CHAPTER -VI

EVALUATION OF VARIOUS CHROMOGENIC REAGENTS IN THE SPECTROPHOTOMETRIC ANALYSIS OF CLOSTEBOL ACETATE

Clostebol (as acetate CSB) is an Anabolic steroids bind to specific receptors present especially in reproductive tissue, muscle and fat (Mooradian & Morley, 1987). The anabolic steroids reduce nitrogen excretion from tissue breakdown in androgen deficient men. They are also responsible for normal male sexual differentiation. The ratio of anabolic ("body-building") effects to androgenic (virilizing) effects may differ among the members of the class, but in practice all agents possess both properties to some degree. There is no clear evidence that anabolic steroids enhance overall athletic performance (Elashoff et al, 1991).

It's officials status has been presented in table 1.01 (p. 2). The structural features, category, certain characteristics, therapeutic importance and commercially available formulations of CSB or compiled in Tables 6.01 (p. 267), 6.02 (p. 268) respectively.

A very few physico-chemical methods appeared in the literature for the assay of CSB in biological fluids and pharmaceutical formulations. Most of them are based on HPLC[300-303], MS[304-307] GLC[308,309] and UV and visible spectrophotometric methods. The analytically useful functional groups in CSB have not been fully exploited for designing suitable visible spectrophotometric methods and so still offer a scope to develop more visible spectrophotometric methods with better sensitivity, selectivity, precision and accuracy. The author has made some attempts in this direction and succeeded in developing two methods (M_9, 4-AP; M_{10}, INH). All these methods have been extended to pharmaceutical formulations as well.

A reported UV spectrophotometric method has been adopted for the determination of CSB in pharmaceutical formulations (tablets), which has been made use of reference method (Table 6.04,p.270) to compare the results obtained by the proposed visible spectrophotometric methods.

Table6.01

Structural features of selected drug

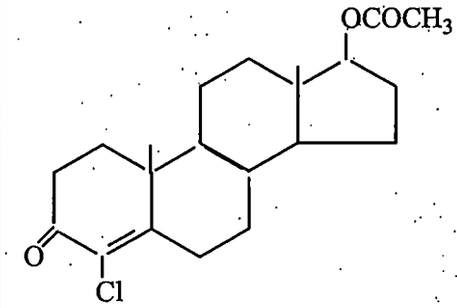
Drug Name / Abbreviation	Category	Chemical Name	Structure, Molecular formula and Molecular Weight	Analytically important moieties / functional groups
Clostebol acetate	Anabolic steroid	4-Chloro-3-oxoandrost-4-en-17 beta-yl acetate, 4-Chloro-17 beta-hydroxyandrost-4-en-3-one acetate, 4-Chloro-17-hydroxy-(17b)-Androst-4-en-3-one.		Alpha,beta-unsaturated ketone, Steroid nucleus, OAC ring in steroid nucleus.

Table 6.02

Physico chemical characteristic and therapeutic importance of Clostebol acetate

Category	Characteristic	Therapeutic importance
Anabolic steroid	Molecular formula - C_{19} H_{27} Cl O_2 Molecular weight - 322.88 State & physical properties - A white crystalline powder Usually having a faint odour Solubility – In soluble in water , Freely soluble in alcohol, partially soluble in NaOH. Storage: Refrigerator UVmax : 323nm	The only legitimate therapeutic indications for anabolic steroids are: a) Replacement of male sex steroids in men who have androgen deficiency, for example as a result of loss of both testes b) The treatment of certain rare forms of aplastic anaemia which are responsive to anabolic androgens c) The drugs have been used in certain countries to counteract catabolic states Clostebol acetate has been applied topically in dermatological and opthalmological preparations.

