CHAPTER 2

Spectrophotometric Determination of Corticosteroids and Their Dosage Forms

2.1 Introduction

Steroids are widely distributed throughout the plant and animal kingdom. The basic structure of the steroid is the cyclopentanoperhydrophenanthrene ring system. The steroids of natural origin include those produced by the adrenal cortex, corpus luteum, placenta, testes, ovaries and liver. The major therapeutic classes of steroids are (i) Adrenocortical hormones (ii) Sex hormones (iii) Cholesterol and related sterols (iv) Cardiac glycosides and (v) Steroidal saponins and sapogenins. The minor changes in steroid molecules can cause extensive changes in biological activity.

The terms adrenocortical steroids, corticosteroids and corticoids are used to designate that they are isolated from adrenal cortex. Thirty such compounds are reported, twenty four of them including thirteen 21-hydroxy-20-keto derivatives belong to the pregnene series. The common structural features of natural corticosteroids or their synthetic analogs have the pregnene skelaton, the \( \Delta^4 \)-3-keto, 20-keto, 21-hydroxyl group and, in most of the cases a hydroxy group at C-11. Corticosteroids with a tertiary hydroxyl group attached to C-17 which influence carbohydrate metabolism are called glucocorticoids whereas the C-17 deoxy derivatives regulate the electrolytes and water metabolism are known as mineralocorticoids.
The adrenocortical steroids are used primarily for their glucocorticoid effects, including immunosuppression, anti-inflammatory and antiallergic activity. The mineralocorticoids are used mainly for treatment of Addison’s disease. They are also useful in the treatment of rheumatoid arthritis, bronchial asthma and bronchial hyper sensitivity.

The naturally occurring corticoids are corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone, 17α-hydroxycorticosterone (hydrocortisone), 17α-hydroxy-11-dehydrocorticosterone (cortisone), 17-hydroxy-11-deoxycorticosterone and aldosterone. Out of these, mainly hydrocortisone and its 11-keto analog (cortisone) as well as their synthetic analogs are used in the therapy. The most important structural changes for enhancement of their therapeutic effects and broadening of their field of application are the following:
(i) Introduction of a double bond at the $\Delta^1$ position to form prednisolone and prednisone from hydrocortisone and cortisone, respectively.

(ii) Introduction of halogens, mainly fluorine, at 6 and/or 9 and other positions e.g. fluorocortisone.

(iii) Introduction of methyl group at 6 or 16 positions and a hydroxyl group at 16 position. For example medrol.

Combination of the above structural changes led to a great number of effective new drugs e.g. Dexamethasone and Betamethasone, Flumethasone and Fluocortolone.

In therapy many of the natural and synthetic corticoids are used in the form of 21, 16 or 17 esters, which possess superior properties such as more efficient absorption or prolong duration of action. For instance, the following 21-esters of prednisolone were proposed: tert-butylacetate, diethylaminoacetate, phosphate, stearoylglycolate, succinate, metasulphobenzoate, and trimethylacetate. The water soluble sodium salts of 21-phosphates or hemisuccinates are of great importance for the preparation of pharmaceutical specialities. Water soluble derivatives can also be obtained by replacing the 21-hydroxy group by amino group. For example, depersolone. $16 \alpha$, 17 $\alpha$-Dihydrocorticosteroids (triamcinolone, fluocinolone etc.) are often used as cyclic ketals formed with acetone and other ketones.
2.1.1 Analysis of corticosteroids

The reported methods of analysis of corticosteroids involve ultraviolet absorption, infrared absorption, NMR spectrometry, polarography, chromatography etc. They are reviewed briefly.

2.1.1.1 Titrimetric Methods

Corticosteroids do not contain functional groups which are easy to determine by titrimetric methods. Moreover, they require the relative large sample size, which excludes the possibility of utilizing titrimetric technique in analysis of dosage forms and in biological or clinical analysis.

Methods for the indirect titrimetric determination based on oxidation of the α-ketol side chain of corticosteroids are reported. The 21-hydroxy group is oxidised to aldehyde with alkaline potassium mercuriiodide and the metallic mercury formed is determined iodometrically. In another variant of the method, the side chain is oxidised with alkaline potassium cupritartrate to form 20-keto-21-carboxylic acid and the resulting copper (I) oxide is determined permanganometrically. A procedure is developed in which copper sulphate is titrated with ethanolic hydrocortisone solution in presence of sodium potassium tartrate, potassium ferricyanide and methylene blue. Esters of corticoids can not be determined by using classical method based on alkaline hydrolysis and...
back titration of the excess alkali with standard acid, as in boiling alkali, the side chain decomposes to yield acidic products.\textsuperscript{86-88} This limitation is overcome by

\[
\text{CH}_2\text{OH} + \text{O}_2 + 2\text{OH}^- \rightarrow \text{COO}^- + \text{HCOO}^- + 2\text{H}_2\text{O}
\]

reducing 20-keto group by sodium hydroxide-sodium borohydride reagent,\textsuperscript{89} prior to the hydrolysis of the 21-ester. The excess alkali, after decomposition of sodium borohydride, is determined by standard acid using phenol red as an indicator.\textsuperscript{89}

A complexometric determination of corticoids using either copper\textsuperscript{90} or lead\textsuperscript{91} picrate at pH 11.5 is also described.\textsuperscript{90-92}

**2.1.1.2 Ultraviolet absorption methods**

The corticoids having $\Delta^4$-3-keto and $\Delta^{1,4}$-3-keto groups show maximum absorption at 240 and 244 nm, respectively in ethanolic medium, with molar absorptivities of about 17000.\textsuperscript{93}

Alkali metal borohydrides reduce the 3-keto group to spectrophotometrically inactive 3-hydroxy derivatives. This forms the basis of differential spectrophotometric method for the determination of $\Delta^4$ and $\Delta^{1,4}$-3-keto steroids.\textsuperscript{94,95}
2.1.1.3 **Spectrophotometric methods**

The various functional groups present in corticosteroid molecules permit the colorimetric determination of these compounds through a wide range of more or less specific reactions. However, each of these reactions is applied to only a limited number of corticosteroids.

(i) **3-Keto group**

Several reagents are suggested for the colorimetric analysis of corticosteroids. Amino, hydrazide and hydrazide derivatives containing amino group react with the carbonyl group of the corticosteroids to form Schiff's base.

The number of keto steroids are determined using 4-nitro or 2,4-dinitro-phenylhydrazine\textsuperscript{96,97} or Girard's reagent T\textsuperscript{98} which form hydrazones with keto steroids. The procedure involving Girard’s reagent T does not give very sensitive results, whereas the other reagents are sensitive but nonspecific.

Colorimetric methods based on the reaction of the active methylene group adjacent to the keto group with reagents such as aromatic di- and tri-nitro derivatives, diethyl oxalate, 2,6-di-ter-butyl-p-cresol etc. are employed for determination of corticosteroids.
(ii) \( \Delta^4 \)-and \( \Delta^1,4 \)-3-keto group

Methods based on the use of isoniazid,\(^9\) 4-amino antipyrine,\(^1\) or dimethylaminooanilene,\(^2\) give positive results only with \( \Delta^4 \)-, \( \Delta^1,4 \)- and \( \Delta^5(10) \)-3-keto steroids. They are selective and are applied to the determination of corticosteroids in pharmaceuticals. Isoniazid when condensed with keto-steroid in acidic medium gives yellow color with an absorption maximum at 380 nm. This method is widely used for the estimation of corticosteroids.

