DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CLOMIPHENE CITRATE AND MELATONIN IN MARKETED FORMULATION

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***Abstract*- RP-HPLC Method was developed and validated for simultaneous estimation of Clomiphene Citrate (CLP) and Melatonin (MLT). Separation was achieved on Shim-pack solar C18 (250mm × 4.6mm, 5µm), Flow rate was 1 ml/min and detection were carried out at 225 nm using ACN: Sodium Dihydrogen Ortho Phosphate Buffer (NaH_2PO_4) (80: 20 v/v) (pH-3 is adjusted with 1% OPA). The linearity for CLP & MLT were 20-60 µg/ml & 1-5 µg/ml respectively. The correlation coefficient (r) were found to be 0.9961 and 0.9969 for CLP and MLT respectively. Limit of detection for CLP and MLT were found to be 0.5187 µg/ml and 0.0131 µg/ml and Limit of quantification were found to be 1.5718 µg/ml and 0.0399 µg/ml respectively. The % assay was found to be 99.69 % and 99.34 % for CLP and MLT respectively. Further % R.S.D. was found to be less than 2 % for repeatability, intraday and Interday study. RP-HPLC method was developed for selection and optimization of mobile phase. All the developed methods are validated according to ICH guidelines. The developed methods were applied successfully for the Quantitative determination of CLP & MLT in tablet dosage form.**

Key Words: **Clomiphene Citrate, Melatonin, Method Development and Validation, RP-HPLC.**

➢ INTRODUCTION:

HPLC is one type of column chromatography that is specifically used in biochemistry and analysis to separate, identify, and quantify the active compounds is called high-performance liquid chromatography, or high-pressure liquid chromatography (HPLC). In the present study attempt is made to estimate the following three drugs simultaneously. clomiphene citrate (2-[p-(2-chloro-1,2-diphenylvinyl) phenoxy] triethylamine citrate) it is Freely soluble in methanol, soluble in ethanol, slightly soluble in acetone, water, and chloroform, and insoluble in ether and melatonin (N-[2-(5-methoxy-1H-indol-3-yl)ethyl]acetamide) it is Soluble in water (0.1 mg/ml), ethanol (8 mg/ml), benzene, chloroform, methanol, DMSO, toluene, and dilute aqueous acid, and very slightly soluble in petroleum ether. For women who are not ovulating and are unable to become pregnant, it is effective.

➢ MATERIALS AND METHODS

- **Apparatus and Instrument**

Model: SHIMADZU LC-2010 CHT, Column: Shim-pack solar C18 (250 mm × 4.6 mm, 5µm), Injector: Auto injector, Detector: UV Detector, Software: LC solution, Electronic analytical balance (Shimadzu), Digital pH meter (Systronic pH system), Ultrasonic cleaner (Athena Technology), Filter paper: Vacuum filter: Membrane filter 0.45 micron, Syringe filter: Membrane filter 0.27micron, Volumetric flask and pipettes

- **Reagents and Materials**

Clomiphene citrate and Melatonin (gift sample from Akums Drug Pharmaceutical Pvt. Ltd., Delhi.), Acetonitrile (HPLC grade, Rankem,Maharashtra), Methanol (HPLC grade, Rankem,Maharashtra), Water (HPLC grade, Rankem,Maharashtra), Orthophosphoric acid (HPLC grade FisherScientific)

- **Selection of wavelength**

Aliquots of 5 ml from working solution of CLP (100 µg/ml) and 3 ml from working solution of MLT (10 µg/ml) were pipette out into two separate 10 ml of volumetric flask and volume was made up to the mark with methanol to get 50 µg/ml of CLP and 3 µg/ml of MLT. Each Solutions of CLP and MLT were scanned between 200-400 nm using UV-Visible Spectrophotometer. Wavelength was selected from the overlay spectra of CLP and MLT.

➢ PREPARATION OF STANDARD AND WORKING SOLUTION

1. Preparation of CLP standard stock solution (1000 µg/ml):

Weigh accurately 10 mg of CLP and transferred to 10 ml volumetric flask. It was dissolved in methanol and volume was made up to the mark with methanol to give a solution containing 1000 µg/ml. Aliquot of 2.5 ml from above standard stock solution was pipetted out into 25 ml of volumetric flask and volume was made up to the mark with methanol to give a solution containing 100 µg/ml.