Table 6.03

Commercially available formulations

Generic Name	Pharmaceutical concern	Formulation	Strength of formulation	Other ingredients	
				Active	Inactive
Clostebol acetate	Steranabol	Tablets	20mg	---	---
	Megagrisenit-Mono[R]	Tablets	15mg	---	---

Table 6.04

Procedure for the assay of CSB in formulations

Pharmaceutical formations	Reference Method
Tablets	An accurately weighed amount of formulation (Tablets powder) equivalent to 100 mg was dissolved in a few ml of methyl alcohol and filtered. The filtrate was evaporated to dryness. The residue was dissolved in distilled water and further diluted to 100 ml with methyl alcohol to obtain concentration of 500 µg/ml. It was further diluted step wise with distilled water to get the concentration of 50 µg/ml. Aliquots of CSB solution 1.0-5.0ml; 50 µg/ml were taken into a series of 5ml-calibrated tubes and made up to the mark with methyl alcohol. The absorbance of each solution was measured at 323nm (Fig. 6.01) against distilled water. The concentration of the drug was computed from its calibration graph (Fig. 6.02).

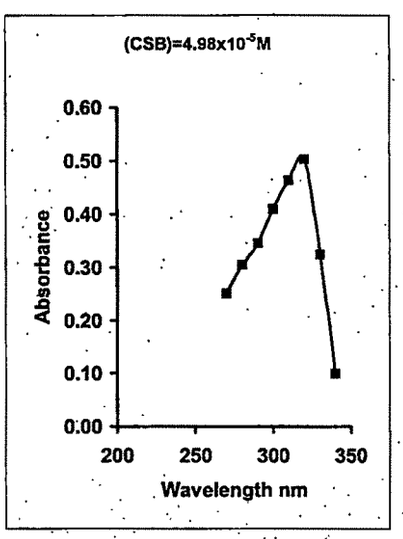


Fig. 6.01 UV Absorption Spectrum of CSB (reference method)

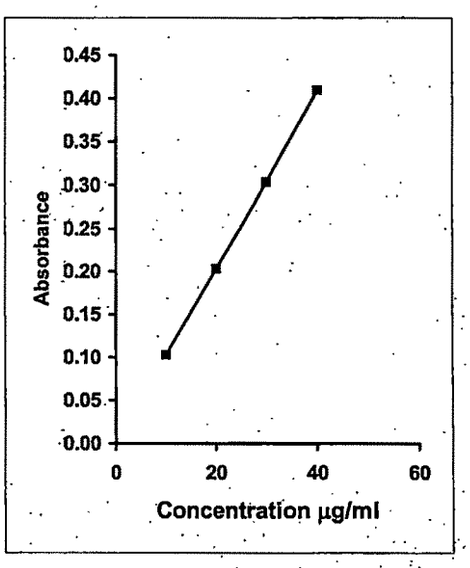


Fig. 6.02 Beer's law plot of CSB (UV reference method)

Experimental:

1. **Instruments used:**

A Elico, UV – Visible digital spectrophotometer with 1cm matched quartz cells were used for the spectral and absorbance measurements. An Elico LI-120 digital pH meter was used for pH measurements.

2. **Preparation of standard drug solutions:**

A 1 mg/ml solution was prepared by dissolving 100 mg of pure CSB in 100ml of distilled water and this stock solution was diluted step wise with distilled water to get the working standard solutions of concentration of 50 µg/ml (M_9); 100µg/ml (M_{10}).

3. **Preparation of reagents:**

All the chemicals and reagents used were of analytical grade and solutions were prepared in triply distilled water, Chloroform or isopropyl alcohol.

Method M_9

4-AP Solution (BDH; 0.5%, 2.45×10^{-2} M)	:	Prepared by dissolving 500 mg of 4-AP in 100 ml of MeOH containing 1 ml of conc.HCl.

Method M_{10}

INHsolution (Sd-Fine chem;0.8%,5.83×10^{-3}M)	:	Prepared by dissolving 800 mg of INH IN 100 ml 100 ml of MeOH containing 1% of conc.HCl.
Sulphuric acid (Qualigens 18M)	:	Used as it is.

Recommended procedures:

After systematic and detailed study of the various parameters involved, as described under results and discussions the following procedures [Methods M_9, 4-AP; INH; M_{10}] were recommended for the assay of CSB in bulk samples and pharmaceutical formulations.

