CHAPTER 5

BIOAVAILABILITY OF CORTICOSTEROID CREAMS.

5.1. In Vivo Methods

In the final analysis, to gain a full insight into the percutaneous absorption process of a drug in a living animal we must determine the permeation in that species. However, particularly in man, this approach is often fraught with experimental and ethical difficulties. Many *in vitro* procedures monitor steady state flux across the intact stratum corneum, whereas *in vivo* methods often employ physiological end points. These may be sensitive to minute amounts of the penetrant entering the skin during the transient phase of diffusion and particularly down the shunt routes. A further complication is that many drug molecules probably penetrate the living skin mainly through the shunt routes, and, *in vitro* procedure which neglects this aspect is potentially misleading. Another severe difficulty with human subjects or living experimental animals is that the techniques determine the extent of absorption, indirectly. In a typical experiment, the investigator applies a radioactive drug in an ointment to the animal's skin and assesses the kinetics of skin permeation from the rate of excretion in the urine. However, the drug may be partially metabolised while crossing the viable tissues of the skin. Body organs may take up a fraction of the penetrant, and a correction for this has sometimes been made by the worker injecting intravenously a single dose and determining urinary excretion[1]. However, sufficient

time must be allowed for collection of all the excreted drug; otherwise the organ storage value may include a sizeable error. For some compounds, binding, slow metabolism and distribution of the medicament to a "deep compartment" may rate-limit the excretion.

When experimental animals are used as models for man, in addition to species differences we must allow for increased permeation because of shaving and we must prevent the animal from ingesting or inhaling part of the dose.

More specialised methods such as human skin window and dermal perfusion were described briefly by Katz and Poulsen[2].

5.1.a. Animal models:

A persistent theme in work on percutaneous absorption is the development of suitable animal models which correlate adequately with man. However, most experimental animals differ significantly from man in the features which affect percutaneous absorption.

An additional limitation arises when the investigation aims primarily to assess the therapeutic activity of a drug formulation. Some workers developed few techniques which produce disease states in animals which are similar to human affliations.[3,4] However, several diseases are common

to dog and man Manna et al.[5] developed an animal model for chronic ulceration, and various workers have produced several types of "clean" wounds using, e.g., cantharidin or suction blisters, adhesive tape stripping, scalpel blade abrasion and sand papering.[6-9]

Nguyen et al.[10] developed a new model for a scaling dermatosis induces hyperproliferation in hairless mice. Scott[11] stated that even when simulated disease states developed, their response to drug may mislead in an extrapolation to man. The situation as summarised by Katz and Poulsen[2] still holds today that animal models are invaluable for the more detailed study of the anatomy, physiology, and biochemistry of the skin, for screening topical agents, and for detecting possible toxic hazards.

5.1.b. Techniques:

5.1.b.1. Observation of a physiological or pharmacological response.

If the penetrant stimulates a biological reaction when it reaches the viable tissues, then this response may provide the basis of determining the penetration kinetics. At least in theory, local allergic, toxic, or physiological reactions may be used and various topical agents affect such skin functions as sweat gland secretion, pigmentation, sebaceous gland activity, vasodilation, vasoconstriction, vascular permeability, epidermal proliferation, and keratinization. The most productive

technique in terms of biopharmaceutical application is the vasoconstrictor or blanching response to topical steroids. Other methods include determination of changes in blood pressure, reduction in pain threshold, production of convulsions, and the red tear response[2].

5.1.b.2. Physical properties of the skin.

A wealth of information is available on methods for measuring diverse physical properties of skin, *in vivo* and *in vitro*, much of it presented in the bioengineering literature. Some of the methods include the measurement of transepidermal water loss, thermal determinations (conductivity, differential scanning colorimetry, surface sorption), mechanical analysis (linear and non-linear viscoelastic techniques), use of ultrasound, classification of function and dimension, spectral analysis, and use of photoacoustic and electrical properties.

Wuepper *et al.*[12] have edited a symposium on photobiology and photomedicine. Tregear[13] wrote a classic review text, and Wilkes *et al.*[14] summarise additional information.

5.1.b.3. Analysis of body tissues or fluids.

Urinary analysis is often used to study percutaneous absorption, and the work of Wurster and Kramer[15], Butler[16] and particularly Feldmann and Maibach[17-19] illustrated its value. However, as emphasized earlier, all the drugs

which penetrate the skin should be accounted for by a "calibration" of the subject with a slow intravenous injection and a simultaneous determination of blood levels. The aim is to allow for all the pharmacokinetic factors inherent in drug absorption, distribution, storage, metabolism, and excretion. This combined procedure is a good method for *in vivo* assessment of topical bioavailability.[20] Neild and Scott[21] analysed Psoralens in the circulating blood. Sved *et al*.[22] detected nitroglycerin. Some of the relevant earlier attempts of plasma analysis include those of Gammell and Morrison[23], Stolar *et al*.[24]. Stelzer *et al*[25] and Hosler *et al*.[26] worked on the pharmacokinetics of topically applied lindane. Horhota and Fung[27] measured nitroglycerin absorbed in the shaved rat.

A combination of blood, urine, and feces analysis was used with rats, monkeys, and human volunteers to examine the percutaneous absorption and excretion of tritium-labelled Diflorasone diacetate, a novel topical steroid[28]. Webster and Noonan[29] observed that in the monkey the free base of antiacne agent was twice as bioavailable as the salt form.

Sometimes the drug has a particular affinity for a specific animal organ, which can be removed and the drug content analysed. Iodine, iodides, and mercury have been investigated in this manner[(30-32)]. Biopsies of organs

and skin tissue may be analysed and even individual sections measured.

5.1.b.4. Surface loss.

Measurements of the rate of loss of a penetrant from an applied vehicle should lead to a determination of the flux of the material into the skin. Malkinson[33] stated that the main use of a loss technique has been to monitor the decrease in radioactivity at the skin surface.

5.1.b.5. Histology.

It is tempting for an experimenter to try to locate the routes through which materials penetrate the skin by examining microscopic sections. However, such an approach can mislead because the drastic treatment applied in preparing the skin sections encourages leaching and translocation of diffusible materials away from their original sites.

Some workers[34-36] observed that certain drugs change the epidermal sulphydryl groups in an easily detectable way. In earlier days workers coloured a penetrant with a dye and then examined skin sections to locate the penetrant. However, physicochemical theory emphasizes that membrane transport occurs at the molecular level with each species partitioning and diffusing separately.

Baker and Kligman[37] used tetrachloro salicylanilide to determine the permeability of the stratum corneum.

Foreman et al.[38] observed by examination of UV fluorescence photomicrographs of hairless hamster skin treated with crude coal tar that the hair follicles in this species provide an important route for the penetration of tar components. In unfixed sections, the follicle, sebaceous gland, and fat cells underlying the dermis contained high concentrations of material.

5.2. Bioassays for Topical Steroids.

Topical corticosteroid bioassays are the most sophisticated and refined of all bioassays which we use to develop and to assess dermatological formulations[39]. In particular, one may not only employ the vasoconstrictor assay to evaluate the intrinsic activity of a topical steroid for correlation with possible clinical anti-inflammatory action; but one may use it also as a test in fundamental biopharmaceutical studies. Thus, the blanching test occupies a unique position among topical bioassays.

Haleblian[39] reviewed the bioassays for topical corticosteroids, anti-bacterials, antifungals, antiyeast preparations, antimitotics, antiperspirants, sun screen agents, antidandruff, anaesthetic - analgesic formulations, antipruritics, antiwart, poison oak/ivy dermatitis and Psoriasis.