2. Preparation of MLT standard stock solution (1000 µg/ml):

Weigh accurately 10 mg of MLT and transferred to 10 ml volumetric flask. It was dissolved in methanol and volume was made up to the mark with methanol to give a solution containing 1000 µg/ml. Aliquot of 1 ml from above standard stock solution was pipetted out into 10 ml of volumetric flask and volume was made up to the mark with methanol to give a solution containing 100 µg/ml. Aliquot of 2.5 ml from above standard stock solution was pipetted out into 25 ml of volumetric flask and volume was made up to the mark with methanol to give a solution containing 10 µg/ml.

3. Preparation of Binary mixture of CLP and MLT:

Aliquots of 5 ml from working solution of CLP (100 µg/ml) and 3 ml from working solution of MLT (10 µg/ml) were taken into common volumetric flask and diluted up to 10 ml with methanol to make final concentration CLP (50 µg/ml) and MLT (3 µg/ml).

➢ Selection of Mobile Phase

Number of trials were taken for the selection of mobile phase.

➢ Preparation of buffer:

Dissolve 7.8 g of Sodium dihydrogen phosphate in 900 ml of water, pH 3 was adjusted with phosphoric acid and dilute up to 1000 ml with the same solvent.

➢ VALIDATION OF PROPOSED HPLC METHOD

1. System Suitability studies

Evaluation of system suitability was done by analyzing six replicate of CLP and MLT in a mixture at concentration of 50 µg/ml of CLP and 3 µg/ml of MLT. The column efficiency, peak asymmetry and resolution were calculated for each replicate.

2. Specificity

Specificity involves quantitative detection of analyte in the presence of those components that may be expected to be part of sample matrix. Specificity of developed method was established by spiking of CLP and MLT in hypothetical placebo (i.e. might be expected to be present) and expressing that analytes peak were not interfered from excipients.

3. Linearity

The linearity response was determined by analyzing 5 independent levels of concentration in the range of 20-60 µg/ml and 1-5 µg/ml for CLP and MLT respectively.

Preparation of Calibration Curves:

Calibration curve for CLP consisted of five different concentrations solution ranging from 20-60 µg/ml. Calibration curve of Peak area vs Conc. was plotted and regression equation was determined and Calibration curve for MLT consisted of five different concentrations solution ranging from 1-5 µg/ml. Calibration curve of Peak area vs Conc. was plotted and regression equation was determined.

4. Precision

a) Repeatability

Repeatability of the developed method was assessed by analyzing samples from the same batch 6 times with standard solutions containing concentrations 50 µg/ml for CLP and 3 µg/ml for MLT and % R.S.D. was calculated.

b) Intraday precision

It was assessed by analyzing samples from the same batch with three standard solutions containing concentrations 40, 50, 60 µg/ml for CLP and 1, 2, 3 µg/ml for MLT. Solutions were analyzed thrice (n=3) on the same day within short interval of time and % R.S.D. was calculated.

c) Interday precision

It was assessed by analyzing samples from the same batch with three standard solutions containing concentrations 40, 50, 60 µg/ml for CLP and 1, 2, 3 µg/ml for MLT. Solutions were analyzed thrice (n=3) on the three different day and% R.S.D. was calculated.

5. Accuracy

Tablet mixture of 50 mg equivalent of CLP was taken into 10 ml of volumetric flask. Methanol was added and sonicated for 2-3 mins and volume was made up to mark with methanol. Filtered the through Whatman filter paper no. 42. Thus, resulting solution gave µg/ml of CLP and 1000 µg/ml of MLT. From the above solution, 1.0 ml was pipette out and transferred to 10 ml volumetric flask and volume was made up to mark with methanol in order to give a solution containing CLP (50 µg/ml) + MLT (3 µg/ml).

