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ANALYTICAL METHOD DEVELOPMENT & VALIDATION FOR CLINDAMYCIN, CLOTRIMAZOLE & TINIDAZOLE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

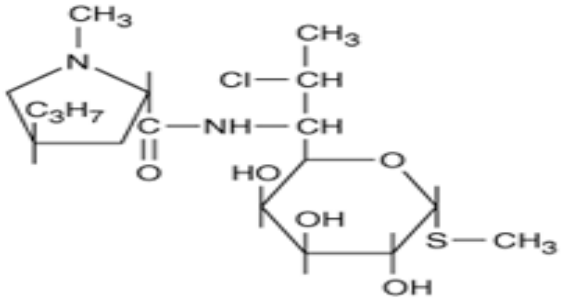
Method A: RP-HPLC Method was studied by using Shimadzu 2010 CHT, Chromatographic separation was achieved using Shiseido C_{18} RP column (250 mm × 4.6 mm i.d., 5µm) kept at ambient temperature, using a mobile phase consisting a mixture of Phosphate buffer (pH 3.0): methanol (60:40 v/v) and pH adjusted with 0.5% orthophosphoric acid at a flow rate of 1.0 ml/min. The detection was made at 210 nm. Retention time was 3.02 min, 4.13 min and 6.98 min for Clindamycin, Clotrimazole And Tinidazole. Linear correlation was obtained between peak area and concentration in the range of 5-15 µg/ml for Clindamycin, Clotrimazole And Tinidazole respectively. The %RSD value was less than 2, for intraday and interday precision. **Method B:** Simultaneous Equation Method for Clindamycin Clotrimazole and Tinidazole was studied by UV-3000+, LAB-INDIA. HCl was used as a solvent. The wavelength selected was 216 nm, 230 nm and 296 nm for Clindamycin, Clotrimazole And Tinidazole respectively. Beer's law is obeyed in the concentration range of 20-100 µg/ml for Clindamycin, Clotrimazole And Tinidazole respectively.

KEYWORDS: Clindamycin, Clotrimazole, Tinidazole, RP-HPLC, UV- Spectroscopy.

INTRODUCTION

Vaginal yeast infection, Commonly known **vaginal thrush** is excessive growth of yeast in the vagina that results in irritation. The most common. Symptom is Discharge of white and thick vaginal discharge that typically does not smell bad, pain with sex, and redness around the vagina. Symptoms worsen just before Commencement of woman's period. It Generally Occurs due to excessive growth of *Candida*. This yeast are present in the vagina in small number.

Clindamycin is chemically methyl 7-chloro-6,7,8-trideoxy-6-[[[(2S,4R)-l -methyl-4-propyl-2-pyrrolidinyl] carbonyl] amino]-1-thio-L-threo-a-D-galactooctopyranoside hydrochloride. [11] Clindamycin is a Lincosamide Antibiotic Drug. Clindamycin inhibits protein synthesis of bacteria by binding to the 50S ribosomal subunits of the bacteria. Specifically, it binds primarily to the 23s RNA subunit.



Clindamycin

Figure 1: Structure of Clindamycin.

Clotrimazole is Chemically 1-[{2-cholrophenyl) diphenylmethyl}]-1H-imidazole. [12] Clotrimazole is an Imidazole Antifungal. Clotrimazole interacts with yeast 14- α demethylase, a cytochrome P-450 enzyme that converts lanosterol to ergosterol, an essential component of the membrane resulting in increased cellular permeability. It also inhibit the transformation of yeasts to mycelial forms and the uptake of purine, impair triglyceride and/or phospholipid biosynthesis, and inhibit the movement of calcium and potassium ions across the cell membranes by blocking the ion transport pathway known as the Gardos Channel.

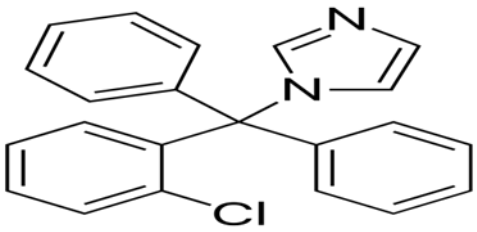


Figure2: Structure of Clotrimazole.

Tinidazole is chemically 1-[2-(ethylsulphonyl)ethyl]-2-methyl-5-nitroimidazole1.[13] Tinidazole is an Antiprotozoal, Nitroimidazole Class drug. Tinidazole is a prodrug and antiprotozoal. The nitro group of Tinidazole is reduced by ferredoxin mediated electron transport system. The free nitro radical generated as a result of this reduction is believed to be responsible for antiprotozoal activity, It is suggested that it the toxic free radicals covalently bind to DNA, causing DNA damage and leading to cell death.

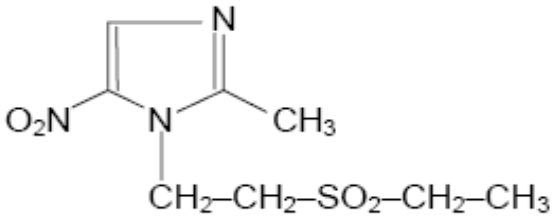


Figure 3: Structure of Tinidazole.

After literature survey and patent search we can conclude that, there are many process patent for manufacturing of individual drug and product patent for manufacturing of dosage form containing either Clindamycin, Clotrimazole or Tinidazole. The dosage form containing Clindamycin, Tinidazole & Clotrimazole in combination is also available in market. So, it is worthwhile to develop a newer, rapid, accurate, precise and simple analytical method for simultaneous estimation of Clindamycin, Clotrimazole & Tinidazole in Pharmaceutical Dosage form.

The aim of this work is, To develop UV spectrophotometric & RP-HPLC method method for simultaneous estimation of Clindamycin, Clotrimazole and Tinidazole.

EXPERIMENTAL WORK

Chemicals and reagents

Clindamycin & Clotrimazole API was gifted by Aroma Remedies, Dabhel Daman, while Tinidazole API was gifted by Vapi Care pharma, Vapi. Marketed dosage form-Clinge Forte (Aristo.Pharma Pvt. Ltd.) was procured from local pharmacy store. Methanol: HPLC Grade was purchased from, Rankem. Water: HPLC Grade was Purchased Lichrosoly-E. Merck (India) Ltd. Mumbai. Ortho Phosphoric acid: Analytical Grade was Purchsed from, E Merck (India) Ltd. Mumbai. Hcl was purchased From Ran Kem

INSTRUMENTATION

Method A: Shimadzu LC 2010 CHT, UV detector, Sheisdo Column C_{18} (250*4.6 mm, 5µm particle size), Auto Injector (Capacity Loop of 10 µl),Software: LC Solution, Digital pH meter (Mfg. by systronic),Ultra Sonicator: Pci ^{TM} Analytics, Pipettes of 1, 2, 5 and 10 ml capacity were used (Borosil),Volumetric flasks of 10, 50 & 100 ml capacity, Measuring cylinders of 100 and 1000 ml capacity (All glassware were calibrated before use).

Method B: A double beam UV-visible Spectrophotometer (LAB INDIA UV-3000+), attached to a computer software UV win 5.2.0, with a spectral width of 2 nm, wavelength accuracy of 0.5 nm and pair of 1 cm matched quartz cells**,** Volumetric flask – 10, 25, 50, 100 ml (Borosil),Pipettes – 1, 2, 5, 10 ml &Electronic Analytical Balance**.** All instruments and glass wares were calibrated.

Method A: RP-HPLC Method Development

Chromatographic conditions

Proper selection of the HPLC method depends upon the nature of the sample (ionic, ionizable or neutral molecule), its molecular weight and solubility. The drugs selected for the present study are polar in nature and hence either reversed phase or ion-pair or ion-exchange chromatography can be used. Reversed phase HPLC was selected for the initial separations because of its simplicity and suitability. To optimize the chromatographic conditions, the effect of chromatographic variables such as mobile phase pH, flow rate, and solvent ratio were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetric factor, and resolution and column efficiency were calculated. The conditions that gave the best resolution, symmetry and capacity factor were selected for estimation.

Table no 1 Chromatographic conditions.

Stationary phase: Sheisdo -C_{18} (250 mm x 4.6 mm, 5 µm)	**Mobile phase**: Potassium Phosphate buffer (pH 3.0): Acetonitrile (60:40 v/v)
pH: pH of mobile phase was adjusted to 3.0 using O-Phosphoric acid	**Flow rate**: 1.0 ml/min
Temperature: Ambient	**Wavelength**: 210 nm

Chromatographic separation
Standard solutions Of Clindamycin, Clotrimazole, And Tinidazole were injected in column with 20 µl micro-syringe. The chromatogram was run for appropriate minutes with mobile phase Potassium Phosphate buffer pH (3.0): Acetonitrile (60:40). The detection was carried out at wavelength 210 nm. The chromatogram was stopped after separation achieved completely.

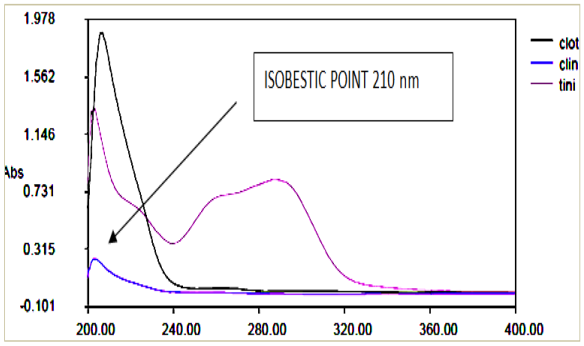


Fig 4: Iso bestic Point at 210nm for CLIN, CLOT & TINI.

Preparation of Standard Solution for Method A
Clindamycin standard stock solution: (100µg/mL)
A 10 mg of Tinidazole was weighed and transferred to a 100 mL volumetric flask. Volume was made up to the mark with methanol

Clotrimazole standard stock solution: (100µg/mL)
A 10mg of Clotrimazole was weighed and transferred to a 100 mL volumetric flask. volume was made up to the mark with methanol

Tinidazole standard stock solution: (100µg/mL)
A 10 mg of Tinidazole was weighed and transferred to a 100 mL volumetric flask. Volume was made up to the mark with methanol. Take 10 ml from this solution and Transfer to 100 ml volumetric flask and made up the volume up to the mark with Methanol.

