CHAPTER 2

MATERIALS AND METHODS

2.1. Materials and their Suppliers.

Raw materials including drugs used in the present investigation along with the source of procurement are tabulated below.

TABLE - 1 : Materials and their Suppliers.

Raw materials	Supplier
Linoxyd CM 1000; Polyethylene glycol monocetyl ether; Polyethylene glycol 400; Polyethylene glycol 1540; Silicone A.F. emulsion; Simethicone and Dimethicone oils, Katrang 350, Katrang FAR21; Katrang Sim, Katrang SD emulsion; (Tween 80, Tween 60) Span 40, Span 60, Sodium Lauryl sulphate, Polyethylene glycol monostearate, Polyoxyethylene stearate, Polyoxyethylene lauryl ether, Glyceryl monostearate, self-emulsifying.	Hico Products Ltd., India.
	Amrut Industrial Products, India.
White bees wax, Triethanolamine, Disodium ededate.	S.D. Fine Chem. Pvt. Ltd., India.
Cetomacrogol 1000; Emulsifying wax. E. (Non-ionic); Cetostearyl alcohol Emulsifying wax, white soft paraffin IP, Microcrystalline wax, Hard paraffin, Heavy liquid paraffin, Light Liquid Paraffin.	Ferri-Chem., India.
	Wax Oils Pvt. Ltd., India.
Chlorocresol	Yojana Enterprise, India.
Isopropylmyristate	Robert-Johnson.,
Thiomersol	Venus Chemicals, India.
Methyl paraben, Propyl paraben	Gaurav Chemicals Pvt. Ltd., India.
Methyl Paraben sodium, X Propyl paraben sodium. X	Alta Lab., India.
Stearyl alcohol (95%), Cetyl alcohol	Aegis Chemical Ind. Ltd., India.
Polyoxyethylene esters (Myrj 52) Polyethylene glycol 4000, Polyethylene glycol 1500.	Techno Products Ltd., India.

TABLE 1 : (Condtd.)

Raw Materials	Supplier
Potassium hydroxide, Benzyl alcohol IP/BP, Borax, Sodium thiosulphate. Sorbitol 70% solution, Propylene glycol, Chloroform G.R. Grade (Spectroscopic grade) Methanol G.R. Grade (Spectroscopic grade), Butylated Hydroxy Toluene, Butylated Hydroxy Anisole.	Sarabhai M. Chemicals, India.
Stearic acid	Bombay Oil Ind. Ltd., India.
2-Pyrrolidone	BDH Chemicals Ltd., Prole, England.
Glycerin	Godrej Chemicals, India.
Castor Oil	Jayant Oil Products, India.
Propylene glycol monostearate	Wilson Laboratory, India.
Tetrazolium blue GR (BTC), Tetramethyl ammonium hydroxide 10% solution	Loba-Chemie Indoaustranal Co., India.
Isoniazid IP	Pfizer Limited, India.
Betamethasone 17-valerate I.P.	Avikon Pharma., India.
Halcinonide USP	Synbiotics Limited, India.
Triacinolone acetonide USP/BP	Rhemilano, Italy.
Fluocinolone acetonide USP	LARK S.P.a., Milano.

Synthesis of new drugs in the field of anti-inflammatory corticosteroids have taken place at a very fast pace during the last twenty five years and specific activity of corticosteroids has been more accurately studied.

In early times, traditional corticosteroids were naturally used also for topical therapy. Later on modifications of the molecular structure lead to the synthesis of a dozen of corticosteroids with increased topical activity.

The chemical structures of the corticosteroids are all very similar and resemble those of androgens and oestrogens. The main corticosteroids used systemically are hydroxy compounds (alcohols). Esterification of corticosteroids at the 17 or 21 positions with fatty acids generally increases the activity on the skin. The formation of the cyclic acetonides at the 16 and 17 positions further increases topical antiinflammatory activity, usually without increasing systemic glucocorticoid activity, and fluorinated corticosteroids also generally have increased topical activity.[1]

Introduction of a 9——fluoro group to the hydrocortisone molecule resulted in a eight fold increase in antiinflammatory activity. The 21-chloro-group also increased antiinflammatory properties.[2]

A brief and concise comparison of the structure and physical properties of the steroidal drugs employed in the present investigation are presented in Table - 2.

2.2.a. Mechanism of action :

The mechanism of action of the corticosteroids is related at least in part to their properties of vaso-constriction, suppression of membrane permeability and the immune response and antimitotic activity. Their vasoconstrictor action decreases extravasation of serum into the skin and inhibits swelling and discomfort. They prevent release of various lytic enzymes that not only extend tissue damage during inflammation but also that generate leukotactic substances, which cause pain and pruritus. This prevention of release is done by their lysosomal membrane stabilizing effect. Suppression of mitotic activity effectively diminishes epidermal hyperplasia, and epidermal and dermal atrophy may result from interference with synthetic pathways. Cortico-steroids interfere with lymphokine stimulation which enhances an immune response; therefore, migration of immune-effector substances to a site of inflammation is limited. The therapeutic usefulness of glucocorticoids in nonendocrine disease states is related to their ability to retard normal inflammatory and immunologic responses. They suppress the inflammatory response whether this is part of a disease process or is the result of mechanical, chemical, or immunologic insult by suppressing circulating lymphocytes and monocytes sensitization of lymphocytes is blocked, and the cell-mediated hypersensitivity reactions (including graft rejection) are inhibited.[7]

Glucocorticoids probably exert their action at multiple sites. They do not block the interaction of antibodies of sensitized lymphocytes and antigen or the release of histamine or kinins that is initiated by this process. Rather, they block the usual tissue responses to these stimuli. Normally, histamine increases capillary permeability with resultant extravasation of fluid and protein and consequent formation of edema, during an antigen-antibody interaction, migration inhibitory factor (MIF) is released from the lymphocytes involved, which inhibits the mobility of macrophages and causes them to accumulate in the surrounding area. Glucocorticoids help to maintain capillary integrity, prevent the macrophage reaction to MIF, inhibit phagocytosis and digestion of antigens, and, in high tissue concentrations may stabilise lysosomal membranes, thus preventing the release of hydrolytic enzymes. By inhibiting the inflammatory process at the cellular level, glucocorticoids decrease its superficial manifestations (eg. heat, redness, tenderness)[8].

2.2.b. <u>Uses (Indication)</u> :

Pharmacological results have stressed the remarkable antiinflammatory, antiphlogistic and antiallergic activity of topical preparation of glucocorticosteroids which can be used clinically in the following indications.

(A) Topical glucocorticosteroids preparations relieve the signs and symptoms of many inflammatory and allergic dermatosis, such as contact, neuro, seborrheic and nummular dermatitis.

(B) Topical glucocorticosteroids preparations can be used in eczemas such as infantile, allergic idiopathic and varicose eczemas.

(C) Topical glucocorticosteroids preparations can be used in pruritus, such as essential, anal and vulvae.

(D) Topical glucocorticosteroids preparations can be used in severe sunburn, nonvenomous insect bites, acute self-limiting eczematous conditions and erythemas.

(E) Topical preparations of glucocorticosteroids can be used in inter trigo, dehydrosis and lichen simplex.

(F) Topical preparations of glucocorticosteroids can be used in localised psoriasis.

The topical application of corticosteroids in ointments and creams often produces dramatic suppression of skin diseases in which inflammation is a prominent feature. However, the diseases may return or be exacerbated when corticosteroids are withdrawn if the cause of the condition is not eliminated or treated.[1,8,9]

2.2.c. Absorption and fate :

Glucocorticosteroids are absorbed from sites of local application such as synovial spaces, the conjunctival sac, and the skin. The absorption may be sufficient, when administration is chronic. Corticosteroids when administered by topical application, particularly under an occlusive dressing or when the skin is broken, sufficient corticosteroid may be absorbed

to give systemic effects. Corticosteroids in the circulation are extensively bound to plasma proteins, mainly to globulin and less so to albumin. The corticosteroid binding globulin has high affinity but low binding capacity, while the albumin has low affinity but large binding capacity. Only unbound corticosteroid has pharmacological effects or is metabolised. The synthetic corticosteroids are less extensively protein bound than hydrocortisone (Cortisol). They also tend to have longer half-lives.

Corticosteroids are metabolised mainly in the liver but also in the kidney, are excreted in the urine. Urinary excretion of 17-hydroxycorticoids is used as an index of adrenal function. The slower metabolism of the synthetic corticosteroids with their lower protein-binding affinity may account for their increased potency compared with the natural corticosteroids.[1,8,9]

2.2.d. <u>Adverse effects of corticosteroids</u> :

Application of corticosteroids to the skin has led to loss of skin collagen and subcutaneous atrophy. The topical application to the eye has produced corneal ulcers, raised intra-ocular pressure, and reduced visual function. In 12 months, 17 patients who had been taking corticosteroids were admitted to a plastic surgery unit on 21 occasions for extensive skin damage from trival accidents.[10]

A review of the hazards of topical corticosteroid application and a reminder that in addition to adverse effect on the skin, eye disease can be induced by topical corticosteroids and systemic absorption may produce adrenal suppression and collapse.[11]

After prolonged topical application of fluorinated corticosteroids for the treatment of rosacea, an aggravation and extension of telangiectasia occurred in 14 patients. Fluorinated corticosteroids should not be used in the topical treatment of rosacea, hydrocortisone preparations appeared to be harmless[12].

Munro,[13] observed adrenal suppression on topical application of corticosteroids. Burry,[14] observed delayed healing. Goldman, and Kitzmiller,[15] have observed atrophy of perianal skin. Franco and Winston[16] have observed facial eruptions in children. Nathan, et al.[17] have studied the death from Cushing syndrome.

