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HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF NIFEDIPINE IN RABBIT PLASMA

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ABSTRACT

A simple, accurate, precise, sensitive, and reproducible isocratic high-performance liquid chromatography method was developed for the determination of Nifedipine in rabbit plasma. Liquid–liquid extraction was used as the sample preparation technique. Carbamazepine was employed as an internal standard (IS). Chromatographic separation was achieved on a Phenomenex C_{18} (5 × 4.0 mm, 5 µm particle size) column using Shimadzu LC20AT system with LC20AD pump at room temperature in isocratic mode. The column effluent was monitored by UV detector (ultraviolet variable wavelength detector, Model SPD-20A Shimadzu, Japan) at 235nm. The mobile phase used was water: acetonitrile:Triflouro acetic acid in the ratio of 40:60:0.1 (v/v/v) at a flow-rate of 1.0 mL min^{-1}. Nominal retention times of Nifidipine and IS were 5.5and 3.8 mins, respectively, with a total run time of 8 min. Method validation was performed according to US Food and Drug Administration bioanalytical guidelines and the results met the acceptance criteria. The calibration curve of Nifidipine in rabbit plasma was linear over the concentration range of 2–1000 ng $mL^{-1}$with a regression coefficient of 0.999. The recovery was about 100.5%and the limit of quantitation (LOQ) was 1.36 ng/mL. The LOD was found to be 4.12 ng/mL .Intra- and inter-run precisions of Nifidipine at a working concentration of 100ng/mL were exemplified with a % RSD of 0.569 and 0.69% respectively. The method was found to be precise, accurate, robust , rugged and specific during the study and is useful for invivo studies of Nifedipine.

Keywords – *Nifedipine, Bioanalytical, Rabbit Plasma , HPLC, Method development, Validation*

1. INTRODUCTION

Nifedipine (NFD) chemically known as 1, 4-dihydro-2, 6-dimethyl-4-(2nitrophenyl)-3, 5-pyridine dicarboxylic acid dimethyl ester is the lead compound of the hydropyridine class of calcium-channel antagonists. It is widely used in the treatment of angina pectoris, hypertension,and other vascular disorders suchas Ray-naud's phenomenon[1-2] .Several methods for the determination of NFD *in vitro* and in human plasma have been described in the literature. This includes a UV spectrophotometer[3] and High performance liquid chromatography (HPLC) with UV, [4-17]Voltammetry[18-20] and spectrophotometry.[21-22] Literature survey revealed that many works have been reported for the estimation of Nifedipine using carbamazepine as internal standard by various analytical techniques like HPLC in

canine plasma, LC-MS/MS-ESI method for simultaneous determination of nifedipine and atenolol in human plasma,[23] HPTLC in human serum,[24] Nifedipine and Atenolol in a Capsule Formulation.[25]

HPLC methods have been widely used for routine quality-control assessment of drugs, because of their sensitivity, repeatability, and specificity.[26] Keeping in view of this, an attempt has been made to develop a easy and convenient RP-HPLC method with simple, precise, accurate, economical method for the estimation of nifedipine in rabbit plasma using carbamazepine as internal standard, which not only useful for researcher but also for the analysts working in the pharmaceutical quality control labs. The RP-HPLC method reported in this study was validated in accordance with the International Conference on Harmonization (ICH) guidelines[27] and best practice. [28-29] Specificity, linearity, precision (repeatability and intermediate precision), accuracy, robustness, limit of detection and limit of quantitation were evaluated

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Pure Nifedipine (NFD) and Carbzamazepine(CBZ) (Internal standard) were obtained as gift samples from, Hetero Drugs ltd., Hyderabad, India, all solvents used were of HPLC grade obtained from Merck, Mumbai India. The chemicals, were of analytical grade purchased from S. D. Fine Chemicals. High purity water was prepared through a Milli-Q water purification system (Millipore, Bedford, MA).