6. LOD and LOQ

The LOD (Limit of detection) was estimated from the set of 5 calibration curves that were used to determine linearity of the method. The LOD was calculated by using the formula:

LOD =3.3 × S.D./ slope

Where, S.D.= Standard deviation of the Y- intercepts of 5 calibration curves

Slope = mean slope of 5 calibration curves

The LOQ (Limit of Quantitation) was estimated from the set of 5 calibration curve that were used to determine linearity of the method. The LOQ was calculated by using the formula:

LOQ = 10 × S.D./Slope

Where, S.D.= standard deviation of the Y- intercepts of 5 calibration curves

Slopes = mean slope of 5 calibration curves

➢ **SIMULTANEOUS ESTIMATION OF CLOMIPHENE CITRATE AND MELATONIN IN TABLET DOSAGE FORM**

For the estimation of drugs in the commercial formulation, twenty tablets were weighed accurately. The average weight was calculated and then crushed to obtain fine powder. A quantity 306 mg of tablet powder equivalent to about 50 mg CLP, 3 mg MLT was transferred to 100 ml volumetric flask, methanol was added and sonicate for 10-15 min, volume was than make up to the mark with methanol (500 µg/ml of CLP and 30 µg/ml MLT) and the solution filtered through Whatman filter paper No.41. This solution was use at stock solution 2 ml of aliquot solution was pipetted out and transferred to a 10 ml volumetric flask. Then the volume made up to the mark with methanol (100 µg/ml of CLP and 6 µg/ml of MLT). Then 5 ml was withdrawn from above working solution and transferred into 10 ml volumetric flask. Then the volume was made up to mark with methanol to get sample solution containing 50 µg/ml of CLP and 3 µg/ml of MLT respectively.

RESULT AND DISCUSSION

➢ **SELECTION OF WAVELENGTH**

Both the drugs showed good absorbance at 225 nm so it was selected for estimation of CLP and MLT.

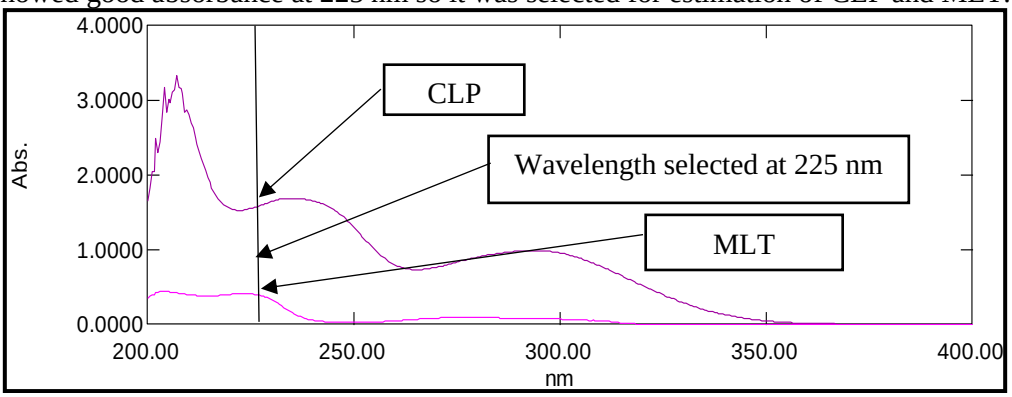


Fig no.1 Overlay UV spectrum of CLP and BEZ showing selection of wavelength detection

➢ **SELECTION OF MOBILE PHASE**

Various trials were taken in different composition for optimizing mobile phase using solvent like Water, Methanol, Acetonitrile and Buffer of pH 3 and 4.

The mobile phase should be sufficiently transparent at the detection of wavelength. Acetonitrile: Sodium Dihydrogen Ortho Phosphate Buffer (NaH_2PO_4) (pH-3 is adjusted with 1% OPA) are the solvent used for mobile phase provided optimum polarity for proper separation and resolution for clomiphene Citrate and Melatonin. The ratio selected is ACN: Sodium Dihydrogen Ortho Phosphate Buffer (NaH_2PO_4) (80: 20 v/v) (pH-3 is adjusted with 1% OPA).