Briggs and Briggs[18] have studied the potential carcinogenicity of some topical preparations in mice.

Howell[19] has commented on an eye disease induced by topically applied corticosteroids, including a warning that the topical use of corticosteroid medications for areas near the eye may result in conjunctival contamination from accumulated amounts of medication.

Brude and Becker[20] have observed the development of catracts and glaucoma with permanent visual defects as a result of indiscriminate prolonged topical application of

corticosteroids to relative ocular irritation associated with contact lenses. They also observed the increased susceptibility to infection such as atypical ringwarm infection (amoebiasis).

The skin may become thin and shiny or violaceous striae may develop due to rupture of subcutaneous collagen-fibers when glucocorticoids are used topically for prolonged periods in intertriginous areas or under occlusive dressings. The long-term topical application of potent fluorinated preparations to the face has been associated with the development of rosacea-like skin eruptions, perioral dermatitis, and acne.[7]

Cutaneous bacterial or yeast infection is the most common complication of topical glucocorticoid therapy.

Acne, hirsutism, menstrual disorders, facial rounding, development of supraclavicular fat pads, weight gain due to increased appetite, headache, pseudotumor cerebri, hypertension, impotence, hyperhidrosis, flushing, vertigo, asthenia, chronic pancreatitis, intestinal perforation, hepatomegaly, hyperlipidemia and acceleration of atherosclerosis have been associated with glucocorticoid therapy.[7]

Negative nitrogen balance is a result of the excessive breakdown of protein caused by glucocorticoids, and hence atrophy and osteoporosis. Glucocorticoids aggravate known diabetes and make latent diabetes chemically apparent.

Glucocorticoids decrease the protection provided by the gastric mucus barrier, interfere with tissue repair, and, in some cases, increase gastric acid and pepsinogen production.[7]

2 3. Review of Method of Analysis :

The drugs selected for the present study are official in many of the pharmacopoeias and assay procedure, for these drugs are also mentioned therein. A brief review of estimation procedure of relevant drugs is presented here.

2.3.a. Spectrophotometric methods :

(Colorimetric analysis).

A variety of colorimetric methods can be used to assay corticosteroids. The usual method of analysis of undecomposed corticosteroid include the tetrazolium blue, in modifications of original method for ∝-ketol steroids,[21-25] is perhaps the most widely used. Reduction of corticosteroids with tetrazolium blue in alkaline medium gives a colour hydrazone formation, which can be quantitised. It measures the reducing power of the ∝-ketol - side chain and is useful for general formulation assays[26-27]. Triamcinolone acetonide gives blue colour and halcinonide gives royal purple colour with tetrazolium blue in alkaline medium. The phenyl hydrazine sulphuric acid-alcohol reaction[28,29] which is also known as the Porter-Silber reaction. Halcinonide reacts with acidic ethanolic 4-nitrophenyl hydrazine[30], after heating and cooling and the addition of sodium hydroxide, to give a brilliant

purple "plum" colour. The isonicotinic acid hydrazide reaction[31], which is known as the Umberger reaction. Reduction of 1,4-diene-3One steroids with methanolic isoniazid[32], produces a yellow hydrazone. Halcinonide has been quantitated in various formulations or as bulk powder by a differential ultraviolet, borohydride reduction assay[34], halcinonide added to 4-amine antipyrine[35], in methanolic hydrochloric acid gives a pale green colour. Halcinonide added to ethanolic tetramethyl ammonium hydroxide[36], and heated, gives a cloudy amber colour. If added to ethanolic tetramethyl ammonium hydroxide[37], and picric acid, an organge-red (tea coloured) solution results. In concentrated sulphuric acid[38], halcinonide gives a deep yellow colour.

Michael *et al.*[39] have studied the corticosteroid determination in skin preparation by a reaction rate method using tetrazolium blue.

Robert *et al.*[40] have described the absorbance - pH relation-ship in the steroid - tetrazolium reaction.

Grahm *et al.*[41] have described a rapid, quantitative analysis of betamethasone and its organic esters at room temperature. They also studied ten corticosteroids using methylene chloride as solvent[42]. Sin *et al.*[43] have described the quantitative colorimetric determination of residual 9-fluore prednisolone and 9 -fluorohydrocortisone in triamcinolone samples. They[44] also studied the determination of triamcinolone and some esters of corticosteroids.

Smith et al.[45] studied the spectrophotometric determination of traiamcinolone acetonide by tetrazolium method and its application to pharmaceutical preparations. Ascione and Pegelin[46] have reported the stabilization of blue tetrazolium assay for triamcinolone. They substituted the chloroform for 60% of the ethanol. Chafetz et al.[46] have described the difference ultraviolet determination of stereids with conjugated ketones chromopheres via. Lithium tetrahydroborate reduction. Rieux et al.[47] have described the specific reactions of glucocorticoids with Dische reagent and its analytical applications to antiinflammatory stereids. They analysed hydrocortisone, cortisone, triamcinolone, Prednisolone, fluocinolone acetonide, glucocorticosteroids gives green colour with Dische reagent, Chafetz et al.[48] have described the colorimetric determination of betamethasone benzoate in topical gel preparation by Lewbarat - Mattox method. Astrakhanova and Kovalenko[49] have described the spectrometric determination of prednisolone in an ointment. The determination was done by treating an extract of the sample with isoniazid or ninhydrin in alkaline medium and measured absorbance at 405 nm. Wang[50] has reported the improvement in quantitative determination of triamcinolone acetonide in creams by colorimetric analysis. Gorog[51] has described the determination of steroids in pharmaceutical formulations. A review is presented of direct spectrophotometric methods, U. V. and colorimetric methods following chemical reactions, fluorimetric, TLC, HPLC and differential pulse polaregraphic

methods. He also outlined[52] the differences in the analysis between industrial-pharmaceutical and biological-clinical steroid and methods are discussed under the heading spectroscopic chromatographic and miscellaneous. Kwan et al.[53] have described the colorimetric determination of prednisolone and its application to dissolution studies. Chatterjee et al.[54] studied the interference of pharmaceutical ingredients in corticosteroid assay by tetrazolium blue reduction method. Heints et al.[55] have described the determination of α-ketolic steroids by reaction with triphenyl tetrazolium chloride. Bundgaard and Hansen[56] have reported the stability indicating properties of some spectrometric assays for corticosteroids. Deodhar and Mehta[57] have reported the colorimetric estimation of prednisolone in pharmaceutical formulations. Shingbal and Prabudesai[58] have described the spectrophotometric estimation of triamcinolone acetonide in its dosage form. Landis[59] has described the rapid determination of corticosteroids in pharmaceuticals by flow-injection analysis. The method is based on the reduction of tetrazolium blue by the steroid in alkaline medium to form a coloured formazan. Bundgaard and Hansen[60] have described a new stability - indicating spectrophotometric method for determination of corticosteroid in aqueous media.

2.2.b. Polarographic analysis :

Halcinonide is reduced in two steps by dimethyl formamide[61]. The 21 α-chloroketo group exhibits a

half-wave reduction potential of -1.17 volts Vs Hg. This is easily distinguished from half-wave potential of -1.62 volts Vs Hg of the Δ^4-3-keto group. The more sensitive technique of differential pulse polarography[62] should also be applicable to halcinonide.

The half wave potential ($E_{\frac{1}{2}}$ Versus standard Calomel electrode) was determined as -1.45 volts in lithium chloride in methanol[63] for triamcinolone acetonide.

Cohen[64] subjected triamcinoline acetonide to polarographic reduction in dimethyl formamide.

Kabasakalian et al.[65] studied the reduction step exhibited by $\Delta^{1,4}$-3-keto steroids usually occurs at more anodic potentials, and is easily discerbible from the reduction step exhibited by the corresponding Δ^4-3-keto steroids.

De Beer et al.[66] have studied the polarographic analysis of corticosteroids. They have described reduction mechanism of halogen containing corticosteroids and analysis of some corticosteroids. They also studied the determination of corticosteroids in single component solutions, suspensions ointments and creams[67].

3.3.c. Fluorescence analysis :

Unlike Δ^4-3-keto steroids, the $\Delta^{1,4}$-3-keto compounds do not exhibit significant sulfuric acid induced fluorescence.[68] Cullen et al.[69] have described the fluorimetric determination of Norgestrel and structurally related steroids. They

described a sensitive procedure, based on sulfuric acid induced fluorescence, for the analysis of norgestrel. Seki and Yamaguchi[70] have described a fluorimetric determination of free glucocorticoids in human urine by high performance liquid chromatography. Yamaguchi and Seki[71] have described the fluorimetric determination of urinary 17-hydroxy corticosteroids using benzamidine. Tokunaga Hiroshi et al.[72] have described the fluorimetric determination of hydrocortisone, prednisolone and cortisone.

2.3.d. Chromatographic analysis :

Quantitative chromatographic methods can be used for identification, quantitative methods for assessment of purity and stability of steroids.

2.3.d.1. Paper chromatographic analysis :

Paper chromatographic Rf values of triamcinolone acetonide and related steroids in a number of solvent systems are reported.[73, 74]

The following detection systems were used.

(1) A modified Haines, Drake ultraviolet - Scaner[73,75].
(2) Isonicotinic acid hydrazide[73,76].
(3) Alkaline tetrazolium blue spray[73,74].

Roberts[77] has described the quantitative determinations of triamcinolone acetonide.

Paper chromatography using Whatman No. 1 paper was once used to determine the homogeneity of halcinonide[78].

