CHAPTER 3

ANALYTICAL METHOD DEVELOPMENT

3.1 Introduction

Analysis of the drug substance to check the integrity, quality, and stability of the drug substances and products within a pharmaceutical industry to be administered to patient is carried out to satisfy both manufacturer companies and the regulatory authority. Chromatography is an analytical technique, which separate a mixture of solutes with help of a mobile phase and stationary phase, based on solutes affinities within the system

In last few decade high performances liquid chromatography (HPLC) became an analyst choice for the separation of pharmaceutical and chemical compounds and the quantitation of the active ingredient and related substances. HPLC method is preferred method especially for the estimation of samples with very low quantity of the drug. The unique property of HPLC to separate and measure the required constituents imparts it high specificity and selectivity desired in analysis of most of the pharmaceutical drugs.

Nicergoline,[(6a*R*,9*R*,10a*S*)-10a-Methoxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo [4, 3-*fg*] quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate Figure 3.1, inhibit the postsynaptic alpha(1)-adrenoceptors present on the vascular smooth muscle which in turn inhibits the vasoconstrictor catecholamine's such as epinephrine and nor epinephrine, resulting in peripheral vasodilatation. Nicergoline is generally available in market at a dose of 30 mg tablet (CHOLERGOL) and film-coated tab (NICERBIUM).

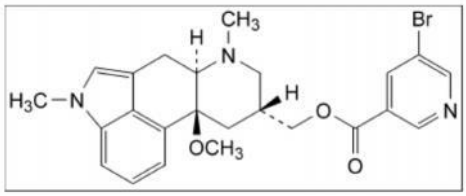

Figure 3.1 Structure of Nicergoline

For routine analysis a simple, rapid and cost effective analytical method is preferred. As literature survey there is no simple UV-spectrophotometric method and HPLC method available for estimation of Nicergoline in bulk drug formulations. Therefore, the objective of the present study was to develop simple, precise, accurate and economic analytical method with better detection range for estimation of Nicergoline in developed nasal formulations, and in vitro dissolution studies during optimization and development studies. The applications will include determination of drug loading, assay, entrapment efficiency in non-conjugated

and stroke homing peptide conjugated micelle of Nicergoline. Therefore it was proposed to develop a simple, reliable, cost-effective HPLC method to analyze Nicergoline.

Once the method is developed analytical method validation is now required by regulatory authorities for marketing authorizations and guidelines have been published. Analytical method has to be validated when it is necessary to verify whether its performance parameters are adequate for estimation of the drug. Analytical method must be validated, (a) when a new method is developed for a specific problem; (b) when indications of established method are changing with time; (c) when an established method is revised to include improvements or to extend it for another purpose; (d) when an optimized method is used in a different laboratory, or with different analysts or different instrumentation; (e) to prove the equality between two methods, i.e. a new method and a standard (1). Different existing reports explain guidelines for the validation of analytical methods, with some reports telling practical minimum necessities (2).Various validation parameters have been suggested by various regulatory agencies which include selectivity/specificity, accuracy, precision, linearity and range, LOD, LOQ, robustness (3, 4). The limits and acceptance criteria of which have been defined by a set of statistical tests and performance parameters. Nicergoline was extracted from micelle formulation using acetonitrile. The developed methods were validated as per ICH guidelines and USP requirements. Suitable statistical tests were performed on validation data. Thus, key in success of drug formulation is a good, rapid and sensitive analytical method developed to analyze the large number of samples collected in development studies. Hence, it is important to develop a rapid, reliable analytical method to accelerate the analytical process.

3.2 Materials and Instruments

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of Shimadzu LC-20 Prominence solvent delivery module, a manual Rheodyne injector with a 20µl fixed loop and SPD-20A Prominence UV-Visible detector. The separation was performed on a Thermo Scientific Corporation C18 column (particle size 5µm, length 250mm X ID 4.6mm; Thermo Scientific, India). Chromatographic data were recorded and processed using Spinchrome Chromatographic Station R CFR Version 2.4.0.193 (Spinchrome Pvt. Ltd., Chennai, India).