Klingman and Mills[40] presented an improved bioassay for assessing comedogenic substances. Pochi[41] considered the assessment of sebaceous gland activity as a useful model for developing antiacne formulations. Gomez and Frust[42] dealt with relevance of the hamster flank organ as a model of the human sebaceous gland and its response to antiandrogens. Gellin[43] reviewed the chemically induced depigmentation in animal models. Leyden et al.[44] presented updated and expanded in vivo methods for determining the efficacy of topical antimicrobial agent.

5.2.a. Antigranuloma.

Some workers evaluated the relative potencies of topical glucocorticoids by standard antigranuloma bioassay.[45,46] This bioassay measures local activity rather than topical action.

5.2.b. Thymus involution.

Some investigators employed the thymolytic anti-inflammatory activity of corticosteroids to predict their possible value as topical steroids.[45,46]

5.2.c. Inflammation.

Since the clinical value of topical corticosteroids lies in their anti-inflammatory properties, several important bioassays developed and then treated inflammatory responses in experimental animals and man.

5.2.c.(1). Croton Oil.

Topical rat ear assay was used by Witkowski and Kligman[47,48] and modified by Dorfman[46] to study the relative potencies of anti-inflammatory compounds. The anti-inflammatory effect of the test compounds may be determined from the relative suppresion of the croton oil induced inflammation. They stated that croton oil irritation in human skin mimics clinical inflammatory conditions. This technique was adopted to human skin.

This bioassay has several advantages as a preliminary screening process. It is simple, non occlusive, and provides dose response data. The procedure yields clues regarding the potential dissociation of topical and systemic drug action, and it is one of the few topical animal screening models[49].

A rank order of corticosteroids evaluated by the suppresion of croton oil induced pustules or kerosene provoked blisters correlated reasonably well with clinical judgements of comparative efficacy, although the grading was not always the same in both the inflammatory tests[50].

Lorenzetti[51] presented data derived from his own experiments or abstracted from the literature on the relative potencies of topically applied steroids in the rat croton oil erythema assay as well as in the human vasocostrictor assay.

5.2.c.(2). Mustard Oil and Nitric acid.

Various concentrations of mustard oil in liquid petrolatum (mainly 80%) and nitric acid in water (usually 15%) were kept in contact with normal skin of volunteers. Corticosteroid ointments were rubbed into the above skin sites at time intervals ranging from 24 hr before to 24 hr after the application of the two primary irritants. The time of appearance of the inflammatory reaction, its rate of progress, and the final degree of response were noted. The inflammatory reaction was graded as erythema, erythemaplus obvious edema, additional formation of papules or vesicles, or necrosis[52]. The major factors which influenced the results were the relationship between the time of steroid application and the induction of inflammation, the duration of steroid contact with the skin site, the concentration of the corticosteroid, the intensity of the inflammatory stimulus, and the thickness of the epidermis.

5.2.c.(3). Tetrahydrofurfuryl alcohol.

Tetrahydrofurfuryl alcohol moderately irriates human skin. It also has the practical advantage that it is an excellent solvent for a wide range of compounds, including steroids. Solutions of the test compounds are applied to the skin under occlusion, and the erythema produced is scored on a 0 to 6 scale. When the erythema reduces in the presence of a steroid, the compound is rated as an

anti-inflammatory agent. The investigators who used this bio-assay concluded that fluorometholone was 40 times more active topically than hydrocortisone, even though the systemic activities were equal, Flurandrenolide was more effective than hydrocortisone acetate.[50,53]

5.2.c.(4). Kerosene.

Steroid preparations were applied under occlusion for 6 hr, and the resulting blanching was scored 1 hr later. The sites were then challenged with irritant patches of kerosene applied for 20 hr, and the responses were graded on a 5 point scale which ranged from 0 for complete absence of pustules or vesicles to 4 for bullae. The rank order of steroid anti-inflammatory efficacy correlated reasonably well with clinicians' judgements of comparative effectiveness. Zeynoun and Kurban[54] used the same technique to evaluate steroid creams and to correlate the results with double blind clinical studies.

5.2.c.(5). Lipopolysaccharide.

Heite et al.[55] evaluated five steroid ointments and two controls for their abilities to suppress the erythema produced by a subcutaneous injection of bacterial lipopolysaccharide into human skin. Hydrocortisone was ineffective in this bioassay.

5.2.c.(6). Histamine.

Reddy and singh[53] assessed the abilities of previously

applied alcoholic solutions of steroids to reduce the size of histamine wheals induced in human skin by the pin-prick method.

5.2.c.(7). Experimentally produced eczematous reactions: allergens.

Several early attempts to produce useful models of allergic contact eczema so as to simulate clinical syndromes were only partially successful.

Haxthausen[56] used electrophoretic studies. Scott[57] induced eczematous lesions in guinea pigs by sensitising them with dinitrochlorobenzene and then evaluated the efficacy of six commercial steroid ointments.

Evans et al.[58] sensitised mice by painting the abdomen with oxazolone in olive oil. Seven days latter the animals were challenged on one ear with oxazolone in acetone. Graded doses of steroids dissolved in the oxazolone solution were applied to the opposite ear. Activity of the corticosteroids was expressed as percentage of inhibition of ear weight gain in treated versus control ears, or as relative ear weight among the ears treated with various steroids.

Young et al.[59] concluded that betamethasone valerate acted as an anticorticosteroid in this assay in the rat, as well as in the antilymphocyte bioassay.

Kepel et al.[60] used a method in which the sensitised

animals are challenged at the ear by rubbing in undiluted antigen. After 24 hr, the developed lesion is treated with a steroid gel. The end point is the reduction in weight of a biopsy from the treated ear with reference to the contralateral control ear.

5.2.c.(8). Tape stripping.

Wells[61] suggested that this technique could be used to study the anti-inflammatory activity of topical steroids. He found that hydrocortisone inhibited the vasodilatation normally produced by 30 strippings of the stratum corneum. The Wells technique was further investigated as a bioassay by Heseltine et al.[62] for hydrocortisone and triamcinolone acetonide. Although tape stripping essentially removes the barrier to steroid penetration through the skin, with all that this implies with respect to biopharmaceutical studies, the method has been employed more recently during the development of corticosteroid formulations.[63,64]

5.2.c.(9). Erythema.

Ultraviolet (UV) light injures the skin in a complex fashion which involves epidermal cell death, increased mitotic index, hyperplasia, cellular exudation, and vasodilation[65]. Since most steroid responsive diseases are inflammatory, a UV erythema suppression bioassay is at least potentially useful for evaluating the relative activities of corticosteroids and their formulations.

Erythema produced in dog and rat skin was inhibited by topical steroids; experiments with guinea pigs yielded contradictory results.[66-68]

Jarvinen[69] discovered that massive oral doses of cortisone reduced somewhat the intensity of erythema produced in human skin by UV, and this work led to investigations which employed topical steroids in human volunteers. Everall and Fisher[70] were the first to evaluate a topical corticosteroid preventing the effect of UV rays on human skin. When corticosteroids were applied before exposure to UV[71,72] or grens rays[73-75], the erythema decreased. Burdick et al.[76] and Haleblian et al.[77] used a combination of the vasoconstrictor test and UV erythema suppression as human bioassays for corticosteroids.

Woodford[78] found that the MED (Minimum erythema dose) varied with the volunteer, the day of testing, and the site of application. Because of these factors, together with the lack of precision of the test, reluctance of volunteers to participate in repeated UV trials, and possible adverse effects of such repeated skin exposure to UV irradiation[79-84], the test should be abandoned for assessing topical steroid activity and bioavailability on human skin in favour of the vasocontrictor assay.

5.2.d. Cytological techniques.

The activity of corticosteroids may be assessed by

their effects on the growth of skin, its components, on its appendages.