Johnson and Flowter[79] have given Rf data for dexamethasone and related steroids utilizing the paper chromatography.

2.3.d.2. Thin layer Chromatography (TLC) :

Roberts[78] has described the separation procedure of halcinonide from its synthetic precursors by TLC, using silica gel GF 254 plates and by using developing solvent of chloroform ethyl acetate (5:1). The Rf values were found.

Experience of TLC of triamcinolone acetonide is summarised.[80,82]

Tatja Na Bicon-fister[83] studied the quantitative separation and estimation of steroid mixture by TLC, he separated and estimated progesterone and estradiol benzoate and progesterone, testosterone propionate and estradiol benzoate in mixture.

Takitani et al.[84] have presented a review, with 134 references over 1977 to mid 1983 and included the discussion of over-pressured TLC, combination TLC with those of HPLC. They have discussed the application of TLC to separate alcohols, phenols, organic acids, aminoacids, amines, steroids, glycerides and drugs.

Van de Vaart et al.[85] have described the application of TLC for the analysis of preparations containing basic, acidic and corticosteroid drugs in several different cream bases.

Vukusic[86] has described the TLC determination of betamethasone dipropionate in semisolid pharmaceutical preparations.

Sinicora and Vach[87] have described the analysis of corticosteroid ointments using TLC.

3.3.d.3. Column chromatographic analysis :

A column partition chromatographic procedure for triamcinolone acetonide and related steroids has been worked out by Poet[88]. Smith et al.[73] have also described a column partition chromatographic procedure for steroids. A generalised system for the prediction of elution curves for corticosteroids based on partition coefficients for a hexane-chloroform-dioxane-water (90:10:40:5) solvent system on diatomaceous earth column was described[89]. A diatomaceous earth column was also used to separate halcinonide from excipients[90].

Novotny[91] have described the new biochemical separations using pre-column derivatization and micro-column liquid chromatography for hydroxy steroids.

Oka[92] has described an on-line extraction, evaporation and injection for liquid-chromatographic determination of serum corticosteroids.

Koupparis et al.[91a] have described the determination of corticosteroid preparation in skin by a reaction-rate method, using column chromatography. They isolated fluocinonide from the sample by column chromatography and determined by modification of tetrazolium blue reaction.

2.3.d.4. High pressure liquid chromatography (HPLC) :

Reverse phase HPLC is used to separate and quantitative bulk and formulated halcinonide[93]. Halcinonide can be separated from Kanalog (triamcinolone acetonide) by HPLC[94].

Gerhord has described the effect of different octadecylsilane columns on mobility of triamcinolone-acetonide by HPLC[95].

Landgraf and Jennings have described the determination of fluocinonide from complex mixture by HPLC technique[96].

Lundmo and Sunde have described the rapid analysis of C_{19} steroid metabolism by HPLC and in-line monitoring of radio activity. They demonstrated that the results agreed well with those by TLC and the HPLC method was rapid.[97]

Saito et al.[98] have described the analysis of corticosteroids in human adrenal tissue by HPLC.

Lake et al.[99] have described the analysis of creams by the application of HPLC.

Carson and Jusko[100] have described the simultaneous analysis of cortexolone and cortisol by HPLC for use in the methyrapone test.

Goto et al.[101] have described the determination of 6-B-hydroxy cortisol in urine by HPLC with fluorescense detection.

Rego et al.[102] have described the simultaneous determination of hydrocortisone and benzyl alcohol in pharmaceutical formulation, by reversed-phases HPLC.

Rehm and Steinigen[103] have described the analytical testing of corticosteroid containing dermal preparations. Twenty five corticosteroids in sixty nine topical preparations were determined by HPLC at 40°C on a column of Nucleosil $10C_{18}$.

Cavina et al.[104] have described the analysis of natural corticosteroids in adrenal extracts and in biological fluids by HPLC.

Finaloy et al.[105] have described the trace-enrichment HPLC technique for determining the dissolution rate of adrenocortical tablets. They analysed betamethasone, dexamethasone and prednisolone, at concentration down to 0.25 µg/ml, by HPLC.

Liwan et al.[106] have described the assay of betamethasone 17-valerate and its degradation product by HPLC. This method permitted the determination of the drug and its separation from the degradation product and free betamethasone. This procedure could also be used for the assay of hydrocortisone 17-butyrate and its analogous degradation products.

Das Gupta[107] has described the quantitative determinations of dexamethasone and dexamethasone sodium phosphate in pharmaceutical dosage forms by HPLC.

Hattori *et al.*[108] have described the HPLC analysis of dexamethasone and chlorpheniramine maleate in ointment.

Juenge and Brower[109] have described the HPLC separation and identification of epimeric 17-ketone impurities in a commercial sample of dexamethasone-sodium phosphate.

Cavina *et al.*[110] have described the analysis of topical corticosteroids in complex pharmaceutical formulations by HPLC.

Kirschbaum[111] has described the HPLC analysis of triamcinolone acetonide and the effect of different octadecylsilane columns on mobility of triamcinolone acetonide.

Kirschbaum *et al.*[112] have studied the HPLC of the topical anti-inflammatory steroid halcinonide.

Lea *et al.*[113] have described the analysis of hydrocortisone acetate ointments and creams by HPLC. This method was suitable for analysis of ointments, but gave consistently low results when applied to creams.

Munson and Wilson[114] have described the HPLC determination of hydrocortisone cypionate. They studied the development method and characterisation of chromatographic behaviour.

Belliardo and Bertolino[115] have described the analysis of dexamethasone acetate in pharmaceutical formulation by HPLC.

Van Dame[116] has described the quantitative determination of steroid acetates in pharmaceutical preparations of tablets and suspension, by HPLC.

Pavli and Dobrovoije[117] have described the analysis of dexamethasone acetate in ointments and suppositories by HPLC.

2.3.d.5. Gas chromatography :

Steroids possessing the C_{17} dihydroxy acetone side chain usually undergo molecular alteration after application to gas liquid chromatography columns to yield as a major product the corresponding 17-ketosteroids.[118]

Cartoni et al.[119] have described the capillary gas chromatographic mass spectrometric detection of anabolic steroids.

Uralets et al.[120] have described the analysis of anabolic steroids in body fluids by capillary gas chromatography with a two channel detection system and a computer.

2.4. Miscellaneous :

Wabba et al.[121] have described the method of analysis on the degradation of dexamethasone in certain pharmaceutical preparations.

Simpson[122] estimated some synthetic glucocorticosteroids in rat muscle.

Monder and Iohan[123] have described the application of polyethylenimine cellulose for the class separation of steroidal carboxylic acids from neutral steroids and pigments in urine.

Kley and Rick[124] have demonstrated the influence of storage and temperature on the determination of steroids in plasma and blood.

Stupnicki[125] has described the direct radio-immuno assay method for steroid hormones.

Alviola et al.[126] have described the densitometric determination of some corticosteroids, like hydrocortisone, prednisolone and betamethasone 17-valerate in topical formulations.

Kruger et al.[127] have described the method of identification of individual steroids in biological matrices by mass-analysed ion-kinetic energy (MIKE) spectrometry.

Gorog et al.[128] have described the simultaneous determination of reduction products of norethisterone acetate.

Huf et al.[129] have described the determination of hydrocortisone acetate in ointment by transmission densitometry.

Belanger et al.[130] have described fast-atom-bombardment mass spectrometry and pharmaceutical analysis of corticosteroids. They have shown that full separation and characterization of the steroids are possible by F.A.B. chemical ionization mass spectrometry.

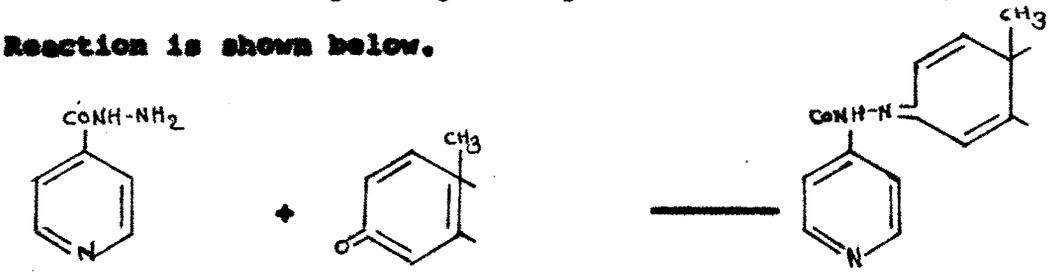
Dekker[131] has studied the stability of corticosteroids under anaerobic conditions. He studied the D-homosteroid corresponding to prednisolone.

Soliman et al.[132] have described the semi-micro titrimetric methods for determination of some corticosteroids in tablets and bulk drugs.

2.5. Method of Analysis :

The determination of Betamethasone 17 valerate is based on the method given in Pharmacopoeia of India[133] 1985 under heading 'Assay of Steroids', A-68. Here instead of aldehyde free alcohol, methanol GR grade (spectroscopic grade) was used. For the determination of Triamcinolone acetonide, Halcinonide and fluocinolone acetonide, isoniazid solution was used as colour development reagent.