Table 3.1 Materials and equipment

Material	Source
Nicergoline	Gift samples from Ivax Pharmaceuticals S.R.O, Opava – Komarov, Cresch Republic.
Water (distilled)	Prepared in laboratory by distillation
TPGS 2000, Poloxamer F 127	Gift samples from gift sample from BASF Mumbai
Stroke Homing Peptide	Santa Cruz Biotechnology, Inc. U.S.A.
Potassium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, potassium hydroxide, sodium chloride, sodium hydroxide	S.D. Fine chemicals, Mumbai, India
HPLC grade methanol, Acetonitrile.	S.D. Fine chemicals, Mumbai, India.
Nuclepore Polycarbonate membrane 2 µm (Diameter 25 mm)	Whatman, USA
Equipments	**Source/Make**
Calibrated pipettes, volumetric flasks, Funnels beakers (250 ml) and other requisite glassware	Schott & Corning Ltd., Mumbai, India
Analytical balance	AX 120, EL 8300, Shimadzu, Japan
pH meter	Pico^{+}Labindia, Mumbai, India
Cyclomixer, magnetic stirrer	Remi Scientific Equipments, Mumbai
Cooling Centrifuge	3K 30, Sigma Laboratory centrifuge, Osterode, GmBH.
Stability oven	Shree Kailash Industries, Vadodara
HPLC	Shimadzu UV-1601, Japan
Vacuum Pump F16	Bharat Vacuum pumps, Bangalore
Bath sonicator	DTC 503, Ultra Sonics
Malvern particle size analyser	Malvern zeta sizer NanoZS, U.K.
Transmission electron microscope	Morgagni, Philips, Netherlands
Differential Scanning Calorimeter	Mettler DSC 20, Mettler Toledo, Switzerland

3.2.1 Selection of Chromatographic Conditions

Estimation of Nicergoline and its metabolite N-desmethylclobazam in biological fluids/tissues have been reported by many researchers (5, 6). With slight modification, the method mentioned in European pharmacopoeia was used for estimation of Nicergoline in formulations and *in vitro* diffusion medium.

3.2.1.1 Column

A C18 column was mentioned in European pharmacopoeia for the analysis of impurities of lipophilic compound Nicergoline. Thus, analysis was carried out using 5 micron C18 Thermo

Scientific (Thermo Scientific Corporation, India) (250 X 4.6 mm) analytical column. The column used gave good resolution in peak size and shape.

3.2.1.2 Mobile Phase

In HPLC method the mobile phase interacts with the drug molecules and stationary phase. The strength of interaction between drug, mobile phase and stationary determine the resolution and the efficacy and efficiency of the separation. Hence, selection of an appropriate mobile phase is important (7). Nicergoline has a log D/P values of 4.3 at pH of 7.4 indicates that the drug has sufficient lipophilicity to allow uniform retention on C-18 column at pH 7. If more than one alternative are available for compositions then the one which results in lower viscosity should be selected since viscosity more than 0.5cps (8) can decrease solvent diffusion coefficients and hence decrease column efficiency (7). Mixture of methanol and acetonitrile with water are generally known to result in such mobile phase giving uniform analyte retention. Since Nicergoline is stable drug only assay method was developed detecting only the presence of drug for which isocratic mobile phase is sufficient to yield required accuracy.

The mobile phase was prepared freshly every day. At the same time this will ensure instrument compatibility which requires that the mobile phase should not alter the characteristics of stationary phase, as it hampers the life span of the column. Further, solutions to be used for HPLC systems must be filtered through 0.45 μm membrane filter to remove any particulate matter to avoid column blockages (8). Air bubbles present in the mobile phase can also occlude in pump or the detector cell and result in irregular performance of the detector or an irregular pumping action. Hence, degassing was carried out using sonicator prior to use.

3.2.1.3 Flow Rate

Generally used mobile phase flow rate is 1-5 ml/min (9). On the other hand, slower flow rates help in increasing the life span of the pump and column and would thus be more cost-effective. Therefore, a flow rate of 1.0 ml/min was selected as reported method in monograph.

3.2.1.4 UV/Vis Detection

A wavelength of 288 nm was chosen for detection of nicergoline, since this region is generally free from solvent interferences and any peaks from excipients resulting in chromatogram with single drug peak with desired reproducibility and accuracy.

Table 3.2 Final chromatographic conditions

1.	Mode of detection	Reverse phase high performance Liquid chromatography
2.	Column	C_{18} (Particle size 5 µm, 4.6 mm x 250mm)
3.	Mobile Phase	MeOH:ACN:KHP 0.01M (pH 7) (50:30:20)
4.	Detector	UV spectrophotometer
5.	λ_{max}	288 nm
6.	Injection Volume	20 µl
7.	Run time	10 mins
8.	Flow rate	1 ml/min

3.3 Reagents

3.3.1 Phosphate Buffer

Potassium dihydrogen Phosphate buffer: 20mM potassium dihydrogen phosphate buffer pH 7 was prepared by dissolving 3.12gm of potassium dihydrogen phosphate in 1000 ml of distilled water and the pH was adjusted.