2.2 HPLC instrumentation and settings

HPLC was performed with a SHIMADZU LC20AT system,with LC20AD pump. The HPLC system was equipped with Lab-Solution data acquisition software. The drug and internal standard were optimally resolved on the stationary phase Octa decyl silane (C_{18}) packing with length 250 mm, internal diameter 4.6 mm (Phenomenex) and particle size 5 µm.. The guard column was Thermo C_{18} (5×4.0 mm, 5 µm particle size). The Mobile phase used for analysis was composed of water, acetonitrile,Triflouro acetic acid (TFA) in the ratio of 40:60:0.1 (v/v/v).The mobile phase was injected into the HPLC apparatus at flow rate of 1ml/min (isocratic pump, Model LC-20AD, Shimadzu, Japan). The detection wave length was 235nm (Ultraviolet variable wavelength detector, Model SPD-20A Shimadzu, Japan). All assays were performed at ambient temperature (30°C).

2.3 Preparation of standard stock and sample solutions

2.3.1 NFD Standard Sample

The standard stock solutions of NFD and internal standard (Carbamazepine) were prepared in Water: Acetonitrile: TFA(60:40:0.1) at a concentration of 1000 ng/mL. The solutions were protected from light with an aluminum foil wrapping and usually freshly prepared solutions were used. A series of spiking standard solutions were prepared in Water: Acetonitrile: TFA(60:40:0.1) from dilutions of the stock to cover the quantitation linear range from 2–1000 ng/mL. A 100 ng/mL solution containing the IS Carbamazepine was also prepared in Water: Acetonitrile: TFA(60:40:0.1) Calibration standard was prepared by spiking a pool of drug free plasma (50 µL for each concentration) with the working standard solution and it was diluted to desired concentration range from 2 to 1000 ng/mL.

2.3.2 Sample treatment and liquid-liquid extraction method

Liquid-liquid extraction (LLE) method was used for sample preparation because of relatively low cost, good extraction efficiency as well as simple procedure. [30] The extraction was carried out with little modidifcations as reported in earlier methods .[31] Rabbit blood samples were allowed to clot and then centrifuged at 3,000 rpm for 10 min. The obtained plasma samples were deep-frozen at 20°C. Aliquots of 1 ml of plasma was taken into a 4-ml centrifuge tubes and protein precipitation was carried out using 100 µL (1.0 M) Hydrochloric acid. . The sampling was carried out under extremely subdued light and all tubes and syringes were wrapped in aluminum

foil because of the photoliability of nifedipine Then, 100 µL of carbamazepine as internal standard was added .Each of the test or standard plasma samples were treated as follows. The samples were vortexed for 1 min. in a 5 mL glass vial and 4 mL of ethyl acetate was added, and vortexed for 30 s, then centrifuged for 10 min at 4,000 rpm. The organic layer was decanted into clean centrifuge tubes and evaporated to dryness at 45°C. The residue was reconstituted with 100 µL of mobile phase, and then 20 µl was injected into the HPLC system using autosampler (SPD-20A Shimadzu, Japan)

2.4 Method validation

The method was validated according to internationally acceptedrecommendations (US Food and Drug Administration,2012)[32]. The method was validated in terms of linearity, precision,accuracy and extraction recovery.

2.4.1 System Suitability

System suitability was determined from sixreplicate injections of the system suitability standard before sample analysis, as the chromatographic systems used for analysis must pass the system suitability limits before sample analysis. The HPLC system was allowed to stabilize for 40 min. Then blank preparation (single injection) and standard preparation (six replicates) are injected. Then, the chromatograms are recorded to evaluate the system suitability parameters like resolution, tailing factor, theoretical plate count and % RSD for peak area [33]

2.4.2 Specificity/ Selectivity

Sensitivity was determined by analyzing control rabbit plasma in replicates (*n* = 6) spiked with the NFD at the lowest level of the calibration standard, that is 2ng/mL. to ensure no interfering peak of NFD and I.S. from endogenous plasma components[34]

2.4.3 Linearity and sensitivity

Calibration curves were plotted as the peak area ratio (drug/internal standard) versus the concentration of NFD in plasma. .Least-squares linear regression method was used to determine the slope, intercept and correlation coefficient. [35]

The limit of detection (LOD), the lowest concentration in a sample that can be detected from background noise but not quantitated was determined using the signal-to-noise ratio (S/N) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples.LOD was calculated by using the Eq. [1]

$$DL=3.3\ \sigma/S \quad \ldots\ldots\ldots \ldots Eq[1]$$

where DL is the detection limit, σ is the standard deviation of intercepts of the regressed lines and S is the average slope of the regression line.

