorbance measurements is the more reliable result because, as already explained , the chance of error in the pk' determination using fluorescence is greater. What both results indicate clearly , though , is that in the presence of CET the acid strength of dithranol is considerably increased, and ionisation is occuring 4-5 pH units lower than the values seen in aqueous solutions without CET present. The implications therefore are that for the pH 5.5 surfactant solutions to be applied to the skin, dithranol in the CET formulation will be ~88% ionised (calculated using Eq.5.7 and pK′= 4.68).

From the above it can be postulated as to how the presence of CET in solution leads to the appearance of ionised dithranol. While it is known that the keto form is the predominant form in chloroform , acetone , dimethylsulphoxide, and pyridine the exact nature of the tautomer in aqueous solution remains ambiguous [Holder & Upadrashta , 1992]. Because of the overall stability of the keto form compared to the enol form it is likely that this is the predominant form in aqueous solutions. It is possible that CET and dithranol molecules come together by a hydrophobic interaction and because of the positive charge carried by CET molecules , the hydrogen bonding responsible for the stability of the keto form would be interfered with. The consequence of this would be a hydrophobically bonded dithranol-CET complex in which dithranol was a stronger acid because of being present in the enol form. Water molecules (or a stronger base e.g OH^-) can then deprotonate the unstable enol form leading to the formation of ionised dithranol [Melo et al , (1983)]. This sequence of events (see scheme 5.1) would be a plausible explanation as to how CET causes the ionisation of dithranol in aqueous solution.

It has previously been shown that surface activity was most marked for CET-dithranol solutions , compared to NaLS-dithranol and Tw-dithranol [Moody & Lubwika, 1992]. A possible explanation for these observations can now be advanced. In scheme 5.1 it is proposed that a dithranol-CET complex is formed. Such a complex would be relatively large thus being able to reduce the surface tension of water to a greater extent than CET molecules alone.

Since it has been shown that the dithranol-CET interaction is pH sensitive, being suppressed at low pH and promoted as pH increases , it was anticipated that the surface activity of such mixtures should be pH sensitive. The results in Fig 5.7 shows that this was indeed the case. At pH 5.5 surface tension measurements of CET solutions done with and without a fixed amount of



Scheme 5.1
The possible interaction between CET and dithranol in aqueous conditions leading to the formation of ionised dithranol.

dithranol (~4.3μg/mL) present show that the extent to which the surface tension of water was reduced was greater in the presence of dithranol. At pH 0.4 (see Fig 5.8) there was little difference between the solutions with and without dithranol. It was also observed that at pH 5.5 there was a reduction in the c.m.c of dithranol-CET solutions (see Fig 5.7). In the absence of CET c.m.c was 0.016% (21 $\pm$1°C) whereas in the presence of CET the c.m.c was reduced to 0.011% (21 $\pm$1°C). This shift in c.m.c is consistent with the idea of a large complex being formed. The observed c.m.c is smaller because less of the large complex would be required to cover the surface of the water compared to the situation at pH 0.4 , where because the dithranol-CET complex is not formed more CET monomers are required to cover the water surface.

In NaLS solutions at pH 5.5 , containing a fixed concentration of dithranol (4.3μg/mL), there was no evidence for an interaction between NaLS and dithranol similar to that seen with CET , though evidence for solubilisation is obtained (see chapter 3). NaLS molecules being negatively charged , it is not all together surprising that no ionising interaction was observed. To investigate the interaction of ionised dithranol with NaLS , the pH was increased to pH 10 , which means that there is > 90% ionisation of all dithranol molecules present. At pH 10 it was observed that there was a reduction in the absorbance at 258nm as NaLS concentration increased. Plotting the absorbance at 258nm against the NaLS concentration gives a curve typical of solubilisation (see Fig 5.9). The indications are that solubilisation is

altering the extent of ionisation , the explanation for this being that ionised dithranol enters the less polar hydocarbon core of the micelles , where some of the ionised molecules convert to the non-ionised form thus the drop in the ionisation peak.

In Tw solutions of pH 5.5 there was no UV spectrophotometric evidence to suggest an interaction with dithranol similar to that observed with CET. In order to investigate the effect of the presence of Tw on ionised dithranol the pH was increased to pH 10. It was observed that introduction of Tw lead to very unstable preparations. It was therefore decided to use solutions of pH 8. Increasing concentrations of Tw were observed to cause a gradual shift to the longer wavelengths of the lamda max (see Table 5.6) of the peak in the 350nm region. This shift in the lamda max is indicative of change in the environment of the dithranol molecules. The environment is becoming less polar, thus the UV spectra tend towards that observed in non-polar solvents such as chloroform (see chapter 3). As Tw concentration increased a shoulder appears at 388nm , this indicates the presence of ionised dithranol. This is confirmed at 258nm where above 0.12% Tw absorbance increased with Tw concentration. A plot of absorbance v Tw concentration (see Fig 5.10) is indicative of the occurrence of solubilisation. The spectrophotometric evidence suggests that on solubilisation the aqueous ionic equilibrium is altered because dithranol molecules enter an environment where there is a small increase in the extent of ionisation.

On the whole it has been shown that surfactants have a pH and type dependent influence on the ionic equilibria of aqueous dithranol. At pH 5.5 CET presence has a marked effect while Tw and NaLS have little or no effect. Currently literature suggests two possible routes for dithranol decomposition (see chapter 4). The consequence of CET's disruption of dithranol's aqueous ionic equilibrium is that the decomposition pattern is observed to change from mainly dimer to mainly danthron (see chapter 4). Current thinking with regard to dithranol's mode of action is that the decomposition process is vital to the effectiveness of the drug. What is not known, however, is which , if any , of the two possible routes is the therapeutically beneficial one. Since the molecular species of dithranol present will influence the decomposition pathway, it follows that surfactants have the potential to influence the effectiveness of dithranol. In the presence of surfactants like CET the danthron pathway is promoted , while in the presence of surfactants like NaLS and Tw the dimer pathway is promoted.

Table 5.1

The UV absorbance of dithranol solutions (2.73μg/mL) in glycine buffer at 25°C.

pH	Absorbance (258nm)
8.1	0.171
8.6	0.295
9.0	0.329
9.4	0.459
9.7	0.501

The absorbance of fully ionised dithranol was 0.642 (determined at pH 11).For non-ionised dithranol it was 0.12 (determined at pH 1).

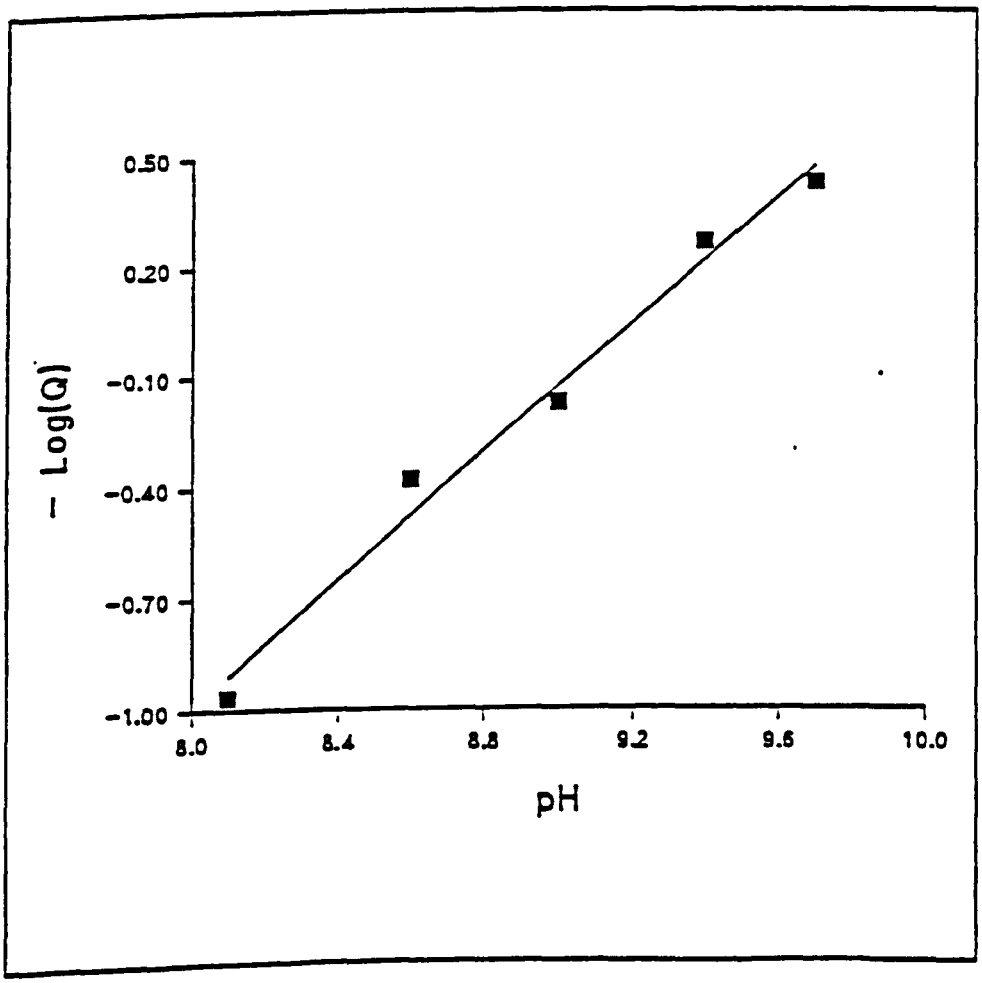


Fig 5.1 The graphical determination of pKa for dithranol using absorbance measurements (see Table 5.1).

y = 0.8660(x) - 7.9245 (correlation = 0.992)

For the equation plotted see Wurster Upadrashta (1988).

Table 5.2

The observed fluoresence of dithranol (10ng/mL) in glycine buffer at 25°C. [Excitation wavelength 390nm, Emission wavelength 518nm].

pH	% fluorescence intensity
6.9	3.0
8.1	21.9
8.5	36.9
9.1	61.5
9.5	69.6

The maximum % fluorescence for the concentration of dithranol used was 84.7 (determined at pH 10.5)

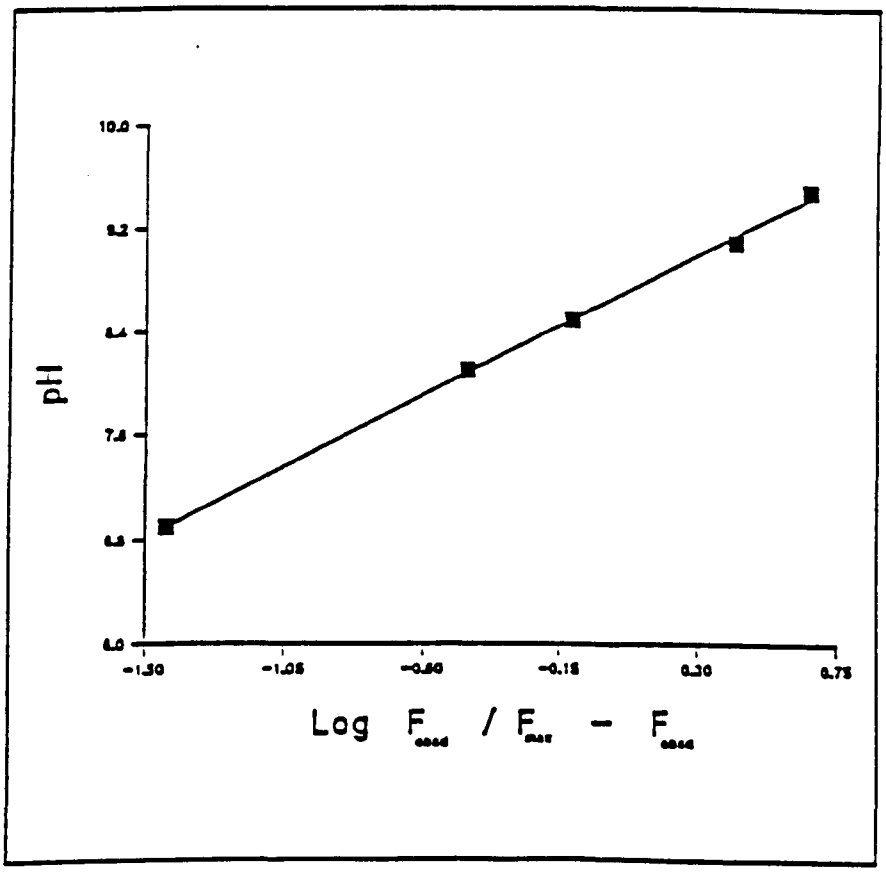


Fig 5.2

The graphical determination of pKa for dithranol using fluorescence measurements (see Table5.2).

y = 1.2197(x) + 8.6435 (correlation = 0.999)

For the equation plotted see Eq.5.1 in the text.

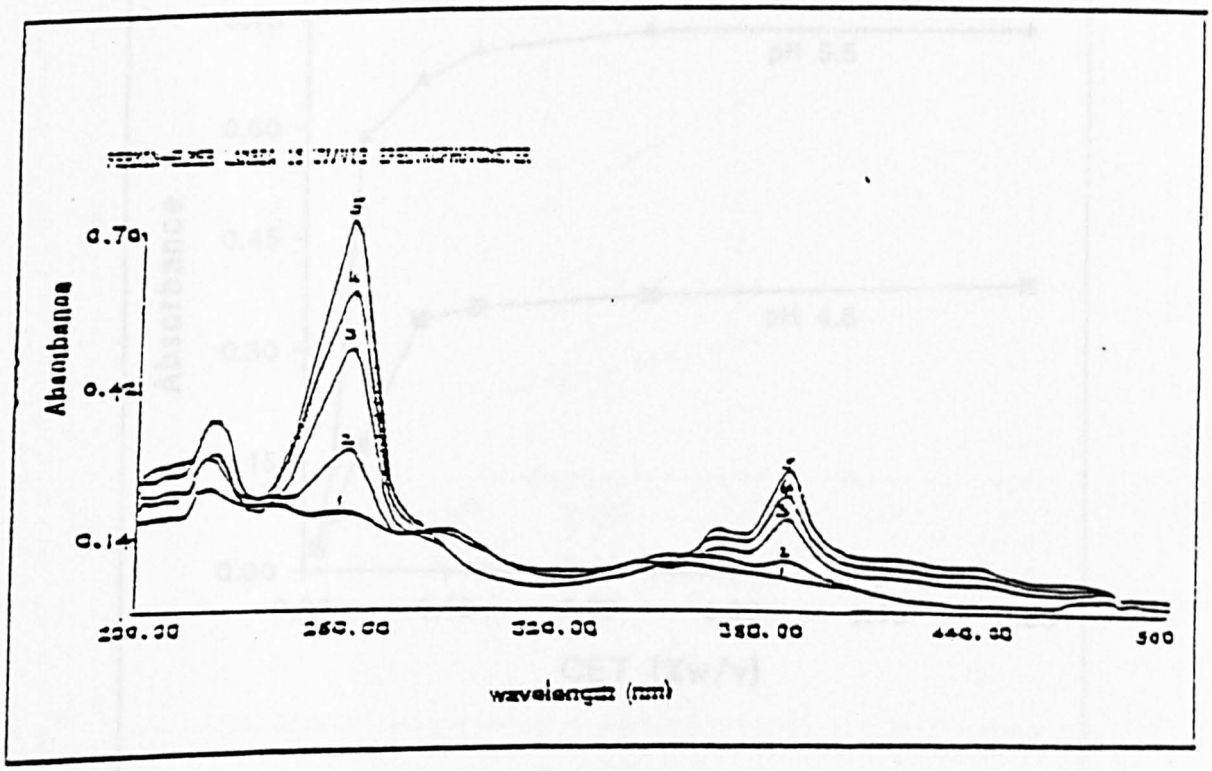


Fig 5.3

The effect of CET concentration on the UV absorbance of dithranol (4.3 μg/ml) in acetate buffer at pH 5.5 at 25°C.

At 258 and 388nm 1 = buffer, 2 = 0.005% CET, 3 = 0.01% CET, 4 = 0.015% CET, 5 = 0.02% CET.