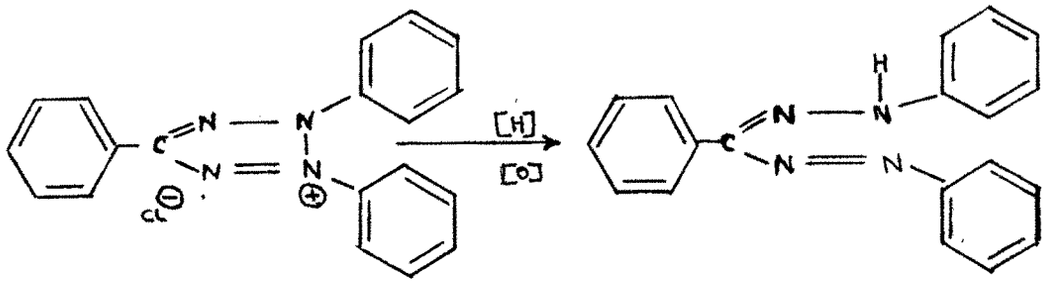
The determinations of Triamcinolone acetonide, Fluocinolone acetonide and halcinonide are based upon the reactions of α,β-unsaturated steroidal ketones ie. the conjungation of the carbonyl group at C_3 with the double bond between C_4 and C_5 in ring A of the steroid nucleous with isoniazid to yield yellow products (Schiff base). Reaction is shown below.



Isoniazid α,β-Unsaturated steroidal ketone.

The determination of Betamethasone 17 valerate is based upon the reaction of tetrazolium blue with C_{17} side chain of Betamethasone 17 valerate.

The α-ketol (-CHOH-CO-) group possesses reducing properties commonly associated with this function. Its reduction of alkaline triphenyl tetrazolium chloride (Tetrazolium blue) to the corresponding reddish brown Formazan.



3.5.a. **Triamcinolone Acetonide :**

Following method was used for in vitro evaluation of various formulations of Triamcinolone acetonide.

Reagents :

a) Chloroform G. R. Grade (spectroscopy)

b) Methanol G. R. Grade (spectroscopy)

c) Isoniazid IP solution.

A 0.1% w/v solution of Isoniazid IP grade was prepared in methanol (G. R. Grade).

1 gm. Isoniazid was added into 1000 ml amber coloured volumetric flask containing 900 ml methanol. Isoniazid was dissolved completely by shaking the flask. 1.25 ml of concentrated hydrochloric acid was added in the above flask. The volume was made to mark with methanol. The solution was mixed well.

Preparation of standard solution :

50 mg of pure Triamcinolone acetonide was weighed accurately and transferred quantitatively into a 100 ml amber coloured volumetric flask. It was dissolved and diluted to volume with chloroform. Exactly 5 ml of this solution was pipetted out into another 100 ml amber coloured volumetric flask and then diluted to volume with chloroform. Exactly (X) ml of above solution was pipetted out one by one into 25 ml amber coloured volumetric flasks. To each volumetric flask 10 ml of Isoniazid solution was added. Flasks were stoppered and the contents mixed by gentle swirling. The solution was kept into the oven at 50 ± 1° for 1 hr. The solution was cooled rapidly, and sufficient chloroform was added to produce 25 ml. The solution was mixed well.

Procedure :

Absorbance of solution of each amber coloured volumetric flask was measured at 415 nm on Beckman Model 35 U.V. and Visible Spectrophotometer in 1 cm cell against the blank treated in same manner. Readings were taken in triplicate and mean of above readings were taken for the calibration curve.

Observations are given in Table 3-1 and the standard calibration curve is plotted in Figure 3-1.

TABLE 3-1 : Calibration Curve Of Triamcinolone Acetonide

(x) ml.	Concentration (µg/ml)	Absorbance at 415 nm			
		I	II	III	Mean
0.1	0.100	0.004	0.003	0.005	0.004
0.2	0.200	0.009	0.008	0.009	0.0087
0.5	0.500	0.021	0.022	0.021	0.0213
1.0	1.000	0.042	0.043	0.041	0.042
2.0	2.000	0.085	0.085	0.085	0.085
3.0	3.000	0.128	0.129	0.127	0.128
4.0	4.000	0.177	0.181	0.178	0.1787
5.0	5.000	0.215	0.216	0.215	0.2153
6.0	6.000	0.253	0.254	0.253	0.2533
7.0	7.000	0.296	0.298	0.296	0.2963
8.0	8.000	0.350	0.352	0.350	0.3506
10.0	10.000	0.421	0.423	0.424	0.4226
12.0	12.000	0.465	0.462	0.467	0.4643

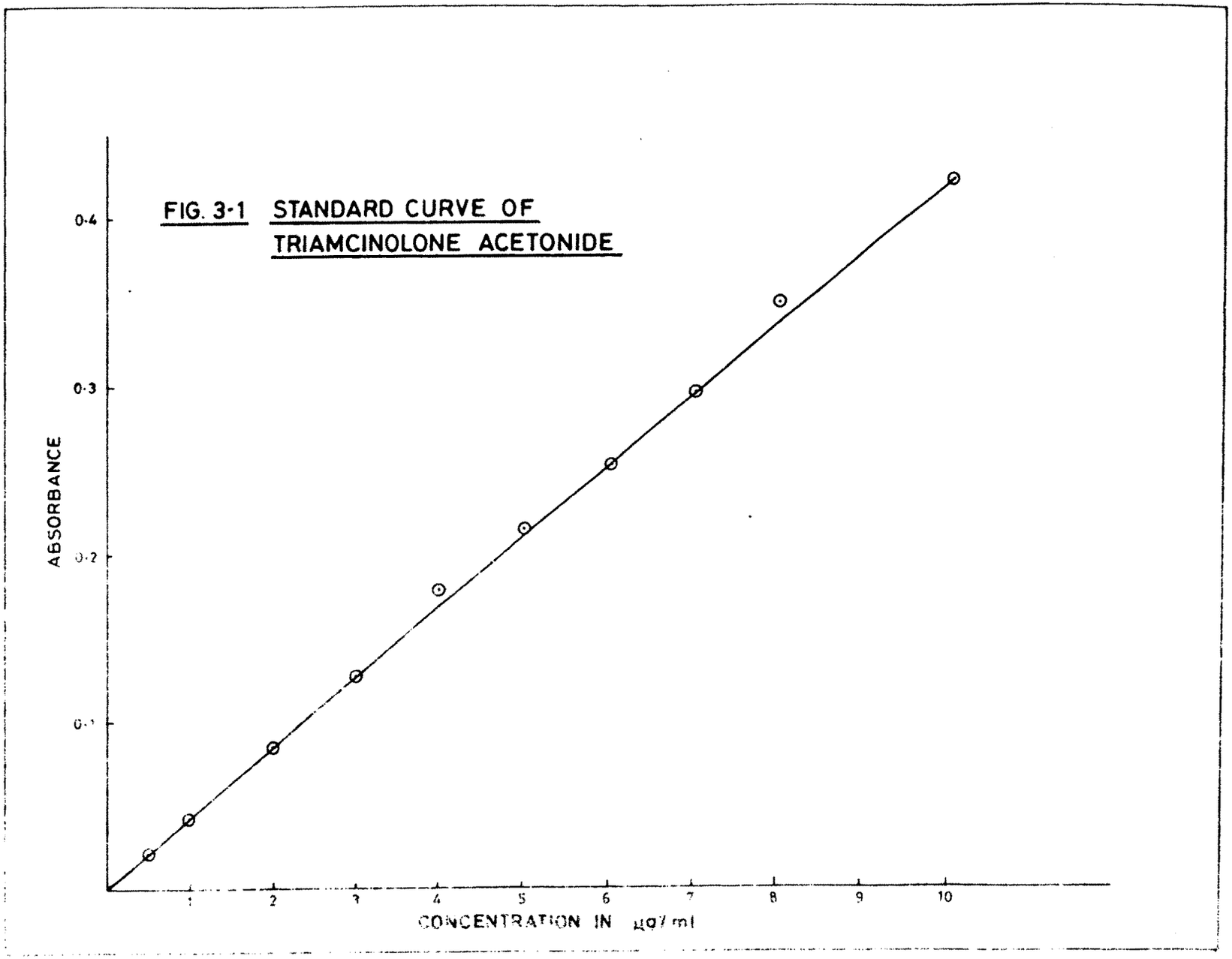


FIG. 3-1 STANDARD CURVE OF TRIAMCINOLONE ACETONIDE
ABSORBANCE
0·4
0·3
0·2
0·1
1
2
3
4
5
6
7
8
9
10
CONCENTRATION IN µg/ml

2.5.b. Betamethasone 17-valerate :

Following method was used for in vitro evaluation of various formulations of betamethasone 17-valerate.

Reagents :

a) Chloroform G.R. Grade (spectroscopy)

b) Methanol G.R. Grade (spectroscopy)

c) Tetrazolium blue solution (G.R. Grade)

A 0.5% w/v solution of Tetrazolium blue was prepared in methanol. This solution was prepared immediately before use.

d) Diluted tetramethyl ammonium hydroxide solution.

2 ml of 10% tetramethyl ammonium hydroxide was diluted to 20 ml with methanol.