5.2.d.(1). Fibroblast inhibition.

Corticosteroids act on many different types of cells. In particular, the fibroblasts of connective tissue respond to an inflammatory stimulus via a series of reactions which glucocorticoids inhibit[85].

Within the inflammatory process fibroblasts manufacture connective tissue components, and they are the cells of origin or transformation for many other cell types. On exposure to steroids, fibroblasts react morphologically e.g., the cytoplasm and nucleus disintegrate and cell replication is inhibited. We can directly correlate both these reactions with the anti-inflammatory activity of corticosteroids[86]. Thus, the potencies of topical steroids have been measured using a sensitive assay based on the *in vitro* inhibition of fibroblast growth when incubated with propylene glycol solutions of the steroids. A plot of fibroblast growth versus log dose of steroid provides a measure of the relative potencies of steroids such as hydrocortisone and fluocinolone acetonide[(86-89)].

The effects of glucocorticosteroids on primary human skin fibroblasts have been studied by Ponec *et al*.[90].

Haleblian[40] tabulated the relative potencies of

steroids such as corticosterone, hydrocortisone, prednisolone, dexamethasone, Paramethasone, triamincinolone acetonide, and fluocinolone acetonide as determined by the antigranuloma, bioassay, thymus involution, and fibroblast inhibition.

5.2.d.(2). Reduction of mitotic rate.

Corticosteroids reduce the mitotic rate in normal human skin[91,92] and in Psoriatic skin[93,94]. Marks et al.[95] developed this tape stripping technique to stimulate mitosis in the dorsal skin of the hairless mouse.

5.2.d.(3). Skin thinning.

Spearman and Jarrett[96] considered that, because thinning of the epidermis follows an ibhibition of epidermal mitosis, measurement of the epidermal mitosis, measurement of the epidermal thickness of the mouse tail in the presence and absence of steroids should prove to be a useful bioassay. Winter and Wilson[97] considered that the back skin of the domestic pig was more like human skin than was mouse skin, and they used the epidermal thinning technique in both pig and man[97,98]. The effect was related to the molecular structure of the corticosteroid, its concentration, and the base used[99]. Delforno et al.[100] showed that corticosteroids reduced viable epidermal cell size, not the number of cell layers.

Dykes and Marks[101] discussed methods for the measurement of skin atrophogenicity of topical steroids. Kirby and Munro[102] used a micrometer to measure the total skin thickness in the mouse ear and the human forearm and found that the magnitude of steroid induced thinning depended on the formulation applied. Jablonska et al.[103] using a histological technique, evaluated skin atrophy in man induced by topical corticosteroids, other workers have employed xeroradiographic, ultrasonic, and stereomicroscopic methods.(104-106)

5.2.d.(4) Inhibition of hair growth.

Corticosteroids inhibit hair growth in rat when they are applied directly to the skin. The bioassay of Whitaker and Baker used this phenomenon by recording the extent and the pattern of regrowth of hair in the clipped, dorsal region of the neck after treatment with steroids.(107,108) This bioassay was used to study the effect of molecular changes in a series of seven hydrocortisone analogs.

5.2.d.(5) Leukocyte challenge.

Corticosteroids inhibit the release of lysosomal enzymes from human and rodent neutrophils. Watson et al.[109] therefore tested seven corticosteroids for their effect on the release of B-glucuronidase when human white cells were challenged with zymosan. The resultant ranking of the

steroids correlated quite well with published vasoconstrictor assays.

5.2.e. Psoriasis bioassay.

Most bioassays fail to deal with spontaneously occuring skin diseases[40]. The psoriasis bioassay was developed for treatment of the disease lesion, and as such it is the nearest approximation to a clinical trial. Chronic stabilised psoriatic patients are selected as test subjects, and steroid preparations are applied under occlusion to the psoriasis plaque. The area is read as either unchanged or converted to normal skin, and the results may be presented as log dose versus percent cleared (probit) graphs.

5.2.f. The Vasoconstrictor test.

It has been known for more than 30 years that certain steroids make human tissues pale. Ashton and Cook[110] reported vasoconstriction in superficial corneal vascularization treated with subconjunctival steroids. The blanching produced by topical application of anti-inflammatory steroids has been used as a valuable bioassay. Mckenzie and Stoughton[111], observed that treating psoriasis with steroids under plastic wrap blanched the lesion and also normal skin, considered that this effect might be used to assess the percutaneous penetration of corticosteroids.

Mckenzie[112] emphasized that the test was most valuable

for demonstrating those compounds that blanched at high dilutions, which correlated with the most effective clinical anti-inflammatory activity.

Further work with alcoholic solutions of steroids developed the test and gave additional insight into the variables which affected the precision and accuracy of the bioassay.

In the meantime, corticosteroids were being tested in solvents other than simple ethanol[113-116].

This bioassay may be employed both to screen new steroids for clinical efficacy and to determine the bioavailability of steroids from topical vehicles. The term "bioavailability" is taken here to mean the relative absorption efficiency for a medicament as determined by the release of the steroid from the formulation and its penetration through the stratum corneum and viable epidermis into the dermis to produce the characteristic vasoconstrictor effect[117].

Thus we may measure the intensity and duration of the steroid induced pallor to assess both the activity of a corticosteroid and its bioavailability from different vehicles, as determined by a pharmacological response[118].

5.2.f.(1). Development of a standard vasoconstrictor test.

In the years since the vasoconstrictor test was

introduced many of its practitioners have proposed their own modifications of the test design. The most important variables have included such features as the method of application, occluded or nonoccluded; the duration of steroid application from 2 hr to 20 hr; and the method assessing the response graded or present/absent, single or multiple readings with time.

5.2.f.(2). Design consideration for the vasoconstrictor test.

To design a standard procedure using the arms of volunteers, many features must be considered. For example, blanching may be inconsistent for people with very short or very narrow forearms, For application sites nearer to the pulse or elbow than 4 cm, and for typists, i.e., persons exercising the forearm muscles during the period of steroid application[118].

Because of the differences between sites on the same forearm[119] preparations should be applied according to randomisation charts. Lower blanching scores and more inter-and intra-subject variation arise with nonocclusion. A short steroid application time of 6 hr provides better differentiation between products than does 12 hr of occlusion. Variations in the applied amount of a cream or ointment between 3 and 8 mg applied over 50 mm of skin do not significantly affect the degree of pallor produced over 96 hr.

Blanching readings should be taken under standard lighting conditions with the arms held horizontally or slightly upward. The blood vessels in some volunteers enlarge when their arms hang downward, and this swelling may obscure the pallors. The pallor may be assessed against untreated skin on a 0 to 4 scale with half point ratings as follows: 0, normal skin; 1, slight pallor of indistinct outline; 2, more intense pallor with at least two corners of the application square outlined; 3, even pallor with a clear outline of the application area; 4, very intense pallor. Only the most potent formulations applied to very sensitive volunteers produce a score of 4 at the peak time of 9 to 12 hr. Pallor should be assessed by observing the response for a minute before allocating scores, when the eye can seek out the less intense sites.

Over the years there have been several attempts to replace a visual method of assessment by an instrumental approach. Greeson et al.[120] modified the Xenon 133 gas clearance technique of Sejrsen to measure cutaneous blood flow alterations caused by flurandrenolide, and they correlated decreased blood flow and vasoconstriction. Tronnier[121] reported the "thermal conductivity" of areas blanched by steroid. Stuttgen[122] reported that blanching was accompanied by a decrease in IR radiation, although Kiraly and Soos[123] could not detect such differences with IR camera. Gibson[124] discussed the disadvantages of visual

standards and the limitations of normal photographic techniques.