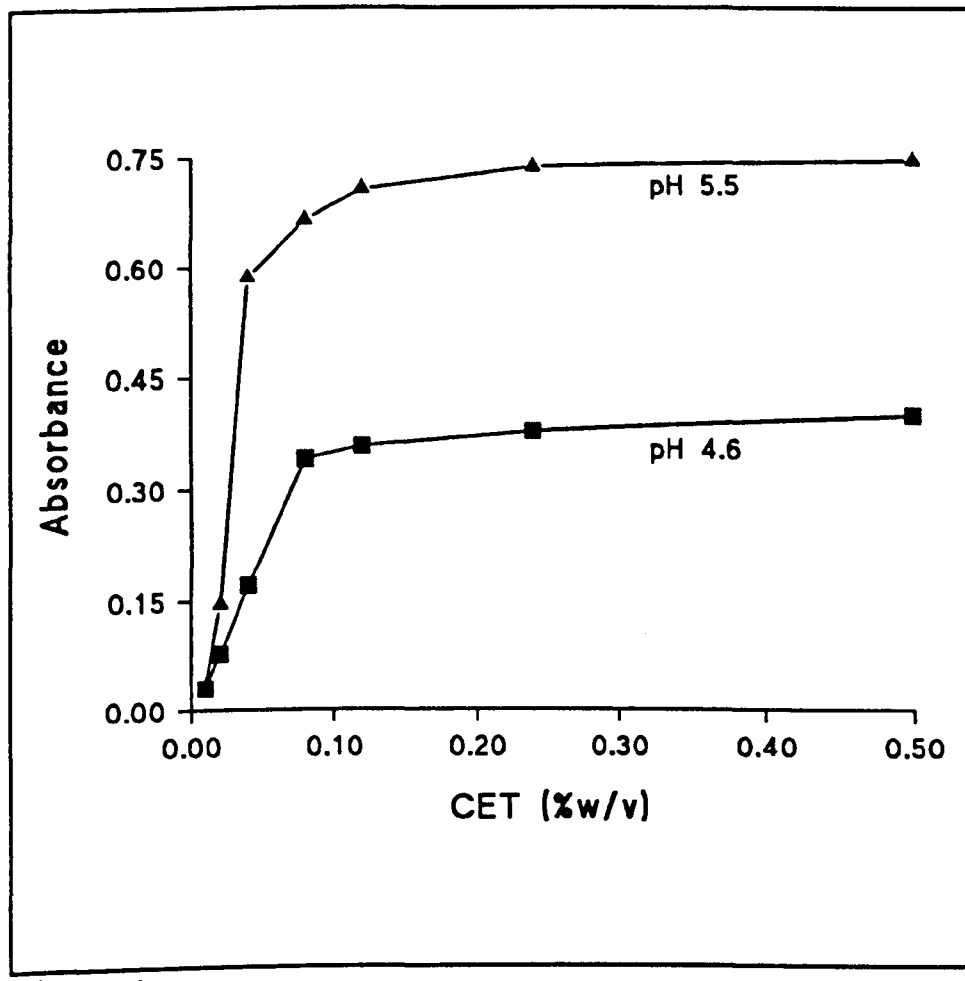


Fig 5.4
The effect of CET concentration at pH 5.5 (▲) and pH 4.6 (■) on the absorbance of dithranol measured at 258nm.

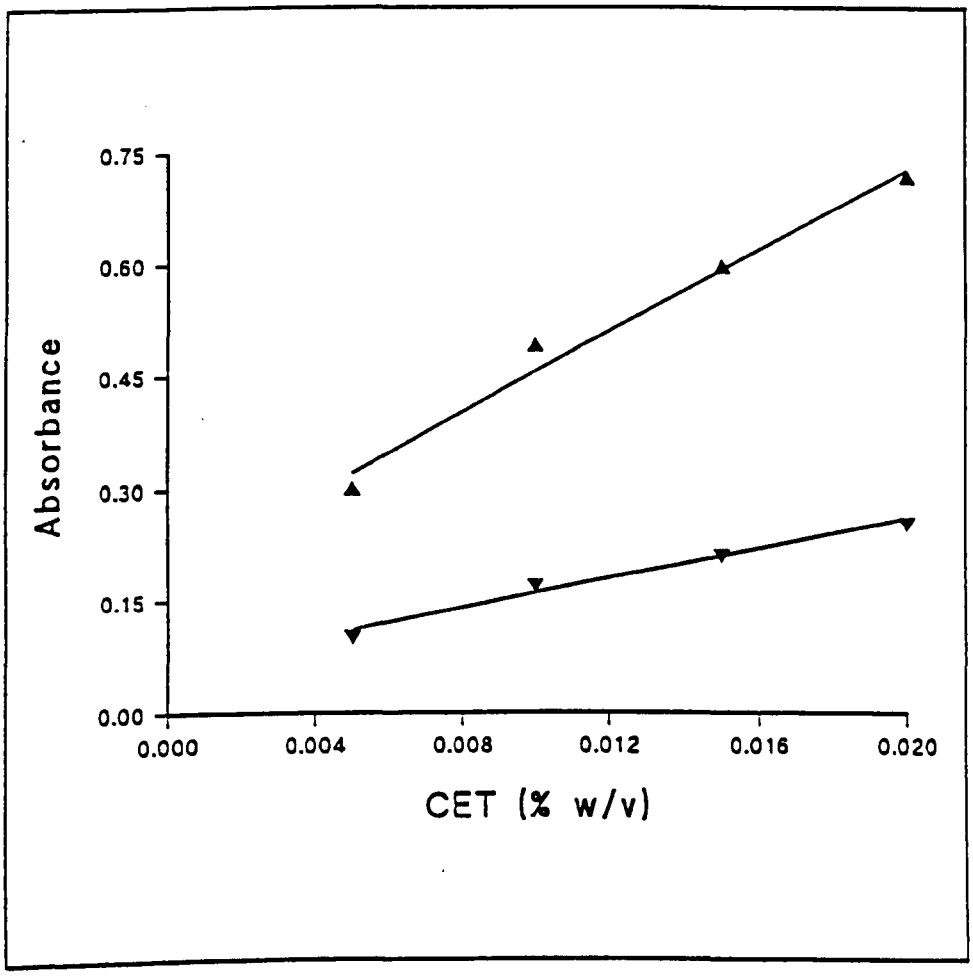


Fig 5.5

The absorbance of dithranol(~4.3μg/mL) in CET solutions of increasing concentration at 25°C. pH of solutions = 5.5. ▲ = 258nm , ▼ = 388nm.

At 258nm y = 27.1800(x) + 0.1845 ; At 388nm , y = 10.0800(x) + 0.0605
(Correlation coefficient = 0.991). (Correlation coefficient = 0.992)

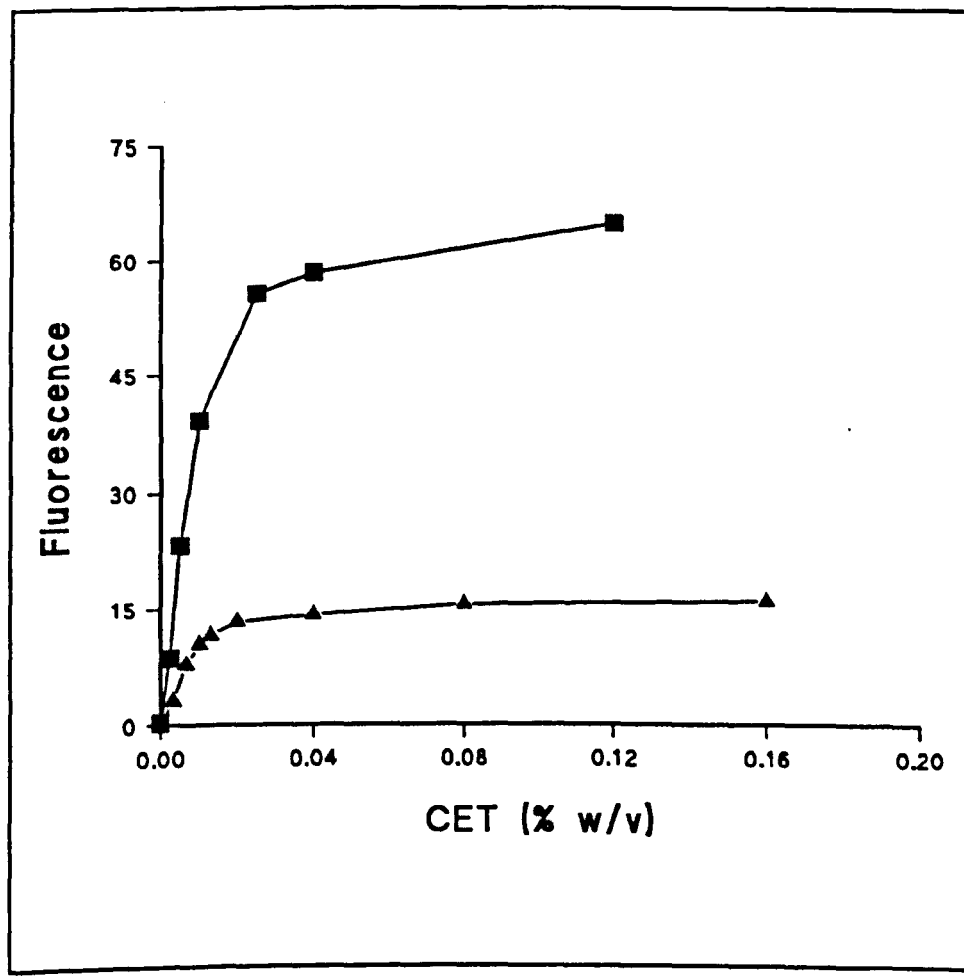


Fig 5.6

The effect of pH and CET concentration on the fluorescence of dithranol (~10ng/mL) in acetate buffer at 25°C. ▲ = pH 4.6 , ■ = pH 5.5

Table 5.3

The effect of dithranol concentration on the absorbance at 258nm in a fixed concentration of CET (0.2% w/v) at pH 5.5.

CET (% w/v)	pH	Dithranol(μg/mL)	Absorbance(258nm)
0.2	5.5	5.0	0.517
0.2	5.5	7.5	0.751
0.2	5.5	10.0	1.022
0.2	5.5	12.5	1.269
0.2	5.5	15.0	1.577

Table 5.4

The effect of pH on absorbance at 258nm of a fixed concentration of dithranol (4.4μg/mL) in 0.2% CET.

CET (0.2%w/v)	pH	Dithranol(μg/mL)	Absorbance (258nm)
0.2	4.00	4.4	0.350
0.2	4.50	4.4	0.486
0.2	4.75	4.4	0.600
0.2	5.00	4.4	0.714
0.2	5.30	4.4	0.802
0.2	5.50	4.4	0.917

Absorbance of fully ionised dithranol (4.4μg/mL) determined at pH 11 = 0.994. The absorbance for non ionised dithranol (pH 0.4) was 0.161. Both were done in the absence of CET.

Table 5.5

The observed % fluorescence of dithranol (10ng/mL) in acetate buffer at 25°C, in the presence of 0.04% w/v CET.

Cetrimide (%w/v)	pH	% Fluorescence
0.04	4.6	42
0.04	4.8	67
0.04	5.0	98
0.04	5.3	131
0.04	5.5	145

Maximum fluorescence (190) was determined using acetate buffer pH 5.7 with 0.3% CET and 0.1% isoascorbic acid.

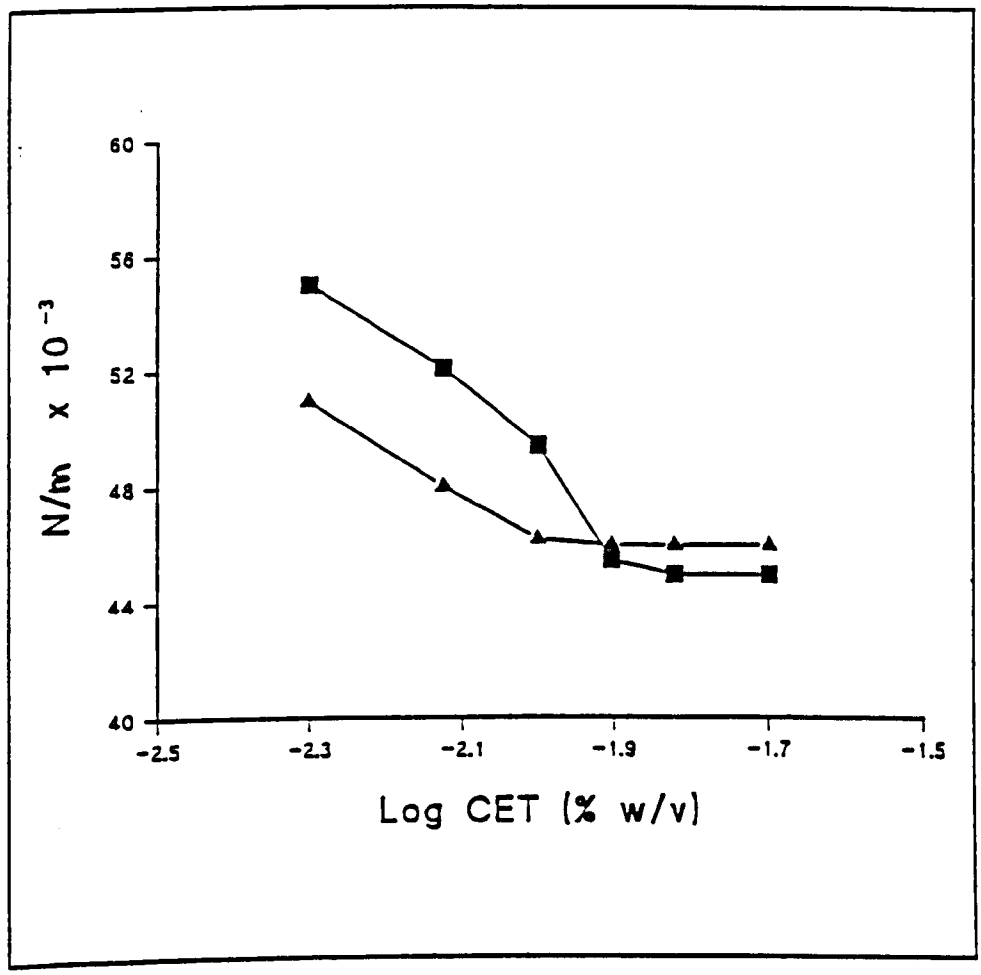


Fig 5.7

Surface tension measurements of CET solutions of pH 5.5 at 25°C. Measurements were made with and without the addition of dithranol (~4.3μg/mL). ■ = no dithranol ▲ = dithranol present.

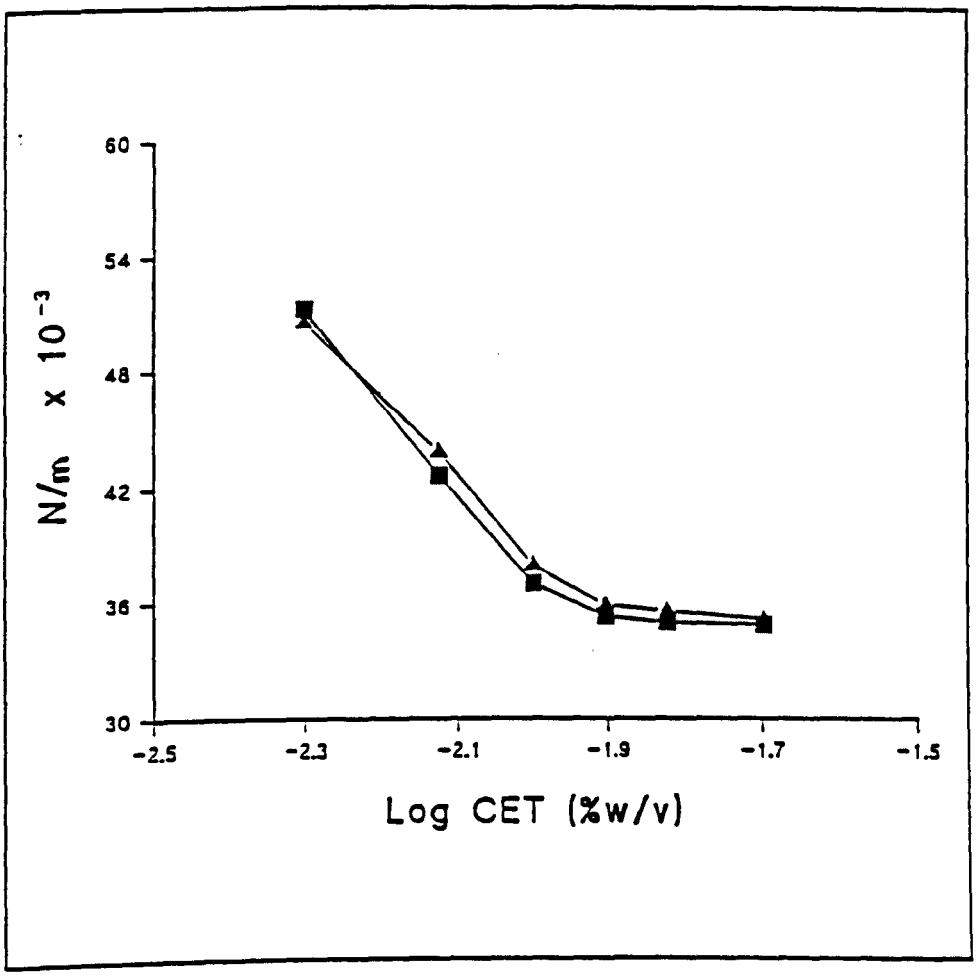


Fig 5.8

Surface tension measurements of CET solutions of pH 0.4 at 25°C. Measurements were made with and without the addition of dithranol (~4.3μg/mL). ▲ = no dithranol ■ = dithranol present.