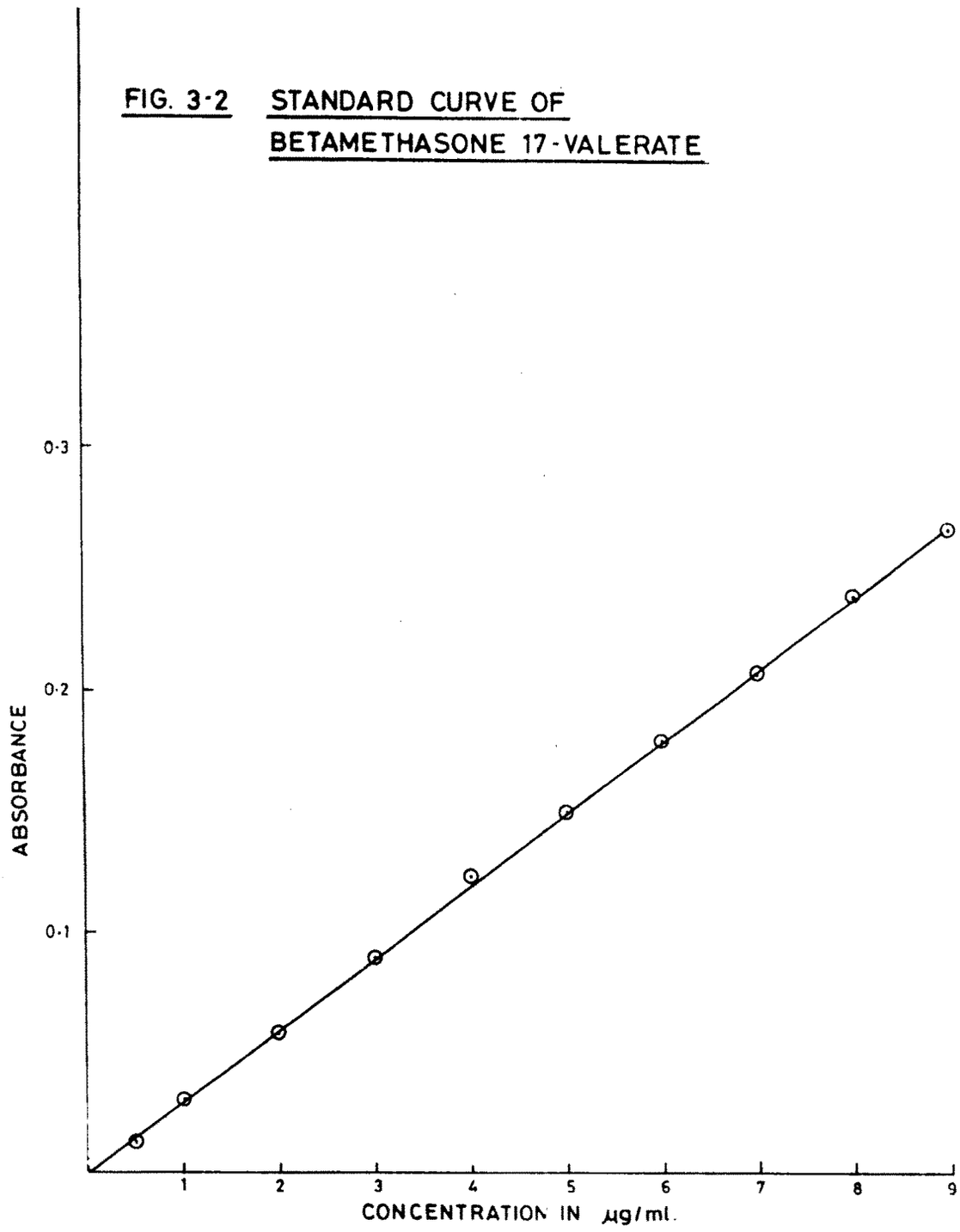
Preparation of standard solutions :

25 mg of pure betamethasone 17-valerate was weighed accurately and transferred quantitatively into a 100 ml amber coloured volumetric flask. It was dissolved and diluted to volume with chloroform. Exactly 5 ml of this solution was pipetted out into another 100 ml amber coloured volumetric flask and then diluted to volume with chloroform. Exactly (X) ml of the above solution was pipetted out one by one into 25 ml amber coloured volumetric flasks. To each volumetric flask 2 ml of the tetrazolium blue solution was added. Immediately after addition of tetrazolium blue solution, 2 ml of dilute tetramethyl ammonium hydroxide solution was added. Flasks were stoppered and the contents was mixed by gentle swirling. The solution was allowed to stand at 25° for

TABLE 3-2 : Calibration Curve Of Betamethasone 17-valerate

(X) ml	Conc. in µg/ml	Absorbance at 525 nm			
		I	II	III	Mean
0.20	0.100	0.003	0.003	0.003	0.003
0.5	0.250	0.009	0.007	0.009	0.0083
1.0	0.500	0.012	0.014	0.013	0.013
2.0	1.000	0.031	0.032	0.031	0.0313
2.5	1.250	0.041	0.041	0.041	0.041
3.0	1.500	0.042	0.044	0.045	0.044
4.0	2.000	0.058	0.059	0.061	0.0593
5.0	2.500	0.072	0.074	0.074	0.0733
6.0	3.000	0.088	0.091	0.092	0.0903
7.5	3.750	0.113	0.114	0.114	0.1136
8.0	4.000	0.130	0.124	0.122	0.1250
9.0	4.500	0.134	0.136	0.134	0.1346
10.0	5.000	0.150	0.149	0.149	0.1493
12.0	6.000	0.178	0.180	0.178	0.1786
14.0	7.000	0.209	0.210	0.207	0.2086
16.0	8.000	0.239	0.241	0.243	0.241
18.0	9.000	0.271	0.268	0.267	0.2686

FIG. 3-2 STANDARD CURVE OF BETAMETHASONE 17-VALERATE



25 min. in dark. Sufficient chloroform was added to make volume. The solution was mixed well.

Procedure :

Absorbance of solution of each amber coloured volumetric flask was measured at 525 nm on Beckman Model 35 U.V. and Visible Spectrophotometer in 1 cm cell against the blank treated in same manner. Reading were taken in triplicate and the mean of the above readings were taken for calibration curve.

Observation are given in Table 3-2 and the Standard Calibration Curve is Plotted in figure 3-2.

2.3.c. **Halcinonide :**

Following method was used for *in vitro* evaluation of various formulations of halcinonide.

Reagents :

a) Chloroform G. R. Grade (spectroscopy)

b) Methanol G. R. Grade (spectroscopy)

c) Isoniazid IP grade solution.

A 0.1% w/v solution of isoniazid was prepared in methanol (G.R. Grade).

1 g Isoniazid IP was added into 1000 ml amber coloured volumetric flask containing 500 ml methanol. Isoniazid was dissolved completely by shaking flask. 1.25 ml of concentrated hydrochloric acid was added in above flask. The volume was made to mark with methanol. The solution was mixed well.

Preparation of standard solution :

50 mg of pure halcinonide was weighed accurately and transferred quantitatively into a 100 ml amber coloured volumetric flask. It was dissolved and diluted to volume with chloroform. Exactly 5 ml of this solution was pipetted out into another 100 ml amber coloured volumetric flask and then diluted to volume with chloroform. Exactly (X) ml of the above solution was pipetted out one by one into 25 ml amber coloured volumetric flasks. To each volumetric flask 10 ml of Isoniazid solution was added. Flasks were stoppered and the contents was mixed by gentle swirling. The solution was kept into the oven at 50 ± 1° for 40 min. The solution was cooled rapidly and sufficient chloroform was added to produce 25 ml. The solution was mixed well.

Procedure :

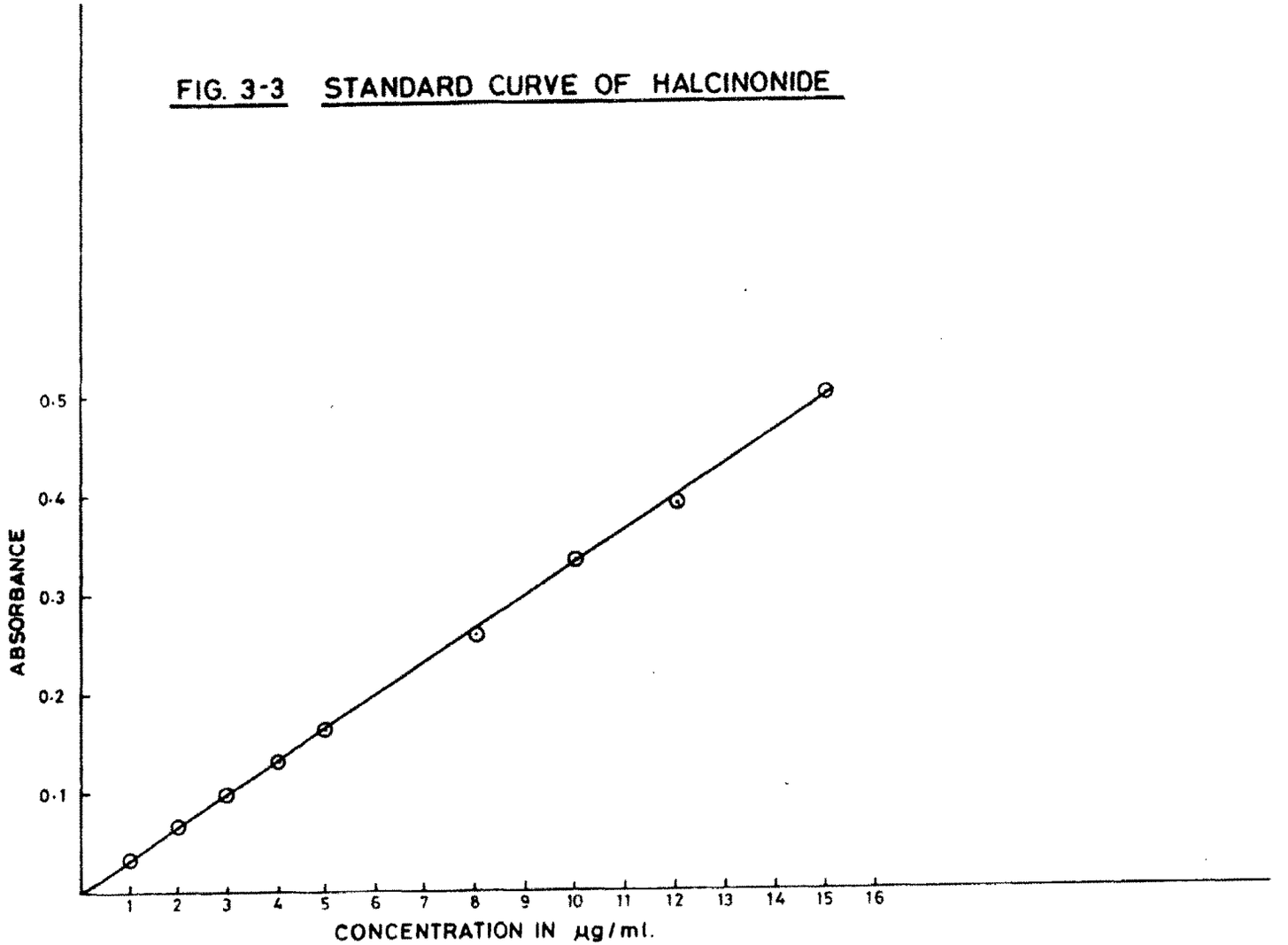
Absorbance of solution of each amber coloured volumetric flask was measured at 377 nm on Beckman Model 35 U.V. and Visible Spectrophotometer in 1 cm cell against the blank treated in same manner. Readings were taken in triplicate and mean of above readings were taken for plotting the Calibration Curve.