The LOQ the lowest concentration of analyte that can be determined with acceptable precision and accuracy was found by analyzing a set of mobile phase and plasma standards with a known concentration of NFD.[38] LOQ was calculated by the Eq. [2]

$$DQ=10\ \sigma/S \quad \ldots\ldots\ldots\ldots\ldots Eq[2]$$

where DQ is the limit of quantification.[37]

2.4.4 Accuracy/Recovery

Accuracy is the degree of agreement between a measured value and the accepted reference value. The accuracy of the method was tested by triplicate samples at 3 different concentrations of 50, 100,150ng/mL .The recovered amount of NFD, %RSD of recovery and % recovery of each concentration were calculated to determine the accuracy. [38]

2.4.5 Precision

The precision of the method was checked by inter day and intraday repeatability and reproducibility. Six replicate injections(n=6) of the standard solution at working concentration of 100ng/mL were injected and % RSD (Relative Standard Deviation) concerning peak area

of the drug was calculated to find system precision whereas method precision was determined by performing assay of sample by the tests of following,

(i) Repeatability (Intra day precision)

Six consecutive injections(n=6) of the sample solution from the same homogeneous mixture at working concentration of 100ng/mL on the same day under similar conditionswere injected into the HPLC system. % RSD for % assay of the drug was caluclated

(ii) Intermediate Precision (Ruggedness / Inter day precision)

Six consecutive injections(n=6) of the sample solution from the same homogeneous mixture at working concentration of 100ng/mL on three consecutive days by three different analysts, were injected and % RSD for % assay of the drug within and between days was caluclated.[98-41]

2.4.6 Robustness

Robustness was evaluated by changing the flow rate by ±0.2 mL, and wavelength by ±1 nm, and analyzing the samples by making above changes in HPLC method. The factors of robustness study are summarized in Table 1.

Table 1: Parameters of Robustness Study

Parameter	Normal	Low	High
Wave length(nm)	235	232	236
Flowrate(ml/min)	1	0.8	1.2

3. RESULTS AND DISCUSSION

3.1 Optimisation of the HPLC method

A novel HPLC method was optimized by using the different compositions of mobile phase, which affected the separation of NFD and I.S. In the present study various mobile phase compositions such as methanol: water, methanol: water: acetonitrile, acetonitrile: water, water: acetonitrile: methanol:triflouro acetic acid were tried but peak did not separate properly. Finally the optimised chromatographic separation of NFD was achieved by using water: acetonitrile:triflouroacetic acid buffer (60: 40:0.1, v/v/v), with a flow rate of 1 mL/min. The optimised chromatographic conditions for the proposed RP-HPLC method were given in **Table 2**

Table 2: Optimized chromatographic conditions of the proposed RP-HPLC method for Nifedipine

Parameter	Chromatographic conditions
Instrument	HPLC SHIMADZU LC20AT system
Column	PhenomenexC_{18} (5×4.0 mm, 5 µm particle size).
Detector	SPD-20A UV-Visible detector
Mobile phase	Water:Acetonitrile:Triflouro acetic acid (40:60:0.1) (v/v/v).
Detection wave length	235 nm
Temperature	Ambient(30°C)
Flow rate	1mL/min
Vol. of Injection loop	20µL
Pump	Model LC-20AD, Shimadzu, Japan
Guard column	Thermo C_{18} (5×4.0 mm, 5 µm particle size).
Diluents Elution	Mobile Phase Isocratic.

3.2 Validation of the analytical method

The method was validated for selectivity, sensitivity, linearity, precision, accuracy and recovery .

3.2.1 System suitability

System suitability was evaluated by replicate (n = 6) injection of the same standard solution containing NFD and the IS.The system suitability assessment for the analytical HPLC method was established by instrument performance parameters such as peak area %RSD, total plate number, USP tailing factor. The number of theoretical plates and the USP tailing factor were within the acceptance criteria of >2000 and ≤2, respectively, indicating the good column efficiency and optimum mobile phase composition as per the data shown in **Table 3**.

Table 3: System suitability data of Nifidipine by RP- HPLC method.