The reaction of 2,6-di-tert-butyl-p-cresol\(^3\) gives pink color with compounds having a saturated ring A, while it gives blue or orange color with \( \Delta^4 \)-and \( \Delta^1,4 \)-3-keto steroids. This reaction is not specific for corticosteroids, however, it is applied with various modifications, to assay corticosteroids in pharmaceuticals.\(^4\)

The reaction with tetramethylammonium hydroxide is specific for \( \Delta^4 \)-3-keto steroids.\(^5\)

(iii) 21-Hydroxy-20-keto(Ketol) group

Reaction based on presence of the ketol groups are more specific for corticosteroids, then the reactions described previously. These methods have been used for the determination of corticosteroids in pharmaceuticals and biological fluids.
Various methods based on the reducing properties of the ketol group are reported. Those performed in acidic or neutral medium are applicable to unesterified derivatives of corticosteroids. In alkaline medium, ketol side chain is very unstable. In presence of oxygen two carboxylic acids are obtained from drug molecule.\textsuperscript{86} However, triamcinolone is not oxidised by oxygen under the alkaline conditions. In presence of potassium hydroxide, formaldehyde is liberated from triamcinolone.\textsuperscript{105} It was postulated that the oxidation is completely hindered by an immediate isomerisation\textsuperscript{106} (a) which is followed by a slow release of formaldehyde (b).\textsuperscript{105}

(a) \[
\begin{align*}
\text{CH}_2\text{OH} \\
\text{CO} \\
\text{OH} \quad \text{OH} \\
\end{align*}
\text{fast} \\
\stackrel{\text{OH}}{\longrightarrow} \\
\begin{align*}
\text{HOH}_2\text{C} \\
\text{C} \\
\text{OH} \\
\text{OH} \\
\end{align*}
\]

(b) \[
\begin{align*}
\text{HOH}_2\text{C} \quad \text{OH} \\
\text{C} \\
\text{OH} \\
\text{OH} \\
\end{align*}
\text{Slow} \\
\stackrel{\text{HCHO}}{\longrightarrow} \\
\begin{align*}
\text{OH} \\
\text{C} \\
\text{OH} \\
\text{OH} \\
\end{align*}
\]
The reducing properties of free ketol group of corticosteroids allow their colorimetric determination in acidic medium with sodium molybdate\textsuperscript{107} and in neutral medium with cupric ion and o-phenylenediamine.\textsuperscript{108} In alkaline medium, corticosteroids are estimated with tetrazolium salt\textsuperscript{109} or with p-nitrosodimethylaniline.\textsuperscript{110,111}

Tetrazolium salts are reduced by ketol group in alkaline medium to the corresponding strongly colored formazans. Triphenyltetrazolium chloride was proposed first as a reagent,\textsuperscript{109} but the result obtained has poor reproducibility.\textsuperscript{112,113} More stable reagents e.g. 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium bromide,\textsuperscript{114} 2,5-diphenyl-3-(p-styrylphenyl)tetrazolium chloride,\textsuperscript{115} and blue tetrazolium\textsuperscript{116} were suggested.

The mechanism of the reaction between corticosteroids and colorless tetrazolium reagent has been proposed by Gorog and Horvath,\textsuperscript{117} Graham et al.,\textsuperscript{118-123} and Mohrle and Schittenhelm.\textsuperscript{12}
The formazan has a maximum absorbance at about 485 nm. Blue tetrazolium forms mainly two colored products viz. red monoformazan (\(\lambda_{\text{max}} 525\ \text{nm}, \ E = 24000\)) and the blue diformazan (\(\lambda_{\text{max}} 590\ \text{nm}, \ E = 42000\)).\textsuperscript{117} Under the usual reaction conditions only the red monoformazan is obtained; the blue diformazan is formed when the reaction is carried out at elevated temperature.

In practice triphenyltetrazolium chloride and blue tetrazolium are used widely. The blue tetrazolium reagent is more sensitive than triphenyltetrazolium chloride. Factors such as pH,\textsuperscript{122} temperature,\textsuperscript{125-127} light,\textsuperscript{113,119} presence of oxygen,\textsuperscript{113,119} concentration of water,\textsuperscript{122,125} solvents,\textsuperscript{118} base\textsuperscript{113,122} and purity\textsuperscript{112,128} as well as stability of reagent\textsuperscript{129} have a significant effect on the course of reaction. Moreover, the nonsteroidal reducing agents\textsuperscript{130,131} give positive reaction. Varying blank reading are obtained in tetrazolium blue procedure.\textsuperscript{113} Further 3-keto group present in the molecule of corticosteroids also reacts with reagent to a slight extent.\textsuperscript{132} Therefore, reagent blanks, standards and samples must be analyzed concurrently. The procedure is nonspecific, time consuming and required carefully controlled conditions for reproducible results.

In case of triamcinolone, which contains the \(16\alpha,17\ \alpha\)-dihydroxy groups, the intensity of color produced with blue tetrazolium is twice that of the corresponding 16-deoxy derivative.\textsuperscript{116,121}
Estimation of 21-esters of corticosteroids requires hydrolysis of ester prior to the reaction with tetrazolium salts.\textsuperscript{109} Readily saponifiable 21-esters of corticosteroid react slowly with blue tetrazolium because they have to hydrolyse first under the reaction conditions.\textsuperscript{133,134} The esters of phosphoric and sulfuric acid do not hydrolyse under the conditions of reaction. They are determined after their enzymatic hydrolysis.\textsuperscript{135}

Steric hindrance of 16-substituents results in a decreased reactivity towards blue tetrazolium.\textsuperscript{121} The reactivity of the inert derivatives can be increased by replacing ethanol by chloroform\textsuperscript{116} or dichloromethane\textsuperscript{118,120,136} as solvent.

The tetrazolium method is recommended in all pharmacopoeia for the determination of corticosteroids. The blue tetrazolium reaction has been automated in several laboratories.\textsuperscript{137-139}

17-Ketol and 17-hydroxy-17-ketol steroids are oxidised by periodic acid to form formaldehyde but 21-esters of corticosteroids do not react under the reaction condition.
The liberated formaldehyde is determined colorimetrically in situ or after its separation. The reaction is also applicable to the estimation of 20,21-dihydroxy and 17,20,21-trihydroxy steroids.

(iv) 17,21-Dihydroxy-20-keto group

All the above described reactions have been applied to analyse 17,21-dihydroxy-20-keto steroids. In Porter-Silber reaction the compound when treated with sulfuric acid gives 21-aldehyde derivative, which is condensed with phenylhydrazine to yield a yellow colored product (\(\lambda_{\text{max}} 410\, \text{nm}\)). The mechanism of reaction is proposed by Lewbart and Mattox. Easily hydrolysable 21-esters of corticosteroids react similarly.
21-Hydroxy-20-keto derivative is oxidised with cupric ion and condensed with phenylhydrazine. This procedure is used for the estimation of betamethasone-17-benzoate in gel formulation. To increase the sensitivity and chromophore stability, 4-hydrizinobenzensulfonic acid-phosphoric acid reagent is recommended.