Preparation of standard solution of combined solution of Clindamycin (10µg/mL), Clotrimazole (10µg/mL) and Tinidazole (10µg/mL)
Take 1 mL from the Clotrimazole stock solution and 1mL from Tinidazole stock solution and 1mL from Clindamycin stock solution and transferred to 10 mL volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

Sample stock solution: (100µg/mL for Clotrimazole and 100µg/mL for Tinidazole and 100µg/mL Clindamycin)
Weigh 370mg of the sample Pessaries and dissolve in 100 ml of mobile phase transferred carefully in a clean and dry 100 ml volumetric flask which contains 1000 µg/mL each of Clotrimazole, Clindamycin and Tinidazole. pipette out 1ml of solution from the above solution make up the volume to 10 ml of mobile phase containing 100 µg/mL ach of Clotrimazole, Clindamycin and Tinidazole. sonicate for 30 minutes. Cooled and diluted to volume with mobile phase and mixed well. Filtered through 0.45µ membrane filter. Further pipette out 1ml of the above was diluted into 10 ml with mobile phase containing 10 µg/mL ach of Clotrimazole, Clindamycin and Tinidazol

Sample working solution (10µg/mL Clindamycin10µg/mL for Clotrimazole and 10µg/mL for Tinidazole,)
Take 1 mL from standard stock solution and transferred to 10 ml volumetric flask and made up volume up to the mark with the mobile phase.

Optimization of Mobile Phase

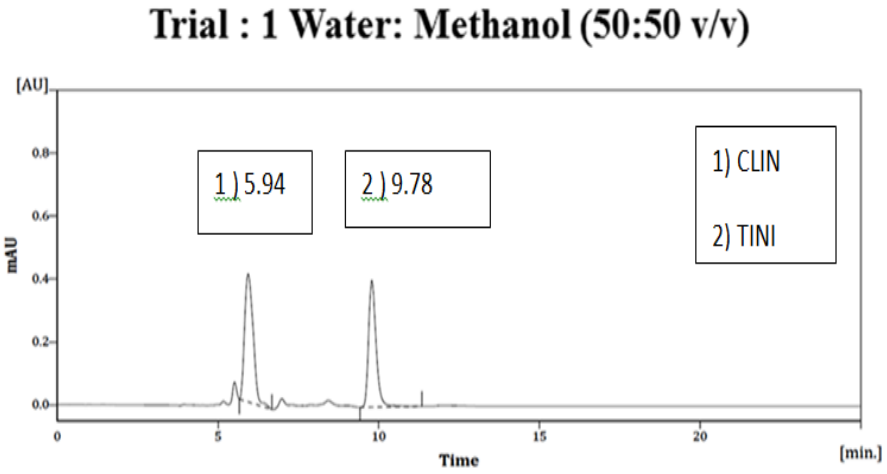


Fig 5: Trial 1 Water: Methanol 50:50v/v.

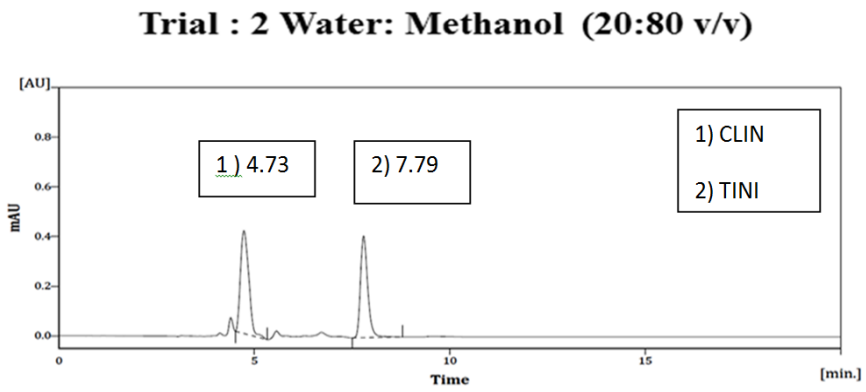


Fig 6: Trial 2 Water: Methanol 20:80v/v.

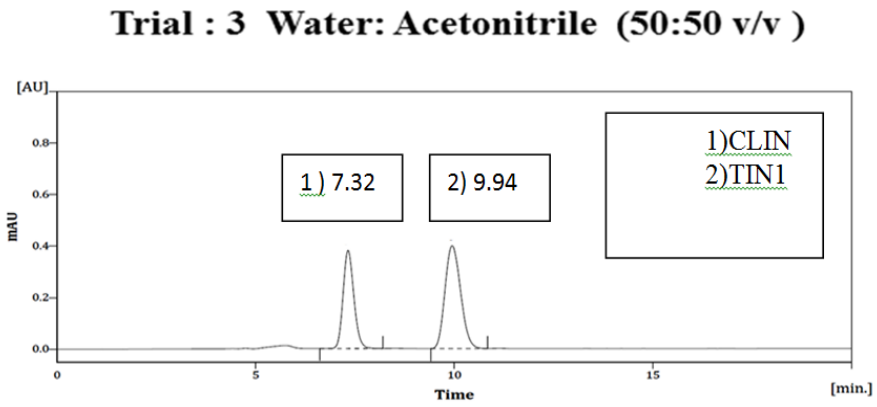


Fig 7: Trial 3 Water: Acetonitrile 50:50v/v.

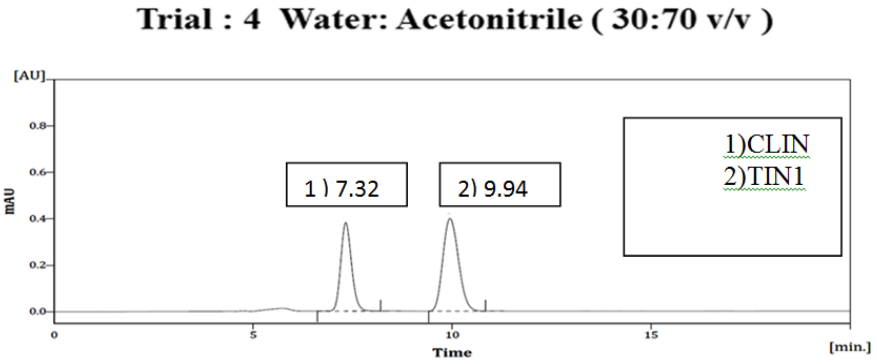


Fig 8: Trial 4 Water: Acetonitrile 30:70v/v

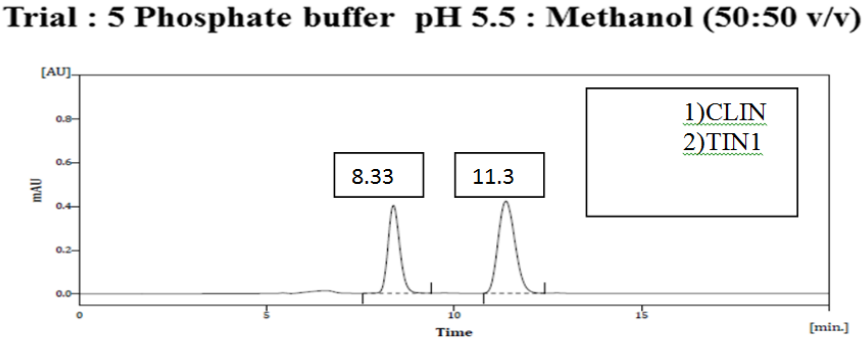


Fig 9: Trial 5: Phosphate Buffer pH 5.5:Methanol 50:50v/v.

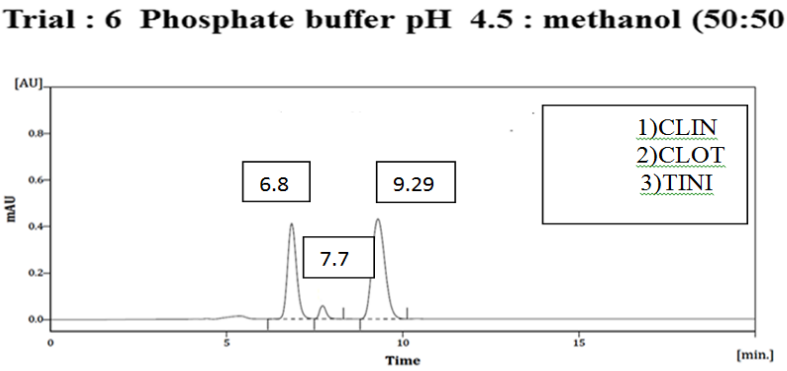


Fig 10: Trial 6 Phosphate Buffer pH 4.5: Methanol 50:50v/v.

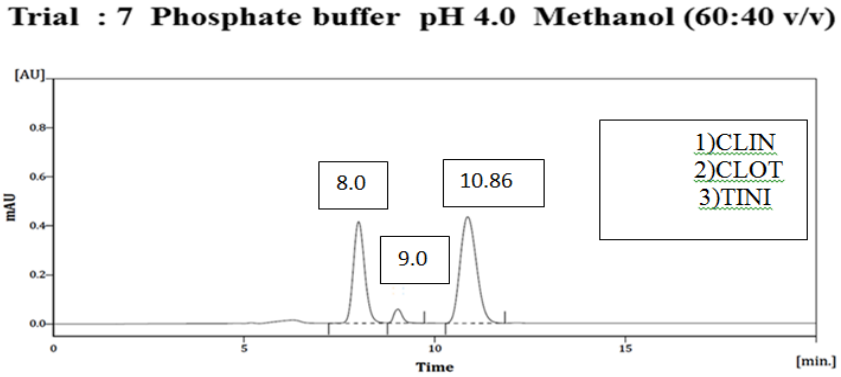


Fig.11: Trial 7 Phosphate Buffer pH 4.0: Methanol 60:40v/v.