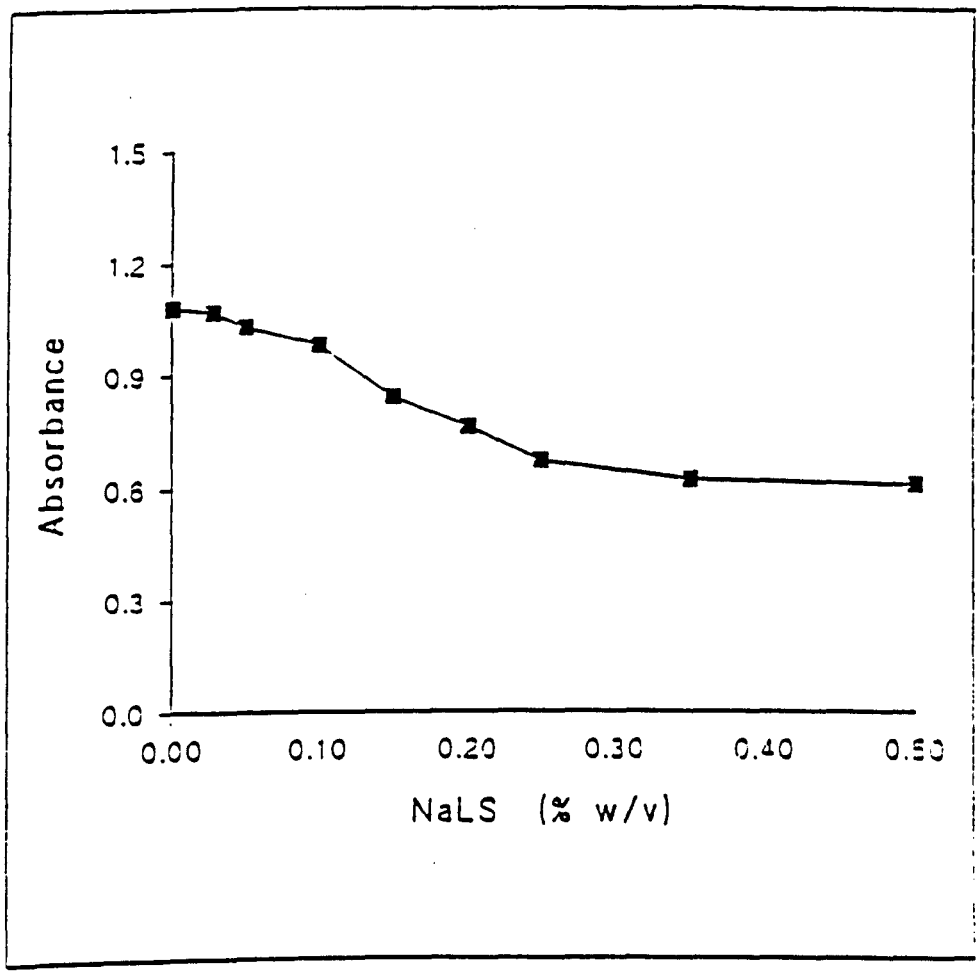


Fig 5.9

The effect of increasing concentrations of NaLS on the absorbance of dithranol (~4.3μg/mL) in glycine buffer pH 10 at 25°C. The absorbance at 258nm is shown

Table 5.6

The effect of Tw on the wavelength of maximum absorbance for dithranol in glycine buffer pH 8 at 25°C.

Tw (%w/v)	wavelength of maximum absorbance (nm)
0.00	350.8
0.01	352.4
0.03	353.4
0.05	354.4
0.08	357.4
0.12	356.8
0.20	366.0
0.30	366.0
0.50	367.2

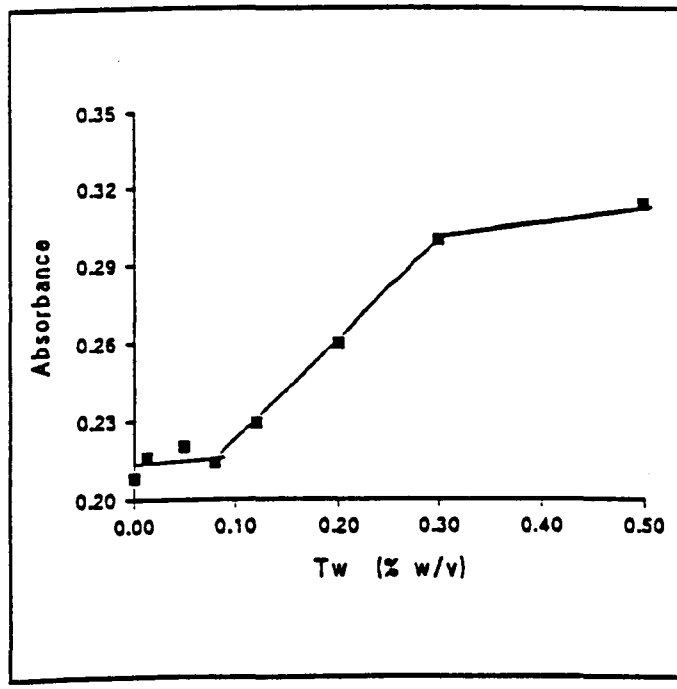


Fig 5.10

The effect of Tw on the absorbance of dithranol(~4.3μg/mL) in glycine buffer pH 8 at 25°C. Absorbance at 258nm is shown.

CHAPTER 6

THE IN VIVO AND IN VITRO INTERACTION OF AQUEOUS SURFACTANT SOLUTIONS OF DITHRANOL WITH SKIN

6.1 Introduction

Solubility investigations (undertaken in the present study) have shown that the aqueous solubility of dithranol can be enhanced by surfactant solubilization , and following stability studies on such solutions conditions for maximum stability have been identified (see chapter 4). In this chapter skin permeation studies will be carried out using these aqueous solutions of dithranol along with hairless mouse , normal human and psoriatic skin. Also to be investigated is the antipsoriatic potential of the dithranol-surfactant preparations using the TPA psoriasis model (see 1.2.3).

6.2 Experimental Methods

6.2.1 Materials

Dithranol , (Sigma Chemical Company)

Sodium dodecyl sulphate (NaLS) approx. 99% pure ,(Sigma Chemical Company).

Hexadecyltrimethylammonium bromide USP (Cetrimide) 99% pure, (Sigma Chemical Company).

Polyoxyethylenesorbitan mono-oleate (Tween 80), (Sigma Chemical Company).

Sodium acetate trihydrate , (Fisons Scientific Equipment)

Sulphuric acid , Rathburn chemicals (Walkerburn , U.K.)

Hydrochloric acid 1M , (May & Baker Laboratory Products) Sodium hydroxide 1M , (May & Baker Laboratory Products)

Acetaldehyde , (May & Baker Laboratory Products)

Diphenylamine , (Sigma Chemical Company).

Calf thymus DNA , (Sigma Chemical Company).

Perchloric acid , (May & Baker Laboratory Products)

12-O-tetradecanoylphorbol-13-acetate (TPA), (Sigma Chemical Company).

All chemicals were used as obtained.

6.2.2 Preparation of the surfactant solutions.

The constituents of the buffer were dissolved in distilled water and then the pH adjusted to 5.5 using 0.4M HCl. The volume was then made up to the

mark using distilled water, such that the final concentrations were 0.2M sodium acetate , 0.5% IAA, 4% NaLS or Tw and 2.5% CET.

The CET and NaLS formulations should be stored above 25°C to prevent precipitation of surfactant especially at concentrations above 3% w/v. Should any precipitation occur the solutions should be warmed and gently shaken.

6.2.3 Skin permeation procedures.

6.2.3.1 In vitro permeation procedure

Skin source.

Skin was obtained from male and female hairless mice (Crl:nu/nu 28 - 42 days old -Charles River UK , LTD). The animals were sacrificed by cervical dislocation and the entire skin removed and stored at -70°C. When required the skin was thawed at room temperature , appropriate sized pieces cut and all subcutaneous fatty tissue removed before mounting the skin in the diffusion cells. Human psoriatic skin was obtained from the lower forearm of a patient with visible psoriasis from Ward 29 of the Dermatology Department of Aberdeen Royal Infirmary. The skin was kept frozen (-70°C) and used within 24 hours.

Permeation procedure

Skin permeation experiments were done using flow through diffusion cells. The type used were those updated in design by Gummer et al (1987) . Some advantages of this design include instantaneous stirring of receptor phase and a water jacket extending up to the level of the skin. (The cells were manufactured to the required specifications by Norlab LTD , Aberdeen (see diagram at end of chapter). Skin was mounted in the diffusion cell with the dermis side in contact with the receptor medium , and the stratum corneum side (effective area $1cm^2$) with the donor solution (2mL). Silicone grease was used to ensure there was no leakage when the two cell compartments were pressed together. Water from a water bath, set at 37°C , was circulated through the water jacket. The flow rate of the receptor medium (methanol 60 , H_2O 38, GAA 2 + 0.5% IAA - 37°C) was set at 0.5mL/min.

6.2.3.2 In vivo normal human skin permeation procedure

Formulation application

Two healthy adult volunteers of African origin (one male and one female both aged 33) were used. The formulations were applied to the forearm skin. To apply the dithranol-surfactant solutions the method recommended by Schaefer and Lamaud (1987) was followed , but instead of using a glass ring a specially designed glass cap (made to required specifications by Norlab LTD, Aberdeen- see diagram at end of chapter.) was used to both delimit the

area ($2cm^2$) to be treated and allow sample withdrawal for analysis. After gluing the cap to the skin (using super glue 4) 0.7ml of the dithranol-surfactant formulation was introduced through a hole in the top of the cap which was then temporarily sealed to prevent escape of material by leakage and evaporation. Samples for analysis (10μL) are withdrawn , using a micropipette through the hole in the top of the cap , at selected time intervals for hplc analysis.

6.2.4 Assessment of the antipsoriatic potential of the surfactant formulations of dithranol

Formulation application to hairless mouse skin in vivo.

The method outlined in 6.2.3.2 was followed with the glass caps being glued to the backs of the mice. 10 μmol of TPA dissolved in 50μl of acetone was then applied to the backs of the animals using a pipette. The top of the glass cap was not sealed for about 10 minutes to allow acetone to evaporate. When both TPA and a dithranol-surfactant preparation were to be applied to the animal's backs ,the surfactant formulation (100μL) was applied after allowing the acetone to evaporate. The caps were then sealed to prevent leakage of the aqueous preparations. The animals were killed (cervical dislocation) 48 hours post treatment. Before killing the animals an inflammation score , based on an arbitrary scale of 0 - 4 , was given (see Table 6.3).The treated area was excised , placed in a sample bottle and frozen at -70°C to prevent enzyme degradation.

6.2.5 DNA quantification using diphenylamine (Burton method).

Aqueous acetaldehyde (1.6%) was prepared by transferring 1mL of commercial acetaldehyde to 50mL of distilled water. Diphenylamine reagent (DPAR) was prepared by transferring 1.5g diphenylamine to a volumetric flask (100mL), 1.5mL concentrated H_2SO_4 is then added to the flask and volume made up to the mark using glacial acetic acid. Before using the DPAR 0.1mL of 1.6% aqueous acetaldehyde is added per 20mL of reagent.

6.2.5.1 Preparation of DNA standards

Calf thymus DNA was weighed (0.0162g) and dissolved in 0.005 M NaOH (25mL). The amount of DNA in solution was quantified spectrophotometrically using the unit definition whereby one unit yields an absorbance at 260nm (A_{260}) of 1 in 1mL of buffer (1cm light path). One unit equals approximately 50μg of DNA (Sigma Chemical Company ,1992).

3mL of 0.005M NaOH were pipetted into a 1cm path absorbance cell , 0.5mL of this was removed and replaced with the DNA solution. The cell contents were mixed by inversion of the cell. The absorbance at 260nm of the resultant solution was 1.03 equivalent to 51.40μg/mL DNA. The concentration of the standard prepared was therefore 51.40 x 6 (dilution factor) = 308.40μg/mL.

The DNA standard was diluted 1:2 using N perchloric acid and then heated

at 70°C for 15 minutes. After heating, aliquotes of the mixture were diluted , using 0.5N perchloric, to give DNA solutions of concentration 10 - 50μg/mL. Each dilution (1mL) was then mixed with DPAR (2mL) and the mixtures incubated at 25 - 30°C for 15 -17 hours after which absorbance was read at 600nm. The calibration graph results are shown in Fig 6.11

6.2.5.2 Determination of DNA content of hairless mouse skin

Frozen hairless mouse skin was left in a closed container was left to thaw at room temperature. Circular discs approximately 2cm^2 were cut and weighed before being placed in 0.5N perchloric acid (3mL). The mixture was then homogenised at high speed for 2 minutes after which it was heated at 70°C for 20 minutes. After the heating stage , 1mL of the skin extract is added to 2mL of DPAR and the mixture incubated at 25 - 30°C for 15 - 17 hours. On running the UV spectrum of the incubated mixture a high background reading was obtained , the problem was resolved by taking 0.5mL of the extract and adding it to 1mL diethylether and then obtaining the UV spectrum . The absorbance at 600nm was recorded (the standards used for calibration graph preparation were also treated in a similar fashion).

6.3 Results/Discussion

Skin permeation characteristics of dithranol have been studied by a number of workers [Kammerau et al 1975 , Schalla et al 1981 , Schaefer et al 1980 , Cavey et al 1985, Wang et al 1987 , Priprem 1991]. Dithranol has been delivered to the skin mainly in ointments , creams and gels and other semisolid or solid (polymeric disk and hydrogel) formulations while , in the main , permeation characteristics have been established by using radioactive-labelled dithranol (C^{14}) and the skin punch biopsy method (see Schaefer and Lamaud , 1987). The skin biopsy method is invasive and radiochemical methods have the disadvantage of being relatively non-specific. This is particularly true of an unstable compound like dithranol. Thus separation is essential to achieve positive identification of dithranol and its decomposition and/or its metabolic products. This is particularly of necessity for kinetic studies. Another disadvantage is the use of radioactive material for in vivo studies. The natural substance would be preferred. Some workers [Wang et al (1987)] have recognised the shortcomings of the radioactivity approach and have used hplc to generate such data using formulations , such as those previously mentioned.

In this study liquid preparations of dithranol were placed in contact with skin and analysis done by hplc. The main advantages of the present approach compared to others is the simplicity of the technique (no formulation release

problems) along with the ability to account for breakdown product formation during the skin permeation process. The use of a liquid formulation of dithranol allows measurements to be made at various time points post application of the dose and hence generation of concentration time profiles for dithranol and its breakdown products during the permeation process. To generate such data using other formulations would require more complex experimental set ups.

In aqueous solution , it has previously been shown that dithranol in the surfactants is expected to produce danthron and dimer in varying amounts. However in contact with the skin it was observed that an unknown peak was repeatedly obtained with a retention time of around 4 minutes (this peak will now be referred to as P_4). This effect was seen with all the dithranol - surfactant formulations and with both hairless mouse and human skin.