Method M_9

Aliquots of standard CSB solution (0.5-3.0ml, 50μg.ml^{-1}) were transferred into a series of 10ml-calibrated tubes. Then 3ml of 4-AP solution was added and kept aside for 15min. Later the solution in each tube was made up to 10 ml with methanol. The absorbance was measured at 395 nm against a similar reagent blank. The amount of CSB was computed from its calibration graph. (Fig. 6.05, p. 278).

Method M_{10}

Delivered aliquots of standard CSB (0.5-2.5ml,100μg.ml^{-1}) solution into a series of 10ml calibrated tubes. Then 2 ml of 0.8% INH solution was added to each tube and heated for 10 min at 60^{0}c. The solution in each tube was cooled and made up to 10 ml with methanol. The absorbance was measured at 450nm against a similar reagent blank. The amount of CSB was computed from its calibration graph. (Fig. 6.06, p. 278).

For pharmaceutical formulations:

An accurately weighed portion of tablet content equivalent to about 100 mg of CSB was dissolved in a few ml of isopropyl alcohol and filtered to get 1mg/ml. The filtrate is evaporated to dryness and dissolved in distilled water. This stock solution (1mg/ml) was further diluted stepwise with distilled water use organic solvent isopropyl alcohol as under CSB. These solutions were analyzed as under procedures described for bulk solutions.

Results and Discussions:

i. Spectral Characteristics:

In order to ascertain the optimum wavelength of maximum absorption (λ_{max}) of the colored species formed in the above methods, specified amounts of CSB were taken and colors were developed separately by following the above procedures. The amounts of CSB present in total volume of colored solutions were 1μg/ml for method (M_9), 2.5μg/ml for method (M_{10}). The absorption spectra were scanned on a spectrophotometer in the wavelength region of 340 to 900nm against similar reagent blank or distilled water. The reagent blank absorption spectrum of each method was also recorded against distilled water. The results were graphically represented in Fig.6.03 to 6.04, p. 274. The absorption curves of the colored species in each method show characteristics absorption maxima (Table 1.03, p.14-18).

ii. Optimum conditions fixation in procedures:

The optimum conditions for the color development of methods (M_9, M_{10}) were established by varying the parameters one at a time, keeping the others fixed and observing the effect produced on the absorbance of the colored species. The following experiments were conducted for this purpose and the conditions so obtained were incorporated in recommended procedures.

Method M_9:

The method involves the reaction between CSP and 4-AP. The optimum conditions were fixed basing on the study of effects of various parameters, such as volume of 2.45×10^{-2}M 4 -AP solution, volume of solvents used initially and subsequently for final dilution and the stability of colored species were studied and the optical conditions are incorporated in Table 6.05,p.276.

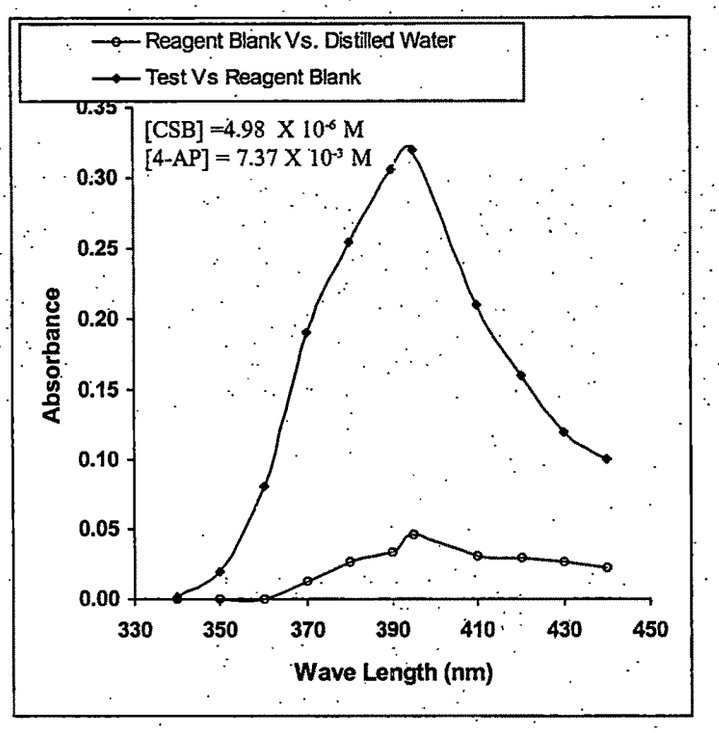


Fig. 6.03 Absorption spectrum of CSB - 4-AP (M_9)