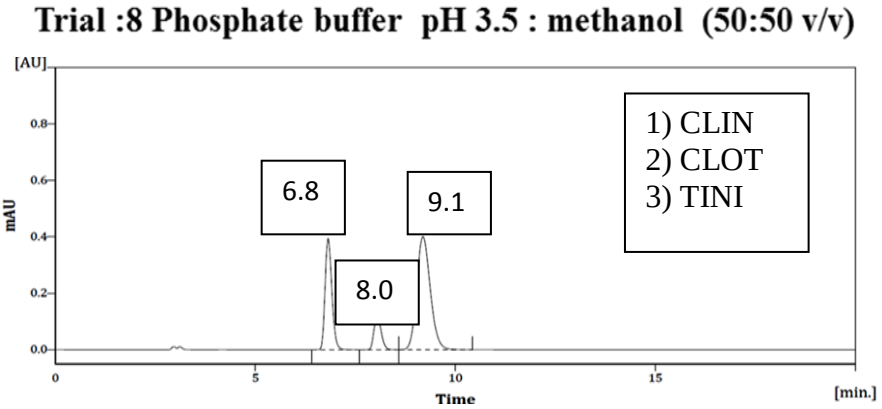


Fig 12 Trial 8 Phosphate Buffer pH 3.5: Methanol 50:50v/v.

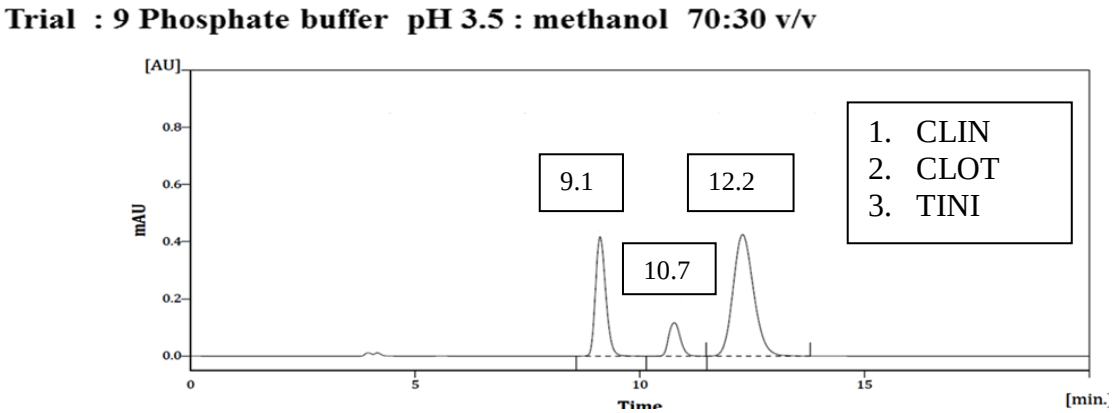


Fig 13 Trial 9 Phosphate Buffer pH 3.5: Methanol 70:30v/.

Validation of RP-HPLC Method

Linearity

The linearity is expressed in term of correlation co-efficient of linear regression analysis. Aliquots of standard solutions Of Clindamycin, Clotrimazole, And Tinidazole in range of 5-15 µg/ml respectively, was prepared from working standard solution and injected to system with stated chromatographic conditions and analysed. The calibration curves of Of Clindamycin, Clotrimazole, And Tinidazole at 210 nm, The graph of peak area obtained versus respective concentration was plotted. The mean area and standard deviation were calculated.

Precision

- **Repeatability**

1 ml of combined working standard solutions (100 µg/ml Of Clindamycin, Clotrimazole, And Tinidazole were transferred into separate 10 ml volumetric flasks and diluted up to mark with mobile phase to get 10 µg/ml of Of Clotrimazole, Clindamycin And Tinidazole) Each concentration was prepared and analyzed. The peak area obtained with each solution was measured and % R.S.D was calculated.

- **Intra-day precision**

Standard solution of Clotrimazole Clindamycin and Tinidazole (5-15 µg/ml) were prepared from working standard solution and injected in to system with stated chromatographic conditions and analyzed on the same day and % R.S.D was calculated.

- **Inter-day precision**

Standard solution of Clotrimazole Clindamycinn and Tinidazole (5-15 µg/ml) were prepared from working standard solutions and injected in to system with stated chromatographic conditions and analyzed on different days and % R.S.D was calculated.

Accuracy

The % recovery experiment was performed by the Standard Addition Method. Fixed amounts of sample mixture of Clotrimazole Clindamycin and Tinidazole (5-15 µg/ml) and increasing amount of its working standard solutions were added at 80, 100 and 120 % level of Clotrimazole (8,10 and 12 µg/ml), Clindamycin (8,10 and 12 µg/ml) and Tinidazole 8,10 and 12 µg/ml). Area of peak obtained with each solution was measured for Clotrimazole Clindamycin and Tinidazole. The mean % recovery from the peak areas was calculated.

LOD and LOQ

The LOD was estimated from the 5 calibration curves. The LOD may be calculated as

LOD = 3.3 × (SD / Slope)

LOQ = 10 × (SD/Slope)

Robustness

The solution containing concentration Of Clindamycin, Clotrimazole, And Tinidazole was analyzed in different flow rate, mobile phase and pH. The peak area obtained with each solution was measured and % RSD was calculated.

Acceptance criteria: % RSD should be less than 2.

Specificity

Specificity is a procedure to detect quantitatively the analyte in the presence of components that may be expected to be present in the sample matrix. While selectivity is the procedure to detect qualitatively the analyte in presence of components that may expected to be present in the sample matrix. Specificity of developed method was established by spiking Of Clindamycin, Clotrimazole, And Tinidazole in hypothetical placebo (i.e. might be expected to be present) and expressing that analytes peak was not interfered from excipients.

Analysis of dosage form

$$\%\text{Assay} = \frac{\text{Area of sample X 100}}{\text{Area of standard}}$$

Method B: UV SPECTROSCOPIC METHOD DEVELOPMENT

Selection of common solvent: HCl was selected as solvent for developing spectral characteristics of CLIN, CLOT &TINI. The selection was made after evaluating the solubility of CLIN, CLOT &TINI in different solvent.

Determination of Absorption Maxima and Selection of Wavelength

By dilution of three standard drug solutions with methanol, solutions containing 10 µg/ml of CLIN, 100 µg/ml of CLOT and 100 µg/ ml of TINI were scanned separately in the range of 200-400 nm to determine the wavelength of maximum absorption for all the drugs. CLIN,CLOT&TINI showed absorbance maxima at 216 nm, 230 nm and 296 nm respectively. The overlain spectra showed λmax of all drugs.

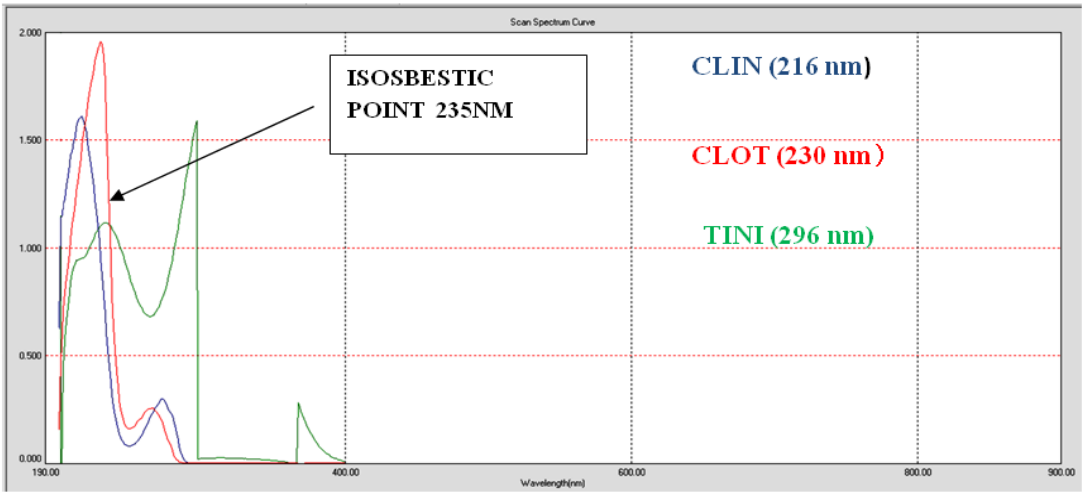


Figure 15: Overlain UV spectra of CLIN, CLOT&TINI showing selection of wavelength for detection.

Preparation of Standard Solution

For Clindamycin (10µg/mL)

Take 1 mL from the Clindamycin stock solution and transferred to 10 ml volumetric flask and volume made up to the mark by methanol and was scanned between 200-400nm

For Clotrimazolek (10µg/mL)

Tae 1 mL from the Clotrimazole stock solution and transferred to 10 mL volumetric flask and volume made up to the mark by methanol and was scanned between 200-400nm

For Tinidazole (10µg/mL)

Take 1 mL from the Tinidazole stock solution and transferred to 10 mL volumetric flask and volume made up to the mark by methanol and was scanned between 200-400nm

Preparation of Stock Solution

Preparation of Clindamycin stock solution: A 100 mg of Clindamycin was weighed and transferred into 100 ml volumetric flask and dissolved in mobile phase and sonicate the flask. The volume was made up to the mark with mobile phase to give 1000 µg/ml.

Working standard solution of Clotrimazole: 2 ml of stock solution was diluted to 10 ml with mobile phase to prepare 200 µg/ml.

Preparation of Clotrimazole stock solution: A 100 mg of Clotrimazole was weighed and transferred into 100 ml volumetric flask and dissolved in mobile phase and sonicate the flask. The volume was made up to the mark with mobile phase to give 1000 µg/ml.

Working standard solution of Clindamycin: 2 ml of stock solution was diluted to 10 ml with mobile phase to prepare 200 µg/ml.

Preparation of Tinidazole stock solution: A 100 mg of Tinidazole was weighed and transferred into 100 ml volumetric flask and dissolved in mobile phase and sonicate the flask. The volume was made up to the mark with mobile phase to give 1000 µg/ml.

Working standard solution of Tinidazole: 2 ml of stock solution was diluted to 10 ml with mobile phase to prepare 200µg/ml.