Various drugs and their metabolites have been reported to interfere with the Porter-Silber procedure. These are carbamazepine, erythromycin, phenazopyridine, methanamine mandelate, acetylspiramycin, leucomycin, rifampicin and chlorpromazine.

The 17,21-dihydroxy-20-keto steroids condensed with 2-hydrizinobenzothiazole or 3-methylbenzothiazol-2-one hydrazone in alkaline medium. The condensed product is reacted with diazonium salt produced in situ by oxidation of the excess of reagent with hydrogen peroxide or ferric chloride to form formazan. The 2-hydrizinobenzothiazole forms bluish violet color while 3-methylbenzothiazol-2-one hydrazone gives blue colored product. The latter reagent is more sensitive.

Colorimetric determination of corticosteroids via splitting of the side chain with strong oxidizing agent e.g. sodium bismuthate or periodic acid is reported. The 17-keto steroid obtained by bismuthate oxidation is determined by
Zimmermann reaction. The method is applied for the estimation of urinary metabolites of corticosteroids. The interference of the other keto groups present in the molecule is eliminated by reducing them with sodium borohydride.

The formaldehyde liberated by sodium bismuthate was also determined by oxalylhydrazide and Cu (II).

2,4-Dinitrophenylhydrazine reacts with corticosteroids in alkaline medium. The reaction is highly sensitive.

2.1.1.4 **Halochromic reaction**

In concentrated sulfuric acid, 11-hydroxycorticosteroids give a product having intense absorbance in the visible region due to the formation of a Δ¹¹-derivatives.

In concentrated hydrochloric acid medium, 11β-hydroxy derivative gives a specific absorption band at 465 nm.

2.1.1.5 **Fluorimetry**

Corticosteroids exhibit fluorescence in a mixture of sulfuric acid-ethanol or sulfuric acid-glacial acetic acid. The fluorescence intensity is affected by structural modifications. The 11-hydroxy group is essential for the appearance of the fluorescence.
The 20-keto-21-aldehyde group obtained by oxidation of $\alpha$-keto side chain with copper (II) acetate on condensation with pyrrole gives a strong fluorescence product.\textsuperscript{167} In another method, formaldehyde obtained by oxidation of corticosteroids is condensed with ammonia and ethyl acetoacetate to give fluorescence.\textsuperscript{168}

2.1.1.6 Polarography

Polarographic technique is mainly used for the determination of steroids in biological fluids and pharmaceutical formulations. Corticosteroids having 4-ene-3-keto or 1,4-diene-3-keto group and their synthetic analogs are analysed polarographically. The classical d.c. polarographic technique has been employed for the determination of prednisolone, prednisone in tablets,\textsuperscript{169} and hydrocortisone in ointments.\textsuperscript{170} Differential pulse polarography has a high sensitivity\textsuperscript{171,172} with working range of 5-40 mcg/ml. It is used to assay the dosage forms of corticosteroids. The procedure does not require prior separation of drug from dosage form.\textsuperscript{171-173} deBoer et al., described in detail the practical\textsuperscript{173-175} and theoretical\textsuperscript{176,177} aspects of the determination of various corticosteroids in single component\textsuperscript{173} and complex\textsuperscript{175} solutions, suspensions, ointments and creams by differential pulse polarography.

2.1.1.7 Chromatography

Column chromatography is used to separate and clean-up of corticosteroids from biochemical materials and pharmaceutical formulations\textsuperscript{178,179} prior to their spectrophotometric,
colorimetric, fluorimetric, gas chromatographic, RIA etc.

estimation.

The paper chromatographic method\textsuperscript{180} finds routine
application in corticosteroids separation before their
identification or quantitative determination.\textsuperscript{181-186}

Inspite of several advantages of paper chromatography
in the corticosteroid analysis, thin-layer chromatography
has superseded it mainly due to its speed and flexibili-

For visualization of the spot, blue tetrazolium
reagent is used.\textsuperscript{190}

Quantitative TLC of corticosteroids followed by UV
spectrophotometric,\textsuperscript{191} blue tetrazolium reaction\textsuperscript{192} or
fluorimetric\textsuperscript{193} techniques are used in their determination.
The scanning of thin-layer chromatogram is used for
corticosteroids determination.\textsuperscript{191,194-196} TLC-densitome-
metric\textsuperscript{197} determination of hydrocortisone acetate in
ointment and hydrocortisone in serum after its derivatisa-
tion with dansyl hydrazine and fluorescence scanning\textsuperscript{198}
are reported.

GC-MS technique for the determination of corticosteroids
is still important due to its selectivity and sensitivity.
Corticosteroids are converted in their corresponding 21-heptafluorobutyryl and/or enolheptafluorobutyryl derivative\textsuperscript{199} which is determined by electron capture detector.

High pressure liquid chromatography (HPLC) becomes one of the most important separation and quantitation methods in analysis of corticosteroids. This field has been reviewed exhaustively by Fitzpatrick.\textsuperscript{200}

In the analysis of corticosteroids, HPLC technique has superseded gas chromatography because the latter is problematic due to heat sensitivity of 17-ketol group. Further, the strong absorption band in UV region of the spectra of all corticosteroids makes possible their detection with fairly high sensitivity. This solves the problems encountered in the analysis in pharmaceutical and biochemical fields, hence many reports are published in the practical application of HPLC.\textsuperscript{200-206} The technique is also suitable for the separation of isomers.\textsuperscript{207-210} Adsorption chromatography and reverse phase partition systems have been extensively used for separation.\textsuperscript{211-215} The corticosteroids present either in biological or pharmaceutical dosage forms can be separated using these systems.

The main application of HPLC in the analysis of corticosteroids is the estimation of their micro-quantities present in ointments or creams which are otherwise difficult
to analyse. By selecting proper chromatographic system the
time consuming clean up procedures may be shortened.\textsuperscript{216}

HPLC is an ideal tool to check stability of cortico-
steroi d formulations and to detect their degradation
products.\textsuperscript{217-227} It is applicable to determine the rate of
dissolution of corticosteroid tablet.\textsuperscript{222}

\subsection{Miscellaneous}

Radioimmunoassay method involves the use of labelled
steroids for their analysis. The subject is reviewed by
Bojesen et al.\textsuperscript{223} The method is used for the determination
of corticosteroids in biological samples.\textsuperscript{224-226}

The enzymatic-photometric or fluorimetric methods
for determination of corticosteroids are based on oxido-
reduction of hydroxy-ketone systems using (3\textbeta)\textsuperscript{20} \alpha-hydroxy-
steroid dehydrogenase.\textsuperscript{227-230} In all cases the reaction is
monitored with the NAD(P) - NADH system.

Corticosteroids are determined by spectrophotometric,\textsuperscript{231}
NMR spectroscopy,\textsuperscript{232} mass spectroscopy\textsuperscript{233-236} and phospho-
rescence techniques.\textsuperscript{237} Recently fast atom bombardment (FAB)
mass spectroscopy\textsuperscript{238} is used for the determination of
corticosteroids in pharmaceutical preparations.
Determination of corticosteroids by acetylacetone method

It has been reported that formaldehyde in aqueous or alcoholic solution reacts with acetylacetone in the presence of ammonium acetate, to afford yellow colored, 3,5-diacetyl-1,4-dihydrolutidine. \(^ {44}\) Corticosteroids having a 17-ketol group are known to liberate formaldehyde on oxidation with sodium periodate. \(^ {156}\) Therefore, it was thought of interest to extend the application of acetylacetone method to determine corticosteroids in bulk powder and their dosage forms.