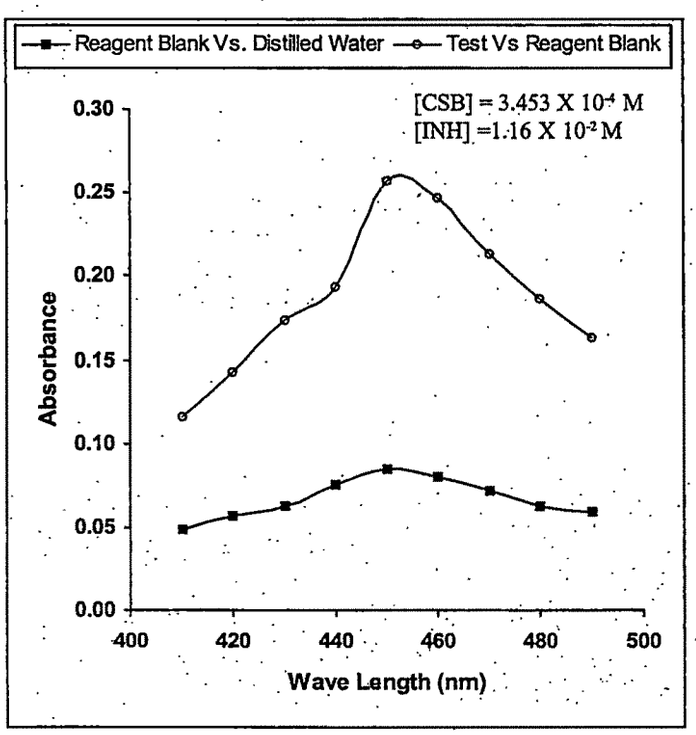


Fig. 6.04 Absorption spectrum of CSB - INH (M_{10})

Method M_{10}:

The method involves the reaction between CSB and INH. The optimum conditions were fixed basing on the study of effects of various parameters, such as volume of $5.83x10^{-3}$M INH solutions, volume of solvents used initially and subsequently for final dilution and the stability of colored species were studied and the optical conditions are incorporated in Table 6.06,p.277.

Optical Characteristics:

In order to test whether the colored species formed in above methods adhere to Beer's law, the absorbance's at appropriate wavelength of a set of solutions containing varying amounts of CSB and specified of amounts of reagents were recorded against the corresponding reagent blanks. The Beer's law plots of these recorded graphically. Beer's law limits, molar absorptivity, Sandell's sensitivity and optimum photometric range for CSB in each method were calculated. Least square regression analysis was carried out for getting the slope, intercept and the correlation coefficient values (Table 6.05a, p. 281).

Precision:

The precision of the proposed methods was ascertained from the absorbance values obtained by actual determination of six replicates of a fixed amount of CSB in total solution. The percent relative standard deviation and percent range of error (at 0.05 and 0.01 confidence limits) were calculated for the proposed methods (Table 6.05a, p. 281).

Accuracy:

To determine the accuracy of each proposed method, different amounts of bulk samples of CSB within the Beer's law limits were taken any analysed by the proposed method. The results (percent error) are recorded in (Table 6.05a, p. 281).

Table 6.05

Optimum conditions established in method M_9 for CSB

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	360-400	395	
Effect of volume ($2.45x10^{-2}$M) of 4-AP IN MeOH and waiting time	2-4 ml room temperature, 15 min	3.0 ml room temperature, 15 min	3ml of 4-AP and 15 min waiting time were preferred for covering broad range in Beer's law limits.
Solvent for final dilution	Methanol	Methanol	MeOH has been found to be suitable for final dilution to give better absorbance values.
Stability period after final dilution	Immediate-40 min	10 min	After 40 min the absorbance of colored species diminish slowly with time.