Observations are given in table 3-3 and the standard curve is plotted in figure 3-3.

TABLE 3-3 : Calibration curve of Halcinonide

(X) ml	Conc. in µg/ml	Absorbance at 377 nm			
		I	II	III	Mean
0.1	0.100	0.003	0.002	0.003	0.003
0.25	0.250	0.008	0.007	0.008	0.008
0.50	0.500	0.016	0.014	0.016	0.015
1.00	1.000	0.033	0.033	0.033	0.033
2.00	2.000	0.066	0.066	0.067	0.066
3.00	3.000	0.095	0.095	0.095	0.095
4.00	4.000	0.129	0.130	0.130	0.130
5.00	5.000	0.162	0.162	0.163	0.162
8.00	8.000	0.258	0.258	0.258	0.258
10.00	10.000	0.334	0.332	0.329	0.332
12.00	12.000	0.393	0.390	0.388	0.390
15.00	15.000	0.499	0.502	0.502	0.501

FIG. 3-3 STANDARD CURVE OF HALCINONIDE



2.5.4. **Fluocinolone Acetonide :**

Following method was used for in vitro evaluation of various formulations of Fluocinolone acetonide.

Reagents :

a) Chloroform G. R. Grade (Spectroscopy)

b) Methanol G. R. Grade (Spectroscopy)

c) Isoniazid IP solution.

A 0.1% w/v solution of Isoniazid was prepared in methanol (G. R. Grade).

1 g. Isoniazid was added into 1000 ml amber coloured volumetric flask containing 500 ml methanol. Isoniazid was dissolved completely by shaking flask. 1.25 ml of concentrated hydrochloric acid was added in the above flask. The volume was made to mark with methanol. The solution was mixed well.

Preparation of Standard solution :

25 mg of pure fluocinolone acetonide was weighed accurately and transferred quantitatively into a 100 ml amber coloured volumetric flask. It was dissolved and diluted to volume with chloroform. Exactly 5 ml of this solution was pipetted out into another 100 ml amber coloured volumetric flask and then diluted to volume with chloroform. Exactly (X) ml of above solution was pipetted out one by one into 25 ml amber coloured volumetric flasks.

TABLE 3-4 : Calibration Curve of Fluocinolone Acetonide

(X) ml	Concentration (mg/ml)	Absorbance at 415 nm			
		I	II	III	Mean
0.1	0.100	0.004	0.003	0.003	0.003
0.2	0.200	0.007	0.006	0.007	0.007
0.5	0.500	0.017	0.017	0.018	0.017
1.0	1.000	0.035	0.035	0.035	0.035
2.0	2.000	0.071	0.070	0.072	0.071
3.0	3.000	0.107	0.107	0.106	0.107
4.0	4.000	0.142	0.141	0.142	0.142
5.0	5.000	0.182	0.180	0.182	0.181
7.0	7.000	0.251	0.251	0.250	0.251
8.0	8.000	0.290	0.290	0.290	0.290
10.0	10.000	0.355	0.355	0.355	0.355
12.0	12.000	0.425	0.425	0.426	0.425

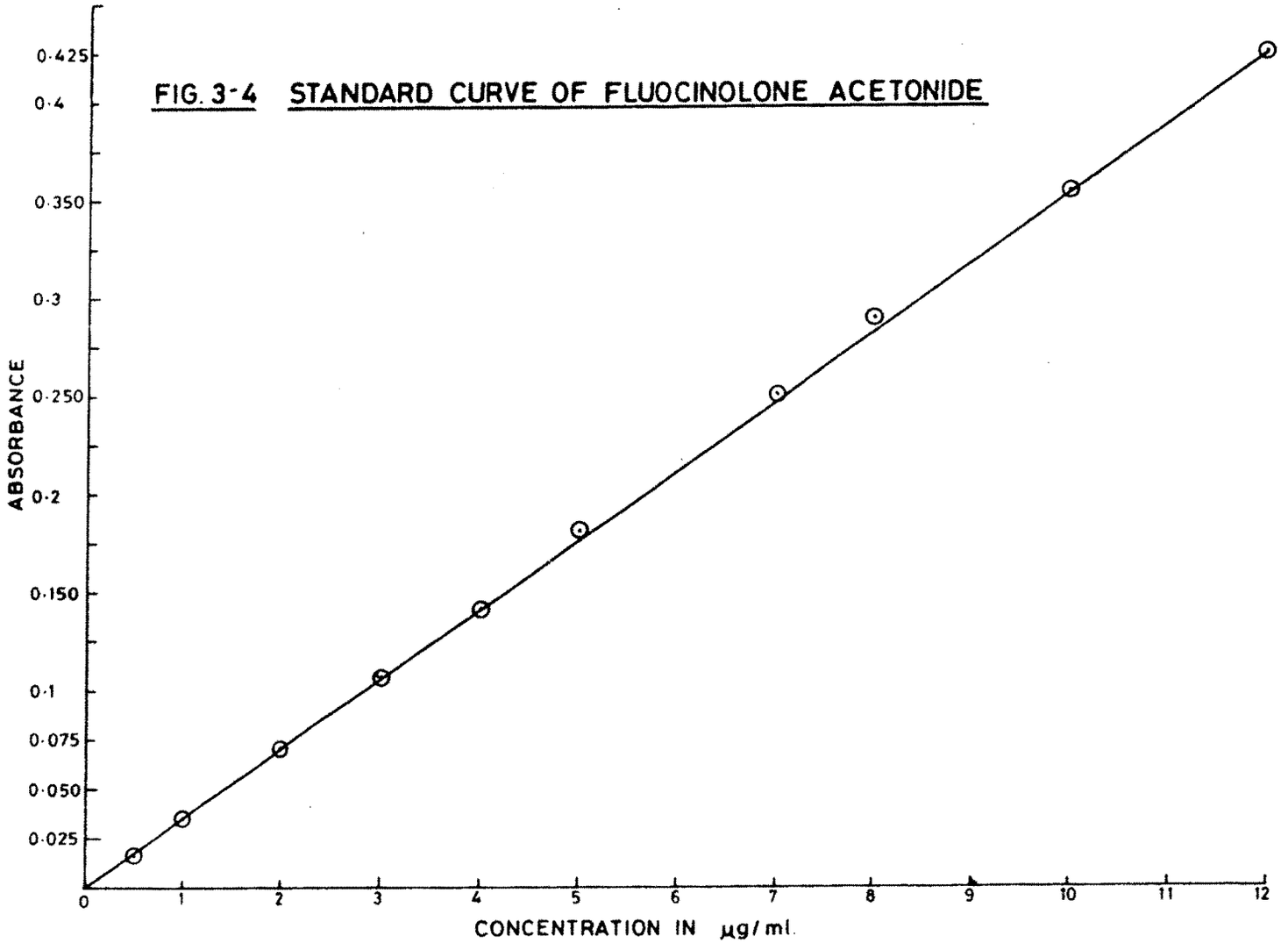


FIG. 3-4 STANDARD CURVE OF FLUOCINOLONE ACETONIDE
ABSORBANCE
0·425
0·4
0·350
0·3
0·250
0·2
0·150
0·1
0·075
0·050
0·025
0
1
2
3
4
5
6
7
8
9
10
11
12
CONCENTRATION IN µg/ml

To each volumetric flask 10 ml of Isoniazid solution was added. Flasks were stoppered and the contents mixed by gentle swirling. The solution was kept into the ovan at 50° for 1 hr. The solution was cooled rapidly, and sufficient chloroform was added to produce 25 ml. The solution was mixed well.

Procedure :

Absorbance of solution of each amber coloured volumetric flask was measured at 415 nm on Beckman Model 35 U.V. and Visible Spectrophotometer in 1 cm cell against the blank treated in same manner. Reading were taken in triplicate and mean of above readings were taken for calibration curve. Observations are given in Table 3-4 and the standard curve is plotted in figure 3-4.

2.6. Interference Study :

As a large number of substances were used in the formulation of creams in the present study, it was thought worthwhile to check for any interference in the estimation of each drug substances.