Reflectometry and colorimetry have been used in anthropological studies of different races, and these methods have received some attention in steroid assessment studies. Simple reflectometry is suitable for measuring changes in pigmentation and has been used to quantify erythema, but the technique lacks sensitivity[124]. Heseltine *et al*.[113] found that the procedure was no improvement over the visual assessment for pallor.

Reid and Brookes[125] assessed blanching with a reflectance spectrometer but found that differentiation between steroid products was difficult. Tring[126] used a similar technique to assess the effects of various treatments, including steroid application, on skin color in Psoriasis. It is possible that reflectance measurements are more satisfactory for determining skin color than for assessing blanching. Considerable success has been achieved by workers determining the logarithm of the inverse of the reflectance of the skin[127].

For optimal value in biopharmaceutical studies, any instrumental method for assessing the blanching response must meet certain criteria. The instrument must not contribute to the response which it measures, nor should it change the physiological or physico-chemical condition

of the skin. Thus, methods which apply even a slight weight to the skin can cause a spurious pallor, and occlusive methods may hydrate the skin and alter the percutaneous absorption of the steroid. The determination must be rapid compared with the time scale of the absorption process. The measurement should be sensitive, reproducible, and accurate, and the technique must be acceptable to the volunteers.

The method has to allow for physiological variables, such as the normal variation of skin temperature during the day[128-130]. Most importantly, any instrumental technique must not be affected by or must compensate for skin imperfections which may alter, for example, a reflectance measurement. At the present time, the only really satisfactory technique employs the trained human eye, which is adept at assessing subtle color differences and at making automatic allowances for skin imperfections. In particular, the eye brain combination can readily compare a steroid application site with the adjacent untreated skin. It is this comparison, rather than some arbitrary fixed standard of pallor, which is the cornerstone of a successful vasoconstrictor assay. Thus, Barry and Woodford[118] found that a visual assessment method was more sensitive and precise than an objective, instrumental method such as reflectance spectroscopy.

The blanching response fades quickly and erratically

if volunteers become hot or their arms become wet; panel members may need to change their life style somewhat during a test period[118].

To use the vasoconstriction assay as a biopharmaccutical technique, it is not essential to know precisely how steroids produce pallor. This is fortunate, as the exact mechanism by which topical corticasteroids blanch skin remains in doubt. Most workers agree that the pallor arises from a vasoconstrictor action and the terms "vasoconstriction", "blanching", and "pallor" are widely used as synonyms.

5.2.f.(3). <u>The Barry - Woodford vasoconstrictor test: Occluded</u>.

Semisolid formulations are stored at room temperature, and the first gram of product from a tube is rejected. Caucasian volunteers are screened for a consistent response to a standard preparation, containing 0.1% betamethasone 17-valerate. They are ranked as good, medium, or poor responders. Ten volunteers are selected to provide a team with a balance of such responders. In subsequent trials, any particular volunteer not available may be replaced by one with a similar sensitivity ranking. This procedure leads to greater consistency of results from trial to trial. No volunteer should have received topical application of a steroid for at least 2 months prior to an investigation.

About 5 ± 1 mg of each product is applied to the washed flexor surface of both forearms. Application sites are paired 7 x 7 mm areas punched out of double sided adhesive Blenderm Polyethylene tape (3 m Medical Products, London); formulations are applied by reference to randomisation tables. The sites are occluded with type S 12 µm. Melinex film (I.C.I. Plastic Division, Welwyn Garden City, United Kingdom) for 6 hr. The sites are then washed with soap and water at skin temperature, dried, and the degree of peller is estimated 10 min later under standard lighting conditions using the 0 to 4 scale.

Readings may then be taken as a function of time to provide data points for skin blanching at 6,7,8,9,12,24, 32,48,72,80 and 96 hr after application. A full blanching profile may then be drawn. All estimations of peller are performed in a double blind manner by an experienced investigator, without reference to application charts. During the test period, as far as possible, volunteers should avoid high temperatures and water.

5.2.2.(4). <u>The Barry - Woodford vasoconstrictor test : Non-occluded</u>.

The nonoccluded design is similar to the occluded tests, except that after application of samples to the skin the Blenderm tapes are removed and a perforated plastic screen is used for 6 hr instead of the polyester film. This design removes the occlusive, hydrating effect of the water-impervious film.

5.2.f.(5). Precision and reproducibility of the Barry-Woodford: Vasoconstrictor test.

For a test which depends on a pharmacological response in humans assessed subjectively, it is important to investigate how results may alter as the panel composition changes and how reproducible the test is from year to year. Sixteen volunteers were employed, six being used throughout as members of every panel and the remainder being called upon as necessary to produce full panels of 10 volunteers.

The occluded blanching test was used, and the results for all volunteers were expressed in a standard manner as the percentage of the total possible score at each time period of assessment. A blanching profile could then be drawn from a plot of percentage total possible score as a function of time.

5.2.f.(6). Dose - response relationships in the vasconstrictor test.

To give confidence in the use of the vasconstrictor test for biopharmaceutical assessments, it is important to establish dose - response relationships. Experiments were therefore performed to see if model preparations which contained different concentrations of steroid in the same base produced graded blanching responses. An essential criterion in this program is that the steroid in each formulation should be entirely in solution. There

would then be no problem with suspended drug dissolving on dilution with placebo vehicle and thus maintaining the thermodynamic activity of the penetrant. The activities and bioavailabilities of many proprietary corticosteroid formulations have been assessed by the single application vasocontrictor test of Barry and Woodford.[131-133] This work ranked the preparations in a classification which often demonstrated comparability between the blanching results and the conclusions of clinical trials.[134-139] The exhaustic account of the literature survey makes it very clear that in the investigation such as the present one, it is highly desirable to evaluate the products for their bioavailability. Besides being a numerous parameter, bioavailability studies on topical formulations would prove the real therapeutic justification and usefulness.

Cream bases selected on the bases of their in vitro evaluation were subjected to bioavailability study employing vasoconstrictor assay.

5.3. Bioavailability of Corticosteroid Creams.

Vasoconstrictor assays on selected corticosteroids creams after in vitro evaluation.

5.3.a. Experimental.

Corticosteroid formulations:

About twenty selected hydrophilic topical steroid

cream formulation, were stored at room temperature for not less than two weeks and the first gram of product obtained from each tube was rejected.

Volunteers.

Ten fair skined volunteers were selected without reference to the sex or steroid sensitivity. None had received topical application of corticosteroids for at least 3 months prior to the investigations and an interval of one month was allowed between trials.

Method.

The blanching test employed was a modification of that described by Barry and Woodford[118] (A) Double-blind technique was used for blanching test. 10±1 mg of cream formulations, were applied to the washed flexor surface of forearms, previously inflammed by four stripping of Johnsonplast adhesive tape. The application sites consisted of 5 mm areas punched out from Johnsonplast adhesive tape (Johnson & Johnson, Bombay, India) to which the formulations were applied by reference to previously prepared randomisation sheets; a total of ten sites were used for each preparation. The sites were occluded with adhesive tape. Thus, each square was converted into an isolated area of steroid application under occlusion. The arms were then bandaged with tube-gauze to ensure a reasonably constant temperature environment.

After 6 hr the bandage, adhesive tapes were removed and the areas were washed with soap and water at skin temperature, were dried, and the degree of pallor was estimated 10 min later by two observers unaware of the position of application of various steroids using a 0 to 4 scale with half-point rating based on that of Pepler, Woodford and Morrison[140] as given below.

0 - normal skin

1 - Slight pallor of indistinct outline

2 - Pallor with at least half circle outline.

3 - Even pallor with a clear outline of the circle.

4 - Very intense pallor.