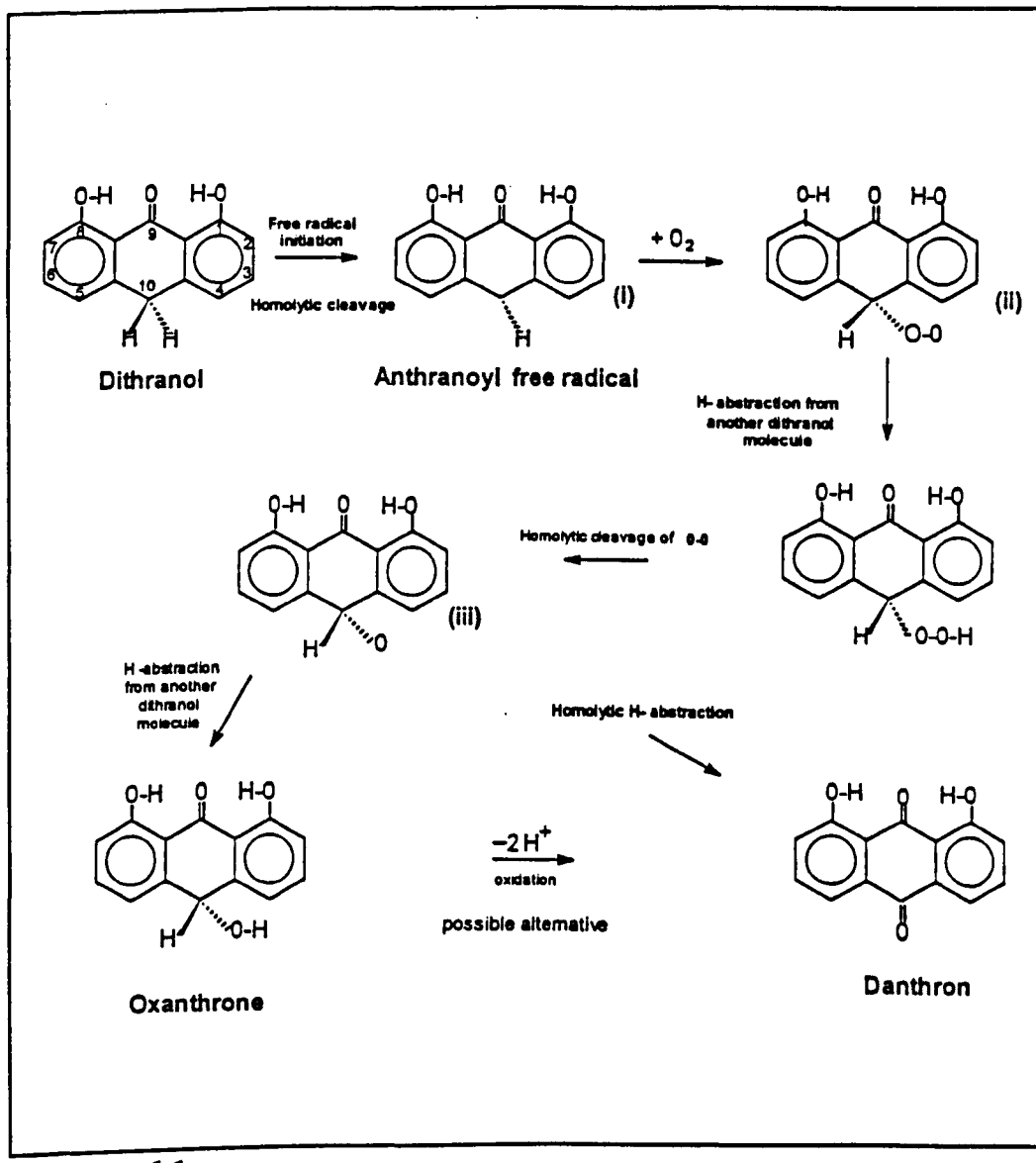
The chromatograms shown in Fig 6.1 were obtained on analysing the DT - surfactant solutions after a period of time in contact with hairless mouse skin . The chromatograms show that the P_4 peak is fully resolved from those of danthron , dithranol and the internal standard. It was therefore possible , by using the hplc column , to isolate the individual fractions as they came of the column. By repeated collection of the P_4 and dithranol fractions enough ($\sim$2mL of each fraction) was collected to enable absorption spectra to be obtained. The spectra for the two fractions are very similar (see Fig 6.2) except that the P_4 spectrum is shifted towards the longer wavelengths. The

retention time of P_4 is shorter than that of dithranol showing that it is a comparatively more polar structure. To elucidate the structure of P_4 larger quantities than those obtained using the hplc column would be necessary to carry out procedures such as molecular weight assignment using mass spectrometry. Time constraints did not allow this to be explored.

Scheme 6.1 shows the proposed reaction pathway for the formation of the oxidation products of dithranol [modified from Holder & Upadrashta (1992)] .It is possible that, once formed , free radical (iii) follows two different pathways, these being homolytic H - abstraction to form danthron and H - abstraction from another dithranol molecule to form an oxanthrone derivative, which can possibly undergo further oxidation to form danthron. Compounds of this structure (oxanthrone derivatives) are known to occur naturally as O-glycosides in cascara bark (Trease and Evans , 1983). An oxanthrone derivative formed in this way would be very similar in structure to dithranol. Further , because of the lack of resonance enhancement due to the saturated nature of the bond at C10 in such a derivative , it can be expected that there is little change in the absorption spectrum relative to dithranol. The spectra shown in Fig 6.2 show that this indeed was the case.

There are no reports in the literature of chromatographic data , obtained during dithranol skin permeation process, of a dithranol metabolite similar to that observed in the present study. This could perhaps be because no skin permeation studies using aqueous solutions of dithranol have been reported.

To check if P_4 production was observed because of having the combination of dithranol, water and skin , dithranol was delivered to the skin in methanol.



Scheme 6.1
The possible reaction pathways for the formation of the oxidation products of dithranol in and on skin [modified from Holder & Upadrashta (1992)]

P_4 formation was still observed. The production of P_4 is therefore not due to the aqueous conditions but more because of dithranol being in contact with the skin. This was further supported by the fact that application of the vehicle blanks did not result in P_4 formation , and also shows that P_4 did not derive from the glass cap or the glue used. Since P_4 was formed on both hairless mouse skin and human skin , it follows that it's formation is not influenced by the presence or absence of sweat glands.

The results presented in Fig 6.4 - 6.7 show the concentration vs time profiles , for periods up to 10 hours , for dithranol and decomposition products when the DT -surfactant formulations were placed in contact with hairless mouse skin . Figures for the conversion of dithranol to P_4 are estimates as the exact nature of P_4 is unknown. The UV spectra shown in Fig 6.2 show that the absorbances at the analytical wavelength (254 nm) for dithranol and P_4 are very similar along with the whole spectrum. Based on these observations it was assumed that the molar absorptivity of the two compounds is also very similar thus estimates for the amounts of P_4 were obtained using the calibration graphs for dithranol. The concentration vs time graphs show that skin surface metabolite formation reached a maximum by 3 and 6 hours in the CET and NaLS and Tw formulations respectively. Table 6.1 shows the distribution and total recovery of dithranol 6 hours after the start for NaLS and Tw , and 3 hours after the start for CET. With all three surfactant formulations recoveries of 87% or greater were obtained. In view of these results the assumptions made in estimating the amounts of P_4 formed during

the permation process , seem reasonable ones to make.

In all three surfactants the amount of dithranol remaining with time was observed to follow first order kinetics and in all cases concentration decline was faster in the presence of skin (see Fig 6.3). In CET the rate of decomposition was increased to 0.322 from 0.161 hr^{-1}, in NaLS to 0.040 from 0.018 hr^{-1} , while in Tw the rate was increased to 0.125 from 0.078 hr-1. This effect of an enhanced rate of decomposition for dithranol when in contact with hairless mouse skin is in general agreement with observations of Hulsebosch & Ponec-Waelsch (1972), who showed that dithranol decomposed at a faster rate when in contact with surfaces such as lint , skin and glass wool.

In the CET formulation dithranol decomposition in solution results in a particularly marked increase in danthron formation and a significant increase in that of dimer. Dimer reaches a maximum in about 3 hours whilst danthron increases linearly with time. Danthron formation both in solution and when in contact with skin was observed to follow zero order kinetics (see Fig 6.4(b)). There was no difference ($p < 0.05$) found between the rate of formation in the solution , and when the solution was in contact with the skin , the two rates being 4.51 and 3.94$\mu g\ hr^{-1}$ respectively. On skin the dimer levels are much reduced while those of danthron are of a similar order to those seen in the solution not in contact with skin.The most significant change is the production of P_4 , at the expense of dimer , over the initial

3 hour period. P_4 reaches a steady state after 3 hours.

In the NaLS formulation dithranol decomposition results in the appearance of both dimer and danthron in solution (see Fig 6.5). The dimer levels are about 5 - 6 times greater than those of danthron. Formation of both reaches a plateau in about 6 hours. In contact with skin a similar pattern for dimer and danthron formation is observed (see Fig 6.5). An increase in the amount of both decomposition products was observed, though in this case dimer levels are about 4 times those of danthron. P_4 formation is also observed, the levels being twice as much as those of danthron. On skin , decomposition product formation reaches a plateau in about 4 hours.

Dithranol decomposition in the Tw formulation results in the formation of more dimer than danthron, the levels being 8 - 9 times those of danthron (see Fig 6.6). Dimer formation reaches a steady state in about 6 hours. A large decrease in the dimer formed was observed due to skin contact , while there was , approximately , a two fold increase in danthron formation (see Fig 6.6(b)). P_4 formation is observed and results show that it is at the expense of dimer.

P_4 showed a pattern of increased formation on the skin surface followed , in the Tw formulation , by a gradual penetration into the skin shown by a steady decline in the concentration on the surface (see Fig 6.7). With the CET and NaLS formulations a plateau region is observed. From the data

presented in Table 6.1 it is seen that in NaLS , dithranol was held on the skin surface where it was protected from decomposition and also only released slowly to the skin. Because of this slow release of dithranol to the skin P_4 formation with NaLS was very slow and any formed tended to stay on the skin surface. This perhaps reflecting the inability of NaLS to enhance the rate of permeation of molecules , when the contact time with skin is short (see later). With the CET formulation more P_4 was formed (about twice that in NaLS) though skin permeation was still relatively slow. Again perhaps because of the fact that it is an ionic surfactant , like NaLS , there was little permeation enhancement over the short contact period. In the non ionic Tw formulation the observed P_4 penetration into the skin is more likely due to the more rapid ability of Tw , as will be discussed , to enhance percutaneous absorption compared to the ionic surfactants.

In the diffusion cells decline in dithranol concentration is either due to penetration into the skin , metabolite formation or both. At the end of a selected time period , establishing the % of the initial amount of dithranol applied , along with the % converted to metabolites , which are still present on the skin surface and subtracting these two figures from the initial amount of dithranol gives an indication of the amount that penetrates into the skin. Carrying this out with the data presented in Fig 6.3 - 6.7 it was found that as regards skin permeation between 3.2 - 10.8% of the initial amount of applied dithranol penetrated the skin using the NaLS formulation. In comparison 10.3 - 27.3% was observed with the CET formulation and 57.6 -

65% in the Tw formulation. Dithranol skin permeation was therefore greatest in the presence of Tw and poorest in the NaLS formulation. It must be pointed out here that these permeation results are mainly indicators of trend. This is because of the limited efficiency of the skin extraction procedure (see chapter 2). Present findings on the skin permeation effect of NaLS , CET and Tw are in general agreement with the findings of Ashton et al (1992) who investigated the effects of surfactants on the transdermal flux of methylnicotinate. They found that B36T (non ionic surfactant) acted rapidly on the skin to produce small increases in the transdermal flux. In contrast the increased transdermal flux produced by the ionic surfactants (sodium lauryl sulphate and cetyltrimethylammonium bromide) occurred over a much longer time scale. The hypothesis put forward by Ashton et al (1988) is that as non ionic surfactants are more lipophillic than ionic ones it is possible that entry into the stratum corneum is faster , and since the effect of surfactant entry into the stratum corneum is a disruption of the barrier function , increases in skin permeability are relatively rapid. This hypothesis is consistent with present findings.

In skin permeation studies , skin permeability coefficient data are usually reported. This was not done in the present study due to the fact that whole skin was used. One factor that governs the permeation of a molecule through the skin is the thickness of the skin preparation. This was demonstrated by Wang et al (1987) using a 1% dithranol microemulsion gel, and normal human skin sectioned to varying thickness. After 8 hours contact no penetration of

dithranol occurred when the skin was 255 μm thick , whereas progressively more dithranol penetrated through skin preparations of 183 , 114 and 80μm respectively. The thickness of the hairless mouse skin used in the present studies was around 400 μm. It was therefore understandable that no dithranol was observed to penetrate through the skin during the course of the experiments.

Compared to hairless mouse skin events ocurring on the surface of normal human skin in vivo took place much faster. This can in part be linked to the fact that the area exposed to the formulations in the human skin experiments was twice that in the hairless mouse skin experiments. In the CET formulation dithranol decomposed at a rate of 8.01 hr^{-1} , while in NaLS the rate was 1.04 compared to 3.59 hr^{-1} in Tw (see fig 6.8). In all three surfactant formulations the major metabolite formed was P_4 (see fig 6.9). No dimer and only very small amounts of danthron were detected in both the Tw and NaLS formulations. With the CET formulation around 3% danthron was formed during the permeation period (1.5 hours). Permeation observation period was short (1.5 hours) for CET due to rapidity of events occurring on the skin surface. For Tw and NaLS observation period was 2.5 hours.

The appearance of P_4 on the surface of normal human skin can also be accounted for by the sequence of events proposed in scheme 6.1. The concentration time profile of P_4 in CET and Tw shows that there was a rapid increase in skin surface P_4 followed by penetration into the skin (see

Fig 6.9). Using the "tail end" of the profiles (all first order kinetics) the rate of penetration of P_4 was calculated. In CET the penetration rate constant was 3.59 hr^{-1} (correlation coefficient 0.995) compared to 0.48 hr^{-1} (correlation coefficient 0.996) in Tw. In NaLS the profile shows that P_4 remained on the skin and did not penetrate into the skin. The difference in the penetration of P_4 into normal human skin compared to hairless mouse skin could , in part , be due to the fact that human skin has sweat glands and hair follicles , providing other possible routes of entry into the skin. In hairless mouse skin these features are absent therefore all penetration is through the strateum corneum , this slower penetration leading to steady states in the P_4 concentration time profiles generated on hairless mouse skin. The fact that twice the area of the hairless mouse skin exposed was used in the human skin experiments can also contribute to the enhanced rates observed.

The results from the human skin experiments show that there was a significant deviation from the decomposition pattern in the formulations when not in contact with skin. Whereas both dimer and danthron were formed in the formulations not in contact with the skin - indicating dithranol decomposition via both danthron and dimer routes , on normal human skin the only decomposition products detected were danthron and P_4, this indicating that dithranol decomposition was ocurring through the danthron route only.

An indication of dithranol permeation into the skin using the DT-surfactant formulations was obtained as was done for hairless mouse skin. The data

used are presented in Fig 6.8 & 6.9 . In the CET formulation skin surface decomposition was pronounced , 70 ± 7% of the initial dithranol was converted to P_4 inside 7 minutes of skin contact. 11 ± 3% was present as unchanged dithranol while 3 ± 2% was converted to danthron . The results indicate that between 72 - 82% of the dithranol applied decomposed on the skin surface (within 7 minutes) while between 4 - 28% penetrated into the skin. Factors contributing to the wide variations observed include differences in skin from person to person and also site differences on the same person .

With the Tw formulation 40 ± 6% of initial dithranol was converted to P_4 in around 47 minutes , at the end of this contact period period 4 ± 2% of unchanged dithranol was still present on the surface. There were only trace amounts of danthron and dimer detected thus indications are that between 42 - 68% of initial dithranol penetrated into the skin during this contact period.

In the NaLS formulation only a small fraction , 10 ± 1.5% of initial dithranol underwent surface conversion to P_4 after 47 minutes contact with the skin. As no danthron and dimer were on the surface an estimate of between 55.5 - 62.5% of starting dithranol penetrated into the skin. There was 31 ± 2% unchanged dithranol at the end of the 47 minute period.

Using an aqueous hydrophillic ointment (oil in water) Kammerau et al (1975) showed that , after 5 hours , 1.25% of the initial dithranol dose (100μg spread over 28cm^2) penetrated the skin. The question of breakdown product

formation on the skin surface was not mentioned , perhaps because the radiochemical approach was used for the study. In comparison to present results the indications are that skin permeation is greater with any one of the surfactant formulations. Major differences in the analytical techniques , however , does not allow conclusive comparisons to be made.

On leaving the DT-Surfactant solutions on the skin for up to 2 hours before removal of the holder it was seen that with the NaLS formulation the skin in contact with the formulation was stained brown. The staining/inflammation only became apparent 24 hours after removal of the solutions. With the Tw and CET formulations this reaction was not observed. These results suggest that there is a possible link between the staining/inflammation reactions observed and the skin surface decomposition patterns in the DT-surfactant solutions. In the NaLS formulation surface decomposition was minimal, thus this formulation facilitates the entry of intact dithranol into the skin/stratum corneum. Melo et al (1983) showed that dithranol can persist in the stratum corneum for up to 24 hours , while other investigators Finnen et al (1984) have shown that the continued presence of dithranol in the stratum corneum for up to 24 hours is necessary for dithranol inflammatory reactions to be seen. Because of the stability of dithranol in the presence of NaLS (see ch4) it is able to persist, intact , for long periods in the skin/stratum corneum giving rise to the staining/inflammation observed. The Tw formulation caused the greatest permeation of intact dithranol into the skin/stratum corneum , but because of a reduced stability in the presence of Tw (compared to NaLS) the

dithranol does not persist for long periods once in the skin, thus no inflammatory reaction is observed. With the CET formulation permeation of intact dithranol is severely hampered due to the degree of skin surface decomposition ,also any dithranol entering the skin/stratum corneum will not persist because of CET presence.

Only one pilot study could be done using psoriatic skin due to the difficulty of obtaining psoriatic skin. In Fig 6.10 the decline of dithranol concentration and formation of metabolites in the CET formulation in contact with psoriatic skin is shown. After 3 hours there was 19.3% of initial dithranol present in the unchanged form on the skin surface , 19.5% was converted to danthron and 4.3% to P_4. The maximum for P_4 conversion was reached within 0.33 hours with none detected after 3 hours. Using the quantities of dithranol and metabolites found on the skin surface, the indications were that 57% of initial dithranol penetrated into the skin. On extraction of the skin danthron (28%) , dimer (17%) , P_4 (3%) and dithranol (1.5%) were found. The distribution of the applied dose of dithranol after 3 hours in contact with the psoriatic skin is summarised in Table 6.2. It is seen in the Table that around 90% of the dithranol dose is accounted for.