Procedure :

The interfering substance was added to a standard solution of corticosteroid which was then evaporated to dryness and the residue dissolved in chloroform G.R. (Spectroscopy grade), prior to colour development. In each case, the absorbance was compared to the absorbance of a standard corticosteroid solution under same condition. The results are given in Table 3-5.

2.7. Results and Discussion :

The calibration curves prepared for triamcinolone acetonide, betamethasone 17-valerate, halcinonide and fluocinolone acetonide are linear plots and all the four drugs can be estimated quantitatively by selected spectrophotometric methods. They follows the Beer's Law at concentration range of 0-10 µg/ml, 0-9 µg/ml, 0-15 µg/ml, 0-12 µg/ml, respectively.

Estimation of drug in presence of large number of ingredients revealed that almost none of the ingredients used in the formulation of creams interfere in the estimation of the steroids incorporated therein, with the exception of Tween 80 and Tween 60 which gives slightly higher absorbance than the normal. However, the interference due to Tween 80 and Tween 60 has been considered insignificant in the present investigation.

REFERENCES

1. Martindale, "The extra Pharmacopoeia", Reynolds E.F., London, 28th Ed., 1982, PP 446-485.
2. Druey, J. and Tripod, J., Med. Chem. (New York), 7, 223-262, 1967, through C.A., 70, 77829e, 1969.
3. "Pharmacopoeia of India", 3rd Ed., Vol. I (A.P.), 1985, The Controller of Publication, Delhi, pp 73-74.
4. "Pharmacopoeia of India", 3rd Ed., Vol. II (Q.Z. & Appendices); 1985, The Controller of Publications, Delhi, p, 527.
5. "Pharmacopoeia of United State", XXIst Ed., 1985 United State Pharmacopoeial Convention, Inc., pp, 114-116, 434, 1077-1079.
6. "Remington's Pharmaceutical Sciences", Mack Publishing Co., Pennisylvania, 15th Ed., 1975, pp, 886-899.
7. "American Medical Association", John Wiley & Sons, Inc., New York; 14th Ed., 1980, pp, 1026-1029.
8. Ibid., 14th Ed., 1980, pp, 619-650.
9. Robert, C., Haynes, Jr., and Joseph, L., "The Pharmacological Basic of Therapeutics,, Goodman L.S. and Gilman A., Ed.," 6th Ed., Mackmillan Publishing Co., New York, 1970, pp, 1478-1503.
10. David, B.J., Brit. Med. J., 2, 614, 1972.
11. Lancet, 2, 487, 1977, through Ref. No. 1.
12. Sneddon, I., Brit. Med. J., 1, 671, 1969.
13. Munro, D.D., Brit. J. Dermatol., 94, 67, 1976.
14. Burry, J.J., Med. J. Aust., 1, 393, 1973.
15. Goldman, L. and Kitzmiller, W., Arch. Dermatol., 107, 611, 1973.

16. Franco, M.L., Weston, W.L., Pediatrics., 64, 36, 1979.

17. Lancet., 1, 207, 1979 through Ref. No. 1.

18. Briggs, M.M., and Briggs M., Brit. J. Dermatol., 3, 135, 1978.

19. Howell, J.B., Arch. Dermatol., 112, 1529, 1976.

20. Burde, R.M. and Becker, B., J. Am. Med. Assoc., 213, 2075, 1970.

21. Mader, W.J., and Buck, R.R., Anal. Chem., 24, 66, 1952.

22. Callahan, J.J., Litterio, F., Britt, E., Rasen, B.D., and Owens, J., J. Pharm. Sci., 51, 333, 1962.

23. Guttman, D.E., J. Pharm. Sci., 55, 919, 1966.

24. Johnson, C.A., King, R., and Vickers, C., Analyst., 85, 714, 1960.

25. Kunze, F.M., and Davis, J.S., J. Pharm. Sci., 53, 1259, 1964.

26. Ascione, P., and Pegolin, C., ibid., 52, 709, 1963.

27. Umeda, M., Tsubota, S., and Kajiita, A., Takamine Kenkyusho Nempo., 14, 87, 1963, through C.A., 53, 4379f, 1963.

28. Porter, C.C., and Silber, R.H., J. Biol. Chem., 185, 201, 1950.

29. Silber, R.H., and Porter, C.C., ibid., 210, 923, 1954.

30. Kimura, M, Nishina, T., and Sakamoto, T., Chem. Pharm. Bull., 15, 454, 1962.

31. Umberger, E.J., and Ferist, A.A., J. Amer. Pharm. Assoc. (Sci. Ed.), 47, 773, 1958.

32. Cingolani, E., Cavina, G. and Amormino, V., Farmaco (Pavia), Pract. Ed., 15, 301, 1960.

33. Ivashkiv, E., J. Pharm. Sci., 51, 698, 1962.

34. Kirschbaum, J., ibid., 67, 275, 1978.

35. Schulz, E.P., Diaz, M. Lopez, G., Guerrero, L. Barrera, H., Pereda, A., and Aquilera, A., Anal. Chem., 36, 1624, 1964.

36. Cross, J., Eisen, M. and Kedersha, R., Anal. Chem., 24, 1049, 1952.

37. Pesez, M., and Bartos, J., "Colorimetric and Fluorimetric Analysis of Organic Compounds and Drugs," Marcel Dekker, New York, 1974, P, 481.

38. Berstein, S., and Lenhard, R., J. Org. Chem., 18, 1146, 1953.

39. Michael, A.K., Kan, M.N., and Howard, V.M., J. Pharm. Sci., 68, 1479, 1979.

40. Robert, R.E., and Charles, T.K., ibid., 62, 103-107, 1972.

41. Grahm, R.E., Biehl, E.R., and Kenner, C.T., ibid., 67, 360, 1978.

42. Grahm, R.E., Biehl, E.R., and Kenner, C.T., ibid., 67, 792, 1978.

43. Sin, C.J., Pan, S.C., and Bennett, R.E., Anal. Chem., 32(6), 669-671, 1960.

44. Mrdy, O., Ceskosl. Farm., 11(5), 255-259, through Anal. Abstr., 9, 5402, 1962.

45. Smith, L.L., and Halwer, M., Anal. Chem. 48, 348, 1959.

46. Chafetz, Lester, Tsilifonis, Dimitric and Riedl Joanne, M., J. Pharm. Sci., 61(1), 148, 1972.

47. Rioux-lacaste, Catherine, and Viel, Claide, Annls. Pharm. Fr., 33 (3-4), 163-170, 1975, through Anal. abstr., 30, 261, 1976.

48. Chafetz, Lester, Tsilifonis, Dimitric, and Moran, Christina, J. Pharm. Sci., 63 (11), 1771, 1974.

49. Astrakhanova, M.M., and Kovalenko, L. I., Farmatsiya, 32(1), 1982, through Anal. Abstr., 43, 505, 1982.

50. Wang, Yunmei., Yaoxue Tongbao, 16(6), 12, 1981, through Anal. Abstr., 43, 409, 1983.

51. Gereg, S., Fresenius, Z., Anal. Chem., 309(2), 97, 1981.

52. Gereg, S., Trends Anal. Chem (Pers. Ed.), 3(6), 157, 1984, through Anal. Abstr., 47, 56, 1985.

53. Kwan, L.C., Schott, H., J. Pharm. Sci., 73(2), 157-161, 1984.

54. Chatterjee, S.K., Sharma, S.C., and Roy, S.K., J. Inst. Chem. (India), 54(4), 184, 1982.

55. Heintz, B., and Kalusa, R., Dtsch. Apoth Ztg., 119(21), 808, 1979, through Anal. Abstr., 38, 199, 1980.

56. Bundgaurd, H., and Hansen, J., Pharm. Weekbl. (Sci. Ed.), 2(4), 1131, 1980, through Anal. Abstr., 40, 648, 1981.

57. Deodhar, R.D., and Mehta, R.C., Indian J. Pharm., 42(4), 126, 1980.

58. Shingbal, D.N., Prabudesai, J.S., Indian Drugs., 22(9), 489, 1985.

59. Landis, John, B., Anal. Chim. Acta., 114, 155, 1980, through Anal. Abstr., 39, 310, 1980.

60. Bundgaard, H., and Hansen, J., Arch. Pharm. Chemi., (Sci. Ed.), 7(1), 19-32, 1979, through Anal. Abstr., 39, 192, 1980.

61. Koey, O., and Smith, C., Personal Communication through "Analytical Profile of Drug Substances," Ed. by Klaus Flory, 8, 1979, pp 251-281.

62. Yadav, R.N., and Teare, F.W., J. Pharm. Sci., 67, 436, 1978.

63. Coy, N. Squibb Institute, Personal Communication, through "Analytical Profile of Drug Substances", Ed. by Klaus Flory, 1, 1972, pp, 379-421.

64. Cohen, A.I., Anal. Chem., 35, 128, 1963.

65. Kobasakalian, P. and McGlotten, J., J. Am. Chem. Soc., 78, 5032, 1956.

66. De Boer, H.S., Van Oort, W.J., and Zuman, P., Anal. Chim. Acta, 120, 31-39, 1980, through Anal. Abstr., 40, 789, 1981.

67. De Boer, H.S., Lansaat, P.H., and Van Oort, W.J., Anal. Chim. Acta., 108, 389-393, 1979, through Anal. Abstr., 38, 200, 1980.