In present work, reaction conditions are modified suitably to develop a method for the estimation of corticosteroids. The effect of various reaction conditions such as concentration of sodium periodate, reagent and corticosteroids, time for oxidation, as well as time for color development on the color intensity are standardised.

Pure samples of hydrocortisone, prednisolone, dexamethasone, betamethasone, triamcinolone and their pharmaceutical formulations are analysed by proposed method. The results compare favourably with those obtained by pharmacopoeial method. \(^ {240}\)
2.2 Experimental

2.2.1 Apparatus

1. Double beam Beckman Model 25 spectrophotometer having two matched cells with 1 cm lightpath was employed for spectral measurements.

2. Corning volumetric flask of 25 mL, 50 mL and 100 mL capacity were used in the study.


2.2.2 Reagents and Materials

Hydrocortisone BP, Hydrocortisone Acetate BP, Prednisolone BP, Dexamethasone BP, Betamethasone BP, Triamcinolone BP, Triamcinolone acetonide USP, Fluocinolone acetonide BP, Dexamethasone sodium phosphate IP, Sodium periodate (SD'S), Acetylacetone (Freshly distilled), Ammonium acetate (ExcelaR), Tetramethylammonium hydroxide (E. Merck), Triphenyltetrazolium chloride (LOBA), Tetrazolium blue (LOBA), Ethanol (aldehyde free), Chloroform (BDH) and double distilled water were used in the study.

The dosage forms of corticosteroids were procured from local market.

2.2.2.1 Preparation of sodium periodate solution

Sodium periodate (2.14 g) was weighed accurately and dissolved in and diluted to 1000 mL with water.
2.2.2.2 Preparation of Reagent solution

The reagent solution was prepared by mixing ammonium acetate (300.0g) dissolved in water (500mL) with freshly distilled acetylacetone (10mL). The final volume was adjusted to 1000 mL with water and kept in refrigerator.

2.2.2.3 Preparation of standard solution of hydrocortisone, prednisolone, dexamethasone, betamethasone, triamcinolone, triamcinolone acetonide, fluocinolone acetonide or hydrocortisone acetate

The drug (25.0mg) was weighed accurately and dissolved in and diluted to 100mL with ethanol. The final solution contained 250mcg of the drug per mL of the solution.

2.2.3 Procedure

2.2.3.1 Hydrocortisone

2.2.3.1.1 Determination of wavelength of maximum absorbance

Standard hydrocortisone solution (2.0mL) was pipetted into 25mL volumetric flask. Sodium periodate solution (3.0mL) was added to it. The reaction mixture was allowed to stand for 5 minutes at 37°C with occasional shaking. The reagent solution (5.0mL) was added to it and mixed thoroughly. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with water or ethanol. The absorbance of the colored solution was scanned on Beckman Model 25 spectrophotometer in the range of 350 to 550 nm against blank.
The blank was prepared similarly in which volume of standard hydrocortisone solution was replaced by an equal volume of ethanol.

Maximum absorbance was obtained at 412nm (Fig.1).

2.2.3.1.2 Lambert-Beer's curve for Hydrocortisone

Standard hydrocortisone solution (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0ml) was pipetted in a series of 25mL volumetric flasks and treated as described under 2.2.3.1.1.

The absorbance of the reaction mixture was measured at 412nm against reagent blank (Fig.2).

2.2.3.1.3 Factors affecting the development of color

(i) Effect of concentration of sodium periodate

Standard hydrocortisone solution (2.0ml) was treated with different volumes of sodium periodate solution and immersed in a water bath at 37°C for 5 minutes. The reagent solution (5.0ml) was added to it and mixed thoroughly. It was allowed to stand at 37°C for 30 minutes and analysed as described under 2.2.3.1.1.

Maximum absorbance was observed in the presence of 2.0ml of sodium periodate solution (Fig.3).
(ii) **Time of reaction (for oxidation)**

Standard hydrocortisone solution (2.0mL) was treated with sodium periodate solution (3.0mL). The reaction mixture was immersed in a water bath at 37°C for different time intervals. The reagent solution (5.0mL) was added to it and analysed as described under 2.2.3.1.1.

Maximum color intensity was obtained after 5 minutes which remained constant for 1 hour (Fig.4).

(iii) **Effect of concentration of reagent solution**

Standard hydrocortisone solution (2.0mL) was pipetted into a series of 25mL volumetric flasks. Sodium periodate solution (3.0mL) was added to each flask. The reaction mixture was allowed to stand for 5 minutes at 37°C with occasional shaking. Different volume of the reagent solution was added to each flask and mixed thoroughly. The reaction mixture was allowed to stand for 30 minutes at 37°C and analysed as described under 2.2.3.1.1.

Maximum color intensity was obtained in the presence of 5.0mL of the reagent solution which remained constant with increase in the volume of the reagent solution (Fig.5).

(iv) **Time for maximum color development and color stability**

Standard hydrocortisone solution (2.0mL) was pipetted into a series of 25mL volumetric flasks. Sodium periodate solution (3.0mL) was added to each flask and immersed in
a water bath at 37°C for 5 minutes. The reagent solution (5.0mL) was added to it. The reaction mixture was allowed to stand for 5, 10, 20, 30, 40, 60 and 120 minutes at 37°C and analysed as described under 2.2.3.1.1.

Maximum absorbance was observed after 30 minutes which remained constant for more than 2 hours (Fig.6).

2.2.3.2 Prednisolone

2.2.3.2.1 Determination of wavelength of maximum absorbance

Standard prednisolone solution (2.0mL) was pipetted in a 25mL volumetric flask. Sodium periodate solution (2.0mL) was added to it. The reaction mixture was allowed to stand for 5 minutes at 37°C with occasional shaking. The reagent solution (5.0mL) was added to it and mixed thoroughly. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with water or ethanol. The absorbance of the colored solution was scanned on Beckman Model 25 spectrophotometer in a range of 350 to 550nm against blank.

The blank was prepared similarly in which volume of standard prednisolone was replaced by an equal volume of ethanol.

Maximum absorbance was obtained at 412nm.
2.2.3.2.2 Lambert-Beer's curve for Prednisolone

Standard prednisolone solution (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0ml) was pipetted in a series of 25ml volumetric flasks and analysed as described under 2.2.3.2.1.

The absorbance of the reaction mixture was measured at 412nm against reagent blank. Lambert-Beer's law was obeyed in concentration range of 5.0 to 40.0 mcg of prednisolone per ml of reaction mixture.

2.2.3.2.3 Factors affecting the development of color

(i) Effect of concentration of sodium periodate

Standard prednisolone solution (2.0mL) was treated with different volumes of sodium periodate solution and was immersed in a water bath at 37°C for 5 minutes. The reagent solution (5.0mL) was added to it and mixed thoroughly. It was allowed to stand at 37°C for 30 minutes and analysed as described under 2.2.3.2.1.

Maximum absorbance was observed in the presence of 2.0mL of sodium periodate solution (Fig.3).

(ii) Time of Reaction (for oxidation)

Standard prednisolone solution (2.0mL) was treated with sodium periodate solution (2.0mL). The reaction mixture was immersed in a water bath at 37°C for different time intervals. The reagent solution (5.0mL) was added to each flask and analysed as described under 2.2.3.2.1.
The maximum color intensity was obtained after 5 minutes which remained constant for 1 hour (Fig.4).