Parameter	Observed value	Acceptance criteria
Peak area (mV.s) ±%RSD	9629.366±0.26	< 2% RSD
Retention time (min)	5.383	-
Tailing factor (Tf) (Assymetry)	1.725	< 2%
Theoretical plates [th.pl] (Efficiency) (N)	2622	> 2000
Theoretical plates per meter [t.p/m]	47,500	-
Resolution	3.755	>2

3.2.2 Specificity

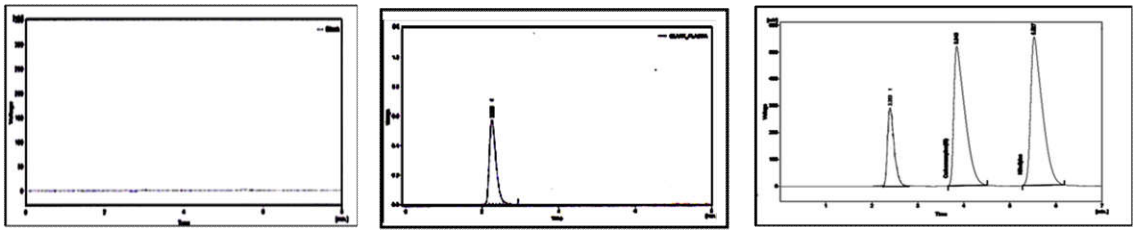
The specificity was evaluated by comparing the chromatogramsof blank plasma and spiked plasma samples (2ng/mL).Comparison of spiked plasma samples and blank plasma showed no significant interfering peaks with the quantification of the analyte and I.S. and the representative chromatogram of plasma samples showed similar chromatographic behaviours as shown in **Fig.1.** The retention times for NFD and Carbamazepine (Internal standard) were approximately 5.5 and 3.8 minutes,respectively. The peaks were sharp and symmetrical with good baseline resolution and minimal tailings, thusfacilitating accurate measurements of the peak-arearatios. No elute of endogenous serum components were found at the retention time of NFD or internal standard.

3.2.3 Linearity and sensitivity

Calibration curve was constructed using peak-area ratio for the NFD and Carbamazepine as a function of the concentration added as shown in **Fig. 2**.The calibration curve was linear with regression correlation coefficients greater than 0.99 (r^2 = 0.999) over the concentration range of 1.010–1000 ng/mL as per the data reported in the **Table 4**.The typical equation (3) of the calibration curve is

y = 0.011 x + 0.013.....**Eq[3]**

Where x represents the rabbit plasma concentration of NFD and y is the peak area ratio of NFD to I.S.. The LOD value for NFD was found to be 76.8ng/mL and LOQ (*n* =6) was 232.76 ng/mL.



a. b. c.

Fig.1: Chromatogram of (a) Blank (b) blank plasma of rabbit (c) Plasma spiked with Nifedipine and Internal standard(CBZ)

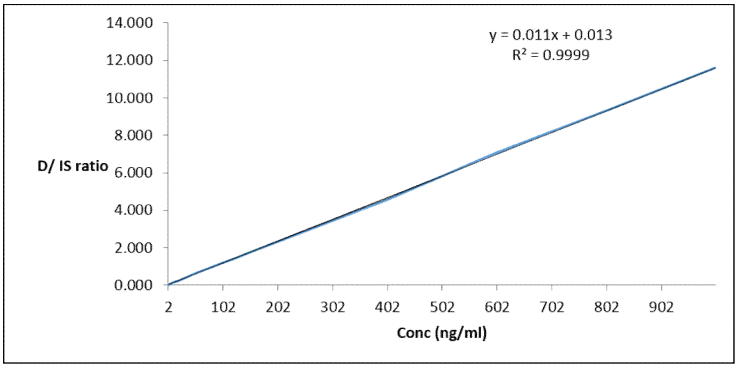


Fig 2: Calibration Curve of Nifedipine

Table 4: Linear regression data of the proposed HPLC method of Nifedipine :

Parameter	Value
Linearity range (µg/mL)	2-1000 ng/ml
Regression equation	Y = 0.011X + 0.013
Slope(b)	0.011
Intercept(a)	0.013
Regression coefficient (r^2)	0.9997
LOQ	1.36 ng/ml
LOD	4.12 ng/ml

3.2.4 Accuracy/Recovery

Approximately 100.5% recovery of NFD was found at three different concentrations 50,100,150ng/mL as per the data shown in the Table 5,which confirms that thes simple LLE procedure efficiently extracts analyte as well as IS from rabbit plasma.