When a comparison is made with the results obtained using CET formulation on normal human skin the immediate obvious difference is the skin surface decomposition pattern. With normal skin the major metabolite (70%) was P_4 , whereas with psoriatic skin much smaller amounts (4.3%) of P_4 were

observed. On normal skin 3% danthron formation was observed compared to 20% on psoriatic skin. There was also a difference in the skin permeation pattern. With normal skin it was estimated that between 4 - 28% of initial dithranol penetrated into the skin compared to 57% with psoriatic skin. The results indicate that the penetration barrier presented by normal skin is greater than that of psoriatic skin. Wang et al (1987) using 1% Westragel and involved psoriatic skin also found that dithranol skin penetration was enhanced in the diseased skin compared to the normal skin. A number of reports in the literature concerning the penetration kinetics of dithranol using barrier compromised skin (stripped skin) all show that penetration is enhanced compared to intact skin. [Cavey et al (1985) , Schalla et al (1981), Scheafer et al (1980)]. On comparing the concentration vs time profiles for dithranol and breakdown products in the CET formulation in contact with human psoriatic skin and normal human skin ,it is seen that on psoriatic skin the kinetics for dithranol penetration/decomposition were zero order , which is different from the first order kinetics followed on normal human skin, in the formulation when not in contact with skin , and also hairless mouse skin. On both skin types (normal and psoritic human skin) danthron and P_4 were the major breakdown products detected - indicating that dithranol decomposition ocurred via the danthron pathway. The results indicate that the state of the skin barrier had a bearing on the kinetics for dithranol penetration/ decomposition. It can therefore be anticipated that there could be variation in the kinetics for skin surface decomposition of dithranol with the severity of the psoriasis.

To decide whether or not a particular formulation possesses any potential as an antipsoriatic preparation, it must be demonstrated that the formulation in question is capable of suppressing one or more of the biological events present in an animal model of psoriasis. Cyclosporine A , which is effective in the treatment of psoriasis was shown to block the pleiotypic responses of mouse skin to TPA treatment involving biochemical events , inflammatory cell infiltration and epidermal hyperplasia [Gupta et al,1989, Gschwendt et al, 1988]. In the present study the antipsoriatic effect of the dithranol-surfactant formulations was assessed in the TPA hairless mouse skin psoriasis model. A visual inspection of the TPA-induced inflammatory response and determination of DNA content were decided as the indicators to be used.

The results in Table 6.3 show that at a strength of 4% only the CET blank produced a mild inflammatory response, seen after 24 hours. With NaLS , Tw and acetone the mouse skin showed no obvious signs of inflammation after this period. Treatment of the skin with TPA resulted in marked inflammation of the treated area, this being seen after 48 hours. Treating the skin with TPA followed by dithranol (80μg/mL) in acetone resulted in the inflammation observed after 48 hours being reduced from severe to moderate. TPA followed by dithranol (80 & 40μg/mL) in 4% NaLS resulted, in both cases , in the TPA-induced inflammatory response being reduced from severe to moderate. With dithranol (40μg/mL) in 4% Tw the TPA-induced inflammatory response was reduced from severe to mild. With the CET formulation (dithranol 40μg/mL in 2.5% CET) areas of inflammation outwith the

confines of the cap were evident. This indicated TPA loss to surrounding areas. Underneath the cap , however, the TPA-induced inflammation remained severe. Suggesting that CET negated the effect of dithranol.

By visual inspection alone the conclusion that could be reached regarding the NaLS formulations (80 and 40μg/mL dithranol) was that NaLS was without effect on the anti-TPA effect of dithranol. With both formulations TPA-induced inflammation was reduced from severe to moderate (cf dithranol 80μg/mL in acetone).

With the Tw formulation the result obtained suggests a very successful reduction in the TPA-induced inflammatory response , the response being reduced from severe to mild. However , since only one animal was treated with this formulation and given the problematic nature of the experiment this sample size of one reduces the reliability of the result. It could be that the inflammation was mild after 48 hours because of loss of TPA from the treated area, although no signs of inflammation were present in surrounding skin.

The CET formulation had the most obvious effect. The anti-TPA effect of dithranol was negated. This was concluded by the fact that no evidence for the reduction of the inflammatory response to TPA treatment of the skin

under the cap was present.

The results for the DNA content of normal hairless mouse skin determined using diphenylamine (Burton , 1966) are shown in Table 6.4. The Table shows that a DNA content of 4.9 ± 0.84 μg/mg of skin was determined (skin from eight different animals). In Table 6.5 the DNA content of the skin treated with either TPA or a dithranol -surfactant formulation is shown. Following the treatment of hairless mouse skin with TPA an increase in DNA synthesis is expected. On comparing the DNA content determined for untreated skin against that determined for TPA treated skin, no significant difference ($p < 0.05$) between the two types was found. The same result was obtained on comparing untreated skin and skin treated with TPA and the dithranol-surfactant formulations. The results obtained can either mean that TPA treatment did not influence DNA synthesis or that the DNA quantification method was not capable of showing changes in DNA synthesis at the levels that it occurred. Inadequate sensitivity of the DNA quantifiaction method is the more probable case.

Using the diphenylamine method (Burton ,1966) for DNA quantification present results show that μg quantities of DNA were required for the preparation of the DNA calibration graph. In Fig 6.11 a y-intercept value of 0.194 is obtained. The absorbance at 600nm of the complex formed between DPAR and DNA (10μg/mL) was 0.451 thus a 1:10 dilution (1μg/mL) would give an absorbance below that of a solution with no DNA present (0.194). The

detection limit for this diphenylamine method was determined to be in the region of 3μg/mL. The conclusion reached is that if DNA changes occurred at nanogram levels the quantification procedure was incapable of detecting this. Gupta et al (1989) reported the use of the Burton method in quantifying DNA synthesis, but the results of their findings were not indicated.

A number of practical problems were encountered during the course of the experiments. These are listed below.

(1) Because of the cost of the materials , especially TPA and hairless mice , sample sizes had to be small.

(2) There was a problem with ensuring that the agents applied to the mice remained in contact with the skin. This was because once the caps were glued to the backs of the animals they tended to become extremely agitated some to the point of inflicting self injury in an effort to remove the cap. This behaviour of the animals therefore led to variation in the success with which contact between applied agents and the skin was obtained.

On the whole only the visual effects on TPA-induced inflammation , bearing in mind the practical problems encountered , could serve as a guide to the antipsoriatic potential of the dithranol-surfactant formulations. Alternative approaches that could have been taken but were not , due to time and financial constraints , would have been to monitor the activity of enzymes such as ornithine decarboxylase and protein kinase C and also a measure of the thickness of the epidermis [Gupta et al ,1989] these being used as indicators

of changes in cell multiplication following various treatments.

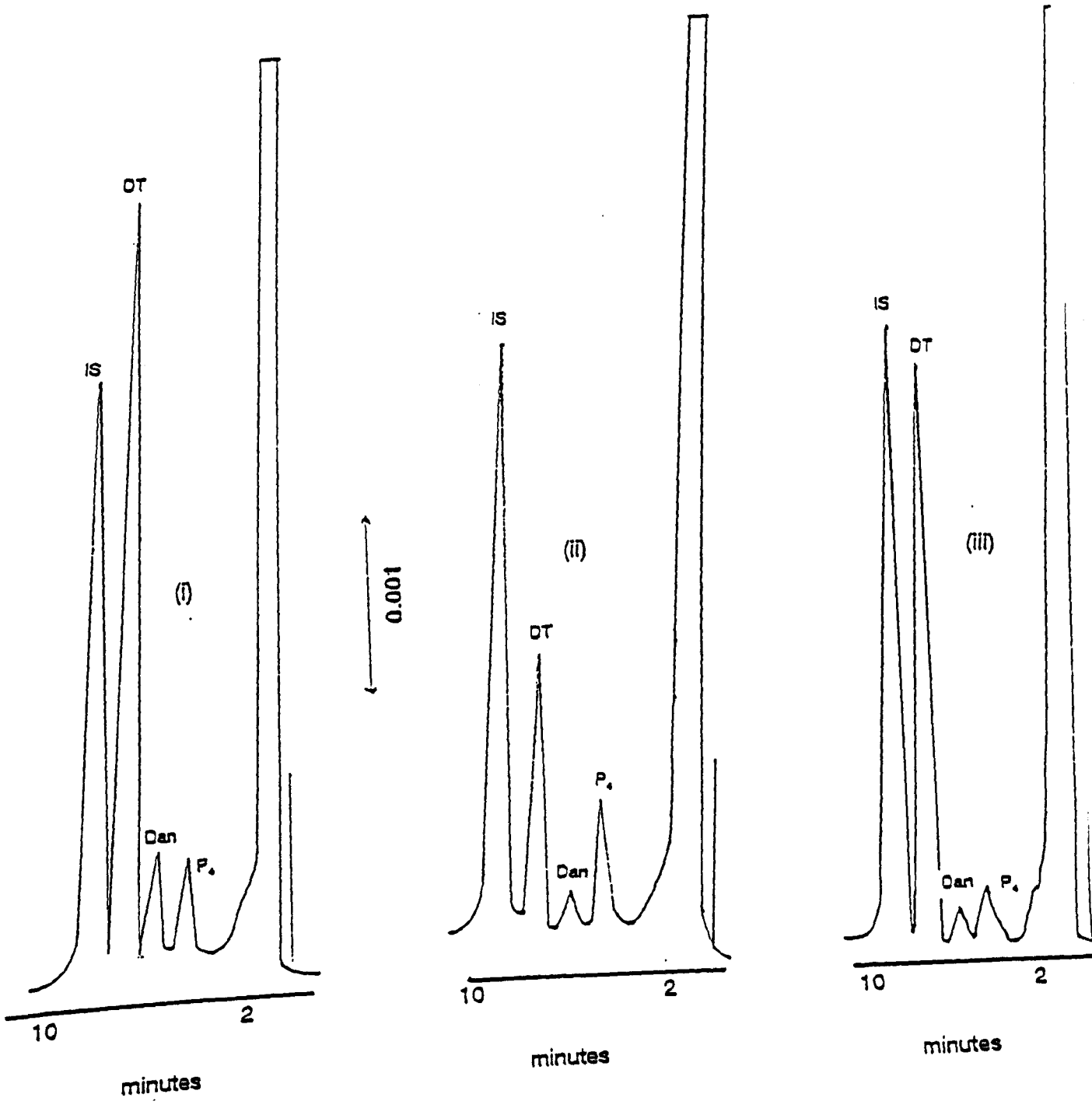


Fig 6.1

Chromatograms of the dithranol-surfactant formulations after varying contact periods with hairless mouse skin (i) Dithranol (100µg/mL) in 2.5% CET + 0.5% IAA pH 5.5 after 1 hour contact time; (ii) Dithranol (40µg/mL) in 4% Tw + 0.5% IAA pH 5.5 after 6 hours ; (iii) Dithranol (40µg/mL) in 4% NaLS + 0.5% IAA pH 5.5 after 6 hours.

DT = dithranol , Dan = danthron IS = internal standard.

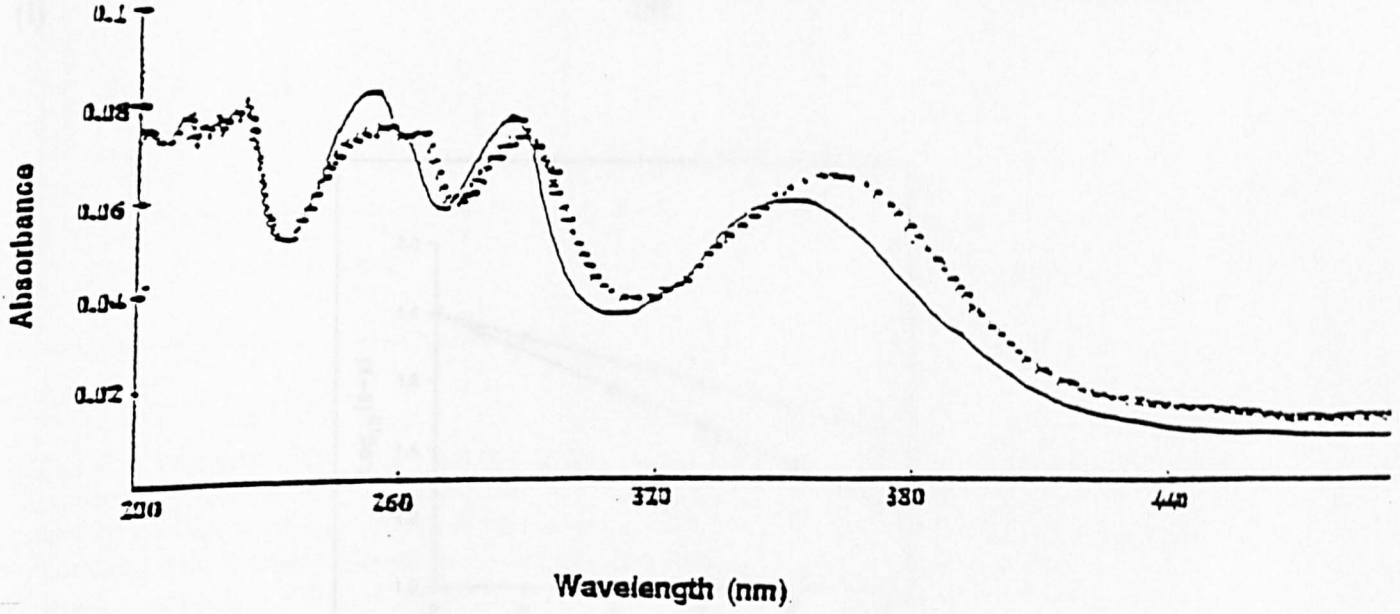
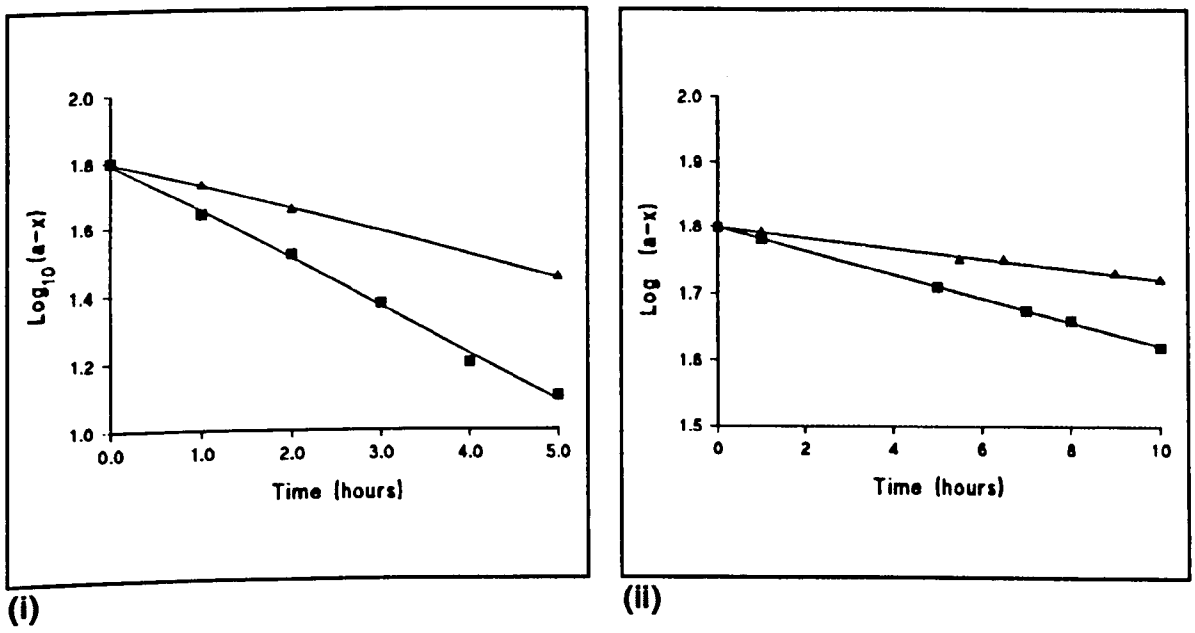


Fig 6.2

The UV spectra of the P_4 and dithranol fractions obtained after Hplc chromatography of the Tw formulation after 6 hours contact with hairless mouse skin.