(iii) Effect of concentration of reagent solution

Standard prednisolone solution (2.0mL) was pipetted into a series of 25mL volumetric flasks. Sodium periodate solution (2.0mL) was added to each flask. The reaction mixture was allowed to stand for 5 minutes at 37°C with occasional shaking. Different volume of the reagent solution was added to each flask and mixed thoroughly. The reaction mixture was immersed in a water bath at 37°C for 30 minutes and analysed as described under 2.2.3.2.1.

Maximum color intensity was obtained in the presence of 5.0mL of reagent solution which remained constant with increase in the volume of reagent solution (Fig.5).

(iv) Time for maximum color development and color stability

Standard prednisolone solution (2.0mL) was pipetted into a series of 25mL volumetric flasks and was treated with sodium periodate solution (2.0mL) at 37°C for 5 minutes. The reagent solution (5.0mL) were added to each flask and mixed thoroughly. The reaction mixture was allowed to stand for 5, 10, 20, 30, 40, 60 and 120 minutes at 37°C. The absorbance of the colored solution was measured at 412nm against reagent blank as described under 2.2.3.2.1.

Maximum absorbance was observed after 30 minutes which remained constant for more than one hour (Fig.6).
2.2.3.3 Dexamethasone

2.2.3.3.1 Determination of wavelength of maximum absorbance

Standard dexamethasone solution (3.0mL) was pipetted into 25mL volumetric flask. Sodium periodate solution (6.0mL) was added to it. The reaction mixture was allowed to stand for 30 minutes at 37°C with occasional shaking. The reagent solution (5.0mL) was added to it and mixed thoroughly. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with water or ethanol. The absorbance of the colored solution was scanned on Beckman Model 25 spectrophotometer in the range of 350 to 550nm against blank.

The blank was prepared similarly in which volume of standard dexamethasone solution was replaced by an equal volume of ethanol.

Maximum absorbance was obtained at 412nm.

2.2.3.3.2 Lambert-Beer's curve for Dexamethasone

Standard dexamethasone solution (0.5, 1.0, 1.5, 2.0, 3.0, 4.0mL) was pipetted in a series of 25mL volumetric flasks and analysed as described under 2.2.3.3.1.

The absorbance of the reaction mixture was measured at 412nm against reagent blank. The Lambert-Beer's law was obeyed in concentration range of 5.0 to 40.0mcg of dexamethasone per mL of reaction mixture.
2.2.3.3.3 Factors affecting the development of color

(i) Effect of concentration of sodium periodate

Standard dexamethasone solution (3.0mL) was treated with different volume of sodium periodate solution and allowed to stand at 37°C for 30 minutes. The reagent solution (5.0mL) was added to each flask and mixed thoroughly. It was allowed to stand at 37°C for 30 minutes. The volume was adjusted to 25mL with water or ethanol. The absorbance of the reaction mixture was measured at 412nm against reagent blank.

Maximum absorbance was observed in the presence of 6.0mL of sodium periodate solution (Fig.7).

(ii) Time of Reaction (for oxidation)

Standard dexamethasone solution (3.0mL) was treated with sodium periodate solution (6.0mL). The reaction mixture was immersed in a water bath at 37°C for different time intervals. The reagent solution (5.0mL) was added to each flask and analysed as described under 2.2.3.3.1.

Maximum absorbance was obtained after 30 minutes which remained constant for 1 hour (Fig.8).

(iii) Effect of concentration of reagent solution

Standard dexamethasone solution (3.0mL) was pipetted into a series of 25mL volumetric flasks. Sodium periodate solution (6.0mL) was added to each flask. The reaction
mixture was allowed to stand for 30 minutes at 37°C with occasional shaking. Different volume of the reagent solution was added to each flask, mixed thoroughly and analysed as described under 2.2.3.3.1.

Maximum color intensity was obtained in the presence of 5.0mL of the reagent solution which remained constant with increase in the volume of the reagent solution (Fig.9).

(iv) **Time for maximum color development and color stability**

Standard dexamethasone solution (3.0mL) was pipetted into a series of 25mL volumetric flasks. Sodium periodate solution (6.0mL) was added to each flask and the reaction mixture was allowed to stand for 30 minutes at 37°C. The reagent solution (5.0mL) was added to it and mixed thoroughly. The reaction mixture was allowed to stand for 5, 10, 20, 30, 40, 60 and 120 minutes in a water bath at 37°C. The volume was adjusted to the mark with water or ethanol. The absorbance of the solution was measured at 412nm against reagent blank.

Maximum absorbance was observed after 30 minutes which remained constant for more than 2 hours (Fig.10).

2.2.3.4 **Betamethasone**

2.2.3.4.1 **Determination of wavelength of maximum absorbance**

Standard betamethasone solution (3.0mL) was pipetted into 25mL volumetric flask. Sodium periodate solution (6.0mL)
was added to it. The reaction mixture was allowed to stand for 1½ hours at 37°C with occasional shaking. The reagent solution (5.0mL) was added to it and mixed thoroughly. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with water or ethanol. The absorbance of the colored solution was scanned on Beckman Model 25 Spectrophotometer in range of 350 to 550nm against blank.

The blank was prepared similarly in which volume of standard betamethasone solution was replaced by an equal volume of ethanol.

Maximum absorbance was obtained at 412nm.

2.2.3.4.2 **Lambert-Beer's curve for betamethasone**

Standard betamethasone solution (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0mL) was pipetted in a series of 25mL volumetric flasks and analysed as described under 2.2.3.4.1.

The absorbance of the reaction mixture was measured at 412 nm against reagent blank. Lambert-Beer's law was obeyed in concentration range of 5.0 to 40.0mcg of betamethasone per mL of reaction mixture.
2.2.3.4.3 Factors affecting the development of color

(i) Effect of concentration of sodium periodate

Standard betamethasone solution (3.0mL) was treated with different volume of sodium periodate solution and allowed to stand at 37°C for 1½ hours. The reagent solution (5.0mL) was added to each flask and mixed thoroughly. It was allowed to stand at 37°C for 30 minutes. The volume was adjusted to 25mL with water or ethanol. The absorbance of the reaction mixture was measured at 412nm against reagent blank.

Maximum absorbance was observed in the presence of 6.0mL of sodium periodate solution (fig.7).

(ii) Time of Reaction (for oxidation)

Standard betamethasone solution (3.0mL) was treated with sodium periodate solution (6.0mL). The reaction mixture was immersed in a water bath at 37°C for different time intervals. The reagent solution (5.0mL) was added to each flask and analysed as described under 2.2.3.4.1.

Maximum absorbance was obtained after 1½ hours which remained constant for more than 2 hours (Fig.8).

(iii) Effect of concentration of reagent solution

Standard betamethasone solution (3.0mL) was pipetted into a series of 25mL volumetric flasks. Sodium periodate solution (6.0mL) was added to each flask. The reaction mixture was allowed to stand for 1½ hours at 37°C with
occasional shaking. Different volume of the reagent solution was added to each flask and mixed thoroughly. The reaction mixture was allowed to stand for 30 minutes at 37°C. The volume was adjusted to the mark with water or ethanol. The absorbance of the colored solution was measured at 412nm against blank.