3.2.5 Precision

The percent relative standard deviation (% RSD) was within the acceptable criteria of not more than 2.0, indicated that the method is reproducible and good with high precision. The low RSD values indicated the high degree of correctness of method and reflected the inter-day reproducibility as per Table 5.

Table 5: Data of Recovery/Accuracy of the Method

Levels	Amount added (ng/mL)	Nifedipine			Carbamazepine(IS)		
		Amount recovered Mean±SD (n=3)	%Mean Recovery±SD (n=3)	%RSD	Amount recovered Mean±SD (n=3)	%Mean Recovery±SD (n=3)	%RSD
50%	50	50.239±0.008	100.477±0.017	0.017	50.25±0.008	100.499±0.016	0.016
100%	100	100.5±0.007	100.5±0.007	0.007	100.502±.01	100.502±.01	0.01
150%	150	150.744±0.06	100.496±0.004	0.004	150.752±0.008	100.502±0.006	0.005

a) System precision

Six replicate injections of the standard solution at working concentrationof 100 ng/mL have shown peak area of the drug with % RSD (Relative Standard Deviation) less than 2 which indicated the acceptable reproducibility and thereby the precision of the system as shown in Table 6.

b) Method precision (Intra day precision)

Six consecutive injections of the sample from the same homogeneous mixture at working concentration of 100 ng/mL showed % assay of NFD with % RSD less than 2 (Table 6) indicated that the method developed is more precise by the test of repeatability (Intra day precision) and hence can be understood that the method gives consistently reproducible results.

c) Intermediate Precision (Ruggedness / Inter day precision)

Six consecutive injections of the sample solution from the same homogeneous mixture at working concentrationof 100 ng/mL on three consecutive days by three different analysts, showed % RSD less than 2 for % assay of the drug within and between days, which indicated the method developed is inter day precise / rugged as per ther data given in the Table 7.

Table 6: Data of System precision and Intraday precision

N	RT(min)	Area(mV.s)	%assay of Nifedipine
1	5.38	9729.366	95.232
2	5.41	9719.642	96.568
3	5.34	9761.950	95.841
4	5.30	9734.101	96.756
5	5.31	9785.23	96.075
6	5.57	9753.75	96.271
Avg	5.385	9747.34	96.124
S.D	0.0997	24.3134	0.547
%RSD	1.8524	0.24940	0.569

Table 7: Data of Interday precision

N	% Assay of Nifedipine		
	Day1 Analyst 1	Day2 Analyst 2	Day 3 Analyst3
1	95.26	95.07	95.36
2	96.55	96.58	96.58
3	96.14	99.59	95.79
4	96.75	96.84	96.69
5	96.02	96.06	96.14
6	96.02	96.4	96.39
Avg	100.12	96.09	96.16
S.D	0.539	0.66	0.505
%RSD	0.54	0.69	0.52

3.2.6 Robustness

It was observed that there were no marked changes in chromatograms with the small changes in analytical parameters like change in wavelength and flow rate. It demonstrated that the developed method was robust in nature and reliable based on the results of robustness study given in Table 8.

Table 8: Results of robustness study

Chromatographic conditions	Normal	Variation	RT(min)	Area(mV.s)	%Assay
Wavelength (nm)	235	232	5.51	9484.992	96.45
		236	5.51	9684.992	96.16
Flowrate (ml/min)	1	0.8	5.59	9582.627	96.62
		1.2	5.51	9684.992	95.81

4. CONCLUSION

The present study aimed at the development and validation of an isocratic HPLC method for the quantitative analysis of NFD in rabbit plasma using carbamazepine as internal standard.The extraction of NFD was done by a rapid liquid–liquid extraction method. The method was validated as per USFDA guidelines in terms of specificity, accuracy, precision, linearity, robustness, limit of detection and limit of quantitation. The method was found to be accurate and precise, as indicated by recovery studies close to 100 and %RSD is not

more than 2. The developed method was short with faster run time /elution of NFD less than 6 min and specific with no interferences of blank matrix interfering with the quantification of NFD.The method was proven to be highly sensitive and linear making it suitable and attractive procedure in high-throughput bioanalysis of NFD suggesting its further applicability in pharmacokinetic linearity studies.

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