Procedure for Determination of Wavelength for Measurement

Simultaneous Equation Method

Aliquot of 1.0 ml of standard solution of CLOT, CLIN& TINI (200 µg/ml) were pipetted out into three separate 10 ml volumetric flask and volume was adjusted to the mark with HCl to get 200 µg/ml of CLOT, CLIN& TINI Each solution was scanned between 200 - 400 nm against HCl as a blank reagent. Wavelengths were selected from the overlay spectra of CLOT, CLIN& TINI.

Preparation of Calibration Curve

Calibration Curve for the CLIN (20-100 µg/ml)

Appropriate volume (1, 2, 3, 4 and 5 ml) of aliquot from standard CLIN stock solution was transferred to different volumetric flasks of 10 ml capacity. The volume was adjusted to the mark with the Methanol to obtain concentration of 20, 40, 60, 80, and 100µg/ml. The curve of each solution against the HCl was recorded. Absorbance at 216 nm, 230 nm and 296 nm was measured and the plot of absorbance vs. concentration was plotted. The straight-line equation was determined.

Calibration Curve for the CLOT (20-100 µg/ml) Appropriate volume (1, 2, 3, 4 and 5 ml) of aliquot from standard CLOT stock solution was transferred to different volumetric flasks of 10 ml capacity. The volume was adjusted to the mark with the HCl to obtain concentration of 10 20, 40, 60, 80, and 100µg/ml The curve of each solution against the HCl was recorded. Absorbance at 216 nm, 230 nm and 296 nm was measured and the plot of absorbance vs. concentration was plotted. The straight-line equation was determined.

Calibration Curve for the TINI (20-100 µg/ml)

Appropriate volume (1, 2, 3, 4 and 5 ml) of aliquot from standard ROS stock solution was transferred to different volumetric flasks of 10 ml capacity. The volume was adjusted to the mark with the HCl to obtain concentration of 20, 40, 60, 80, and 100µg/ml. The curve of each solution against the Methanol was recorded. Absorbance at 216 nm, 230 nm and 296 nm was measured and the plot of absorbance vs. concentration was plotted. The straight-line equation was determined.

VALIDATION OF UV SPECTROSCOPIC METHOD

Linearity and Range

Procedure: The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of 20-100 µg/ml for CLIN, CLOT & TINI µg/ml respectively (n=5). The calibration curve of absorbance vs. respective concentration was plotted and correlation coefficient and regression line equations for CLIN, CLOT & TINI were calculated.

Precision

- **Repeatability**

Procedure: Aliquots of 1.0 ml of standard stock solution of CLIN, CLOT & TINI (200µg/ml). respectively were transferred to a series(n=6) of 10 ml volumetric flask transferred to the series(n=6) of 10 ml volumetric flask. The volume was adjusted up to mark with HCl to get 20 µg/ml solution of CLIN, CLOT & TINI solutions. The absorbance of solution was measured spectroscopically six times and % RSD was calculated.

- **Intraday precision**

Procedure: Aliquots of 1, 2 and 3 ml of standard stock solution of CLIN, CLOT & TINI (200µg/ml) were transferred to a series(n=3) of 10 ml volumetric flask. The volume was adjusted up to mark with HCl to get 20, 40 and 60 µg/ml solution of CLIN, CLOT & TINI (200µg/ml) The absorbance of solution was measured spectroscopically three times and % RSD was calculated.

- **Interday Precision**

Procedure: Aliquots of 1, 2 and 3 ml of standard stock solution of CLIN, CLOT & TINI (200µg/ml)) were transferred to a series (n=3) of 10 ml volumetric flask. The volume was adjusted up to mark with HCl to get 20, 40 and 60 CLIN, CLOT & TINI µg/ml solution. Solution was analyzed 3 times on the 3 different day using spectrophotometry and % RSD was calculated.

Accuracy

Procedure: The % recovery experiment was performed by the Standard Addition Method. Fixed amounts of sample mixture of Clindamycin (10 µg/ml), Clotrimazole (10 µg/ml) and Tinidazole (10µg/ml) and increasing amount of its working standard solutions were added at 80, 100 and 120 % level of Clindamycin (8,10 and 12 µg/ml), clotrimazole (8,10 and 12 µg/ml) and Tinidazole (8,10 and 12 µg/ml) Area of peak obtained with each solution was measured for clindamycin, clotrimazole and Tinidazole. The mean % recovery from the peak areas was calculated.

LOD and LOQ

The LOD was estimated from the 5 calibration curves. The LOD may be calculated as

$$\textbf{LOD} = 3.3 \times (\textbf{SD} / \textbf{Slope})$$

$$\textbf{LOQ} = 10 \times (\textbf{SD/Slope})$$

Assay of Pharmaceutical Dosage Form

The absorbance was measured at 216 nm for CLIN, 230 nm for CLOT and 296 nm for CLOT for quantification of CLIN, CLOT & TINI. The amount of CLIN, CLOT & TINI present in the sample solution were determined by the substituted in the following equation to obtain the concentration,

C_{CLIN}= $(A1(ay_2az_3-az_2ay_3)-ay_1(A_2az_3-az_2A_3)+az_1(A_2ay_3-ay_2A_3)/ax_1(ay_2az_3-az_2ay_3)-ay_1(ax_2az_3-az_2ax_3)+az_1(ax_2ay_3-ay_2ax_3)$……(1),

C_{CLOT}= $(ax_1(A_2az_3-az_2A_3)-A_1(ax_2az_3-az_2ax_3)+az1(ax_2A_3-A_2ax_3)/ax_1(ay_2az_3-az_2ay_3)-ay_1(ax_2az_3-az_2ax_3)+az_1(ax_2ay_3-ay_2ax_3)$…… (2), and

C_{TINI}= $(ax_1(ay_2A_3-A_2ay_3)-ay_1(ax_2A_3-A_2ax_3)+A_1(ax_2ay_3-ay_2ax_3)/ax_1(ay_2az_3-az_2ay_3)-ay_1(ax_2az_3-az_2ax_3)+az_1(ax_2ay_3-ay_2ax_3)$…… (3),

Where,

C_{CLIN}, C_{CLOT} and C_{TINI} are the concentrations of CLIN, CLOT & TINI respectively in mixture and in sample solutions,

A_1, A_2 and A_3 are the absorbances of sample at 216, 230 and 296 nm, respectively,

ax_1, ax_2 and ax_3 are the absorptivity of CLIN at 216, 230 and 296, respectively,

ay_1, ay_2 and ay_3 are the absorptivity of CLOT at 216, 230 and 296 nm, respectively, az_1, az_2 and az_3 are the absorptivity of TINI at 216, 230 and 243 nm, respectively.

RESULT AND DISCUSSION

METHOD

Validation

Linearity and Range

The linearity range for CLIN, CLOT & TINI was found to be 5-15 µg/ml depicted in Table no 1, 2 and 3, Figure 17, 18 and 19,

Chromatogram of CLIN, CLOT & TINI depicted in Figure 16

Correlation co-efficient for calibration curve of CLIN, CLOT, Was found to be 0.999, 0.999 and for it was found TINI 0.998 respectively.

The regression line equation for Clindamycin is as follows:

$$Y=118.8x+17.30$$

The regression line equation for clotrimazole is as follows:

$$Y=346.7x+2.36$$

The regression line equation Tinidazole is as follows:

Y=969.4x+101.6

Where,

Y=corresponding peak area

x=concentration of Clindamycin, Clotrimazole & Tinidazole in µg/ml.

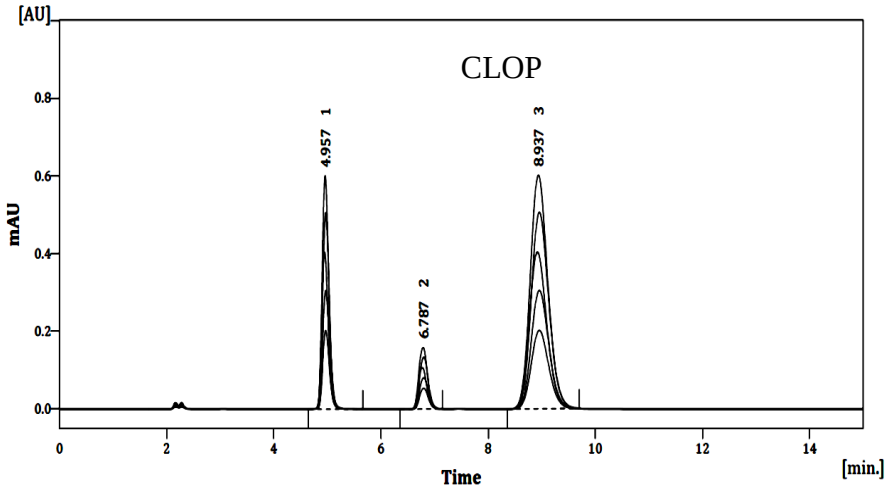


Figure 16: Chromatogram of different concentrations of ternary mixture of, CLIN, CLOT and TINI.

Table No 1. Linearity data for clindamycin

SR NO	CONCENTRATION (µg/ml)	AREA MEAN±S.D (n=3)	% R.S.D
1	5	604.476 ± 1.31	0.264740035
2	7.5	911.21±1.06	0.106037881
3	10	1209.5± 21.92	0.108285919
4	12.5	1533.7± 29.70	0.149311115
5	15	1796.51 ± 6.38	0.12753

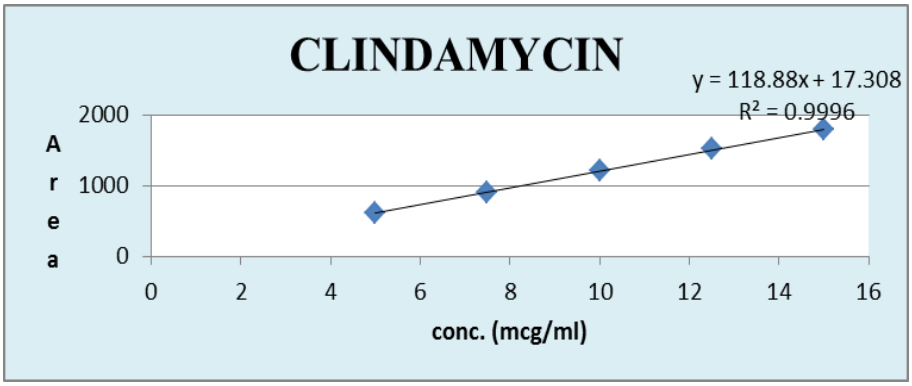


Figure 17: Calibration curve of CLIN.