3.3.2 Stock Solution of Drug

Accurately weighed (10 mg) Nicergoline was transferred to 10 ml volumetric flask. Small quantity of Acetonitrile was added to ensure complete dissolution of Nicergoline and finally volume was made up to the mark with Acetonitrile (1 mg/ml solution).

3.3.3 Standard Solutions of Drugs

From the above solution, 5ml of solution was withdrawn accurately with the help of pipette and transferred to 50ml volumetric flask. Volume was made up to the mark with Acetonitrile to make stock solution (100µg/ml). A series of standard solutions with concentrations in the

range of 10-100 µg/ml were obtained by further dilution of the stock solution with Acetonitrile.

The mobile phase was filtered through a 0.2 µm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. Prior to injecting solutions, the column was equilibrated for 60 minutes with the mobile phase flowing through the system. Injections were made in the sequence of blank, placebo, reference standard and test solution. When injecting the test solution system suitability was performed by injecting six injection of reference standard to give an RSD of below 2% and peak parameters within the acceptance criteria. Each solution was injected in triplicate.

3.4 Preparation of Calibration Curve

Calibration curve of nicergoline was developed by preparing suitable dilutions from the stock solution to obtain solutions of concentrations 0.1, 2, 10, 20, 30, 40, 50, 80, 100 µg/mL in ACN. In order to develop a robust calibration equation averaging all modes of variation, three stock solutions were prepared on three different days by accurately weighing 10 mg of Nicergoline. From which triplicates of each concentration standards were prepared which gave a total value of n=9 for each calibration standard. The samples are sonicated for 15 minutes. Respective blank solutions were prepared accordingly. Analysis was done by using HPLC UV-detector.

3.5 Method Validation

The developed method was validated according to standard guidelines (1). Various validation parameter of the developed method were determined as per the protocols mentioned.

3.5.1 Specificity and selectivity

Specificity and selectivity of the proposed method was established by injecting diluents, placebo formulation and drug solution of 40µg/mL from the pure drug stock solution. The chromatograms of diluents and placebo were checked for any interference at Rt of drug and on the peak parameters.

3.5.2 Accuracy

Accuracy is a measure of the closeness between the true and measured values of a sample (10). Accuracy and bias were evaluated by making repeat measurements of three samples of varying concentration. For this purpose, different quality control (QC) levels of drug concentrations were selected from the calibration curve [lower quality control sample (LQC) = 7.5µg/mL, medium quality control sample (MQC1) = 17.5 µg/mL, MQC 2 = 77.5 µg/mL and higher quality control sample (HQC) = 87.5 µg/mL] were prepared from independent stock solution and analyzed (n=3). Accuracy was assessed by calculating mean percentage recovery and percentage bias (% bias). % Bias was calculated as,

$$\% \text{ Bias} = [(\text{predicted conc.} - \text{Nominal conc.})/\text{Nominal conc.}] \times 100.$$

The accuracy was further supported by performing method of standard addition. In this study, different concentrations of pure drug in acetonitrile were added to a known pre-analyzed formulation sample and the total concentration was determined using the proposed methods (n = 3). The percent recovery of the added pure drug was calculated as, % Recovery = [(Cv – Cu)/Ca] × 100, where Cv is the total drug concentration measured after standard addition; Cu, drug concentration in the formulation; Ca - drug concentration added to formulation. Percentage recovery for nicergoline by the proposed method was found in the range of 98.95 to 101.74%. The results have been reported in (**Table 3.4 and Table 3.5**)

3.5.3 Linearity

The linearity of the analytical method is ability to obtain the test results which are directly proportional to the concentration of the analyte in the sample. To establish linearity of the proposed method, nine separate series of solutions of the drug (0.1–100 µg/ml in acetonitrile were prepared from the stock solutions and analyzed.

3.5.4 Range

The range is interval between the lower and upper analyte concentration, for which it has been demonstrated that the analytical procedure has a suitable level of accuracy. The range is obtained from the linearity studies. The % RSD at each concentration selected should be less than 2%.