P_4 (xxxxx) ; Dithranol (————)



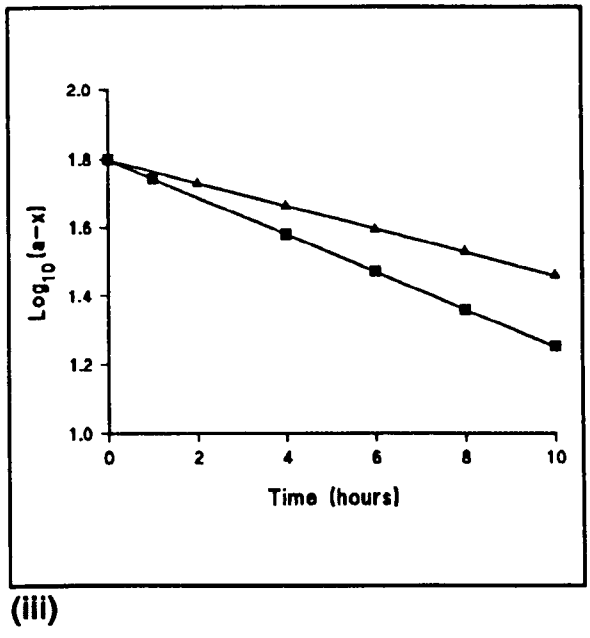
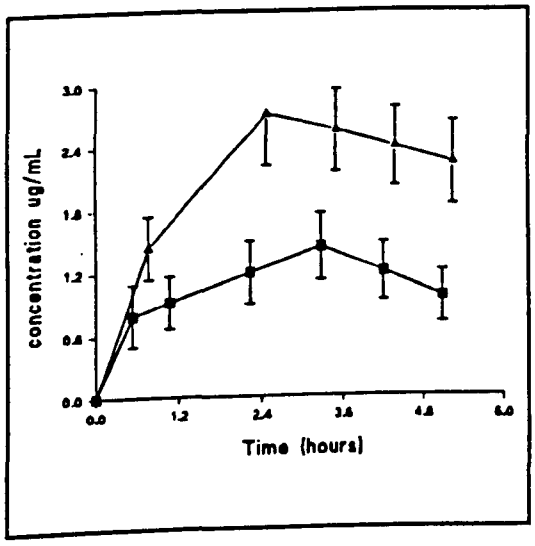
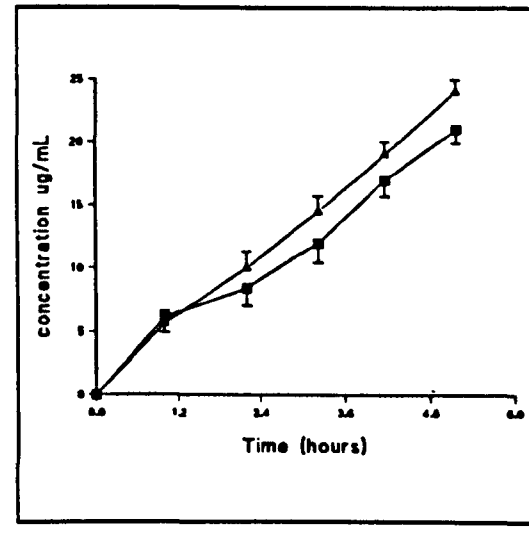


Fig 6.3
First order plots for the decomposition of dithranol (~63μg/ml) in (i) 2.5% CET, 0.5% IAA, pH 5.5 (ii) 4% NaLS, 0.5% IAA, pH 5.5 and (iii) 4% Tw, 0.5% IAA, pH 5.5, at 37°C both in contact, and not in contact, with hairless mouse skin. ■ skin contact, ▲ no skin contact.

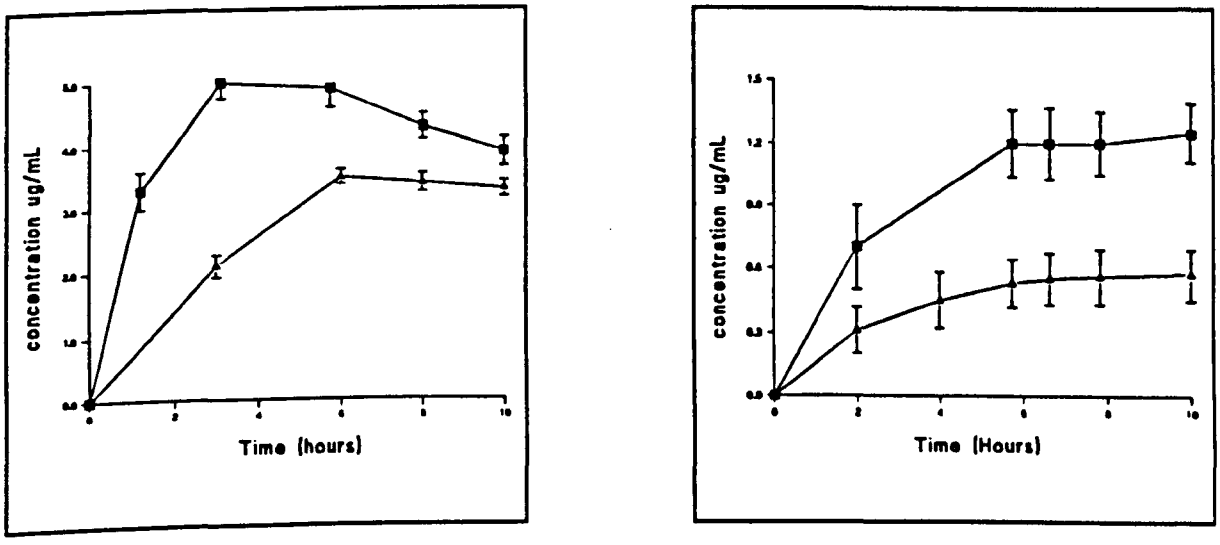


(a)



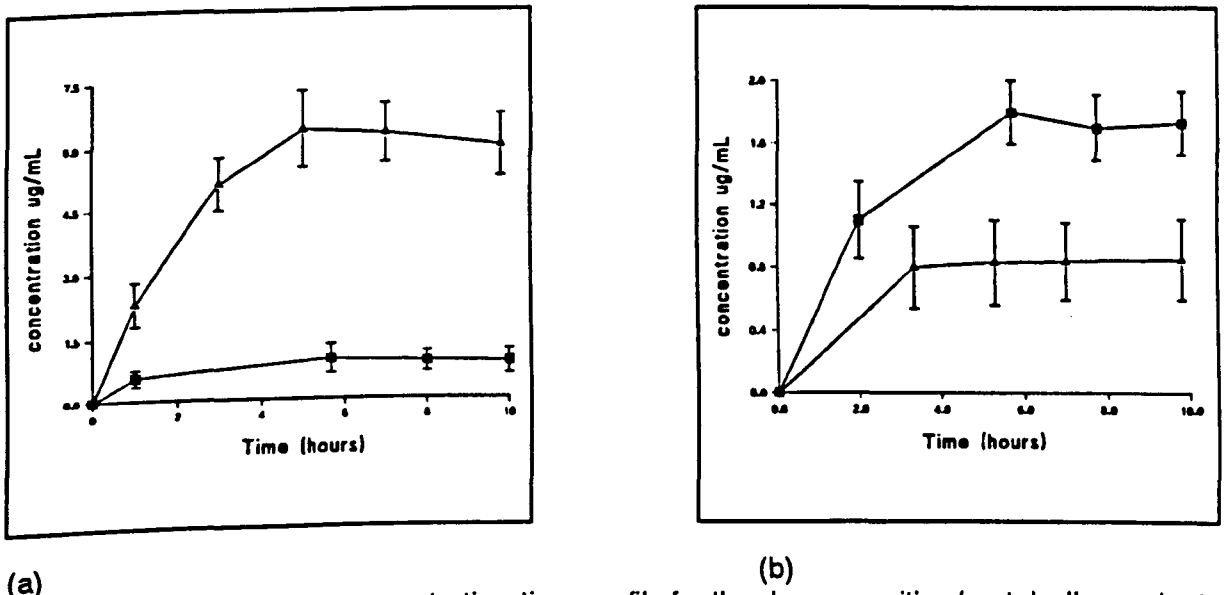
(b)

Fig 6.4 (a) and (b) The concentration - time profiles for the decomposition/metabolic products of dithranol (~63μg/mL) in 2.5% CET , 0.5% IAA , pH 5.5 solution at 37°C. Graphs are shown for a) dimer , b) danthron production for the same solution in contact and not in contact, with hairless mouse skin. Results are shown as mean ± SEM (n=3).
■ skin contact, ▲ no skin contact.



(a) (b)

Fig 6.5 (a) and (b) The concentration-time profiles for the decomposition/metabolic products of dithranol (~63µg/mL) in 4% NaLS, 0.5% IAA, pH 5.5 solution at 37°C. Graphs are shown are shown for a) dimer and b) danthron production for the same solution in contact, and not in contact with hairless mouse skin. Results are shown as mean ± SEM (n=3).
■ skin contact, ▲ no skin contact



(a) (b)

Fig 6.6 (a) and (b) The concentration-time profile for the decomposition/metabolic products of dithranol (~63μg/mL) in 4% Tw, 0.5% IAA , pH 5.5 solution at 37°C.Graphs are shown for a)dimer , b) danthron production for the same solution in contact, and not in contact, withhairless mouse skin. Results are shown as mean ± SEM (n=3).
■ skin contact, ▲ no skin contact.

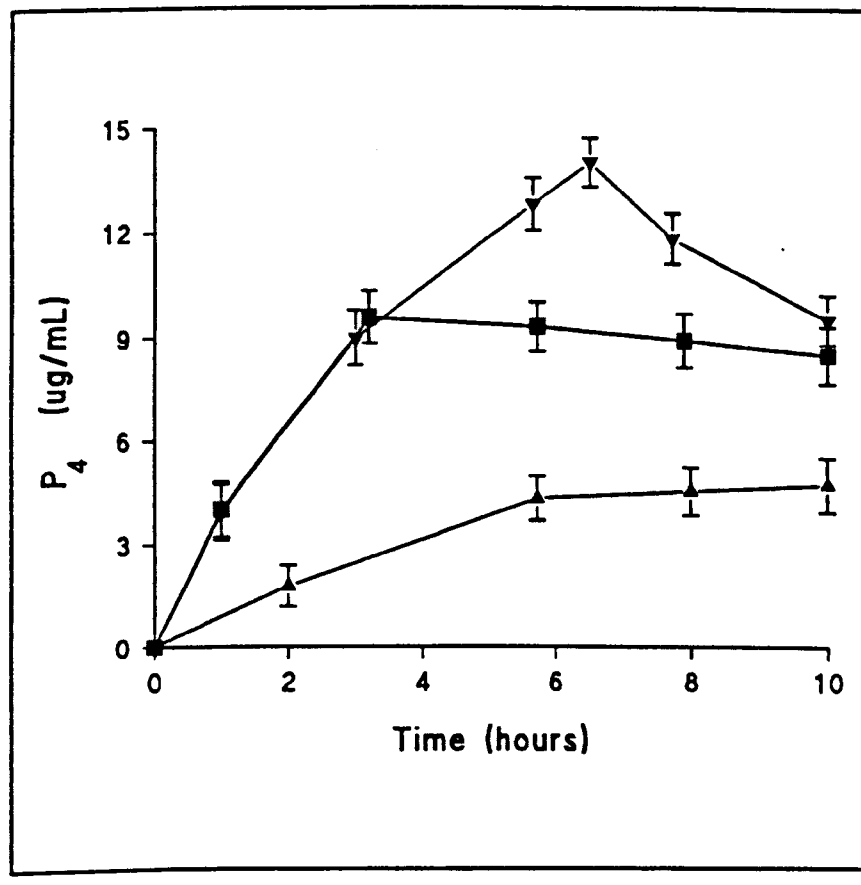


Fig 6.7 The concentration-time profile for the formation of P_4 in the surfactant solutions in Fig 6.3-6.5 in contact with hairless mouse skin at 37°C. Results are shown as mean ± SEM (n=3). ▲ 4% NaLS, ▼ 4% Tw, ■ 2.5% CET.

(a)

	DT	Dan	P4	DM
Skin surface	81±1	1.5±0.5	6±1	6.3±0.5
Skin tissue	3±1	-	-	-
Recovery	92%			

(b)

	DT	Dan	P4	DM
Skin surface	39±3	21±2	13±2.5	1±3
Skin tissue	4±1.5	2±0.5	3±0.5	3±1
Recovery	86.1%			

(c)

	DT	Dan	P4	DM
Skin surface	39±5.2	3±0.6	19±2	1.5±0.8
Skin tissue	12±3	1.5±0.2	10±2	-
Recovery	87%			

Table 6.1

The amount (% of initial dithranol applied) of dithranol and its metabolites on and in hairless mouse skin for dithranol delivered in a)NaLS after 6 hours, b)CET after 3 hours and c) Tw after 6 hours

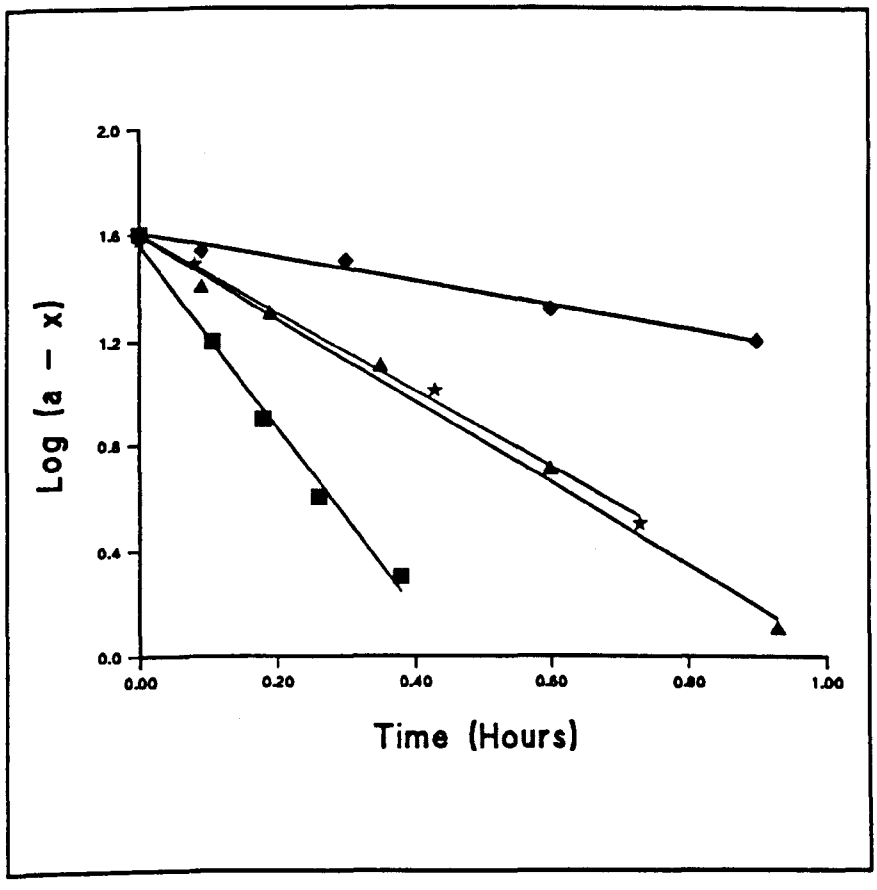


Fig 6.8
First order plots for the decomposition of dithranol (~40µg/mL) in the dithranol-surfactant formulations in contact with normal human skin in vivo. ■ 2.5% CET, ▲ 4% Tw, ◆ 4% Nals, * MeOH.

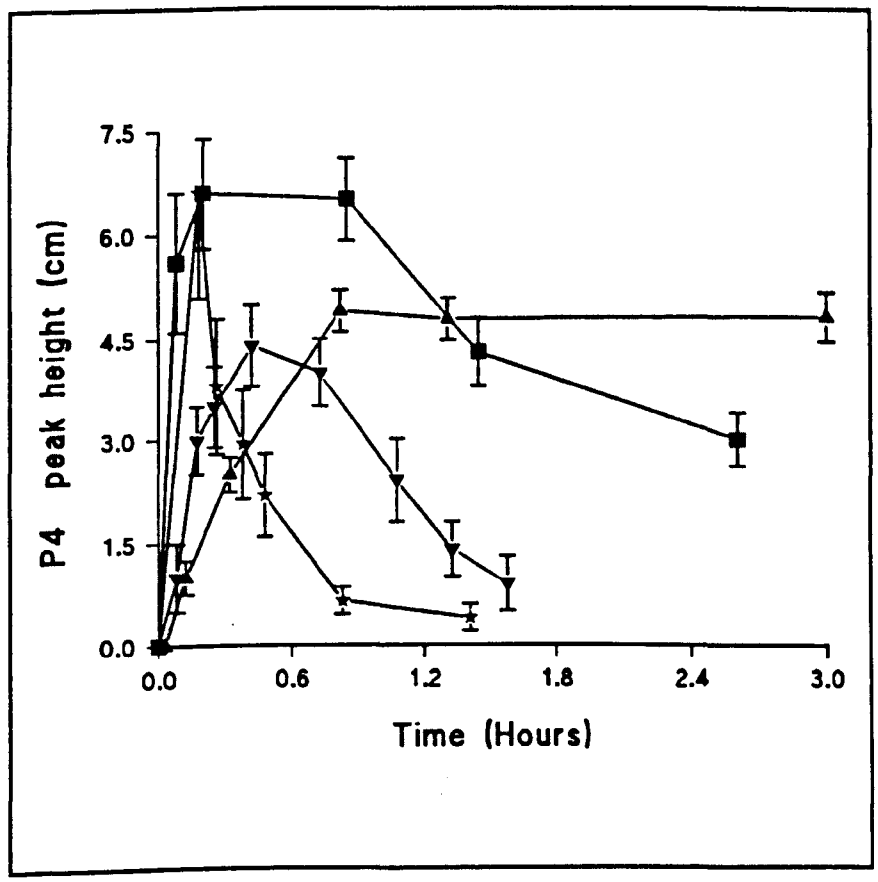


Fig 6.9
The in vivo formation of P4 from dithranol (~40µg/mL) in isoascorbic acid stabilised (0.5%) surfactant solutions in contact with normal human skin.The results are presented as mean ± SEM (n=3). * 2.5% CET, ■ 4% Tw, ▲ 4% NaLS, ▼ MeOH.