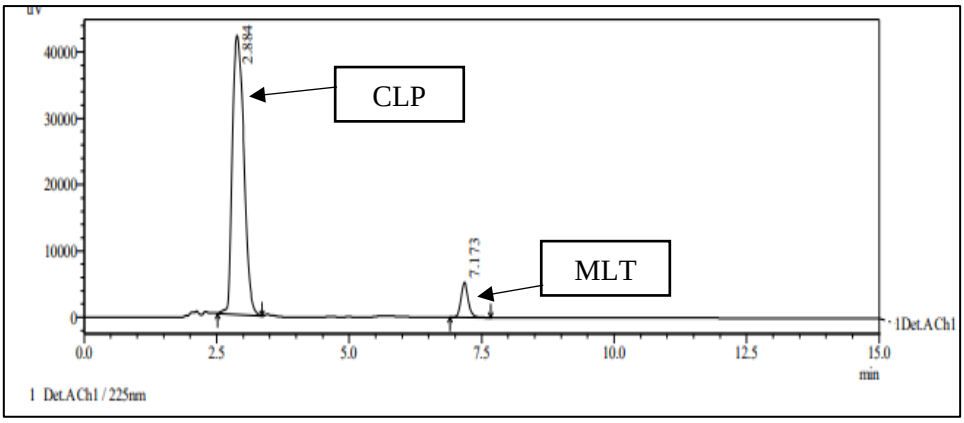
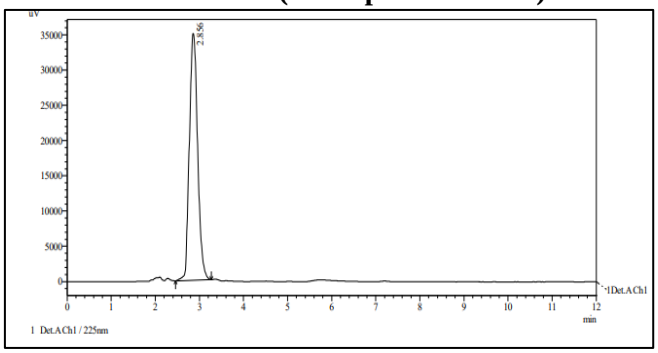


Fig no.2 Chromatogram of CLP and MLT in ACN: Buffer (NaH_2PO_4) (80: 20 v/v) (pH -3 is adjusted with 1% OPA)

Confirmation-1 (Clomiphene Citrate)



Confirmation-2 (Melatonin)

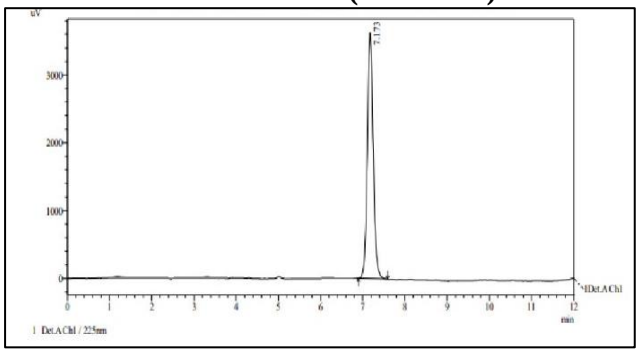


Fig no.3 Chromatogram of CLP

Fig no.4 Chromatogram of MLT

Development of Method

After optimization of mobile phase, final optimized chromatographic conditions are given in table:

Table no.1 Optimized Chromatographic Conditions

Sr. No.	Parameters	Conditions
1	Mobile phase	ACN: Sodium Dihydrogen Ortho Phosphate Buffer (NaH_2PO_4) (80: 20 v/v) (pH 3 adjusted with 1% OPA)
2	Flow Rate	1 ml/min
3	Run Time	15 min
4	Volume of Injection	10 µL
5	Detection of wavelength	225 nm
6	Diluent	Methanol

1. System Suitability Data

Table no.2 System suitability data for CLP and MLT

Drugs	Parameters	Mean ± S.D. (n=5)	%R.S.D.
CLP	Retention Time	2.836 ± 0.0193	0.6831
	Theoretical Plate	6894 ± 57.002	0.8268
	Tailing Factor	1.245 ± 0.0063	0.5097
MLT	Retention Time	7.173 ± 0.0094	0.1316
	Theoretical Plate	83196 ± 277.43	0.3334
	Tailing Factor	1.144 ± 0.0043	0.3757
	Resolution	13.980 ± 0.1379	0.9868