Table 6.06

Optimum conditions established in method M_{10} for CSB

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	400-460	450	
Effect of volume ($5.83x10^{-3}$M) of INH in MeOH and heating (time and temp).	1-3 ml, 60°C, 5-15 min	2.0 ml, 60°C, 10 min	Addition of INH (2ml) and heating (60°C, 10min) have been found to be necessary to cover broad range in Beer's law limits.
Solvent for final dilution	Methanol	Methanol	MeOH has been found to be suitable for final dilution to give better absorbance values.
Stability period after final dilution	Immediate-50 min	Immediate	After the stability period, the absorbance of colored species decreased slowly.

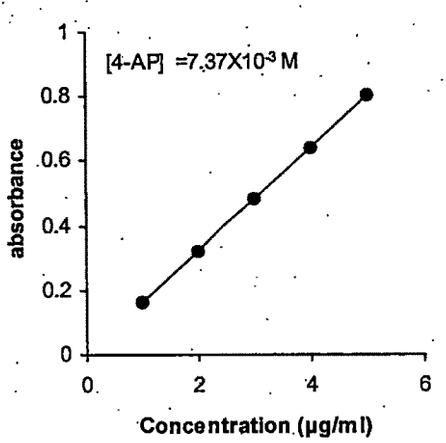


Fig.6.05 : Beer's Law plot of CSB with 4-APsystem (M_9)

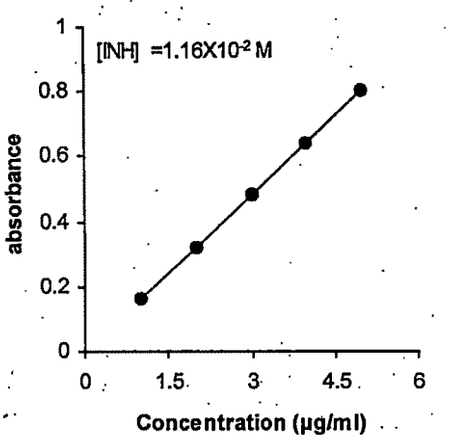


Fig.6.06 : Beer's Law plot of CSB with INH system (M_{10})

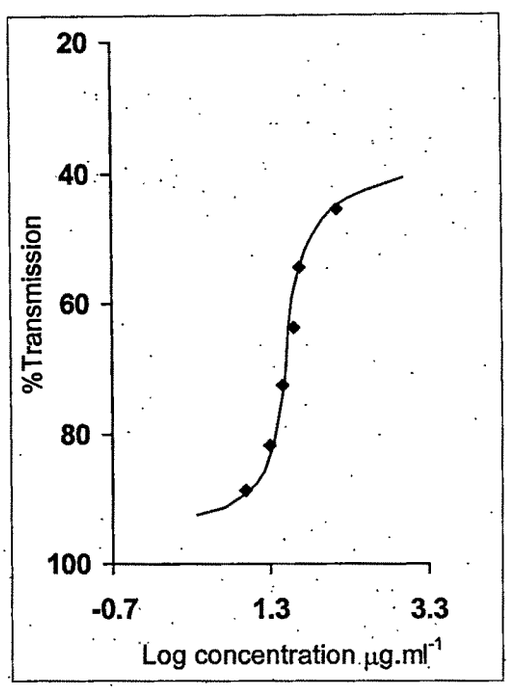


Fig 6.07 Ringbom plot of CSB with 4-AP (M_9)