Table No. 2. Linearity data for clotrimazole.

Sr no	CONCENTRATION (µg/ml)	AREA MEAN±S.D (n=3)	% R.S.D
1	5	1732.8±22.34	1.28
2	7.5	2573.1±1.023	0.03
3	10	3473.5±1.50	0.04
4	12.5	4376.3±2.07	0.04
5	15	5178.3± 2.05	0.03

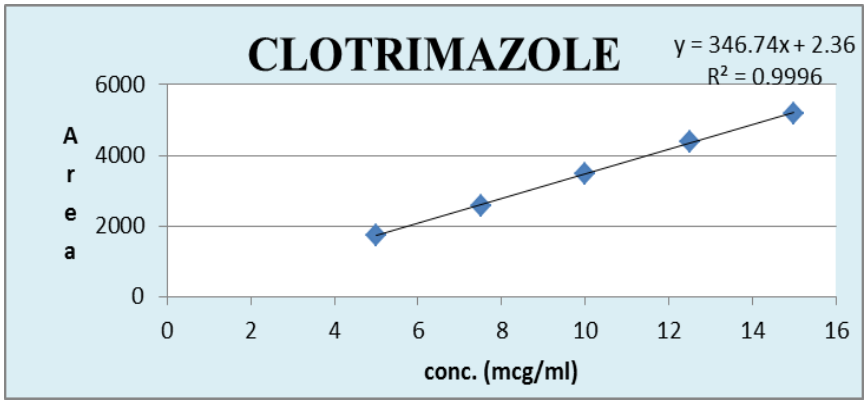


Figure 18: Calibration curve of CLOT.

Table No. 3: Linearity data for Tinidazole.

SR NO	CONCENTRATION (µg/ml)	AREA MEAN±S.D (n=5)	% R.S.D
1	5	4630±25.9	0.56
2	7.5	7089±3.6	0.05
3	10	9261.7±158.22	1.70
4	12.5	1163.6±50.08	4.30
5	15	13956.7±92.91	0.65

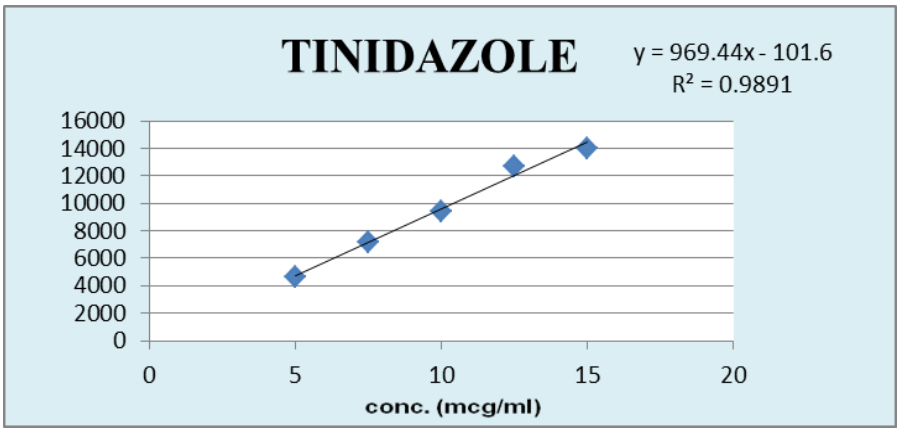


Figure 19: Calibration curve of TINI.

Specificity

The specificity of the method was ascertained by analyzing standard drugs and sample of, CLIN, CLOT &TINI. The results suggested that proposed method is specific, the excipient present in the formulation does not affect the result. The chromatogram taken by running only with mobile phase and after injection of the sample and standard are given in below Figure 20, 21 and 22.

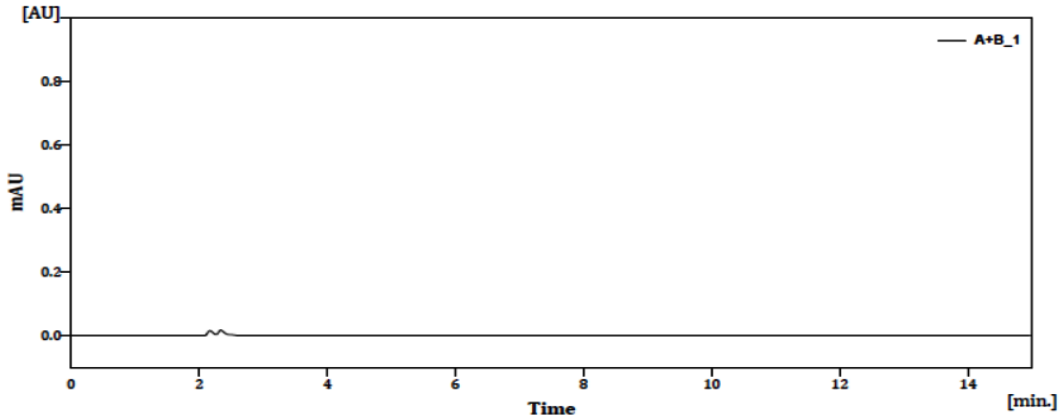


Figure 20: Chromatogram of mobile phase.

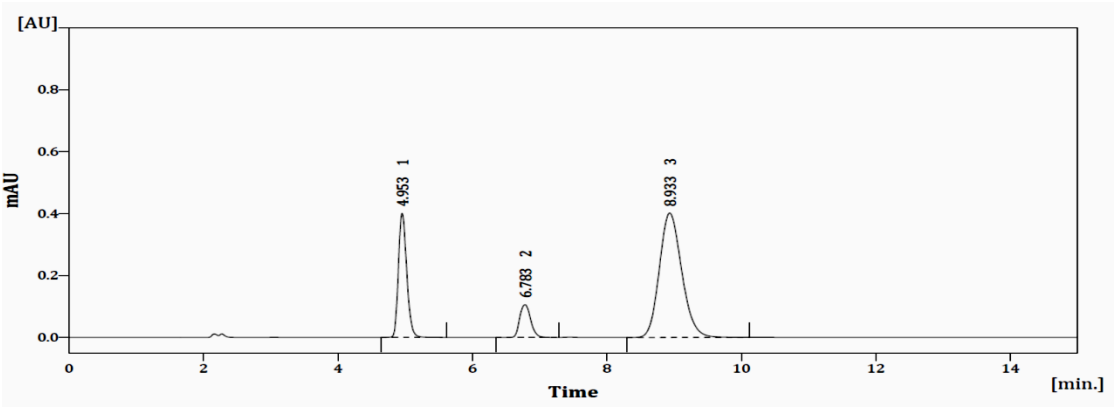


Figure 21: Chromatogram of CLIN, CLOT &TINI of std. solution.

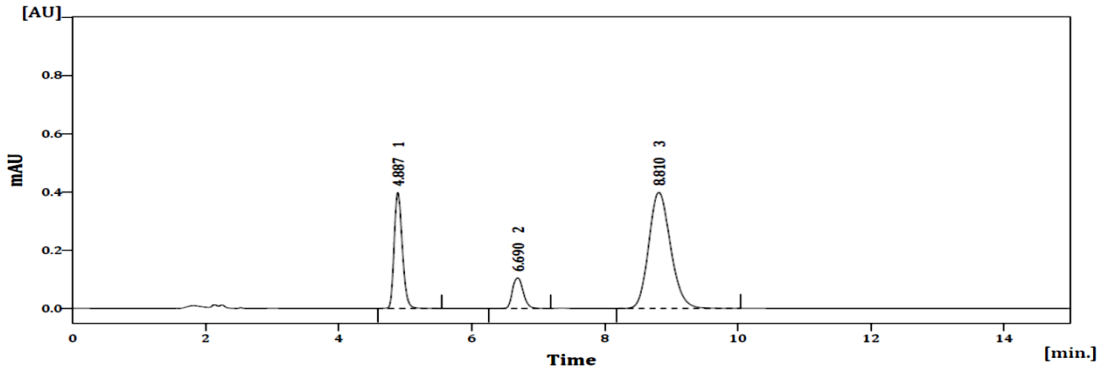


Figure 22: Chromatogram of CLIN, CLOT & TINI of sample solution.

3. Precision

- **Repeatability:** The data for repeatability of peak area measurement for CLIN CLOT, &TINI based on six measurements concentration of CLIN, CLOT & TINI in linearity range are depicted in Table no 4.

Table No 4: Repeatability data for, CLIN, CLOT &TINI.

Drug	Concentration (µg/ml)	Peak area Mean ± S.D (n=6)	% R.S.D
CLIN	10	3462.38 ±27.16	0.78
CLOT	10	1202.02 ± 9.18	0.76
TINI	10	9336.35 ± 85.4	0.91

2. Intraday precision: The data for intraday precision for CLIN, CLOT &TINI is shown in Table 5. The % R.S.D for intraday precision was found to be for for CLIN, CLOT & for TINI

Table No 5: Intraday Precision data for Estimation of CLIN, CLOT, &TINI.

Drug	Conc. (µg/ml)	Intraday precision	
		Peak Area Mean ± S.D (n=3)	% R.S.D
CLIN	5	626.10 ± 8.83	1.41
	10	1181.23±22.24	1.88
	15	9319.13 ± 1181.23	1.20
CLOT	**5**	1774.31 ± 22.25	1.25
	10	3445.99 ± 34.64	1.00
	15	5217.26 ± 70.69	1.35
TINI	5	4741.87 ± 31.75	1.09
	10	1820.71 ± 25.24	1.38
	15	1432.28±25.19	1.75

3. Interday precision: The data for intraday precision for CLIN, CLOT &TINI is shown in Table no. 6.

Table No. 6: Interday Precision data for Estimation of CLIN CLOT & TINI.