2. Specificity

The specificity of the method was determined by analysing standard drugs and sample of CLP and MLT. The results suggested that proposed method is specific, the excipients present in the synthetic mixture does not affect the result. The chromatogram taken by running only with the mobile phase, placebo and after injection of the sample are given in figures below

Fig.5 Chromatogram of Placebo

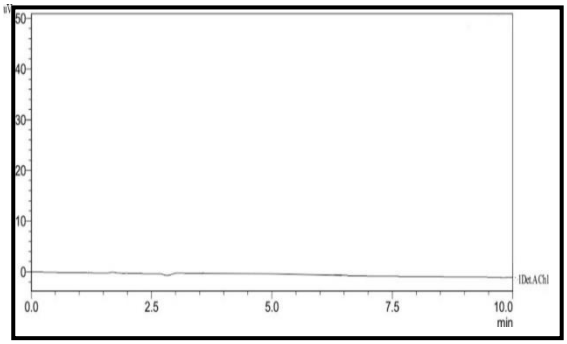
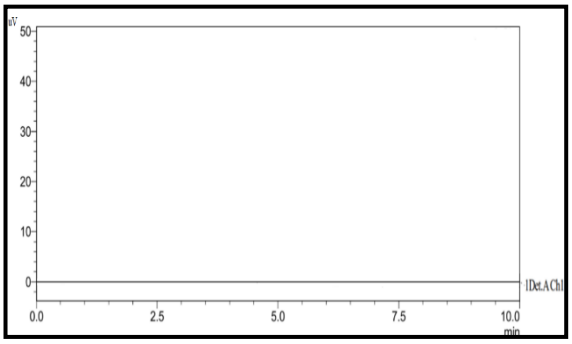


Fig.6 Chromatogram of Mobile Phase



CHROMATOGRAMS IN OPTIMISED CHROMATOGRAPHIC CONDITION

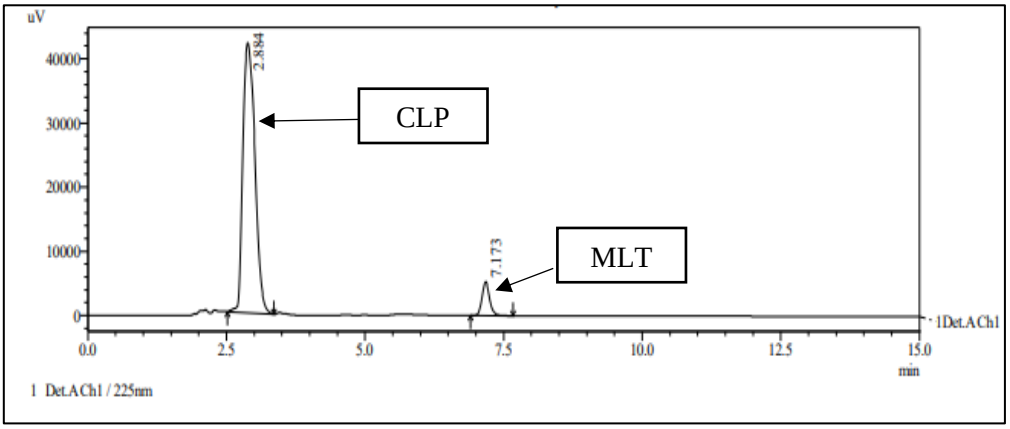


Fig.7 Chromatogram of CLP (50 µg/ml) and MLT (3 µg/ml)

3. Linearity

The linearity study was carried out for both drugs at five different concentration levels. The linearity of CLP and MLT was in the range of 20-60 µg/ml and 1-5 µg/ml are depicted in table.