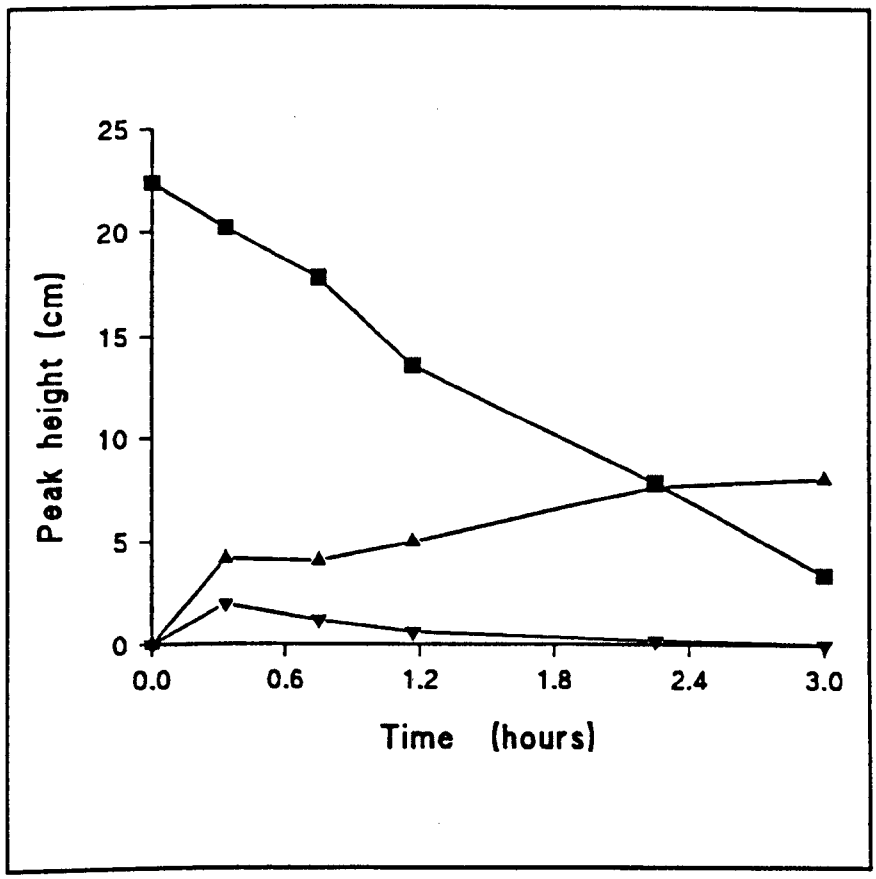


Fig 6.10
The concentration-time profile for the decomposition and metabolite formation from dithranol (~46μg/mL) in a 2.5% CET solution with 0.5% IAA, at pH 5.5 , temperature 37°C in contact with psoriatic skin. Changes in the peak heights with time of 1:100 dilutions of the decomposing solution are shown. ■ Dithranol, ▲ danthron , ▼ P_4.

Table 6.2

The amount (% of initial dithranol applied) of dithranol and its metabolites on and in psoriatic skin 3 hours after application of the CET formulation.

	DT	Dan	P4	DM
Skin surface	19.3	19.5	4.3	trace
Skin tissue	1.5	28.0	3.0	17.0
Recovery	90.6%			

Table 6.3

The degree of visibly evident inflammation of hairless mouse skin 24 and 48 hours after treatment with TPA alone, TPA and a dithranol solution or vehicle only.

Treatment[a]	[b]Inflammation score after 24 hours	after 48 hours
A	0	
4% NaLS	0	
4% Tw	0	
4% CET	2	
Acetone	0	
TPA alone		4
TPA + B		3
TPA + C		3
TPA + D		3
TPA + E		2
TPA + F		4

[a] Treatments. A = no treatment, B = dithranol 80μg/mL in acetone, C = dithranol 40μg/mL in 4% NaLS, D = dithranol 80μg/mL in 4% NaLS, E = dithranol 40μg/mL in 4% Tw, F = dithranol 80μg/mL in 2.5% CET.

[b] Inflammation score. 0 = no inflammation , 1 = traces of inflammation, 2 = mild inflammation, 3 = moderate inflammation. 4 = severe inflammation
In all cases dose of TPA was 10μmol delivered in 50μL of acetone.

Table 6.4

The DNA content of hairless mouse skin, determined using the diphenylamine method.Skin from 8 different animals was used.

Wet weight of skin (mg)	Total DNA extracted (μg)	DNA content μg/mg skin
75.3	300.6	4.0
77.4	368.9	4.8
84.7	476.0	5.6
85.3	380.0	4.5
81.7	446.8	5.4
75.9	276.8	3.6
101.9	531.5	5.2
103.6	632.0	6.1

Average DNA content = 4.9 ± 0.84 μg/mg of skin.

Table 6.5

The DNA content of hairless mouse skin 48 hours after treatment with either TPA only or TPA and a formulation of dithranol.

Treatment	DNA content μg/mg skin (wet weight)
A	4.9 ± 0.84
TPA alone	4.7 ± 0.67
TPA + B	4.5 ± 0.38
TPA + C	4.4 ± 0.27
TPA + D	4.9 ± 0.48
TPA + E	4.6 ± 0.58
TPA + F	4.7 ± 0.51

Results are presented as mean ± SEM. In all cases the dose of TPA was 10μmol delivered in 50μL of acetone. See Table 6.2 for treatment code.

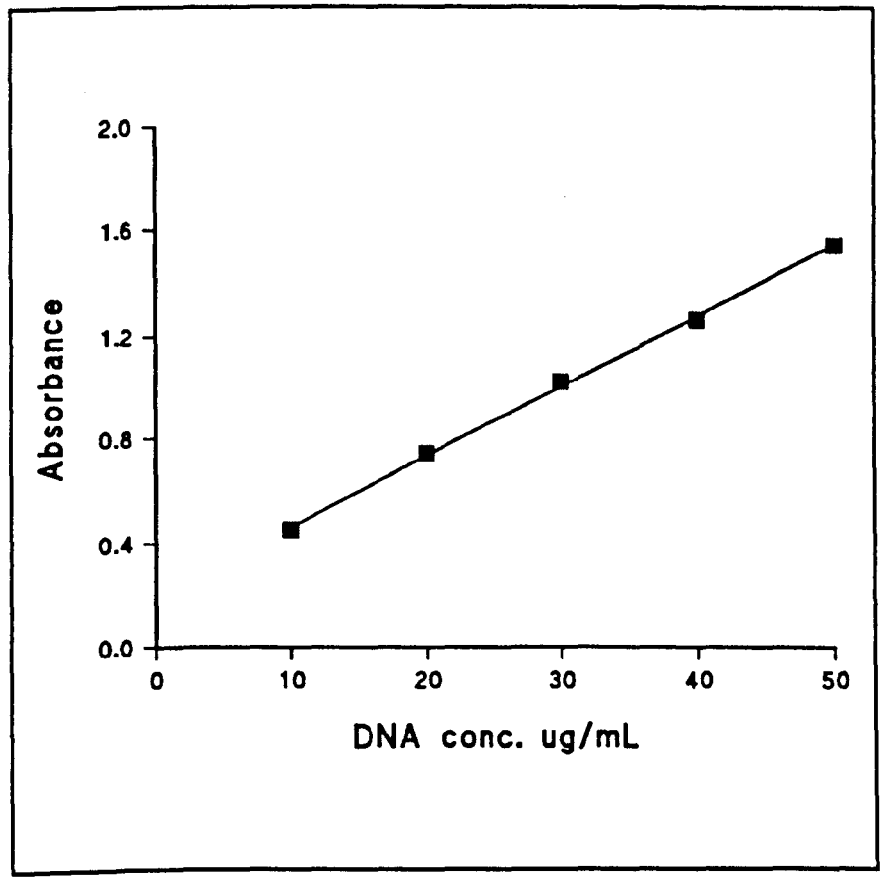
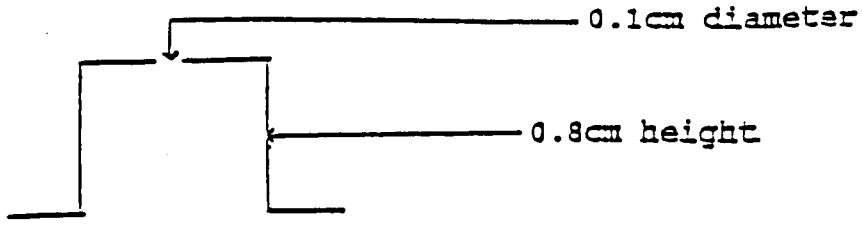


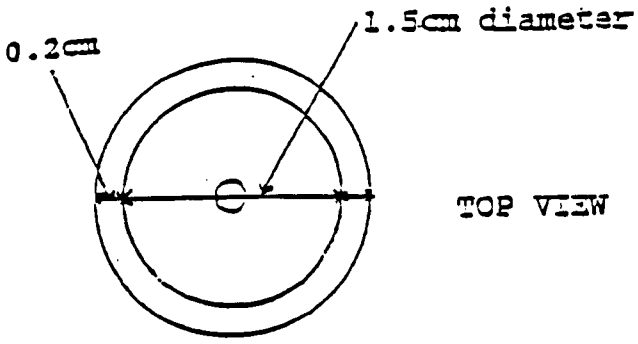
Fig 6.11

DNA calibration graph using the diphenylamine method (y = 0.026980(X) + 0.193800).

A.

Diagram of the glass cap used for the application of the dithranol-surfactant solutions

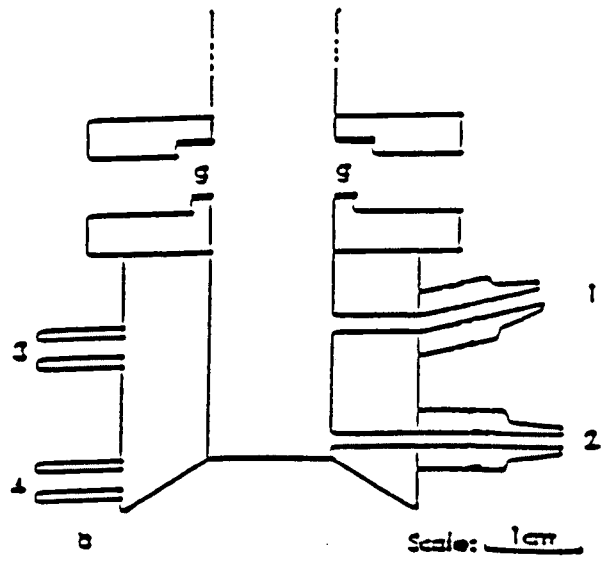




NB not drawn to scale

B Diagram of Diffusion cell used.

1= Receptor phase outlet
2= Receptor phase inlet
3= water jacket outlet
4= water jacket inlet
g = ground glass



CHAPTER 7

CLINICAL STUDY

7.1 Introduction

Dithranol has been used for the treatment of psoriasis for over 100 years now. It is the most potent topical preparation available for this condition. The chemistry of dithranol is related to aspects of therapy, mechanism of action and side effects. Transformation to an ionised form is critical to its effect in psoriasis. This transformation releases free radicals and is not only closely linked with its therapeutic effect but also with the production of metabolites which stain skin and clothing. There are two possible pathways proposed for the oxidation/metabolism of dithranol. One pathway results in the formation of dimer , while the other ends with the formation of danthron. In the present study both in vitro and in vivo work (see chapter 6) have shown that the there was a surfactant dependent alteration in the skin surface metabolism / permeation of dithranol , delivered to the skin as an aqueous surfactant solution. In the presence of the cationic surfactant , cetrimide , (present in antiseptic creams and shampoos) metabolism of dithranol is such that more of the ionised form is produced this resulting in conversion being directed away from the dimer pathway and also leads to the production of a hitherto undescribed metabolite (P_4). It was also established that there was no staining

and irritation when the dithranol-CET formulation was left in contact with normal human skin for two hours before removal.

The aim of this pilot study is to assess whether the surfactant influences on metabolism lead to any useful enhancement of dithranol use in clinical practice. Because of time restrictions the clinical evaluation of only one of the proposed formulations was possible. CET was chosen because of its obvious potential to produce danthron and P_4 and little dimer on human skin, this would allow the establishment of the relevance to the antipsoriatic effect of the danthron pathway. The working hypothesis was that by combining dithranol with CET , irritancy and staining would be reduced and efficacy increased. The study was based in the Dermatology Department of Aberdeen Royal Infirmary.

7.2 Methods

7.2.1 Materials

Dithocream 0.25% , 2% w/v CET solution pH 5.5 , this solution was prepared as per 6.2.2

7.2.2 Patients/volunteers

Patients with psoriasis undergoing dithranol treatment as in-patients or out-patients at Aberdeen Royal Hospitals Trust were selected .Four plaques of psoriasis were identified and scored seperately for thickness, scaling, erythema, irritancy and staining (see 7.2.3).

7.2.3 Treatment protocol

Patch 1 This was treated with an even coverage of dithrocream 0.25% rubbed in and left on for 8-12 hours.

Patch 2 Treated with CET solution applied with a cotton bud twice to ensure a good soaking.

Patch 3 Treated with the same amount of dithrocream for 8-12 hours as for patch 1. The dithrocream was then removed and CET applied as above.

Patch 4 Treated with CET solution painted on twice, the area then dabbed dry and dithrocream applied.

7.2.4 Clinical assessment - This was done at the start (Day 1) and after two days (Day 3) of daily administration of the treatments above. The treated areas were clinically assessed for thickness, scaling , erythema , irritancy , and staining. Each modality was graded on a 0 - 4 scale of increasing severity (0 = absent , 1 = trace , 2 = mild , 3 = moderate , 4 = severe).

7.3 Results/Discussion

Patient 1	1		2		3		4	
Treatment	Dithranol		Cetrimide		Dithranol Cetrimide		Cetrimide dithranol	
	Day1	Day3	Day1	Day3	Day1	Day3	Day1	Day3
Thickness	1	0	1	1	1	1	1	0
Scaling	2	0	2	2	2	1	2	1
Erythema	2	4	2	1	2	2	2	3
Irritancy	0	3	0	0	0	1	0	2
Staining	0	3	0	0	0	1	0	2
Total	5	10	5	4	5	6	5	8

Patient 2.

	1	2	3	4
Treatment	Dithranol	Cetrimide	Dithranol Cetrimide	Cetrimide Dithranol
	Day1 Day3	Day1 Day3	Day1 Day3	Day1 Day3
Thickness	2 0	2 2	2 2	2 1
Scaling	2 0	2 2	2 1	2 1
Erythema	2 3	2 2	2 1	2 1
Irritancy	0 3	0 0	0 2	0 2
Staining	0 3	0 0	0 2	0 2
Total	6 9	6 6	6 8	6 7

Table 7.1

Tables (patient 1 & patient 2) showing the effect on side effects and therapeutic outcome of applying CET before and after dithranol.

The tables of results show that in both patients , for the areas treated with dithrocream only , there was an increase in the total score. In patient 1 the increase was 100% while in patient 2 it was 60% . The main reason for the increase in the total score in both patients was the increase in erythema , irritancy and staining (EIS score). In both patients treatment with dithrocream alone resulted in a thickness and scaling score (TS score) of 0. The TS scores of 0 in both patients after the treatment period shows that clinical improvement of the plaques was good. Accompanying the improvement in the psoriasis , however , were the side effects of erythema , irritancy , and staining as indicated by the increase in the EIS score.

On the areas treated with CET only, it was seen that CET had no effect on the plaques. In patient 1 , there was a small reduction in the overall total score , this being due to a 20% reduction in the erythema score.