Readings were taken after a further 3,6 and 18 hr provide data points for skin blanching at 6,9,12 and 24 hr after application and recorded in Table 7 - 1. All estimations of pallor were performed in a double-blind manner without reference to the application charts. Throughout the duration of the tests the volunteers avoided elevated temperatures. (environmental or those produced by exercise) and did not allow their arms to come into contact with water.

The whole experiment was conducted in consultation and with a dermatologist and a physician. The photographs of the whole experiment shown in figure 7-1 to 7-7.

TABLE 7-1 : Bioavailability Study on Selected Triamcinolone Acetonide creams by Blanching Test.

Cream Nos.	Degree of Blanching at each reading							
	6 hr.		9 hr.		12 hr.		24 hr.	
	A	B	A	B	A	B	A	B
1.	29.0	36.5	26.0	32.5	24.0	30.0	8.0	10.0
5.	16.0	20.0	24.0	30.0	23.0	29.0	9.0	11.5
13.	14.0	17.5	22.0	27.5	12.0	15.0	Nil	Nil
25.	16.0	20.0	25.0	31.5	18.0	22.5	8.0	10.0
27.	18.0	22.5	22.0	27.5	20.0	25.0	10.0	12.5

A = Score.

B = Percentage maximum possible score.

FIG. 8-1

PERCENTAGE OF TOTAL POSSIBLE SCORE AS A FUNCTION OF TIME FOR TRIAMCINOLONE ACETONIDE CREAMS

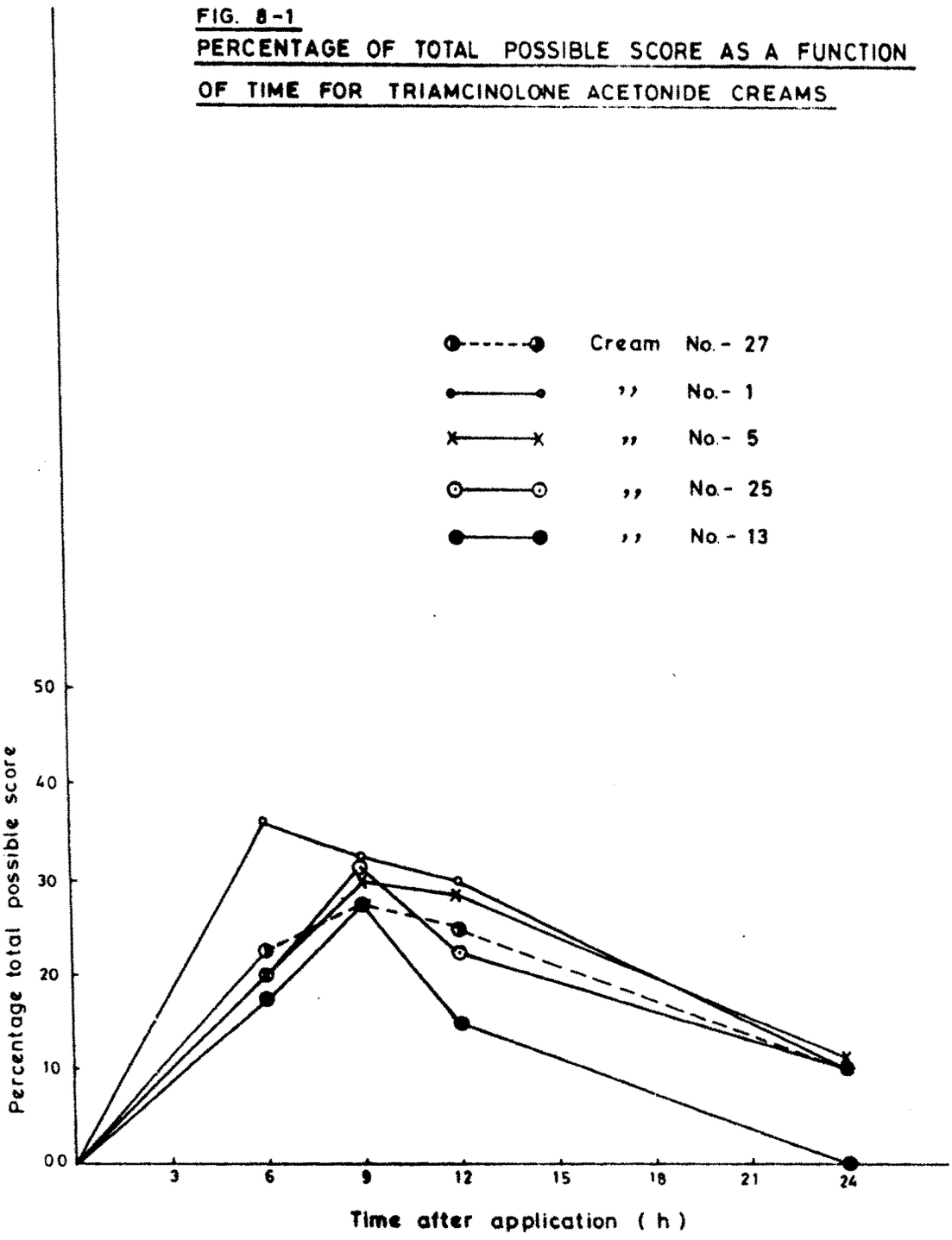

TABLE 7-2 : Bioavailability Study on Selected Betamethasone 17-valerate Creams by Blanching Test.

Cream Nos.	Degree of Blanching at each reading							
	6 hr.		9 hr.		12 hr.		24 hr.	
	A	B	A	B	A	B	A	B
1.	36.0	45.0	49.0	61.5	42.0	65.0	24.0	30.0
5.	34.0	42.5	46.0	57.5	44.0	55.0	19.0	24.0
7.	25.0	31.5	33.0	41.5	35.0	44.0	21.0	26.5
13.	22.0	27.5	30.0	37.5	21.0	26.5	7.0	9.0
27.	32.0	40.0	43.0	54.0	44.0	55.0	26.0	32.5

A = Score.

B = Percentage maximum possible score.