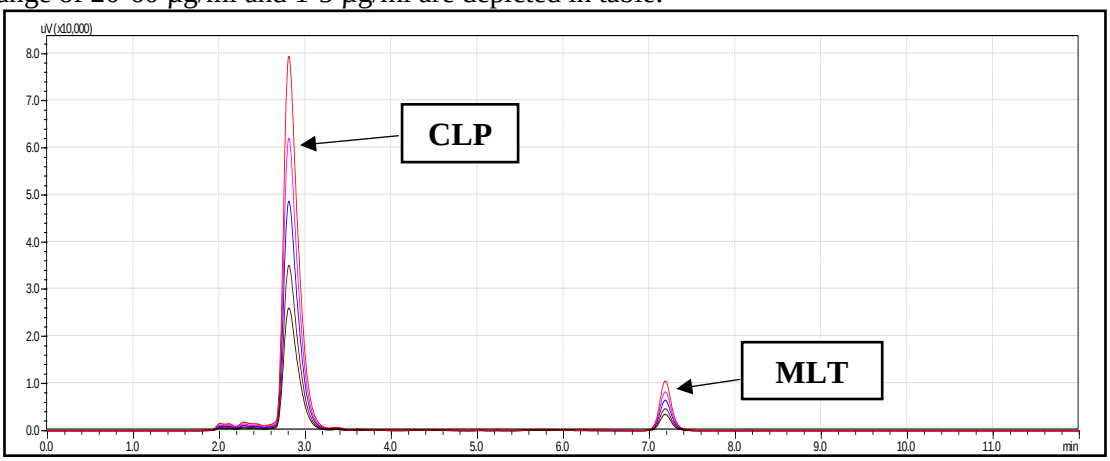


Fig.8 Overlain Chromatogram of CLP (20-60 µg/ml) and ML (1-5 µg/ml)

Table no.3 Linearity data of CLP and MLT

Sr. No.	CLP			MLT		
	Conc (µg/mL)	Mean Peak Area ± S.D. (n=5)	% R.S.D.	Conc (µg/mL)	Mean Peak Area ± S.D. (n=5)	% R.S.D.
1	20	25544 ± 2039.22	0.7983	1	12944 ± 90.270	0.6973
2	30	35384 ± 2201.77	0.6222	2	28339 ± 95.714	0.3377

3	40	49568 ± 2284.56	0.4608	3	47338 ± 104.08	0.2198
4	50	62674 ± 2302.25	0.3673	4	66578 ± 114.97	0.1726
5	60	79700 ± 2318.33	0.2908	5	90641 ± 125.94	0.1389

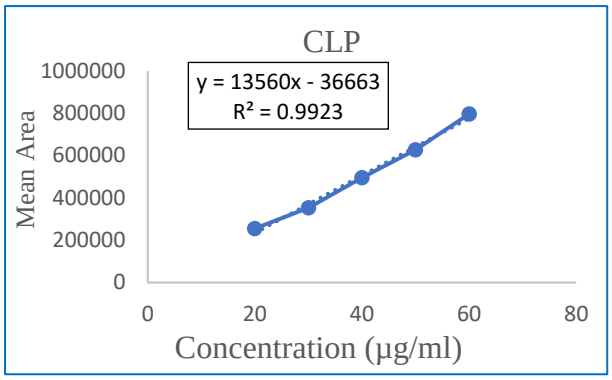


Fig.9 Calibration curve of CLP

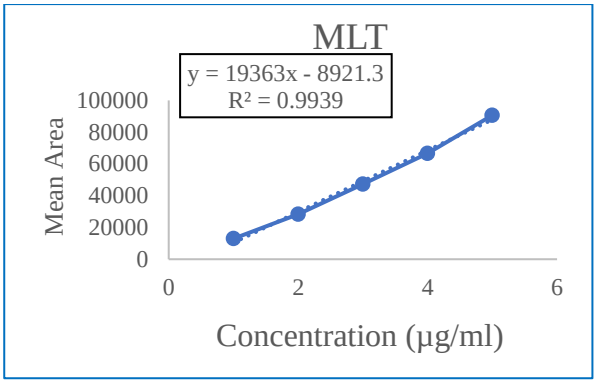


Fig.10 Calibration curve of MLT

4. Precision

a) Repeatability

The data of repeatability for CLP and MLT are depicted in

Table no.4 Repeatability data for CLP and MLT

Drugs	Concentration (µg/mL)	Mean Peak Area ± S.D. (n=6)	% R.S.D.
CLP	50	62674 ± 2532.4	0.4040
MLT	3	47421 ± 166.43	0.3509