3.5.5 Precision

Repeatability was determined in terms of System precision, Method precision and Intermediate precision. The system precision was determined as repeatability of the six replicates of standard solution (40 ppm) and noting the effect on peak parameters i.e. tailing factor, % RSD and confidence interval. In method precision a homogenous sample of a single batch was injected six times to check whether the method is giving consistent results for a single batch overcoming the variables of drug extraction procedure and sample processing. Intermediate precision was carried to determine the degree of reproducibility of the test results obtained at different conditions e.g. time, analyst etc. The % RSD and influence on peak parameters was checked. The values of RSD were calculated to determine the precision (**Table 3.6**). Percent RSD for system precision, method precision and intermediate precision are 1.29, 1.25 and 1.07 respectively. The low % RSD values revealed that the proposed method is robust.

To determine the amount of NG entrapped in the micelle, 4 mg/ml of micelle were added to 4 ml of acetonitrile and subjected to shaking at room temperature for 30 min for complete disruption of micelle and removal of drug from the micelle. The resulting system was centrifuged at 5000 rpm for 15min to remove the precipitated components. The supernatant was further diluted suitably with acetonitrile and estimated using developed HPLC method.

3.5.6 Limit of detection (LOD) and quantification (LOQ)

LOD is the lowest analyte that is detectable above the baseline noise of the system and LOQ is a measure of the level of analyte that can be measured with the required accuracy and precision (10). The USP method was selected for the determinations of the requisite validation parameters. This method describes the LOQ as having a signal to noise ratio of 10:1 and the LOD as having a signal to noise ratio of 2:1 or 3:1. Although, this concept is widely used, it must be noted that these values are likely to vary with changes in detector, which may include deterioration of the detector lamp on prolonged use (11). Repeat measurements (n=6) of blank injections were performed and height of response at retention time of drug was taken as noise. The DL and QL of Nicergoline by the proposed methods were determined using calibration standards. DL and QL were calculated as 3.3r/S and 10r/S,

respectively, where S is the slope of the calibration curve and r is the standard deviation of y-intercept of regression equation (12).

3.5.7 Robustness

The robustness of an analytical method is its capacity to remain unaffected by small variations in method parameters carried out purposely. The variations involve the change in pH, volume, flow rate, column temperature (13). A known concentration was injected at following conditions and effects on % recovery, % RSD were noted.

i. pH : ± 0.2 units
i. Volume
    a. 51:29:20
    b. 49:31:20
    c. 51:30:19
    d. 49:30:21

ii. Flow rate: ± 0.2 ml/min

3.5.8 Solution Stability

The solution stability was performed to determine reproducibility of the method when using standard and sample solutions after date of preparation. The % RSD with respect to initial concentration was reported. The results are reliable up to time point with < 5% RSD in case of assay method.

3.6 Result and Discussion

3.6.1 Method Development

As per the pharmacopoeial method ACN: MeOH: KHP were used at the ratio of 30:35:35 giving a drug peak at 27 min. In order to reduce the retention time of drug buffer content was decreased and organic content in the mobile phase was increased which leads to decrease in retention time to around 8 min. The ratio of aqueous phase: organic phase plays an important role with respect to retention time and optimal proportions of ACN: MeOH: KHP were determined by assessing the impact of varying the organic composition of the mobile phase. Increase in MeOH in mobile phase composition resulted in decreased retention time for nicergoline; however, still broader peaks were obtained. By adjusting the ratio of ACN:buffer

good resolution and sharp peaks were observed. The proportion of MeOH: ACN:KHP 0.01 M pH 7 was selected as optimal in the ratio of 50:30:20%v/v. Prior to use the mobile phase was filtered through a 0.45 µm filter and degassed by ultrasonication. But, still the shape of peak was not sharp. In some reports it is proved that molarity of buffer plays an important role in retention time as well as peak shape. It was observed that as we go from 0.05 M KHP to 0.01 M KHP we got sharp, well resolved peaks for nicergoline exhibiting no tailing. As per pharmacopoeial method when pH of buffer was made using triethylamine to pH 7.0 and it produced peaks with good shape and resolution in a short retention time.

The variables investigated indicate that buffer molarity and ratio of organic: aqueous phase plays a significant effect on peak shape and retention time of Nicergoline in solution. The choice of a suitable wavelength is also important, in terms of method precision, selectivity and sensitivity. The chromatographic conditions were optimized to yield a well-resolved peak with a reasonable retention time. Theoretical plates were > 5000, meaning that the method has good resolution.

