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DETERMINATION OF NIFEDIPINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY AND ITS VALIDATION

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ABSTRACT

A selective, rapid and sensitive high-performance liquid chromatography–tandem mass spectrometry (HPLC -MS/MS) method was developed and validated for the quantification of nifedipine in human plasma. Nimodipine was used as an internal standard, sample pretreatment involved a simple solid phase extraction using HLB Cartridge. The analysis was carried out on a Hypersil, gold C18 column (50 mm×4.6 mm, 5µ) with flow rate of 0.6 mL/min. The mobile phase was consisting of 0.1% formic acid buffer and acetonitrile in the ratio of 30:70 v/v. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode. Linear calibration curves were obtained in the concentration range of 0.5 to 100 ng/mL. The intra- and inter-day precision (R.S.D.) and accuracy results were within the accepted limits as per USFDA guidelines.

KEYWORDS: Nifedipine; Nimodipine; Solid–phase extraction (SPE); LC–MS/MS; Multiple reaction monitoring (MRM) mode; Method validation.

INTRODUCTION

Nifedipine has been formulated as both a long- and short-acting 1,4-dihydropyridine calcium channel blocker with a synonym of 1, 4-dihydro-2, 6-dimethyl-4-(2-nitrophenyl)-3, 5-pyridine dicarboxylic acid dimethyl ester.[1] It acts primarily on vascular smooth muscle cells by stabilizing voltage-gated L-type calcium channels in their inactive conformation. By inhibiting the influx of calcium in smooth muscle cells, nifedipine prevents calcium-dependent myocyte contraction and vasoconstriction. A second proposed mechanism for the drug's vasodilatory effects involves pH-dependent inhibition of calcium influx via inhibition of smooth muscle carbonic anhydrase. Nifedipine is used to prevent certain types of chest pain (angina).[2] Nifedipine, a highly non-polar compound, is absorbed completely from the gastrointestinal tract, predominantly from the jejunum, but has a very low bioavailability mainly due to pre-systemic metabolism.[3] According to Kleinbloesem et al., 1984, following absorption, nifedipine is further metabolized in the small intestine and liver to more polar compounds which are primarily eliminated by the kidney.[4] As per earlier reports nifedipine is a photolabile compound, undergoing oxidative biotransformation in human body into pharmacologically inactive metabolites (Ohkubo et al., 1992).[5] Acording to Schmid et al., and Akira et al., 1988,[6-7] the earlier analytical methods for determining nifedipine levels in biological fluids were either gas chromatographic (GC) alone or combined with spectrometric analysis.[8] Even though some of these methods required microliter volumes and their lower limit of detection could go as low as 2 ng/mL, they suffer from a lack of specificity and selectivity. An efficient bioanalytical method should rapid, simple and consume less sample volume for analysis. Also, it should be specific and selective to avoid possible inferences at mass transition of analyte and the internal standard.[9-29] The reported methods were suffering from lack of sensitivity, use of more sample volume, longer chromatographic run time and employs non-deuterated compounds as internal standards which may results poor precision and accuracy values, where compensation for matrix effect is not possible.

In the present work, recently developed high-performance liquid chromatography–tandem mass spectrometry (HPLC -MS/MS) method for the determination of nifedipine in human plasma has been established. A better method with reduced ion suppression/enhancement, superior sensitivity, satisfactory resolution and selectivity was observed when compared with the published methods.

EXPERIMENTAL

Reagents and chemicals

Working standards of nifedipine (99.0%) and nimodipine (98.2%) were obtained from Sigma Aldrich (Bangalore, India). Chemical structures of nifedipine and nimodipine were shown in Fig. 1. LC-MS grade methanol and acetonitrile were obtained from J.T. Baker (Phillipsburg, USA). Analytical grade ammonium formate and formic acid were procured from Merck Ltd (Mumbai, India). Blank human plasma was obtained from Deccan's Pathological Lab's (Hyderabad, India).