FIG. 8-2

PERCENTAGE OF TOTAL POSSIBLE SCORE AS A FUNCTION OF TIME FOR BETAMETHASONE 17-VALERATE CREAMS

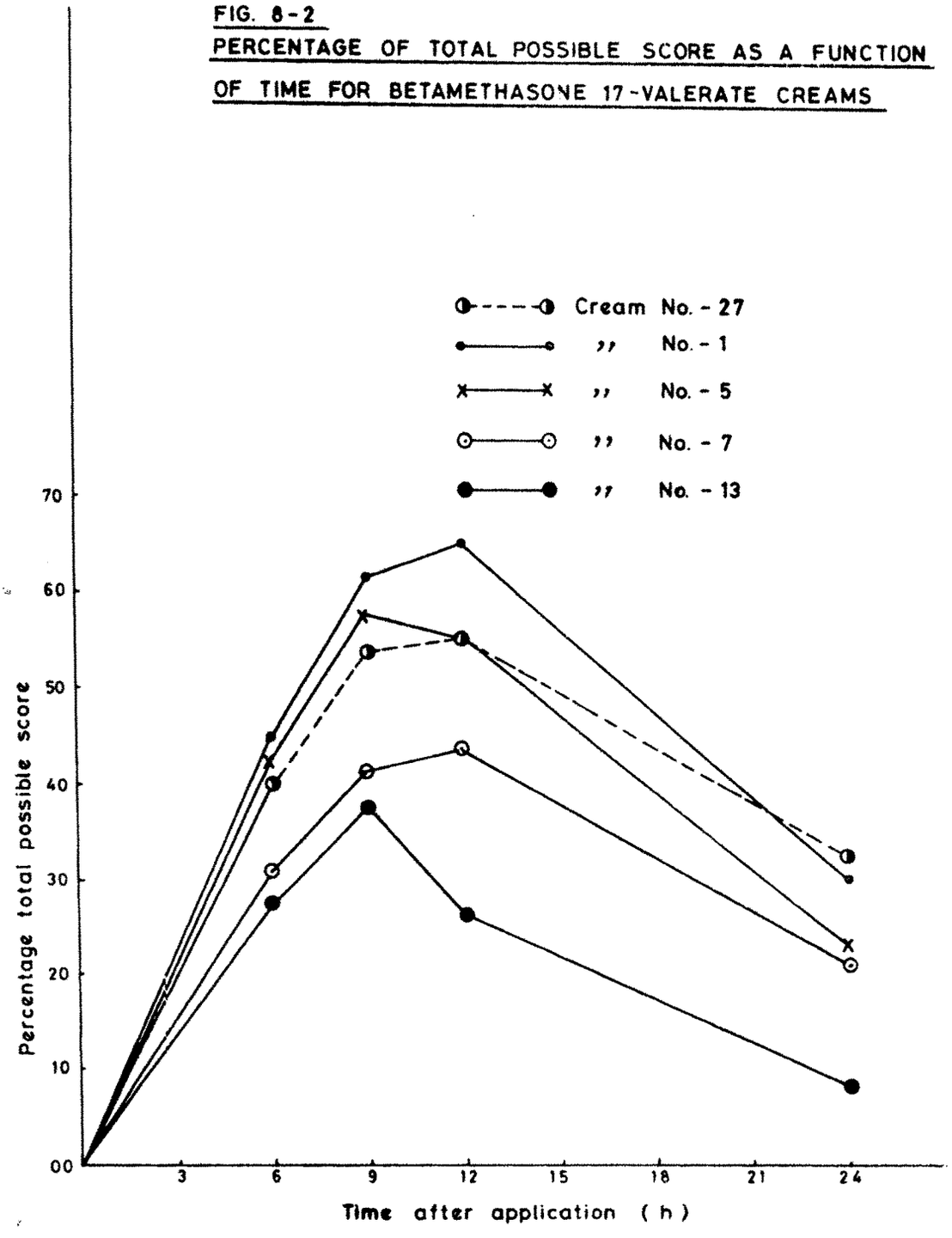

TABLE 7-3 : Bioavailability Study on Selected Halcinonide Creams by Blanching Test.

Cream Nos.	Degree of Blanching at each reading							
	6 hr.		9 hr.		12 hr.		24 hr.	
	A	B	A	B	A	B	A	B
1.	49.0	61.5	56.0	70.0	52.0	65	42.0	52.5
5.	46.0	57.5	52.0	65.0	43.0	54.0	38.0	47.5
13.	24.0	30.0	32.0	40.0	21.0	26.5	2.0	2.5
25.	32.0	40.0	40.0	50.0	40.0	50.0	37.0	46.5
27.	38.0	47.5	46.0	57.5	44.0	55.0	29.0	36.5

A = Score.

B = Percentage maximum possible score.

FIG. 8-3

PERCENTAGE OF TOTAL POSSIBLE SCORE AS A FUNCTION OF TIME FOR HALCINONIDE CREAMS

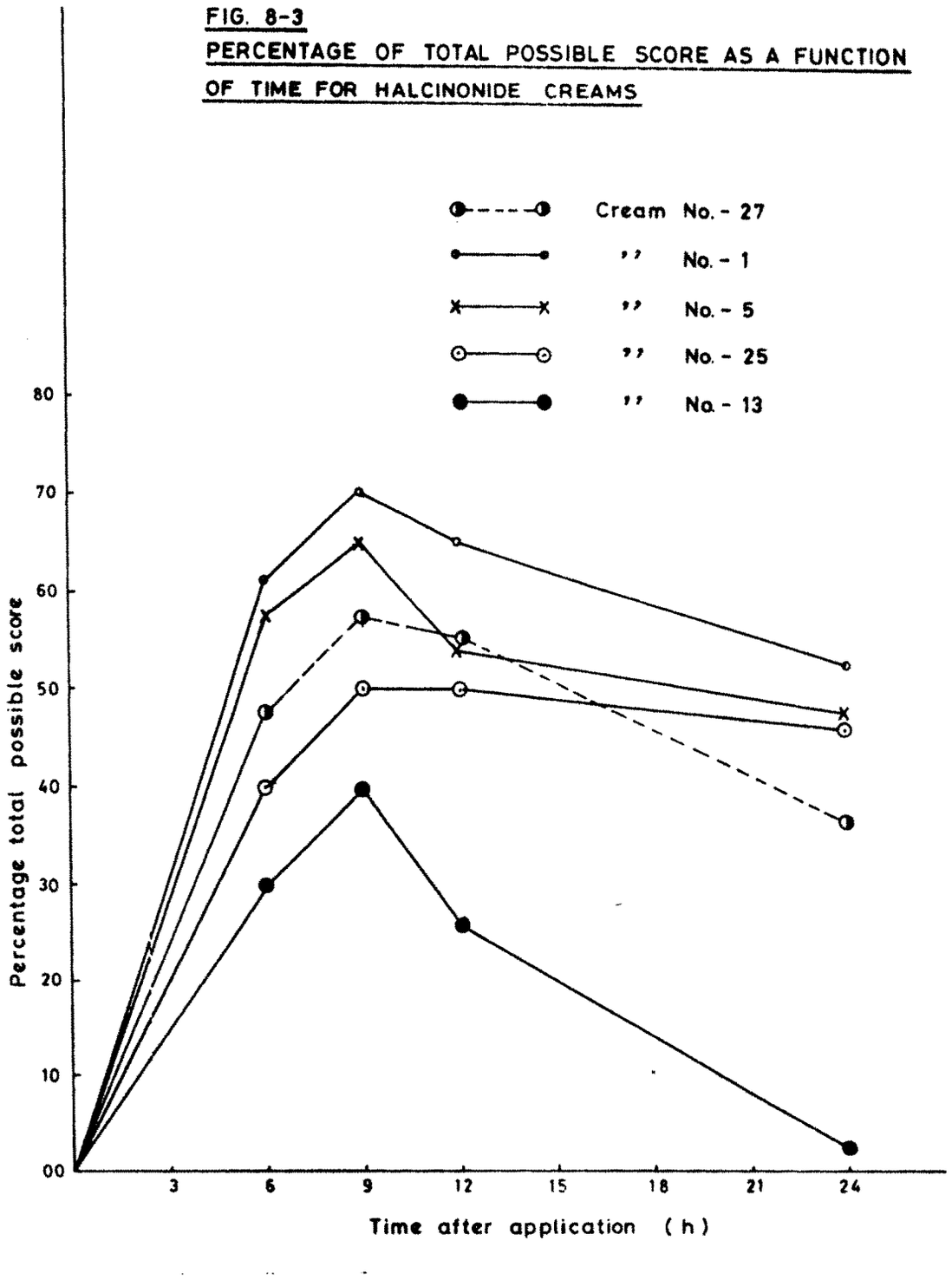

TABLE 7-4 : Bioavailability Study on Selected Flucinolone Acetonide Creams by Blanching Test.

Cream Nos.	Degree of Blanching at each reading							
	6 hr.		9 hr.		12 hr.		24 hr.	
	A	B	A	B	A	B	A	B
1.	34.0	42.5	48.0	60.0	28.0	35.0	20.0	25.0
5.	30.0	37.5	34.0	42.5	42.0	52.5	15.0	19.0
7.	24.0	30.0	32.0	40.0	34.0	42.5	18.0	22.5
13.	20.0	25.0	28.0	35.0	32.0	40.0	10.0	12.5
25.	28.0	35.0	42.0	52.5	40.0	50.0	8.0	10.0
27.	32.0	40.0	40.0	50.0	40.0	50.0	22.0	27.5

A = Score.