Maximum color intensity was obtained in the presence of 5.0ml of the reagent solution which remained constant with increase in the volume of the reagent solution (Fig.9).

(iv) **Time for maximum color development and color stability**

Standard betamethasone solution (3.0ml) was pipetted in a series of 25ml volumetric flasks. Sodium periodate solution (6.0ml) was added to each flask and allowed to stand for 1½ hours at 37°C. The reagent solution (5.0ml) was added to each flask and mixed thoroughly. The reaction mixture was allowed to stand for 5, 10, 20, 30, 40, 60 and 120 minutes at 37°C. The volume was adjusted to the mark with water or ethanol. The absorbance of the colored solution was measured at 412nm against reagent blank.

Maximum absorbance was observed after 30 minutes which remained constant for more than 2 hours (Fig.10).
2.2.3.5 Triamcinolone

2.2.3.5.1 Determination of wavelength of maximum absorbance

Standard triamcinolone solution (3.0mL) was pipetted into 25mL volumetric flask. The reagent solution (5.0mL) was added to it and mixed thoroughly. Sodium periodate solution (3.0mL) was added to it. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with water or ethanol. The absorbance of the colored solution was scanned on Beckman Model 25 spectrophotometer in the range of 350 to 550nm against blank.

The blank was prepared similarly in which volume of standard triamcinolone solution was replaced by an equal volume of ethanol.

Maximum absorbance was obtained at 412nm.

2.2.3.5.2 Lambert-Beer's curve for triamcinolone

Standard triamcinolone solution (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0mL) was pipetted in a series of 25mL volumetric flasks and analysed as described under 2.2.3.5.1.

The absorbance of the reaction mixture was measured at 412nm against reagent blank. The Lambert-Beer's law was obeyed in concentration range of 5.0 to 40.0mcg of triamcinolone per mL of reaction mixture.
2.2.3.5.3 Factors affecting the development of color

(i) Effect of concentration of reagent solution

Standard triamcinolone solution (3.0mL) was pipetted into 25mL volumetric flask. Different volume of reagent solution was added to each flask and mixed thoroughly. Sodium periodate solution (3.0mL) was added to each flask and was allowed to stand at 37°C for 30 minutes. The volume was adjusted to the mark with water or ethanol. The absorbance of the reaction mixture was measured at 412nm against reagent blank.

Maximum absorbance was observed in the presence of 5.0mL of reagent solution which remained constant with increase in the volume of the reagent solution (Fig.11).

(ii) Effect of concentration of sodium periodate

Standard triamcinolone solution (3.0mL) was treated with the reagent solution (3.0mL). Different volume of sodium periodate solution was added to each flask and was allowed to stand at 37°C for 30 minutes. The volume was adjusted to 25mL with water or ethanol. The absorbance of the reaction mixture was measured at 412nm against reagent blank.

Maximum absorbance was observed in the presence of 3.0mL of sodium periodate solution (Fig.12).
(iii) **Time for maximum color development and color stability**

Standard triamcinolone solution (3.0mL) was pipetted into a series of 25mL volumetric flasks. The reagent solution (5.0mL) and sodium periodate solution (3.0mL) were added to each flask. The reaction mixture was allowed to stand for 5, 10, 20, 30, 40, 60 and 120 minutes at 37°C and analysed as described under 2.2.3.5.1.

Maximum absorbance was observed after 30 minutes which remained constant for more than 2 hours (Fig.13).

2.2.3.6 **Determination of hydrocortisone acetate, dexamethasone sodium phosphate, triamcinolone acetonide or fluocinolone acetonide**

Standard drug solution (3.0mL) was pipetted into 25mL volumetric flask. Sodium periodate solution (3.0mL) was added to it. The reaction mixture was allowed to stand for 30 minutes at 37°C with occasional shaking. The reagent solution (5.0mL) was added to it and mixed thoroughly. It was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with water. The absorbance of the colored solution was measured at 412nm against reagent blank.

No appreciable absorbance was observed in any of the drug.
2.2.4.1 **Analysis of Hydrocortisone**

Hydrocortisone (ca 25mg) was weighed accurately and dissolved in and diluted to 100mL with ethanol. The solution (2.0mL) was analysed as described under 2.2.3.1.1.

The amount of hydrocortisone was determined by referring to the standard curve (Table IV).

2.2.4.2 **Analysis of Prednisolone**

Prednisolone (ca 25mg) was weighed accurately and dissolved in and diluted to 100mL with ethanol. The solution (2.0mL) was analysed as described under 2.2.3.2.1.

The amount of prednisolone was determined by referring to the standard curve (Table IV).

2.2.4.3 **Analysis of Dexamethasone**

Dexamethasone (ca 25mg) was weighed accurately and dissolved in and diluted to 100mL with ethanol. The resulting solution (3.0mL) was analysed as described under 2.2.3.3.1.

The amount of dexamethasone was determined by referring to the standard curve (Table IV).

2.2.4.4 **Analysis of Betamethasone**

Betamethasone (ca 25mg) was weighed accurately and dissolved in and diluted to 100mL with ethanol. The diluted solution (3.0mL) was analysed as described under 2.2.3.4.1.
The amount of betamethasone was determined by referring to the standard curve (Table IV).

2.2.4.5 Analysis of Triamcinolone

Triamcinolone (ca 25mg) was weighed accurately and dissolved in and diluted to 100mL with ethanol. The solution (3.0mL) was analysed as described under 2.2.3.5.1.

The amount of triamcinolone was determined by referring to the standard curve (Table IV).

2.2.4.6 Analysis of Prednisolone tablets

Twenty tablets were weighed and powdered. The powder equivalent to prednisolone (ca 10mg) was weighed accurately and mixed with ethanol (25mL) and shaken occasionally for 30 minutes and filtered through Whatman No.41 filter paper. The residue was washed thoroughly with ethanol. The filtrate and washings were combined in a 50mL volumetric flask and diluted to the mark with ethanol. The solution (3.0mL) was pipetted in 25mL volumetric flask. Sodium periodate solution (2.0mL) was added to it. The reaction mixture was allowed to stand for 5 minutes at 37°C with occasional shaking. The reagent solution (5.0mL) was added to it and mixed thoroughly. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with ethanol. The absorbance of the reaction mixture was measured at 412nm against reagent blank.
The amount of prednisolone was determined by referring to the standard curve (Table IV).

2.2.4.7 **Analysis of Dexamethasone tablets**

Twenty tablets were weighed and powdered. The powder equivalent to dexamethasone (ca. 10mg) was weighed accurately and shaken with ethanol (25mL) for 30 minutes and filtered through Whatman No.41 filter paper. The residue was washed thoroughly with ethanol. The filtrate and washings were combined in a 50mL volumetric flask and diluted to the mark with ethanol. The solution (3.0mL) was pipetted in a 25mL volumetric flask. Sodium periodate solution (6.0mL) was added to it. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The reagent solution (5.0mL) was added to it and mixed thoroughly. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with ethanol. The absorbance of the colored solution was measured at 412nm against reagent blank.

The amount of dexamethasone was determined by referring to the standard curve (Table IV).