On treating the skin first with dithranol then followed by CET , there was a 20 and 30% increase in the total scores in patient 1 and patient 2 respectively (see Tables). In both patients the increase was due to increased irritancy and staining. This protocol did not have a great effect on erythema. In patient 1 erythema was reduced from moderate to mild while it remained unchanged in patient 2. When the effectiveness of dithranol is compared this protocol was found to be inferior to the dithrocream alone treatment. In patient 1 the TS score for dithrocream alone was reduced from 3 (Day 1) to 0 (Day 3) compared to a reduction from 3 (Day 1) to 2 (Day 3). The pattern in patient 2 was similar. The protocol however was found to be superior in terms of reducing side effects. In both patients the EIS scores on Day 3 were lower than those for dithrocream alone. In patient 1 the EIS score on Day 3 (for dithranol followed by CET) was 4 compared to 10 for dithrocream only, while in patient 2 , the EIS scores were 5 compared to 9. The overall observation for the dithranol-CET protocol was that while side effects were effectively reduced, the therapeutic effect of dithranol was also compromised.

On treating the skin with CET first then followed by dithranol it was observed in patient 1 that the TS score was reduced from 3 (Day 1) to 1

(Day 3) , and in patient 2 from 4 to 2 (same time period). The indication is that , with regard to therapeutic outcome , CET followed by dithranol was better than the other way round. The CET-dithranol protocol , however , was also inferior to dithrocream alone. The EIS scores on Day 3 show that in patient 1 the side effects of the CET-dithranol protocol were more severe than those from the dithranol-CET protocol a score of 8 compared to 6 was obtained respectively. In patient 2 there was no difference in the severity of side effects between the two protocols. For both protocols a score of five was obtained.

When the three protocols involving dithranol are compared the results show that the presence of CET , whether applied before or after dithranol , reduced side effects but also compromised the antipsoriatic effect of dithranol. This observation is in agreement with the in vivo results obtained for the dithranol-CET formulation using the TPA psoriasis model (see chapter 6).

In chapter 6 it was shown that when a dithranol-CET solution was placed in contact with human psoriatic skin (in vitro) , danthron was the predominant breakdown product following decomposition of dithranol. Based on the present in vivo results (bearing in mind limitations due to small sample sizes) it can be said that decomposition along the danthron pathway was not therapeutically benificial. Lawrence et al (1987) showed that wiping dithranol treated areas with KOH (pH 14) resulted in very effective reduction of inflammatory side effects with no compromise of the antipsoriatic effect of dithranol. The KOH effect

was said to result because it caused a rapid chemical inactivation of dithranol. At alkaline pH dithranol decomposition , not in contact with skin , is along the danthron pathway (preliminary work done in this study). It can therefore be expected that the KOH effect on dithranol therapy would be of a similar pattern to that seen for CET. This was true with respect to reduction in side effects , but not with regard to the effect of the two agents on the therapeutic outcome of dithranol. Unlike Lawrence et al (1987) , our observations with CET were that effectiveness was definitely reduced. The decomposition pattern of dithranol on psoriatic skin at alkaline pH is not known thus it becomes difficult to advance reliable reasons for why there was a difference between the two agents. Also the present patient sample size (2) means the CET results have to be treated as tentative.

CHAPTER 8

CONCLUSIONS

8.1 The new knowledge gained from the present work

There is more information available in the literature on the reactivity of dithranol in non aqueous systems as opposed to aqueous conditions. This , in the main , is because of the poor water solubility of dithranol, its poor stability and the lack of sensitive analytical techniques - particularly for the quantification of the dimer. This latter problem has been overcome in this study by the use of a modified hplc system.

This is the first reported quantitative study of the kinetics of dithranol decomposition to danthron and dimer in aqueous conditions particularly in the presence of surfactants.

8.1.1 Stability of dithranol in aqueous solutions

Decomposition of dithranol follows first order kinetics , with the resultant formation of danthron and dimer. At pH 5.5 decomposition half-life was 1.09 hours. When pH was reduced to pH 0.4 the half-life was increased to 18.8 hours.

8.1.2 Solubility and stability of dithranol in the presence of various surfactants

Dithranol has been shown to be solubilised but to different extent by different types of surfactants. It has also been observed that the choice of surfactant and its concentration has relevance to the observed stability profile. In NaLS and Tw at pH 5.5 and in CET at pH 0.4 the enhanced solubility is due to micellar solubilization with the amount of dithranol in solution (above the surfactant c.m.c) being linearly dependent on the per cent of surfactant present. In CET solutions (excess CET) the acid dissociation constant of dithranol is drastically reduced from 9.12 to 4.68 (these values were established using both absorbance and fluorescence measurements). This increase in the acid strength of dithranol occurs due to a complexation interaction between dithranol and CET. Dithranol is therefore ~88% ionised in CET solutions of pH 5.5. Since ionised dithranol is the more soluble molecular species, solutions of a higher dithranol concentration compared to the concentrations achieved in NaLS and Tw pH 5.5 are possible (see Fig 3.11). For the purpose of increased amounts of dithranol in aqueous solution formulation in CET solutions would be ideal. The major problem though is is that such solutions are unstable because dithranol is present in the less stable ionised form. Precautions such as antioxidant addition are necessary if shelf life is to be prolonged (see Table 4.12).

In all three surfactants the solubilization of dithranol offered a degree of protection from decomposition. Plots of k_{obsd} vs surfactant concentration were

bell shaped. Below the c.m.c values of 0.24% for NaLS pH 5.5 , 0.014% for Tw pH 5.5 and 0.09% for CET pH 0.4 increases in the surfactant concentration resulted in increases in the decomposition rate constant, whereas above the c.m.c values increases of surfactant concentration resulted in reduced rates of decomposition (see Fig 4.13 - 4.15).

8.1.3 Pathways of degradation , the influence of pH , metal ions and surfactant presence

Currently it is proposed that there are two routes by which dithranol will decompose. One pathway ends in the formation of dimer , normally non ionised dithranol will decompose by this route. The second pathway ends in the formation of danthron and ionised dithranol will normally decompose by this route. Dithranol is a weak acid with a pKa of around 9.12 (present study) thus at pH 5.5 it is virtually completely non ionised. On decomposition at this pH ~94% dimer and ~4% danthron were formed. Dithranol decomposition in aqueous solutions of alkaline pH were studied by Upadrashta & Wurster (1988) who found that the main decomposition product (especially at pH 9 and above) was danthron.

Metal ion presence influences dithranol degradation by increasing the rate at which dimer is formed while danthron formation is suppressed (see Table 4.7). Metal ions therefore provide a means by which dithranol decomposition can be controlled such that dimer formation is selectively enhanced.

In CET solutions decomposition is mainly via the danthron pathway even at a pH as low as pH 5.5. This is because in a CET solution (pH 5.5) dithranol is ~88% ionised (see above). In both NaLS and Tw the degradation pattern remains as observed in plain buffer. This is because these two surfactants have little influence on the molecular species of dithranol present at pH 5.5. Surfactant presence, therefore, can also be used as a means of controlling the degradation pathway of dithranol allowing an assessment of the effect of the degradation pathway on the therapeutic outcome of dithranol.

8.2 The stability/metabolism/permeation profile of dithranol delivered to various skin types (hairless mouse , normal human and human psoriatic skin) using surfactant solutions of dithranol.

An extraction procedure based on the use of a mixture of trichloro acetic acid and methanol as an extracting solvent to be used where appropriate , for the quantification of dithranol and its metabolites/decomposition products in hairless mouse skin (in vitro experiments) , was developed and validated. By using aqueous surfactant solutions of dithranol and hplc as the analytical technique the concentration vs time profile for dithranol and decomposition products in the formulation in contact with the skin during permeation experiments were established. Such profiles ,unavailable till now, allowed various kinetic calculations to be performed. In all three surfactants the amount of dithranol remaining with time was observed to follow first order kinetics and in all cases concentration decline was faster in the presence of skin (see Fig 6.3). In CET the rate of decomposition was increased to 0.32 hr^{-1} from 0.16 hr^{-1} , in NaLS to 0.04 from 0.02 hr^{-1} and to 0.12 from 0.08 hr^{-1}

in Tw.

The profiles also revealed that an as yet unknown metabolite/decomposition product (P_4) was formed due to decomposition of dithranol in contact with skin. The short retention time of this metabolite shows that it is more polar than either dithranol or danthron, while the UV spectrum suggests a structure similar to dithranol (see Fig 6.2). There are no reports in the literature of a similar metabolite. This is most likely due to the fact that no work has been reported where the skin surface surface concentration vs time profile for dithranol and decomposition products is monitored.

The results obtained from the permeation experiments on normal human skin in vivo show that there is a significant deviation from the decomposition pattern of dithranol in the formulations when not in contact with skin. Whereas both dimer and danthron were formed in the formulations when not in contact with the skin - indicating decomposition via both the dimer and danthron pathway - on normal human skin the only decomposition products detected were danthron and P_4, this indicating dithranol decomposition was via the danthron pathway only. This is significant as it gives a direct indication of what is expected in a "real life" situation.

The concentration vs time profiles also show that the choice of surfactant will influence permeation characteristics. With hairless mouse skin between 3.2 - 10.8% of the dithranol applied using the NaLS formulation penetrated into

the skin, with the CET formulation about 10 - 27% penetrated into the skin compared to 57 - 65% with the Tw formulation. Using normal human skin dithranol penetration into the skin from the NaLS formulation was between 55.5 - 62.5% of dose applied, whereas with Tw penetration was between 42 - 68% compared to between 4 - 28% with CET. Permeation of dithranol into human psoriatic skin in vitro using the CET formulation was around 57% of applied dose. This increased penetration (cf normal human skin) is consistent with permeation profiles for dithranol using diseased skin [Wang et al , 1987].

8.3 The antipsoriatic potential of the surfactant solutions of dithranol

Using the TPA/hairless mouse psoriasis model and DNA quantification as a marker for cell division it was difficult to judge the antipsoriatic potential of the surfactant formulations because no difference ($p<0.05$) was found in the DNA content of treated and untreated skin. The indications were that visual inflammatory responses to TPA treatment occurred without cell division taking place. It is not known why this happened, it could perhaps be a perculiarity of the species of mice used or perhaps an inadequate TPA dose.

The effect of the surfactant formulations on the inflammatory responses present in the TPA/hairless mouse psoriasis model could be judged visually. The indications here were that the NaLS and Tw formulations were capable of blocking the inflammatory response to TPA to the same degree as an equivalent amount of dithranol delivered in acetone. With the CET formulation

the anti-inflammatory effects of dithranol in the psoriasis model were negated. This particular model can therefore be used to study the anti-inflammation effects of dithranol formulations. The CET result obtained using the TPA/hairless mouse psoriasis model is in agreement with the results of a brief clinical trial aimed at evaluating the effect of CET on the action of dithranol in patients with psoriasis. The trial showed that using a solution of CET to cleanse the skin before or after treatment with dithranol reduces side effects but also compromises its therapeutic action.

During the course of the study a number of areas requiring futher reseach were identified.

(1) Further investigations are required to establish the exact nature of the P_4.

(2) Because of the problems encountered using conscious hairless mice in experiments aimed at establishing the antipsoriatic potential of surfactant solutions of dithranol, the possibity of using anaesthetized mice should be investigated.

(3) Using patients with psoriasis and metal ions to promote dimer formation, the relevance of the dimer decomposition pathway of dithranol to the therapeutic effect of dithranol needs to be investigated. A metal ion solution can be painted over the area to be treated before the application of a

conventional formulation of dithranol e.g dithrocream.

(4) There is also a need to establish the effect on the therapeutic outcome of dithranol , of cleansing the skin with NaLS and Tw before and after dithranol treatment.

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THE EFFECT OF SURFACTANTS ON THE SOLUBILITY AND STABILITY OF AQUEOUS SOLUTIONS OF DITHRANOL

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Dithranol is widely used and is clinically effective in the treatment of psoriasis (Seville 1981). Problems arise in its use because of its instability, its staining effects and its irritancy particularly on normal skin (Cavey et al 1985). The principal decomposition products, danthrone and dimer, are less effective than dithranol and may be responsible for its side effects. Short contact therapy is recommended to prevent side effects (Seville 1981). Therefore, since surfactants are known both to solubilise drugs and effect skin penetration, in this study the effect of sodium lauryl sulphate (NaLS), cetrimide (CET) and Tween 80 (Tw) on the solubility and stability of dithranol in water was evaluated as a preliminary to the development of aqueous low dose formulations for dithranol. Quantification of the dithranol in solution and its decomposition products was done using a previously developed HPLC system (Priprem 1991).

Saturation solubility of dithranol in water at 25°C (1.6 $\mu g\ mL^{-1}$) was increased by solubilization with the surfactants the effect observed being concentration and type dependent. For NaLS (0.01M) the saturation solubility was 3.6$\mu g\ mL^{-1}$; CET (0.01M), 4.8$\mu g\ mL^{-1}$; Tw ($1x10^{-3}$M), 3.2$\mu g\ mL^{-1}$.

Choice of surfactant markedly affected the stability profile. Using CET at 0.05M, dithranol decomposition was rapid, complete decomposition taking place within 3 hrs (see Fig.1). With both NaLS and Tw less than 10% decomposition was observed over the 3 h period. Complete degradation required 30 h for the former and 40 h for the latter. With all the surfactants both dimer and danthrone were formed. In all cases the dimer rapidly exceeded its saturation solubility in the surfactants (~ 1$\mu g\ mL^{-1}$) and precipitated out. The ratio of dimer to danthrone produced after complete decomposition was 3:2 for CET and 4:1 for both NaLS and Tw. Surface tension measurements indicated that surface activity was most marked with cetrimide - dithranol solutions. This may account for the more rapid decomposition of dithranol in CET.

For all three dithranol surfactant solutions inclusion of 0.1% isoascorbic acid (IAA) as antioxidant markedly reduced the rate of decomposition, particularly dimer production. Complete degradation of dithranol in CET was extended to 18 h; NaLS, 100 h; Tw, 150 h.

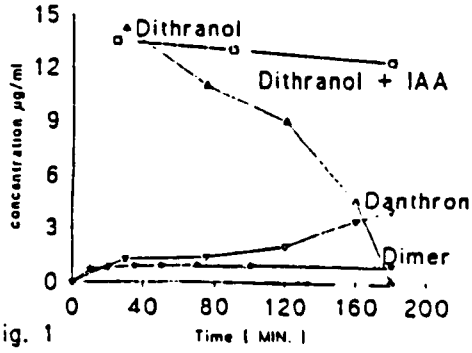

Fig. 1

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PHYSICOCHEMICAL PROPERTIES OF DITHRANOL IN AQUEOUS SOLUTIONS OF SURFACTANTS

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Previously we have shown that choice of surfactant had a marked effect on the solubility and the stability of dithranol (DT) in aqueous solutions of about pH 3.0 (Moody & Lubwika 1992). DT was least stable but most soluble in solutions containing cetrimide (CET) compared to those containing sodium lauryl sulphate (NaLS) or Tween 80 (Tw). In the present studies, at 36°C, measurement of spectroscopic changes caused by change in pH give a possible explanation for these observed differences.

Both the absorption and the fluorescence characteristics of DT change with pH. The absorption spectrum for the non-ionized and the ionised form are markedly different and only the ionized form is fluorescent (Melo et al 1983). These observations were used to determine the pKa of dithranol in water using buffers of constant ionic strength (I=0.5) both by absorption (Upadrashta & Wurster 1988) and fluorescence measurements (ex. 390nm; em. 518nm). Fluorescence has the advantage of being more sensitive (100 times) and it is specific for the ionised form. Both methods (n=3) gave a pK_a value of about 6.30 when determined using universal buffer.

DT solutions (100 ng ml^{-1}) containing 0.02% CET but of different pH values, eg 3.0 and 5.0, produce a marked pH and time dependent fluorescence response (see Fig.1). Increase in the CET concentration at a particular pH increased ionization eg 0.04% CET at pH 3 approximately doubled the response. These, and other results suggest that complex formation is occurring between DT and CET. The equilibrium may be represented as:

$$D + CET = D'CET + H^+$$

For a fixed CET concentration a plot of pH vs log $F_{\infty}/F_{max} - F_{\infty}$, where F_{∞} is the observed fluorescence response at that pH and F_{max} the maximum response for fully ionized DT, gave a straight line of intercept pK'where K' is the apparent stability constant e.g. for 0.02% CET the pK' was 3.33. Thus CET is promoting the ionization of DT at pH values well below its expected pKa, the effect observed being pH and CET dependent. The same effects were not observed with NaLS or Tw the pK_a for DT being unaffected by their presence.

Since the ionized form of DT is its most soluble but least stable form (it rapidly decomposes to danthron), the enhancement in aqueous solubility which is associated with a decrease in stability in CET relative to NaLS and Tw is now explainable.

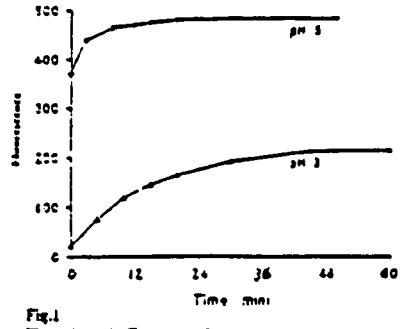

Fig.1
The effect of pH on the fluorescence characteristics of dithranol in universal buffer + 0.02% w/v CET at 36°C.

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