3.6.2 Calibration Standard

Area for different drug concentrations is shown in Table 3.3. At all concentration levels the SD was low and the % RSD was less than 2.12. Linearity range was found to be 2 – 100 µg/ml. The linear regression equation obtained was y = 11.25x + 0.500; with regression coefficient of 0.999 (Figure 3.2,Figure 3.3).

Table 3.3 Peak area for different concentration of nicergoline

Concentration (µg/ml)	Area	SD	%RSD
2	22.307	0.47	2.12
10	113.364	0.90	0.79
20	224.499	2.81	1.24
30	336.716	4.14	1.23
40	451.149	3.62	0.81
50	561.108	3.94	0.70
80	897.578	2.07	0.23
100	1130.699	2.16	0.19

*n=3

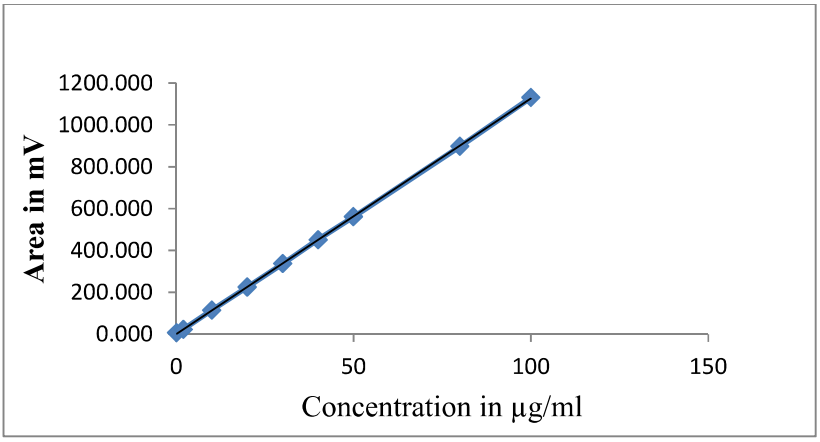

Figure 3.2 Calibration curve of Nicergoline.

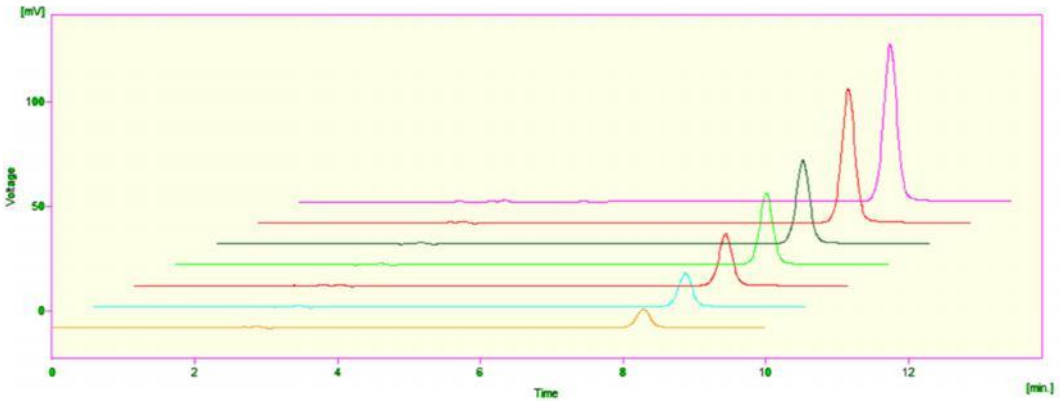

Figure 3.3 Chromatogram of calibration curve.

3.6.3 Accuracy

All three QC levels (LQC, MQC and HQC) showed an accuracy (% bias) ranging from -0.743 to 0.069. The mean % recovery values and their low SD values represent the accuracy of the method (**Table 3.4**). In the standard addition method, the mean % recovery analyte were in the range of 99.33 to 102.13 (**Table 3.5**). This result further established the validity and reliability of the proposed method.