B = Percentage maximum possible score.

FIG. 8-4

PERCENTAGE OF TOTAL POSSIBLE SCORE AS A FUNCTION OF TIME FOR FLUOCINOLONE ACETONIDE CREAMS

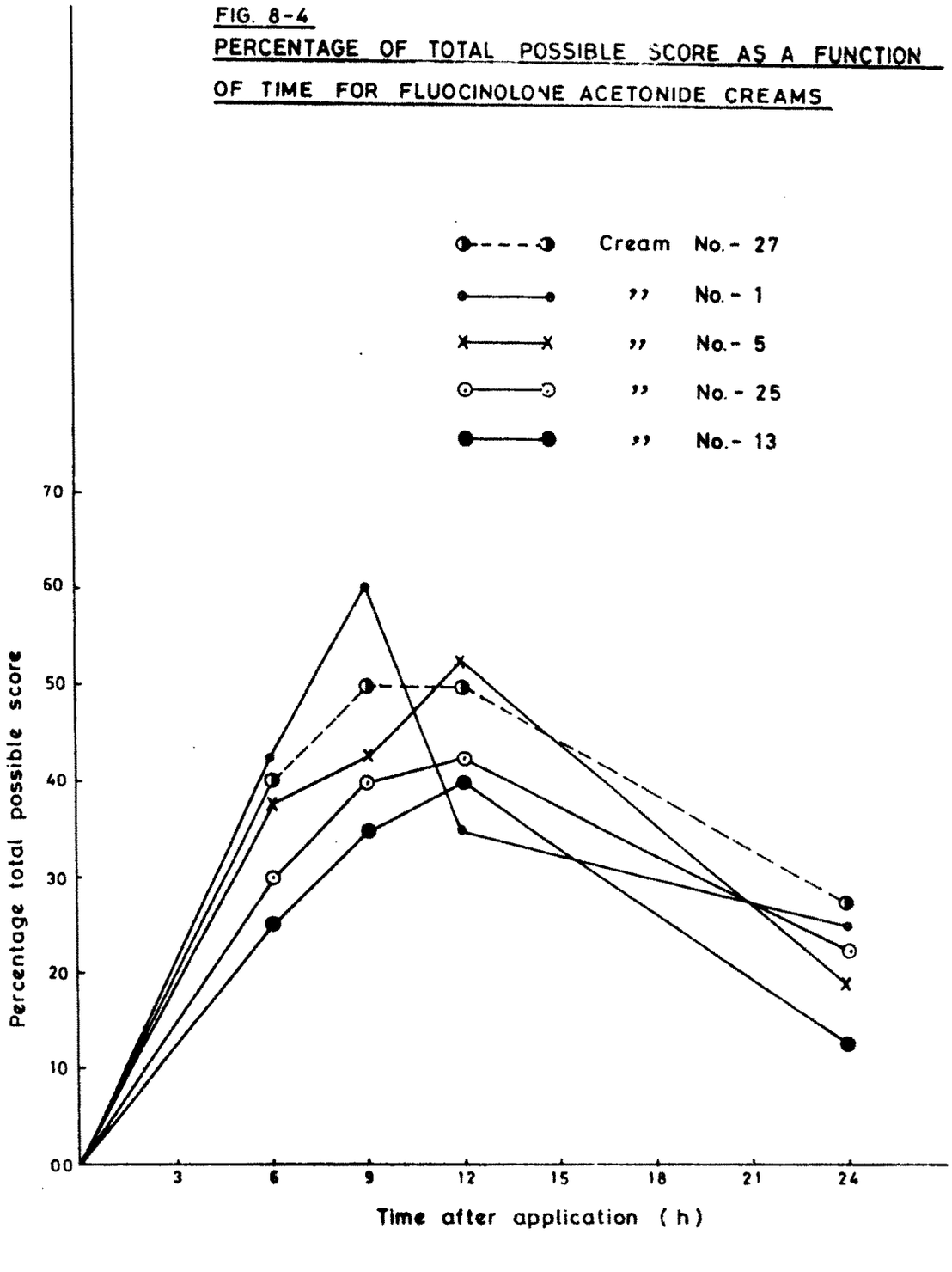

TABLE 7-5 : Approximate Area under the Percentage Response versus Time to 24 hr. Curve of all Selected Creams.

Cream Nos.	Corticosteroids			
	TA	BV	HAL	FA
1.	75.86 cm^2	138.42 cm^2	182.34 cm^2	112.00 cm^2
5.	64.83 cm^2	127.17 cm^2	161.66 cm^2	99.31 cm^2
7.	--	102.07 cm^2	--	--
13.	38.90 cm^2	69.52 cm^2	66.40 cm^2	82.76 cm^2
25.	57.93 cm^2	--	139.31 cm^2	112.83 cm^2
27.	59.31 cm^2	149.00 cm^2	145.93 cm^2	120.83 cm^2

TA = Triamcinolone acetonide.

BV = Betamethasone 17-valerate.

HAL = Halcinonide.

FA = Fluocinolone acetonide.

-- = indicates not taken for study.

5.4. Primary Skin Irritation Study :

The Pharmaceutical or Cosmetic Chemist, involved with the problems of topical formulations, is often concerned with the potential of his product for skin irritation and sensitivity and confused by the number of possible local toxicity tests. The ultimate goal of toxicity studies is to ensure safety or harmlessness under the proposed use conditions[141].

No ideal single test procedure exists which will adequately measure irritation and sensitivity potential. There is rather a spectrum of tests to be selected by the trained toxicologist who can secure data and translate it into probable effects in man, and balance these effects against the intended use and possible misuse of the product. Primary skin irritation test is simpler and less expensive. The further experimental design should be geared to indicate the possible type injury, the extent or seriousness of the injury, and the margin of safety that prevails under the most extreme conditions of use.[142]

While opinions concerning the value of animal studies vary,[143-145] no responsible person would dispense with animal skin tests. If properly, judged, on the basis of degree of variability and not on a "yes-no" basis, animal tests help to predict danger of severe human irritation and, to some degree, the concentrations that may safely be tolerated by man.

The tests have their greatest value as comparative tools. The data, with adequate controls, are related to other substances whose hazards have been defined by time and experience. In addition, toxic doses provide an estimate of the margin of safety under normal usage.[142]

IRRITATION REACTIONS :

Primary irritants are substances which damage skin by direct cytotoxic action.[146] Mild primary irritants exert their reactions after repeated exposure or over-exposure. Mild irritation may become chronic or cumulative after a number of exposures resulting in "skin fatigue"[147].

Skin Irritation :

Methods devised to measure skin irritancy are adequate for screening out stronger and moderate irritants, but are virtually insensitive in the low irritancy range. Materials are either placed in single contact with human or animal skin for varied time periods, at varied concentrations, or repeatedly used. These tests determine not only primary irritation but also "skin fatigue", when the skin reacts due to a succession of insults of a subthreshold intensity.

The skins of the mouse, rat, guinea pig, rabbit, miniature pig, and sheep have been used to screen compounds for primary irritant activity. Histologically, their skin do not closely resemble human skin, but from a responsive point of view, they all show changes when irritated. The rabbit and guinea pig are most frequently

chosen.[148] Materials which cause simple primary irritation of the skin of rabbits generally can be expected to cause a similar response in humans, but not necessarily in all humans. If appreciable edema occurs in rabbit skin, the material should be suspected as a possible vesicant human skin.