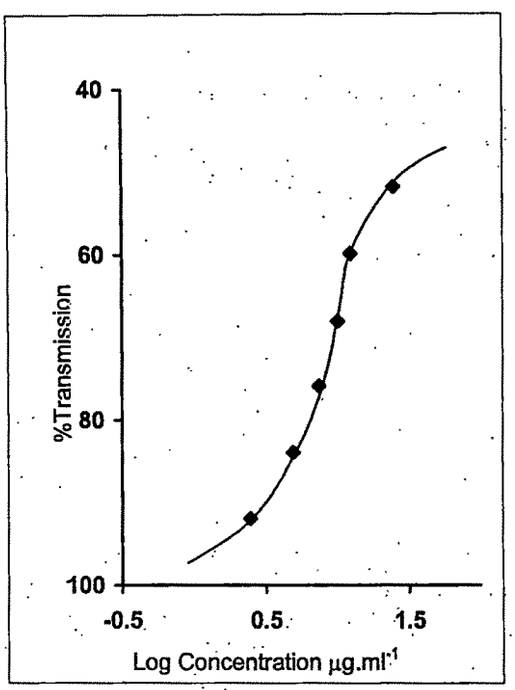


Fig 6.08 Ringbom plot of CSB with INH (M_{10})

Interference studies:

The effect of wide range of excipients and other active ingredients usually present in the formulations for the assay of CSB in methods (M_9& M_{10}) under optimum conditions were investigated. The commonly used excipients and other active ingredients usually present in formulations did not interfere even if they were present in amounts than they usually exist in formulations.

Analysis of formulations:

Commercial formulations (tablets) containing CSB were successfully analyzed by the proposed methods. The values obtained by the proposed and reference methods for formulations were compared statistically with F and t tests and found not to different significantly. The results were summarized in (Table 6.08a, p. 282). Percent recoveries were determined by adding standard drug to prenalysed formulations. The results of the recovery experiments by the proposed methods are also listed in (Table 6.08a, p. 282)

Chemistry of the colored species:

The chemistry of the colored species formed in each one of the proposed methods for the assay of CSB has been presented in chapter VI.

Conclusions:

The proposed methods exploit the various functional groups in CSB molecule. The decreasing order of sensitivity (ϵ_{max}) and λ_{max} among the proposed methods are (M_{10}>M_9) and (M_{10}> M_9) respectively. The concomitants, which do not contain the functional groups chosen in the present investigation, do not interfere in the color development by proposed methods. Thus the proposed methods are simple, sensitive or selective with reasonable precision and accuracy and constitute better alternatives to the reported ones in the assay of CSB in bulk form and pharmaceutical formulations.

Table 6.05a

Optical and regression characteristics, precision and accuracy of the proposed methods for CSB

Parameter	M_9	M_{10}
λ_{max} (nm)	395	450
Beer's law limits (μg/ml)	1-5	1-5
Detection limit (μg/ml)	0.196	0.471
Molar absorptivity (1 $mol^{-1}.cm^{-1}$)	8.356×10^3	1.857×10^4
Sandell's sensitivity (μg.cm^{-2}/0.001 absorbance unit)	0.1352	0.1032
Optimum photometric range (μg/ml)	12.6-30	8.318-20
Regression equation (Y=a+bc)		
slope (b)	0.1245	0.1701
Standard deviation on slope (S_b)	2.445×10^{-3}	0.8069×10^{-2}
Intercept (a)	-3×10^{-3}	1.01×10^{-2}
Standard deviation on intercept (S_a)	8.110×10^{-3}	2.676×10^{-1}
Standard error on estimation (S_e)	7.733×10^{-2}	2.551×10^{-2}
Correlation coefficient (r)	0.9995	0.9905
Relative standard deviation (%)*	0.5464	0.4176
% Range of error (confidence limits)		
0.05 level	0.6283	0.4802
0.01 level	0.9852	0.7531
% error in Bulk samples **	0.126	0.139

* Average of six determinations considered

** Average of three determinations

Table 6.08a

Assay of CSB in Pharmaceutical Formulations

Formulations*	Amount taken (mg)	Amount found by proposed Methods**		Reference method	Percentage recovery by proposed methods***	
		M_9	M_{10}		M_9	M_{10}
Tablet I	20	19.73±0.83 F=1.394 t=0.706	21.23±0.78 F=0.551 t=1.941	20.13±0.98	99.9±0.5	99.8±0.35
Tablet II	20	19.67±0.78 F=1.452 t=0.406	18.75±0.82 F=1.314 t=2.153	19.92±0.94	99.8±0.6	99.7±0.33
Tablet III	15	15.15±0.48 F=1.722 t=0.865	14.78±0.53 F=2.667 t=0.521	14.97±0.63	99.89±0.48	99.56±0.84
Tablet IV	15	14.76±0.61 F=1.635 t=0.709	15.98±0.59 F=1.747 t=1.996	15.08±0.78	99.86±0.74	99.54±0.32

* Tablets from four different pharmaceutical companies.