Table 3.4 Accuracy data of developed method

Level	Theoretical Concentration µg/ml	Observed Concentration µg/ml	% Mean recovery	% RSD	Bias (%)
LQC	7.50	7.51	100.06	2.01	-0.133
MQC 1	17.50	17.63	99.33	2.19	-0.743
MQC 2	77.50	77.48	102.13	1.87	0.026
HQC	87.50	87.44	99.93	2.73	0.069

Table 3.5 Standard addition data of developed method

Conc. of drug in formulations (µg/ml)	Conc. of pure drug added (µg/ml)	Observed conc. of drug (µg/ml)	% Mean Recovery
7.5	2	9.4	98.95
7.5	4	11.7	101.74
7.5	6	13.3	98.52
7.5	10	17.4	99.43
7.5	12	19.6	100.51

3.6.4 Precision

In the repeatability study, % RSD ranged from 1.29 to 2.00. % RSD values were significantly low for system precision with values of 1.29 signifying that HPLC system had high level of repeatability. On the other hand the method precision showed that analyte could be reproducibly determined from a single batch with the developed sample processing method. Finally, intermediate precision, with intra-day variation not more than 2.94 % and inter-day variation not more than 2.06% (**Table 3.6 and Table 3.7**), lower % RSD values indicated intermediate precision of the method.

Table 3.6 Data of system precision and method precision study

Sr. No	Area of system precision samples injected	Area of method precision samples injected
1.	461.22	465.26
2.	452.71	444.82
3.	450.64	458.26
4.	456.37	462.02
5.	448.62	454.82
6.	444.74	442.91
Mean	452.38	454.68
SD	5.82	9.103
RSD	1.29	2.00
Confidence interval	446.27 – 458.49	440.92 – 470.11

Table 3.7 Results of intermediate precision

Conc.(µg/ml)	Observed Conc. (µg/ml) ±SD		% RSD	
	Intraday Precision	**Inter day Precision**	**Intraday Precision**	**Inter day Precision**
7.50	7.35 ± 0.216	7.62 ± 0.200	2.94	2.06
17.50	17.05 ± 0.323	17.81 ± 0.149	1.90	0.84
77.50	77.11 ± 1.472	77.52 ±0.992	1.92	1.28
87.50	86.30 ± 1.521	87.59 ± 1.173	1.73	1.34

3.6.5 LOD and LOQ

DL and QL were found to be 1.55µg/ml and 4.71µg/ml, respectively. The % RSD of the area responses are less than 2% (**Table 3.8**).

Table 3.8 LOD and LOQ determination

Sr. No	Blank (Height of Peak at Drug Rt)
1.	0.114
2.	0.102
3.	0.115
4.	0.099
5.	0.109
Mean	0.107

3.6.6 Robustness

The method was found to be robust as variation of flow rate, pH, mobile phase composition did not affect % recovery significantly. Similarly variation in the analytical instrument did not affect % recovery significantly, so the proposed method was found to be rugged as well (**Table 3.9 and Table 3.10**).

Table 3.9 Data of robustness of developed method

Parameters	Sample	% Recovery	RSD
Flow rate	0.8 ml/min	99.61 ± 1.64	1.65
	1.2 ml/min	101.88 ± 1.82	1.79
pH of mobile phase	pH 6.8	98.38 ± 1.54	1.57
	pH 7.2	99.53 ± 1.49	1.50
Mobile phase composition (MeOH:ACN:KHP 0.01M (pH 7)	51:29:20	100.73 ± 1.71	1.70
	49:31:20	99.28 ± 1.27	1.28
	51:30:19	100.59 ± 1.98	1.97
	49:30:21	101.01 ± 1.66	1.64

Table 3.10 Data of ruggedness of developed method

Conc.(µg/ml)	Mean % Recovery ± SD			
	Instrument I		Instrument II	
	Mean % Recovery	SD	% Mean Recovery	SD
7.5	103.03	2.98	102.957	1.39
17.5	105.86	3.82	101.986	3.78
67.5	99.63	0.54	99.150	1.35
77.5	97.97	0.57	98.219	1.16
87.5	99.32	0.75	99.648	1.20

3.6.7 Solution Stability

The results of solution stability (**Table 3.11**) showed that prepared samples were stable since % RSD was less that 5 % with respect to initial sample. Therefore the samples can be analyzed after storing for 48 hr.

Table 3.11 Data of solution stability study

Sample	% RSD with respect to initial
Initial	-------
8 hr	1.5
16 hr	2.1
24 hr	2.5
36 hr	2.8
48 hr	3.3

3.7 Conclusion

The developed analytical method was found to be accurate with respect to all the validation parameters and therefore was considered suitable for the analysis of the NG during the course of formulation development of micelle nano-constructs to determine drug loading, assay, entrapment efficiency of Nicergoline.

3.8 References

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