Draize[149] proposed the most widely used skin irritation test. Patch tests are read of suspected irritants on the abraded and intact skin of rabbits.

Roudabush[150] compared the dermal effects of a large number of diverse organic compounds in the guinea pig and rabbit. Levenstein and Welven[151] claimed a close correlation between the response to graded patch tests in the rabbit and observation noted in man. Carter and Griffith[152] pointed out the pit falls in placing sole reliance on animal data for assessing primary irritant hazard.

Justice, Travers, and Vinson[153] used human arm immersion, human patch tests, repeat mouse patch tests, and water transmission through rat and rabbit excised skin.

5.4.a. Experimental :

Primary skin irritation test was conducted on albino rabbits (Sarabhai Research Centre, Baroda) weighing 1.5-2.0 kg. Three males and three females were used per group, and the animals were maintained in a temperature controlled room (26°± 2°) throughout the experiment. The food consisting of soaked and Lucern green and water

TABLE 7-4 : Draize Evaluation of Skin Reactions.

1. Erythema and Eschar formation	Maximum score	Score			
		Intact skin		Abraded skin	
		24 hrs.	72 hrs.	24 hrs.	72 hrs.
No Erythema	0				
Very slight erythema	1				
Well defined erythema	2				
Moderate to severe erythema	3				
Severe erythema	4				
Total possible Erythema score	4 (Four)	Zero in all creams.			

2. Odema formation	Maximum score	24 hrs.	72 hrs.	24 hrs.	72hrs.
No odema	0				
Very slight Odema	1				
Well defined Odema	2				
Moderate to severe Odema	3				
Severe Odema	4				
Total possible Odema score	4 (Four)	Zero in all creams.			

was allowed at libidum.

The skin of the rabbit was shaved on the previous evening and on the day of the experiment the test cream base was applied on the abraded and intact skin. One gram of cream base or cream was taken for each site.

Gauze Patch was secured over each medicated area by means of adhesive tape. The whole area was covered with an appropriate sleeve for 24 hr.

The gauze patch was removed after 24 hr and each site was examined carefully. The reaction was evaluated according to Draize's method at 24 and 72 hr.

5.5. Results and Discussion :

It is most interesting to see that the different cream bases containing same corticosteroid drug producing different degree of vasoconstriction, but the difference may be taken to be an indication of the value of formulation in introducing the drug to the epidermal surface in the most favourable way for penetration into the skin. This observation indicates that the formulation can influence the overall efficacy of a topical formulation.

The results of the blanching test of corticosteroid formulations are shown in Table 7-1 to 7-4 as the total score for each formulation and as a percentage of the total possible score of 80, at each time period. The method of calculation was as follows : Maximum score per site = 4, for two arms, 4x2 = 8; for ten volunteers, 8 x 10 = 80.

The Blanching Profiles for all selected formulations are shown in figure 8-1 to 8-4, separated and occasionally dotted for clarity and ease of discussion. Curves were drawn with the help of experimental points. Nearly all the formulations gave curves with a peak in 9-12 hours region with a marked fall-off thereafter, notable exception being triamcinolone acetonide cream No. 1 where the scores decreased continuously after 6 hr, as shown in figure 8-1.

Comparisons between the various cream formulations have so far been made in terms of one set of action, maximum response and duration of action. Poulsen has suggested that all these parameters can be examined on a logical basis by considering the "area-under-the-curve" (AUC) value of response versus time curve, since this would roughly approximate other pharmacokinetic methods commonly employed to estimate biological availability from oral and parenteral dosage forms. This value is then an indication of the overall effectiveness of the corticosteroids in the blanching test. One criticism of applying this technique to the present data is that the area-under-the-curve has been calculated only to 24 hr, and some formulations still show relatively high responses at this time. However, as is experienced by the few results, the responses of all the formulations fall off after this time and the error involved in using the area upto only 24 hr will therefore be relatively small.

The area-under-the-curves in Figure 8-1 to 8-4 was obtained by drawing, cutting and weighing the papers and

empressed in units of "percentage of the total possible score x h." Table 7-5 shows the area-under-the-curve values of all the selected formulations, facilitating direct comparison of formulations using a biological response - time parameter. These values confirm the trend described from purely visual inspection of the graphs.

Halcinonide cream No. 1 has a very high value, producing a more intense degree of vasoconstriction, whereas triamcinolone acetonide creams are much less effective.

Triamcinolone acetonide creams showed a very poor effect (Table 7-1) compared to other corticosteroid formulations. Results in the table show that Cream Nos. 5, 13, 25 and 27 showed a good response at 9 h and Cream No. 1 showed a good response at 6 h and fell away at later readings.

The AUC values for triamcinolone acetonide creams (Table 7-5) show that the relative potency decreased in order : Cream No. 1 > 5 > 27 > 25 > 13.

For betamethasone 17-valerate formulations, Cream Nos. 1, 27 and 7 showed a good response at 12 h and Cream Nos. 5 & 13 showed a good response at 9h., but response fell down at later readings.

The AUC values for betamethasone 17-valerate creams (Table 7-5) show that the relative potency decreased in the order : Cream No. 27 > 1 > 5 > 7 > 13.

All the halcinonide formulations showed a good response at 9 h., but the response fell down at later

readings except in the case of cream No. 25 which appeared to have a prolonged action.

The AUC values for halcinonide creams (Table 7-5) show that the relative potency decreased in the order : Cream Nos. 1 > 5 > 27 > 25 > 13.

For fluocinolone acetonide formulations, Cream No. 1 showed a good response at 9 h. Cream Nos. 5, 13 and 25 showed a good response at 12 h and Cream No. 27 after giving good response at 9h., the response sustained upto 12h and fell down at later readings.

The AUC values for fluocinolone acetonide creams (Table 7-5) show that the relative potency decreased in order :
Cream Nos. 27 > 25 > 1 > 5 > 13.

Comparing the blanching results with <u>in vitro</u> release in laboratory designed model and liberation of corticosteroids through sartorius ointment chamber assembly, Cream No. 13 showed very poor results in all the creams in blanching readings. Cream No. 7 containing betamethasone 17-valerate has also shown a poor blanching readings. Other creams have shown comparable results with <u>in vitro</u> release except in fluocinolone acetonide creams, where Cream No. 27 and 25 gave more blanching readings than Cream No. 1, and in betamethasone 17-valerate creams where cream No. 27 gave more blanching readings than cream No. 1.

The blanching test has been used as a laboratory test to evaluate the efficacy of several topical corticosteroid formulations. Generally the results substantiate

the impressions of their efficacy in clinical practice. This assay demonstrates clearly that the degree of vaso-constrictor activity produced by a steroid, reflecting the degree of skin penetration, may be influenced considerably by the composition of the base in which it is formulated.

The percentage response of triamcinolone acetonide creams at 24 hr does not exhibit marked differences except Cream No. 13. In the formulations of betamethasone 17-valerate, halcinonide and fluocinolone acetonide creams the differences at 24 hr are slightly higher than triamcinolone acetonide creams, but in all the formulations the percentage response of Cream No. 13 exhibited marked differences.

Thus from this part of the work it could be concluded that halcinonide formulations produced a better blanching of skin than the corresponding formulations; followed by betamethasone 17-valerate, fluocinolone acetonide and triamcinolone acetonide formulations. TA cream Nos. 1 and 5, BV cream Nos. 27 and 1, HAL cream Nos. 1 and 5 and FA cream No. 27, 25 and 1 are the best topical formulations, as they may allow the steroid to exert the maximum effect.

Primary skin irritation test in rabbits (Table 7-6) show that non of the selected cream is irritant to the intact and abraded skin of rabbits. Thus from this part of the work it is concluded that it is safe to use the all selected creams.

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