Drug	Conc. (µg/ml)	Interday precision	
		Peak Area Mean ± S.D (n=3)	% R.S.D
CLIN	5	625.25 ± 16.34	2.61
	10	1232.77± 34.94	2.82
	15	1840.91 ± 52.68	2.86
CLOT	**5**	1735.52 ± 51.58	2.97
	10	3321.30 ± 97.76	2.94
	15	5272.4 ± 154.15	2.92
TINI	5	4472.68 ± 116.07	2.59
	10	9168.26 ±237.49	2.59
	15	14598.75 ± 394.98	2.70

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation (CLINGEN FORTE) at three level of standard addition. The results are shown in Table no. 7, 8 & 9.

Table No. 7: Recovery data for CLIN.

% RECOVERY	TARGET CONC µg/ml)	SPIKED CONC	FINAL CONC	AMOUNT RECOVERED	% ASSAY	MEAN ± SD
80%	5	4	9	4.04	101.05	100.73 ± 0.28
	5	4	9	4.02	100.50	
	5	4	9	4.03	100.64	
100%	5	5	10	5.04	100.80	101.12 ± 0.75
	5	5	10	5.10	101.98	
	5	5	10	5.03	100.59	
120%	5	6	11	6.03	100.57	100.39 ± 0.82
	5	6	11	5.97	99.49	
	5	6	11	6.07	101.11	

Table No. 8: Recovery data for CLOT.

% RECOVERY	TARGET CONC (µg/ml)	SPIKED CONC	FINAL CONC	AMOUNT RECOVERED	% ASSAY	MEAN ±SD
80%	5	4	9	6.06	96.96	96.91±1.04
	5	4	9	6.12	97.92	
	5	4	9	5.99	95.84	
100%	5	5	10	6.06	96.96	86.30±9.28
	5	5	10	5.12	81.88	
	5	5	10	5.00	80.06	
120%	5	6	11	6.06	96.91	96.54±0.58
	5	6	11	5.99	95.87	
	5	6	11	6.05	96.84	

Table No. 9: Recovery data for TINI.

% RECOVERY	TARGET CONC.µg/ml)	SPIKED CONC	FINAL CONC	AMOUNT RECOVERED	% ASSAY	MEAN ± SD
80%	5	4	9	3.97	99.33	99.00 ±1.51
	5	4	9	4.01	100.32	
	5	4	9	3.89	97.35	
100%	5	5	10	5.07	101.47	101.46±1.21
	5	5	10	5.13	102.66	
	5	5	10	5.01	100.24	
120%	5	6	11	6.29	104.83	104.06±0.69
	5	6	11	6.21	103.50	
	5	6	11	6.23	103.83	

LOD and LOQ

Calibration curve was repeated for three times and the standard deviation (SD) of the intercepts was calculated. The LOD & LOQ were calculated as follows:

LOD=3.3*SD/slope of calibration curve

LOQ=10*SD/slope of calibration curve

Table No. 10: LOD and LOQ data for CLIN, CLIN & TINI.

Parameters	CLIN(µg/ml)	CLOT (µg/ml)	TINI (µg/ml)
Mean slope	10.77	32.7	46.12
LOD	0.29	0.31	1.57
LOQ	0.90	0.94	4.77

Robustness

All the three drugs were analyzed in different flow rate, % of Mobile phase and pH. The peak area obtained shown in Table no 11,12 & 13 for CLIN, CLOT & TINI respectively.

Table no 11: Robustness data for CLIN.

Sr. No	CLIN (10µg/ml)					
	pH:3		Mobile Phase		Flow Rate:1 ml/min	
	pH 3.2 (+0.2 units)	pH 2.8 (-0.2 units)	Mobile phase (+2%)	Mobile phase (-2%)	Flow rate 1.2 ml/min (+0.2 units)	Flow rate 0.8 ml/min (-0.2 units)
1	1207.83	1179.83	1187.43	1200.63	1229.53	1173.09
2	1216.31	1180.29	1199.31	1219.91	1241.84	1181.33
3	1207.79	1187.03	1201.72	1210.14	1243.37	1191.95
Mean	1210.64	1182.38	1196.15	1210.72	11822.12	1173.09
SD	4.90	4.03	7.64	9.64	9.45	9.4
%R.S.D	0.40	0.34	0.63	0.79	0.61	0.8

Table no 12: Robustness data for CLOT.

Sr. No	CLOT (10µg/ml)					
	pH:3		Mobile Phase		Flow Rate:1 ml/min	
	pH 3.2 (+0.2 units)	pH 2.8 (-0.2 units)	Mobile phase (+2%)	Mobile phase (-2%)	Flow rate 1.2 ml/min (+0.2 units)	Flow rate 0.8 ml/min (-0.2)
1	3480.58	3401.67	3421.80	3404.37	3380.46	3543.1
2	3500.93	3401.27	3436.62	3406.01	3392.03	3578.6
3	3480.50	3408.30	3462.858	3471.26	3434.77	3603.6
Mean	3487.3	3403.75	3440.42	3409.21	3402.42	3575.11
S.D	11.77	3.9	20.79	7.01	28.60	30.43
%R.S.D	0.33	0.11	0.60	0.20	0.84	0.85

Table no 13 Robustness data for TINI.

Sr. No	TINI (10µg/ml)					
	pH:3		Mobile Phase		Flow Rate:1 ml/min	
	pH 3.2 (+0.2 units)	pH 2.8 (-0.2 units)	Mobile phase (+2%)	Mobile phase (-2%)	Flow rate 1.2 ml/min (+0.2 units)	Flow rate 0.8 ml/min (-0.2 units)
1	9396.62	9016.57	9197.82	9410.01	9086.87	9505.47
2	9406.40	9182.47	9330.31	9431.7	9117.38	9606.06
3	9321.64	9160.66	9348.84	9414.71	9272.97	9708.97
Mean	9374.89	9119.90	9292.32	9418.8	9159.07	9606.85
S.D	46.36	90.14	82.36	11.40	99.81	101.75
%RSD	0.49	0.259	0.88	0.12	1.08	1.05

Application to Pharmaceutical Dosage Form/ Applicability of The Method

Applicability of the proposed method was tested by analysing the commercially available vaginal suppository formulation Clingen forte. The assay results were comparable to labeled value of each drug in dosage form. These results indicate that the developed method is accurate, precise and simple. It can be used in the routine quality control of dosage form in industries. The results are shown in Table no. 14.

Table no. 14 Analysis of Marketed Formulation

Formulation (Pessaries)	Pessaries amount (mg)			Amount found (mg)			% Assay		
	CLIN	CLOT	TINI	CLIN	CLOT	TINI	CLIN ± S.D (n=3)	CLOT ± S.D (n=3)	TINI± S.D (n=3)
1	100	100	100	96.65	96.67	96.80	96.80 ± 0.37	96.89 ± 0.39	97.01 ± 0.48
2	100	100	100	97.22	97.34	97.56			
3	100	100	100	96.53	96.66	96.67			

METHOD B

Linearity and Range

Linear correlation was obtained between absorbance versus concentration of CLIN,CLOT&TINI in range of 20-100 µg/ml. The Linearity Curves and Calibration curves of these three drugs were shown in Figure 21, 22 and Figure 23. The results of the calibration curve were shown in Table no. 15, 16 and 17.

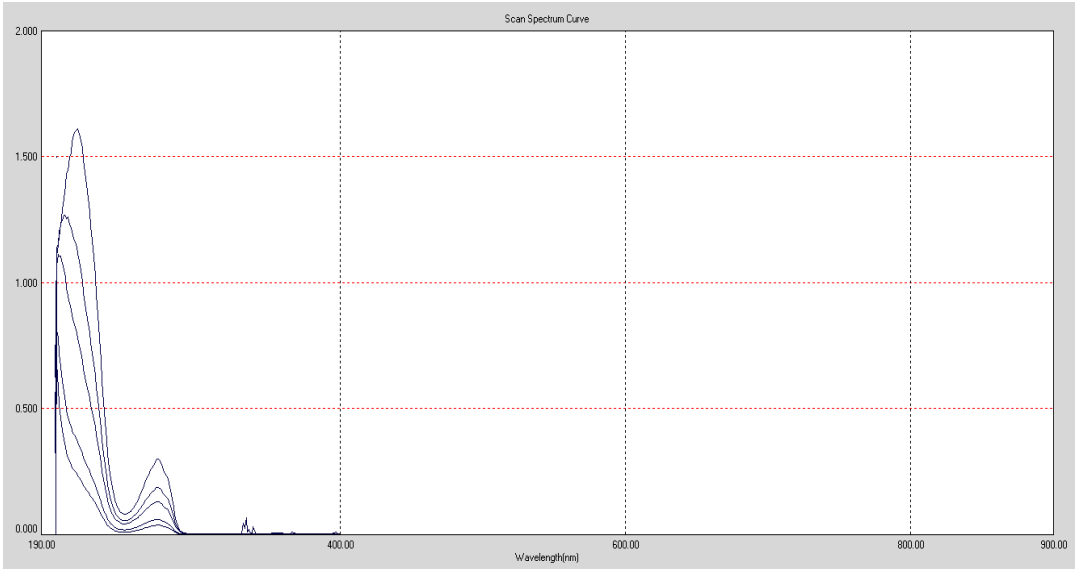


Figure 21 Overlain Spectra of CLIN at 216 nm.