68. Noujaim, A.A., and Jeffery, D.A., Can. J. Pharm. Sci., 5, 26, 1970.

69. Cullen, L.F., Rutgers, J.G., Lucchesi, P.A., and Papariello, G.J., J. Pharm. Sci., 57, 1857, 1968.

70. Seki, T., Yamaguchi, Y., Bunseki Kagaku, 33(7), 1984, through Anal. Abstr., 47, 438, 1985.

71. Yamaguchi, Y., Seki, T., Anal. Biochem., 142(1), 204, 1984.

72. Tokunuga, Hiroshi, Kimura, Toshio, and Kanamura, Jiro., Yakugaku Zasshi, 100 (2), 200, 1980, through Anal. Abstr., 39, 1980.

73. Smith, L.L., Foell, T., Demaio, R. and Halwer, M., J. Am. Pharm. Assoc. (Sci. Ed.), 48, 528, 1959, and Smith, L.L. and Foell, T., J. Chromatogr., 3, 381, 1960.

74. Pan, S.C., J. Chromatogr., 9, 81, 1962.

75. Von, A.E. and Weber, R., Helv. Chim. Acta., 39, 1664, 1956.

76. Smith, L.L., and Foell, T., Anal. Chem. 31, 102, 1959.

77. Roberts, H.R., and Florey, K., J. Pharm. Sci., 51, 794, 1962.

78. Roberts, H. Personal Communication, through Ref. No. 61.

79. Johnson, C.A., and Fowler, S., J. Pharm. Pharmacol. 16, Supp., 17T, 1964.

80. Hall, A., J. Pharmacol., 16, Suppl. 9T, 1964.

81. Clifford, C.J., Wilkinson, J.V., and Wragg, J. S., J. Pharm. Pharmacol. 16, Suppl. 11T, 1964.

82. Sonanini, D., Hofstetter, R., Anker, L. and Muhlemann, H., Pharm. Acta. Helv., 40, 302, 1965.

83. Tatja, Na Bican-fister, J. Pharm. Sci., 57, 169, 1968.

84. Takitani, S., Sano, A., Suzuki, M., Bunseki, 4, 267, 1984, through Anal. Abstr., 47, 111, 1985.

85. Van de Vaart, F.J., Hulshoff, A., and Indemans, A.W.M., Pharm. Weekbl. (Sci. Eds.), 5(3), 113, 1983, through Anal. Abstr., 45, 501, 1983.

86. Vukusic, IVO, J. Chromatogr., 243(1), 131, 1982.

87. Simicora, D., and Vach, S., Farmaceuticky obz., 42(5), 217-221, through Anal. abstr., 25, 4067, 1973.

88. Poet, R., Squibb Institute, Personal Communication., through Ref. No. 63.

89. Graham, R.E., Williams, P.A., Kenner, C.T., J. Pharm. Sci., 59, 1473, 1970.

90. Ivashkiv, E., and Poet, R., Personal Communication, through 'Analytical Profile of drug substances.', Ed. by Klaus Flory 2, 1973, pp163-197.

91. Novotny, M., Karlsson, K.E., Konishi, M., Alasandro, M., J. Chromatogr., 292(1), 159, 1984.

91a. Koupparis, M.A., Wakzak, K.M., and Malmstadt, H.V., J. Pharm. Sci., 68(12), 1479, 1979.

92. Oka, K., Minagawa, K., Hava, S., Noguchi, M., Matsuoka, Y., Kono, M., Irimajiri, S., Anal. Chem., 56(1), 24, 1984.

93. Fairbrother, J.E., in "Assay of Drugs and other Trace Compounds in Biological fluids", Ed. by E. Reid., "Methodological Developments in Biochemistry", Vol. 5, Longman Group, London, 1976, P. 141.

94. Gordon, G., and Wood, P.R., Anal. Div. Chem. Soc., 14, 30, 1977.

95. Gerhord, Levy, J. Pharm. Sci., 69, 481, 1980.

96. Landgraf, W.C., and Jennings, E.C., J. Pharm. Sci., 62, 278, 1972.

97. Lundmo, P.I., Sunde, A., J. Chromatogr Biomed. Appl., 33, 289, 1984, through Anal. Abstr., 47, 136, 1985.

98. Saito, Z., Mimoh, N., Hifumi, S., Takeda, R., Clin. Chim. Acta. 131(3), 239, 1983, through Anal. Abstr., 46, 193, 1984.

99. Lake, O.A., Hulshoff, A, Van de Vaart, F.J. and Indemans, A.W.M., Pharm. Weekbl. (Sci. Ed.). 5(1), 15, 1983, through Anal. Abstr., 45, 177, 1983.

100. Carson, S.W., Jusko, W.J., J. Chromatogr. Biomed. Appl., 31, 345-350, 1984, through Anal. Abstr., 46, 1061, 1984.

101. Goto, J., Shamsa, F., Nambara, T., J. Liq. Chromatogr., 6(11), 1977-1985, 1983, through Anal. Abstr., 46, 648, 1984.

102. Rego, Albert, and Nelson, Brett., J. Pharm. Sci., 71(11), 1219, 1982.

103. Rehm, Klaus, Dieter; and Steinigen, Manfred, Pharm. ztg., 127(16), 888, 1982, through Anal. Abstr., 44, 357, 1983.

104. Cavina, G., Moretti, G., Alimenti, R., and Gallinella, R., J. Chromatogr., 175(1), 125, 1979.

105. Finlay, G., Jefferies, J.P., and Sharma, S.P., J. Pharm. Pharmacol., 31(9), 634, 1979.

106. Liwan, P.A., Irwin, W.J., and Yip, Y.W., J. Chromatogr., 176(3), 399, 1979.

107. Das Gupta, V., J. Pharm. Sci., 68(7), 926-928, 1979.

108. Hattori, T., Washio, Y., Kamiya, N., Itoh, Y., and Inove, M., Yakugaku Zasshi., 99(5), 537, 1979, through Anal. Abstr., 38, 82, 1980.

109. Juenge, Eric C., and Brower, James F., J. Pharm. Sci., 68(5), 551, 1979.

110. Cavina, G., Moretti, G., Gallinella, B., Alimenti, R., and Barchiesi, R., Bull. Chim. Farm., 117(9), 534-544, 1978, through Anal. Abstr., 38, 82, 1980.

111. Kirschbaum, Joel, J. Pharm. Sci., 69(4), 841-842, 1980.

112. Kirschbaum, Joel, Poet, Raymond; Bush, Karen, and Petrie, Glenn, J. Chromatogr., 190(2), 481-485, 1980, through Anal. Abstr., 39, 573, 1980.

113. Lea, A.R., Kennedy, J.M., and Low, G.K.C., J. Chromatogr., 198(1), 41, 1980.

114. Munson, J.W., and Wilson, T.D., J. Pharm. Sci., 70(2), 177, 1981.

115. Belliardo, F., and Bertolino, A., J. Lig. Chromatogr., 4(2), 293, 1981.

116. Van Dame, H.C., J. Assoc. Off. Anal. Chem., 63(6), 1184, 1980, through Anal. Abstr., 40, 789, 1981.

117. Pavli, V., and Dobrovoljc, R., Farm. Vestn., 30(2), 129, 1979, through Anal. Abstr., 40, 789, 1981.

118. Vanden Heuvel, W.J.A. "Theory and Applications of Gas Chromatography in Industry and Medicine", ed. by Kroman, H. S. and Bender, S.R., Greene and Stratton, New York, 1968, PP 120-134.

119. Cartoni, G.P., Ciardi, M., Giarrusso, A., Rosati, F., J. Chromatogr., 279, 515-522, 1983.

120. Uralets, V.P., Semenova, V.A., and Yakushin, M.A., ibid., 279, 695-707, 1983.

121. Wabba, S.R., Amin, S.W., and Rofael, N., J. Pharm. Sci., 57, 1231-1234, 1968.

122. Simpson, P.M., J. Chromatogr., 77(1), 161-174, 1973, through Anal. Abstr., 25, 3225, 1975.

123. Monder, C., Iohan, F., Anal. Biochem., 139(1), 237-242, 1984, through Anal. Abstr., 47, 46, 1985.

124. Kley, H.K., Rick, W., J. Clin. Chem. Clin. Biochem., 22(5), 371-378, 1984, through Anal. Abstr., 47, 136, 1985.

125. Stupnicki, R., Zesz. Probl. Postepow Nauk. Roln., 261, 37-41, 1983, through, Anal. Abstr., 47, 136, 1985.

126. Alviola, K., Haikala, R., and Hosia, L., Acta. Pharm. Fenn., 90(3), 275-282, 1981, through Anal. Abstr., 42, 652, 1982.

127. Kruger, T.L., Kondrat, R.W., Joseph, K.T., and Cooks, R.G., Anal. Bio. Chem., 96(1), 104-112, 1979, through Anal. Abstr., 38, 431, 1980.

128. Gorog, S., Lauko, A., Herenyi, B., Acta. Chim. Acad. Sci., 100(1-4), 377-382, 1979, through Anal. Abstr., 38, 320, 1980.

129. Huf, F.A., Harberts, J.C.M., and Span, M.A., Pharm. Weekbl., 114(25), 660-663, 1979, through Anal. Abstr., 38, 82, 1980.

130. Belanger, J., Lodge, B.A., Parc, J.R.J., Latontaine, P., J. Pharm., Biomed. Anal., 3(1), 81-86, 1985, through Anal. Abstr., 48, 1986, 2E 33.

131. Dekker, D., Pharm., Weekbl. (Sci. Ed.), 2(2), 567-572, 1980 throu h t ., , 573, 1980.

132. Soliman, R., and Belal, S., Pharmazie, 35(3), 153-155, 1980, through Anal. Abstr., 39, 436, 1980.

133. "Pharmacopoeia of India", 3rd Ed., Vol. II (Appendix 3), 1985, The Controller of Publication, Delhi, A-68.