2.2.4.8 **Analysis of Betamethasone tablets**

Twenty tablets were weighed and powdered. The powder equivalent to betamethasone (ca. 10mg) was weighed accurately and mixed with ethanol (25mL) and shaken for 30 minutes and
filtered through Whatman No.41 filter paper. The residue was washed thoroughly with ethanol. The filtrate and washings were combined in a 50mL volumetric flask and diluted to the mark with ethanol. The solution (3.0mL) was pipetted in a 25mL volumetric flask. Sodium periodate solution (6.0mL) was added to it. The reaction mixture was allowed to stand for 1 ½ hour at 37°C with occasional shaking. The reagent solution (5.0mL) was added to it and mixed thoroughly. The reaction mixture was allowed to stand at 37°C for 30 minutes. The volume was adjusted to the mark with ethanol. The absorbance of the colored solution was measured at 412nm against reagent blank.

The amount of betamethasone was determined by referring to the standard curve (Table IV).

2.2.4.9 Analysis of Triamcinolone tablets

Twenty tablets were weighed and powdered. The powder equivalent to triamcinolone (ca 10mg) was weighed accurately and mixed with ethanol (25mL) and shaken for 30 minutes and filtered through Whatman No.41 filter paper. The residue was washed thoroughly with ethanol. The filtrate and washings were combined in a 50mL volumetric flask and diluted to the mark with ethanol. The solution (3.0mL) was pipetted in a 25mL volumetric flask. The reagent solution (5.0mL) was added to it and mixed thoroughly. Sodium periodate solution (3.0mL) was added to it. The reaction mixture was immersed in a water bath at 37°C for 30 minutes. The volume was adjusted to the mark with ethanol. The absorbance of the
The amount of triamcinolone was determined by referring to the standard curve (Table IV).

2.2.4.10 Analysis of Hydrocortisone ointment

Ointment equivalent to 20.0 mg of hydrocortisone was weighed accurately and transferred to a conical flask. Ethanol (20.0mL) was added and heated on a waterbath to melt ointment base and mix thoroughly. It was cooled in an ice bath and filtered through Whatman No.40 filter paper into a 100 mL volumetric flask. The process was repeated with ethanol (20x3) and diluted to 100mL with the same solvent. The solution (3.0mL) was analysed as described under 2.2.3.1.1.

The amount of hydrocortisone was determined by referring to the standard curve (Table IV).

2.2.5.1 Rate of oxidation of corticosteroids

Standard solution of corticosteroid (2.0mL) was treated with sodium periodate solution (5.0mL) in a 25mL volumetric flask and allowed to stand at 37°C for 5 minutes. The reagent solution (5.0mL) was added to it and allowed to stand for 30 minutes at 37°C. The volume was adjusted to the mark with water. The absorbance of the reaction mixture was measured at 412nm against reagent blank. The absorbance is given in the Table V.
2.3 Results and Discussion

It is important to detect decomposition and analytical interference in the determination of pharmaceutical corticosteroid preparations. A number of methods have been reported for the estimation of corticosteroids. The usual methods employed for the analysis of corticosteroids involve the blue tetrazolium reaction (BT), the phenylhydrazine reaction (PH) (Porter-Silber method), the isonicotinic acid hydrazide reaction (INH) and UV spectrophotometry.

The BT and PH reagents react with the C-17 side chain while the INH and UV methods involve $\alpha-\beta$ unsaturated ketone at C-3, as shown in Scheme I. Thus the reactions in these methods take place with different pharmacophoric groups of the molecule. Therefore, they can be employed to detect and distinguish the decomposition products.
The most common type of decomposition of corticosteroids encountered in pharmaceutical preparations is the oxidation of C-17 side chain. The degradation products formed by oxidation of the C-17 side chain are neutral or acidic in character. Therefore, sample clean-up is required prior to the estimation of corticosteroids.

The corticosteroids and their decomposition products contain \( \alpha, \beta \) unsaturated carbonyl group in ring A having \( \lambda_{\text{max}} \) at 240nm. The INH reaction involves the ketonic group to form hydrazone. Therefore, UV as well as INH method cannot be used for the determination of corticosteroids in presence of their decomposition products. The methods based on reaction with C-17 ketol side chain are more specific and are widely used for the estimation of corticosteroids. The most popular procedures are blue tetrazolium and Porter-Silber method.

Porter-Silber method is applicable in case of C-17 hydroxyl steroids except triamcinolone. However, various combination drugs or their metabolites interfere in the procedure.

The blue tetrazolium method is recommended in most of the pharmacopoeias for the assay of corticosteroids and their formulations. However, the reaction is subjected to interference by number of organic and inorganic compounds. Water affects the pH and polarity of the medium resulting in decrease in rate of the reaction with blue tetrazolium.
Extraction with ethanol removes acidic decomposition products and water along with the steroids from the sample. Therefore, chloroform has been suggested as an extracting solvent which extract steroids selectively. Moreover, temperature, light, presence of oxygen, base strength, time of reaction etc. effect significantly the course of reaction. The method is time consuming and gives poor reproducibility of the results. It was, therefore, thought of interest to establish a simple, accurate, and precise method for the estimation of corticosteroids based on the C-17 side chain.

It is known that corticosteroids liberate formaldehyde on periodate oxidation. The formaldehyde formed is determined colorimetrically after its separation. Shah reported the determination of formaldehyde in situ using acetylacetone.

\[
\begin{align*}
\text{HCHO} & \\
\text{H}_3\text{C.COCH}_2\text{C}_ & + \text{CH}_2\text{CO.CH}_3 & \rightarrow & \text{H}_3\text{COC} & \text{H} & \text{CO.CH}_3 \\
\text{H}_3\text{C.CO} & \text{O.C.CH}_3 & & \text{H}_3\text{C.N} & \text{CH}_3 & \text{NH}_3
\end{align*}
\]

3,5-Diacetyl-1,4-dihydrolutidine
In the proposed procedure, corticosteroid is oxidised with sodium periodate to liberate formaldehyde. The reaction mixture is treated with acetylacetone in presence of ammonium acetate. The yellow colored products formed has wavelength of maximum absorbance at 412nm. The color is stable for more than 2 hour.

Various parameters of the reaction such as concentration of sodium periodate, time required for oxidation, reagent concentration and time for maximum color development for prednisolone, hydrocortisone, dexamethasone, betamethasone and triamcinolone were studied to obtain maximum color intensity (Fig. 3-13). The concentration range, molar absorptivity, number of moles of formaldehyde produced per molecule of corticosteroid and standard deviation are calculated (Table II and III).

It is evident from the data (Table II) that the oxidation of corticosteroids proceeds to about 80-90%.

Pure samples of hydrocortisone, prednisolone, dexamethasone, betamethasone and triamcinolone and their pharmaceutical formulations were assayed by the proposed method. The results are in good agreement with those obtained by the pharmacopeial method\textsuperscript{192,240} (Table IV).
During the analysis of tablets of corticosteroids it was observed that the reaction mixture when finally diluted with water gives turbidity. Therefore, the method was modified in which the final dilution was made with ethanol instead of water.

None of the usual diluents, lubricants and solvents employed in the formulations interfer in the analysis of corticosteroids by the proposed procedure.

Further the oxidation products and the excess of periodate do not affect the course of reaction between formaldehyde and acetylacetone. Therefore, the method permits the determination of formaldehyde in situ. This makes the method simple and rapid.