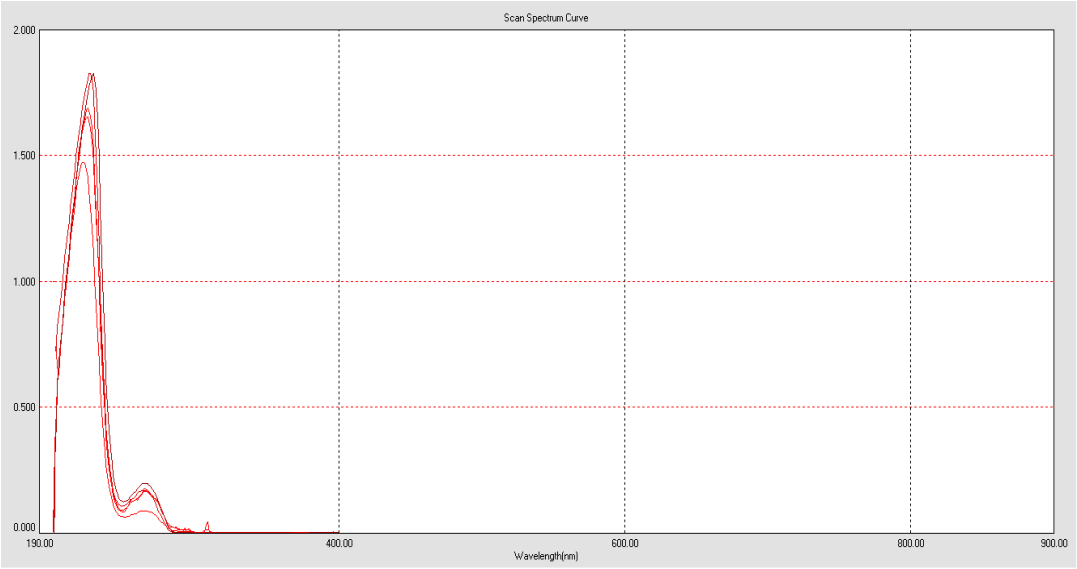


Figure 22: Overlain Spectra of CLOT at 230 nm.

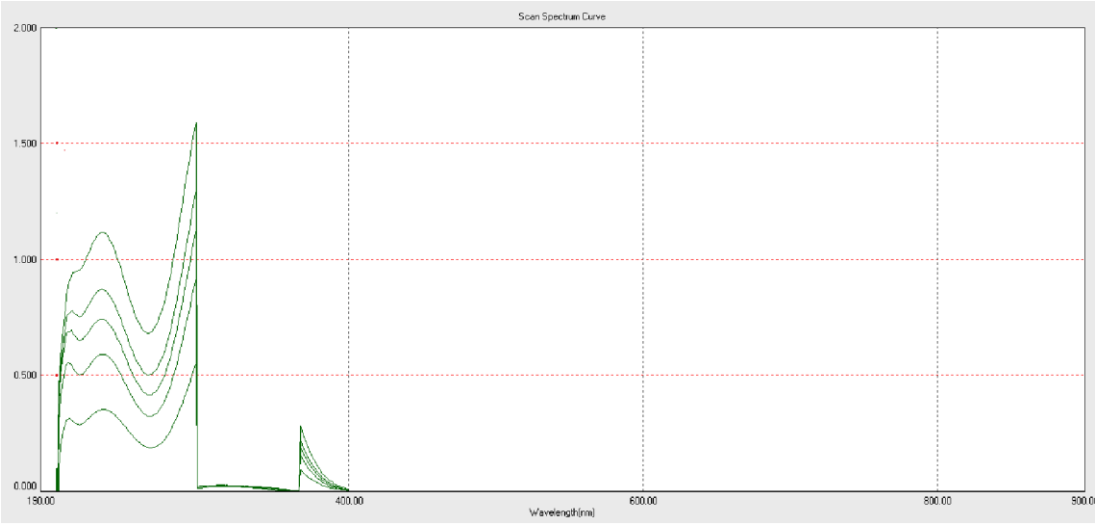


Figure 23 Overlain Spectra of TINI at 296 nm.

Table No 14 Result of Calibration Reading for CLIN at 216 nm

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	0.5±0.0083	2.1
40	0.75± 0.019	0.2
60	1.1± 0.021	1.7
80	1.3± 0.035	1.1
100	1.6± 0.015	0.97

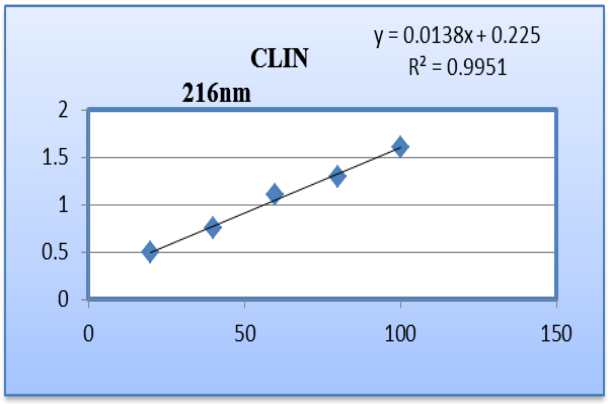


Figure 24 Calibration Curve of CLIN at 216 nm.

Table No. 15 Result of Calibration Reading for CLIN at 230 nm.

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	0.1±0.015	13.1
40	0.2±0.019	8.6
60	0.3±0.015	4.9
80	0.4±0.015	3.7
100	0.61±0.019	3.0

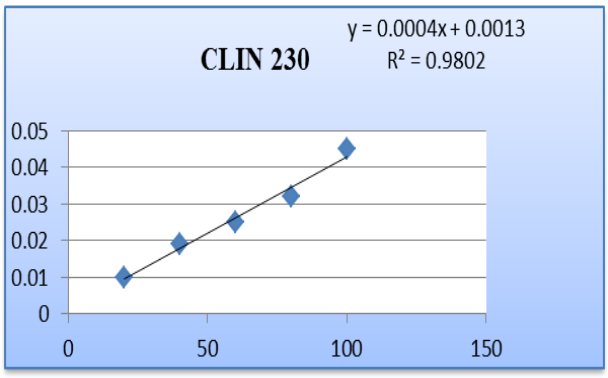


Figure 25: Calibration Curve of CLIN at 230 nm.

Table No. 16: Result of Calibration Reading for CLIN at 296 nm

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	0.01±0.0019	15.7
40	0.019±0.0014	7.1
60	0.025±0.0008	3.3
80	0.032±0.0019	5.9
100	0.045±0.002	4.7

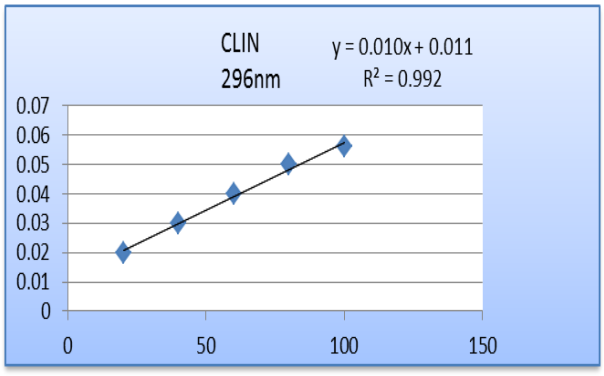


Figure 26: Calibration Curve of CLIN at 296 nm

Table No. 17 Result of Calibration Reading for CLOT at 216 nm.

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	1.37 ± 0.015	1.16
40	1.41 ± 0.016	1.35
60	1.45 ± 0.018	1.24
80	1.5 ± 0.024	1.56
100	1.53 ± 0.023	1.52

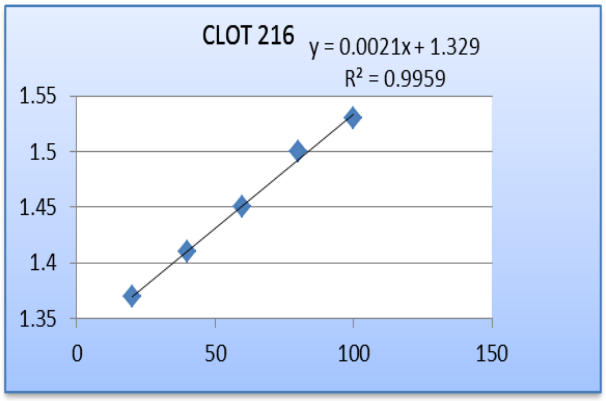


Figure No. 27: Calibration Curve of CLOT at 216 nm.

Table No. 18: Result of Calibration Reading for CLOT at 230 nm.

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	1.47±0.015	0.9
40	1.6±0.034	1.4
60	1.7±0.023	0.4
80	1.85±0.018	0.7
100	1.96±0.063	1.5

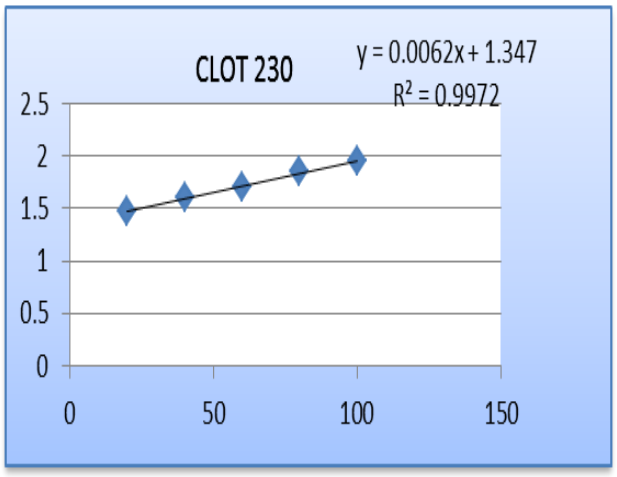


Figure No.28: Calibration Curve of CLOT at 230nm.

Table No. 19: Result of Calibration Reading for CLOT at 296 nm.

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	0.02±0.0019	8.4
40	0.03±0.015	4.9
60	0.04±0.002	4.8
80	0.05±0.0019	3.6
100	0.06±0.0023	4.0

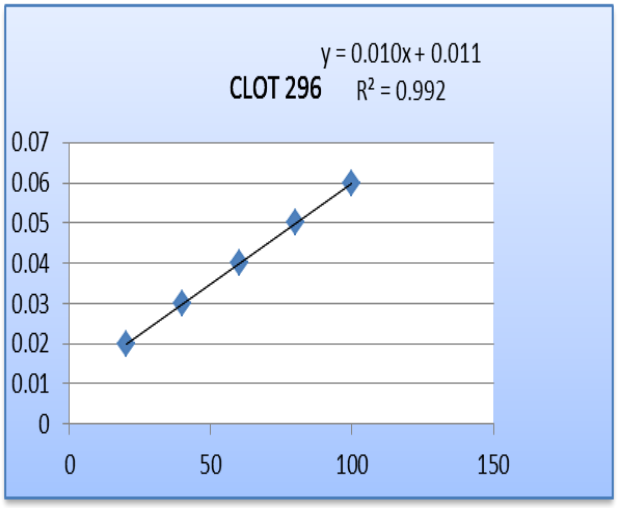


Figure No 29: Calibration Curve of CLOT at 296 nm.