b) Intraday and Interday Precision

The data for intraday Interday Precision for CLP and MLT are depicted in

Table no.5 Intraday precision for CLP and MLT

Drugs	Intraday Precision			Interday Precision		
	Conc. (µg/mL)	Mean Peak Area ± S.D. (n=3)	%R.S.D.	Conc (µg/mL)	Mean Peak Area ± S.D. (n=3)	%R.S.D.
CLP	40	496072 ± 3069.56	0.6187	40	496160 ± 3559.87	0.7174
	50	626776 ± 3166.62	0.5052	50	627971 ± 3935.25	0.6266
	60	796901 ± 3182.88	0.3994	60	798552 ± 3591.90	0.4498
MLT	2	28425 ± 163.62	0.5756	2	28442 ± 197.084	0.6929
	3	47366 ± 206.11	0.4351	3	47408 ± 242.229	0.5109
	4	66626 ± 211.24	0.3170	4	66736 ± 270.463	0.4052

5. ACCURACY

Table no.6 Accuracy data for CLP and MLT

Drugs	Level	Amount of sample (µg/ml)	Amount of std spiked (µg/ml)	Total amount	Mean PeakArea ± S.D.(n=3)	Amount of sample found (µg/ml)	% Recovery
CLP	0%	25	0	25	37442 ± 110.54	24.86	99.44
	80%	25	20	45	64583 ± 119.54	44.94	99.87
	100%	25	25	50	71281 ± 148.38	49.93	99.86
	120%	25	30	55	77469 ± 232.38	54.50	99.10
MLT	0%	1.5	0	1.5	32686 ± 156.03	1.48	99.06
	80%	1.5	1.2	2.7	61298 ± 186.03	2.69	99.95
	100%	1.5	1.5	3	66474 ±197.49	2.97	99.11
	120%	1.5	1.8	3.3	72648 ± 215.28	3.28	99.62

6. LOD and LOQ

The LOD for CLP and MLT was found to be 0.5187 µg/ml and 0.0131 µg/ml respectively.
The LOQ for CLP and MLT was found to be 1.5718 µg/ml and 0.0399 µg/ml respectively.

APPLICABILITY OF PROPOSED METHOD

Table no.7 Determination of Assay of CLP and MLT

TABLET	Amount taken (mg/tab)		Amount Obtained (mg/tab)		CLP %Purity ± S.D. (n=5)	MLT %Purity ± S.D. (n=5)
	CLP	MLT	CLP	MLT	CLP	MLT
	50	3	49.84 ± 0.0571	2.98 ± 0.0096	99.69 ± 0.1022	99.34 ± 0.3211

4. CONCLUSION

Based on the results, obtained from the analysis of CLP and MLT in their Tablet dosage form using RP-HPLC Method, it can be concluded that the method has linearity in the range of 20-60 µg/ml for CLP and 1-5 µg/ml for MLT. The regression coefficient (R^2) was found to be 0.9923 and 0.9939 for CLP and MLT and correlation coefficient (r) was found to be 0.9961 and 0.9969 for CLP and MLT at 225 nm respectively. Limit of detection for CLP and MLT were found to be 0.5187 µg/ml and 0.0131 µg/ml and Limit of quantification for CLP and MLT were found to be 1.5718 µg/ml and 0.0399 µg/ml respectively. The % assay was found to be 99.69 % and 99.34 % for CLP and MLT respectively. Further % R.S.D. was found to be less than 2 % for repeatability, intraday and Interday study. Thus, The result of Repeatability, Intraday and Interday variations with low value of % R.S.D. showed that developed methods were precise.

ABBREVIATION:

HPLC - High Performance Liquid Chromatography, CLP- Clomiphene Citrate, MLT-Melatonin, LOD - Limit of Detection, LOQ - Limit of Quantitation, ml – Milliliter, nm – Nanometer, R.S.D - Relative Standard Deviation, RP - Reverse phase, λmax - Maximum Wavelength.

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