Attempts to analyse 21-esters of the corticosteroids hydrocortisone acetate, and dexamethasone sodium phosphate were failed. It seems that the 21-esters of corticosteroids can not be oxidised with periodate to form formaldehyde under the experimental conditions.

The rate of oxidation of C-17 side chain with periodate depends upon the structure of corticosteroids. The substituents on C-16 of the corticosteroid molecule affect its reactivity towards the periodate oxidation. For example the rate of oxidation of dexamethasone or betamethasone
having 16-methyl group is slow as compared to that of hydrocortisone or prednisolone (Table V). This may be due to steric hindrance. The rate of oxidation of the C-17 ketol group is also influenced by the configuration of C-16 methyl group. In case of betamethasone, 16-methyl group and ketol group are in the same plane while in dexamethasone, they are in different planes. Therefore, the rate of formaldehyde formation from latter is higher than former (Table V).

The course of oxidation of ketol group is also affected by the nature of substituents on C-16 and C-17 of corticosteroid molecule. In case of triamcinolone acetonide (I) and fluocinolone acetonide (II), oxidation does not take place appreciably under the reaction condition.

\[
\text{I} \quad X = H \\
\text{II} \quad X = F
\]

This may be due to the steric hindrance by bulkier acetonide group.
In preliminary experiments to determine triamcinolone by the proposed procedure, it was observed that the color intensity decreases with the increase in oxidation time. Therefore, the effect of order of addition of reagents was studied. Maximum color intensity was obtained when the reagent is added prior to the addition of sodium periodate (Table VI). Therefore, the assay procedure is modified in which reagent is added prior to sodium periodate.

It is evident from this study that the method has several advantages over blue tetrazolium procedure. The method is simple, rapid, precise and accurate.
Figure 1: Visible spectrum of the colored products obtained on reacting Hydrocortisone with reagent.
Figure 2: Lambert-Beer's curve for determination of hydrocortisone.
Figure 3: Effect of concentration of sodium periodate.
Figure 4: Effect of time on oxidation of Hydrocortisone and Prednisolone.
Figure 5: Effect of reagent concentration on the color intensity.
Figure 6: Effect of time on color intensity and stability.
Figure 7: Effect of concentration of sodium periodate.
Figure 8: Effect of time on oxidation by sodium periodate.
Figure 9: Effect of reagent concentration on the color intensity.
Figure 10: Effect of time on color intensity and stability.
Figure 11: Effect of reagent concentration on the color intensity.
Figure 12: Effect of concentration of sodium periodate.
Figure 13: Effect of time on color intensity and stability.
<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>Lambert-Beer's conc. range (mcg/mL)</th>
<th>Molar absorptivity</th>
<th>Moles of Formaldehyde Liberated per Molecule of Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>5.0 to 40.0</td>
<td>6887</td>
<td>0.86</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>5.0 to 40.0</td>
<td>7209</td>
<td>0.90</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>5.0 to 40.0</td>
<td>6640</td>
<td>0.82</td>
</tr>
<tr>
<td>Betamethasone</td>
<td>5.0 to 40.0</td>
<td>6868</td>
<td>0.85</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>5.0 to 40.0</td>
<td>4433</td>
<td>0.55</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>0.2 to 2.4</td>
<td>8000</td>
<td>1.00</td>
</tr>
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</table>
### TABLE III

Analysis of Corticosteroids

<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>% Recovery ± SD&lt;sup&gt;a&lt;/sup&gt; by Proposed Method</th>
<th>% Recovery ± SD&lt;sup&gt;a&lt;/sup&gt; by Pharmacopoeial Method&lt;sup&gt;240&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>99.84 ± 0.85</td>
<td>100.50 ± 0.98</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>100.01 ± 0.76</td>
<td>99.81 ± 0.84</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>99.92 ± 0.77</td>
<td>99.77 ± 0.99</td>
</tr>
<tr>
<td>Betamethasone</td>
<td>100.82 ± 0.88</td>
<td>100.09 ± 0.90</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>99.80 ± 0.79</td>
<td>100.80 ± 0.96</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard deviation was calculated from the results of nine determinations.
TABLE IV

Analysis of Corticosteroids and Their Dosage Forms

<table>
<thead>
<tr>
<th>No.</th>
<th>Formulation</th>
<th>Labelled Amount</th>
<th>Recovery a) by Proposed Method</th>
<th>Pharmacopoeial Method 192,240</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Powder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i)</td>
<td>Hydrocortisone</td>
<td>99.25%</td>
<td>99.55%</td>
<td></td>
</tr>
<tr>
<td>(ii)</td>
<td>Prednisolone</td>
<td>99.78%</td>
<td>99.57%</td>
<td></td>
</tr>
<tr>
<td>(iii)</td>
<td>Dexamethasone</td>
<td>100.05%</td>
<td>99.91%</td>
<td></td>
</tr>
<tr>
<td>(iv)</td>
<td>Betamethasone</td>
<td>100.00%</td>
<td>100.51%</td>
<td></td>
</tr>
<tr>
<td>(v)</td>
<td>Triamcinolone</td>
<td>98.25%</td>
<td>98.55%</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Tablet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i)</td>
<td>Prednisolone</td>
<td>5.0mg/tab</td>
<td>5.090</td>
<td>5.030</td>
</tr>
<tr>
<td>(ii)</td>
<td>Dexamethasone</td>
<td>0.5mg/tab</td>
<td>0.477</td>
<td>0.479</td>
</tr>
<tr>
<td>(iii)</td>
<td>Betamethasone</td>
<td>1.0mg/tab</td>
<td>0.990</td>
<td>0.985</td>
</tr>
<tr>
<td>(iv)</td>
<td>Triamcinolone</td>
<td>4.0mg/tab</td>
<td>3.856</td>
<td>3.941</td>
</tr>
<tr>
<td>3.</td>
<td>Ointment</td>
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<td></td>
</tr>
<tr>
<td>(i)</td>
<td>Hydrocortisone</td>
<td>10mg/g</td>
<td>9.783</td>
<td>9.600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ii)</td>
<td>Neomycin</td>
<td>5mg/g</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulfate IP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iii)</td>
<td>Bacitracin</td>
<td>250mg/g</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Average value of five determinations.
<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>Absorbance at 412 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>0.380</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>0.395</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.226</td>
</tr>
<tr>
<td>Betamethasone</td>
<td>0.185</td>
</tr>
</tbody>
</table>
### TABLE VI

**Effect of Order of Addition of the Reagents on Color intensity of Triamcinolone**

<table>
<thead>
<tr>
<th>Time of minutes</th>
<th>Absorbance at 412 nm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>5</td>
<td>0.230</td>
<td>0.180</td>
</tr>
<tr>
<td>10</td>
<td>0.185</td>
<td>0.270</td>
</tr>
<tr>
<td>15</td>
<td>0.177</td>
<td>0.378</td>
</tr>
<tr>
<td>20</td>
<td>0.175</td>
<td>0.416</td>
</tr>
<tr>
<td>25</td>
<td>0.175</td>
<td>0.435</td>
</tr>
<tr>
<td>30</td>
<td>0.175</td>
<td>0.450</td>
</tr>
<tr>
<td>60</td>
<td>0.175</td>
<td>0.450</td>
</tr>
</tbody>
</table>

I Triamcinolone + NaI0$_4$ + Reagent

II Triamcinolone + Reagent + NaI0$_4$