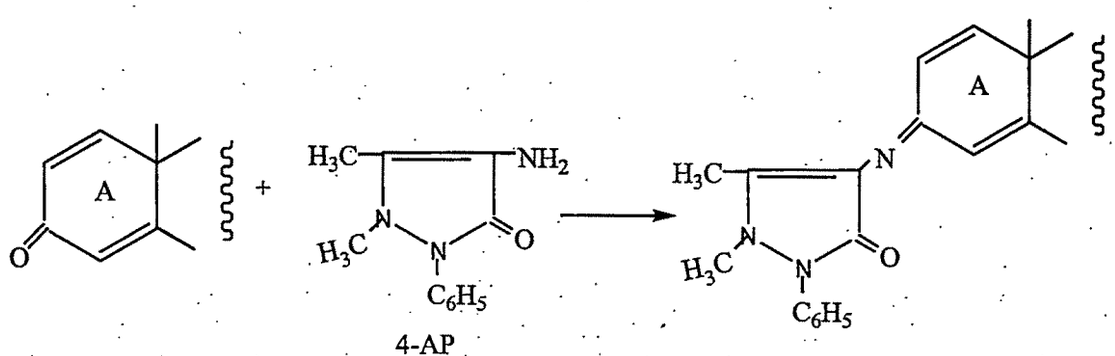
** Average ± standard deviation of six determinations, the t-and F-test values refer to comparison of the proposed method with the reference method. Theoretical values at 95% confidence limit, F = 5.05, t = 2.262

*** Recovery of 10mg added to the preanalysed pharmaceutical formulations (average of three determinations).

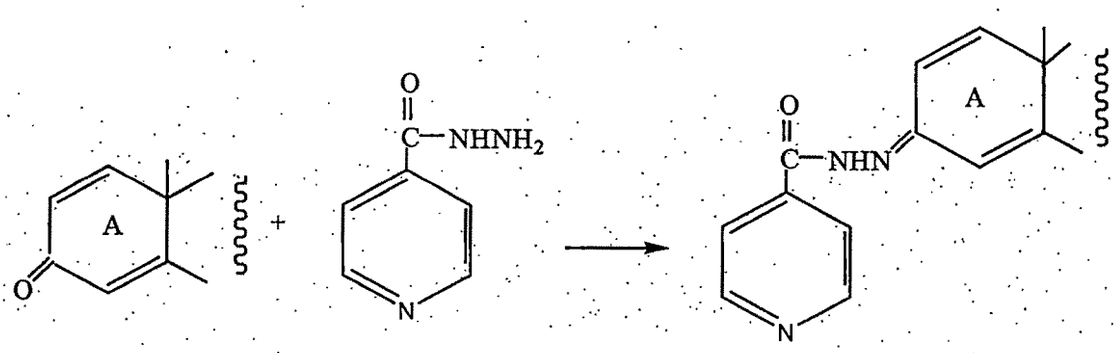
Clostebol acetate is chemically known as 4- Chloro testosterone acetate and responds to typical color reactions characteristic of androgenic steroids containing α, β- unsaturated ketone in ring A besides Cl and 17-hydroxyl exists as acetate.

Method M_9

It is well known that 4-aminophenazone (4-AP), isonicotinic acid hydrazide (INH) give colored hydrazone and schiff base respectively with $\Delta^{1,4}$ or Δ^4-3- ketosteroids. The same reagents (4-AP, M_9; INH, M_{10}) have been used in two methods for the determination of CSB. The colored species formation may be represented as under scheme 6.01&6.02.



Scheme6.01



Scheme6.02