Table No: 20. Result of Calibration Reading for TINI at 216 nm.

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	0.35±0.019	5.6
40	0.53±0.015	2.9
60	0.64±0.018	2.8
80	0.75±0.018	2.4
100	0.88±0.012	1.5

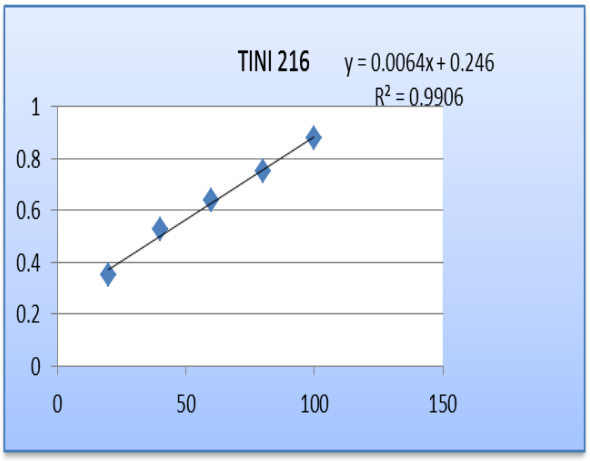


Fig no 30 Calibration Curve of TINI at 216 nm.

Table No: 21 Result of Calibration Reading forTINI at 230 nm.

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	0.3±0.020	6.4
40	0.5±0020	3.9
60	0.7±0.015	2.1
80	0.85±0.019	2.3
100	1.1±0.230	18.5

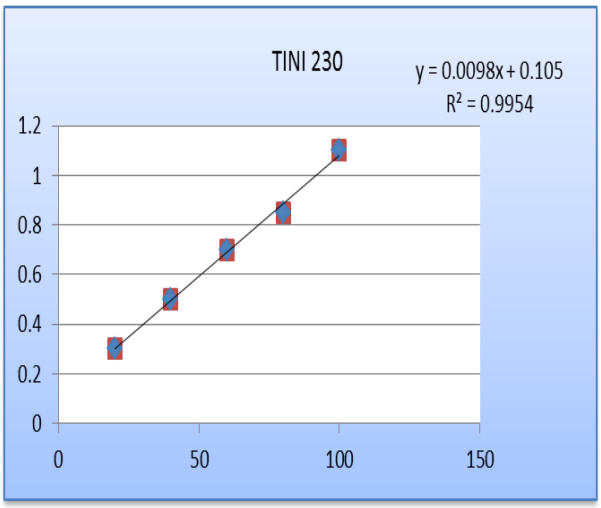


Figure No. 32: Calibration Curve of TINI at 230 nm.

Table No. 22: Result of Calibration Reading for TINI at 296 nm.

Conc. (µg/ml)	Mean Abs. ± S.D. (n=5)	% R.S.D
20	0.6±0.038	6.7
40	0.82±0.049	4.8
60	1.1±0.038	1.4
80	1.3± 0.026	1.9
100	1.5±0.022	1.4

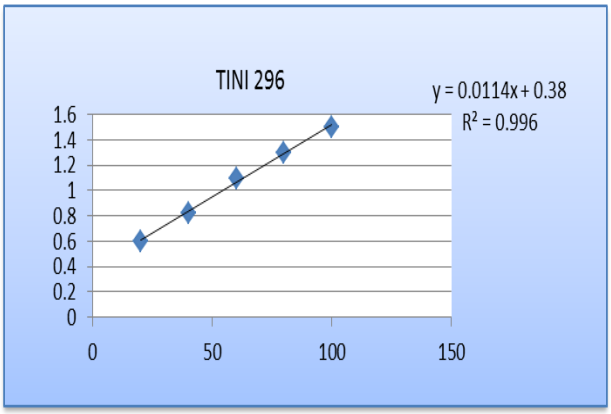


Figure No. 33 Calibration Curve of TINI at 296 nm.

Accuracy (% Recovery)

The % recovery experiment was performed by the Standard Addition Method. Known amounts of standard solutions of CLIN, CLOT&TINI added at 80, 100 and 120 % level to pre quantified sample solutions of CLIN, CLOT&TINI (10µg/ml) respectively Results of recovery studies are shown in Table no. 7.3.10, 7.3.11 &7.3.11.

Table No. 23: Recovery data for CLIN.

% RECOVERY	TARGET CONC.µg/ml)	SPIKED CONC	FINAL CONC	AMOUNT RECOVERED	% ASSAY	MEAN ±S.D
80%	0.2	0.16	0.36	0.17	94.11	94.67± 0.32
	0.2	0.16	0.36	0.168	95.2	
	0.2	0.16	0.36	0.169	94.6	
100%	0.3	0.24	0.54	0.29	103.4	104.68±2.05
	0.3	0.24	0.54	0.28	107.1	
	0.3	0.24	0.54	0.27	103.4	
120%	0.2	0.24	0.44	0.18	108	106± 3.4
	0.2	0.24	0.44	0.18	108	
	0.2	0.24	0.44	0.19	102	

Table No. 24: Recovery data for CLOT.

% RECOVERY	TARGET CONC.µg/ml)	SPIKED CONC	FINAL CONC	AMOUNT RECOVERED	% ASSAY	MEAN
80%	0.2	0.16	0.36	0.17	94.1	94.48 ± 0.56
	0.2	0.16	0.36	0.169	94.67	
	0.2	0.16	0.36+	0.168	94.67	
100%	0.3	0.24	0.54	0.28	107.1	105.911 ±2.13
	0.3	0.24	0.54	0.29	103.4	
	0.3	0.24	0.54	0.28	107.1	
120%	0.2	0.24	0.44	0.19	102	104± 3.4
	0.2	0.24	0.4	0.18	108	
	0.2	0.24	0.44	0.19	102	

Table No. 25: Recovery data for TINI.

% RECOVERY	TARGET CONC.µg/ml)	SPIKED CONC	FINAL CONC	AMOUNT RECOVERED	% ASSAY	MEAN
80%	0.2	0.16	0.36	0.17	94.1	94.67±0.56
	0.2	0.16	0.36	0.168	94.6	
	0.2	0.16	0.36	0.17	95.2	
100%	0.3	0.24	0.54	0.28	107.1	104.67±2.1
	0.3	0.24+	0.54	0.27	103.4	
	0.3	0.24	0.54	0.26	103.5	
120%	0.2	0.24	0.44	0.19	102	106± 3.4
	0.2	0.24	0.44	0.18	108	
	0.2	0.24	0.44	0.17	108	

Precision

The interday and intraday precision of the proposed method was determined by analyzing the corresponding response 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of CLIN, CLOT&TINI (20, 60, 100 µg/ml) and 6 determination for the same concentration of CLIN, CLOT& TINI for repeatability. The results of interday

and intraday precision and repeatability were shown in Table 26 and 27.

Table No. 26 Intraday and Interday Precision data for estimation of CLIN, CLOT & TINI

Drug	Conc. (µg/ml)	Intraday precision		Interday precision	
		Mean ± S.D (n=3)	%R.S.D	Mean ± S.D (n=3)	% R1.S.D
CLIN	20	0.50± 0.011	2.27	0.41± 0.15	3.69
	60	1.12± 0.02	2.24	1.25± 0.045	3.62
	100	1.54± 0.05	2.42	1.54± 0.047	3.07
CLOT	0	1.51± 0.04	2.64	1.45± 0.05	3.44
	60	1.74± 0.045	2.58	1.64± 0.049	3.02
	100	1.95± 0.03	2.44	1.83± 0.055	3.09
TINI	20	0.16± 0.015	2.47	0.53± 0.02	3.67
	60	1.12± 0.025	2.24	1.65± 0.05	3.18
	100	1.52± 0.026	2.11	1.44± 0.05	3.11

Table No. 27: Repeatability data for estimation of CLIN,CLOT&TINI.

Drug	Concentration (µg/ml)	Abs. Mean ± S.D (n=6)	% R.S.D
CLIN	60	1.18 ± 0.03	2.57
CLOT	60	1.72 ± 0.04	2.25
TINI	60	1.13 ± 0.03	2.68

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Calibration curve was repeated for three times and the standard deviation (SD) of the intercepts was calculated. The LOD & LOQ were calculated as follows:

LOD=3.3*SD/slope of calibration curve

LOQ=10*SD/slope of calibration curve

LOD values for CLIN,CLOT&TINI were found to be 6.38,11.8 and 8.8 µg/ml and LOQ values for CLIN,CLOT&TINI were found to be 19.34, 35.7 and 26.9µg/ml at 216 nm, 230 nm and 296 nm.

Application to Pharmaceutical Dosage Form/ Applicability of the Method

Applicability of the proposed method was tested by analysing the commercially available capsule formulation Rosutor gold. The results are shown in Table 6.2.15.

Table no: 28 Analysis of marketed formulation.

Formulation (PESSARIES)	Capsule amount (mg)			Amount found (mg)			% Assay		
	CLIN	CLOT	TINI	CLIN	CLOT	TINI	CLIN± S.D (n=3)	CLOT± S.D (n=3)	TINI± S.D (n=3)
1	100	100	100	96.30	96.05	96.50	96.28 ± 0.25	97.14 ± 0.12	96.18 ± 0.16
2	100	100	100	97.14	97.02	97.26			
3	100	100	100	96.06	96.13	96.37			

The assay results were comparable to labeled value of each drug in tablet dosage form. These results indicate that the developed method is accurate, precise and simple. It can be used in the routine quality control of dosage form in industries.

CONCLUSION

The proposed methods are found to be simple, accurate, and precise with no interference of excipients and impurities. The result of the analysis of pharmaceutical formulation by the proposed method is highly reproducible and reliable and it is in good agreement with the label claim of the drug Both the developed method can be used for routine estimation of Clindamycin, Clotrimazole and Tinidazole respectively in their combined pharmaceutical Dosage Form.

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