Apparatus and operation conditions

Liquid chromatography

Shimadzu HPLC system (Shimadzu, Japan) equipped with cooling autosampler, column oven and high pressure delivery pumps. A Hypersil Gold C18 column (50 mm×4.6 mm, 5µ) was employed for chromatographic separation. The column temperature was maintained at 40°C. The mobile phase consists of 30% ratio of 0.1% formic acid buffer and 70% ratio of acetonitrile (v/v). The flow rate was set at 0.6 mL/min. The auto-sampler was maintained at 10°C, the sample volume injected was 5 µL and the total analytical run time was 2.5 min.

Mass spectrometry

Triple-quadrupole tandem mass spectrometric detection was carried out on tandem quadrupole mass spectrometer (API 4000, Applied Biosystems/MDS SCIEX, USA). The turbo ion spray source was operated with typical settings as follows: ionization mode, positive; curtain gas, 20 psi; nebulizer gas, 35 psi; turbo gas, 55 psi; ion spray voltage, 5500 V; temperature, 450°C. The molecular ions of nifedipine and nimodipine were formed using the declustering potentials of 52 V and 51 V respectively, and their molecular ions were fragmented at collision energy of 25 V and 20 V by collision-activated dissociation with nitrogen as the collision gas at a pressure setting of 5 psi on the instrument. Multiple reaction monitoring (MRM) mode was employed for the quantification: m/z 447.10 → 207.00 for nifedipine and m/z 436.40 → 235.00 for nimodipine. Peak areas for all components were automatically integrated using analyst software version 1.4.2 (Applied Biosystems/MDS SCIEX).

Preparation of standards and quality control samples

Standard stock solutions of nifedipine and nimodipine were prepared in methanol at the concentration of 1.0 mg/mL. The internal standard working solution was diluted with 60%

methanol to get a concentration of 40 ng/mL. The nifedipine solution was then serially diluted with 60% methanol to provide working standard solutions of desired concentrations. All the solutions were stored at 2 to 100°C. Calibration standards were prepared by spiking 1.0 mL of blank human plasma with working standard solutions of nifedipine. The effective concentrations in standard plasma samples were 0.5, 1, 5, 10, 20, 40, 80, 100 ng/mL. One calibration curve was constructed on each analysis day using freshly prepared calibration standards. The quality control samples (QCs) were prepared with blank plasma at low, middle and high concentrations of 1.5, 50, 80 ng/mL. The standards and quality controls were extracted on each day analysis as per the sample extraction procedure described below.

Plasma sample preparation

An aliquot of 300 µL plasma was transferred into a tube containing 30 µL of IS working solution and vertex for proper mixing. The samples were extracted with solid phase extraction technique which includes conditioning and equilibration of cartridge (Oasis, HLB, 30 mg, 1 cc) with 2 mL of methanol and 2 mL of milli-Q water followed by loading the prepared plasma sample. Washing step includes 2 mL of milli-Q water, elutes with 2 mL of methanol and evaporated to dryness under nitrogen at 500°C. The residues were dissolved in 500 µL of reconstitution solution (mobile phase) and reconstituted solution was transferred into 1 mL vials, and 5 µL of samples were injected for LC–MS/MS analysis.

Method validation

Method validation was carried out as per FDA guidelines to assure the method performance by evaluating the calibration curve performance, accuracy and precision of the method (determined by QC performance), stability of the analytes at various test conditions, specificity matrix effect and recovery.

Selectivity

During the validation, blank plasma samples from (8) different lots were evaluated and all plasma lots were found to be satisfactory. There were no interfering peaks in the blank plasma at the retention times of nifedipine and internal standard. During the specificity run, an LLOQ standard was extracted and injected. The responses for the blank plasma from the eight (8) different lots were compared to the LLOQ standard of the analyte and internal standard.

The method is also validated for selectivity; selectivity is the ability of the bio-analytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. In this experiment, interference at one analyte retention time (R.T.) was checked by spiking of highest calibration standard of other analyte of interest and vice versa in six screened blank plasma. Similarly, interference at analyte R.T. was checked by spiking the working range of internal standard in six screened blank plasma.

Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared by making serial dilution of the working stock and assaying standard plasma samples at eight concentrations of nifedipine ranging 0.5- 100 ng/mL. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of nifedipine to I.S. versus the nominal concentration (x) of nifedipine. The calibration curves were constructed by weighted ($1/X^2$) least square linear regression. The lower limit of quantification is defined as the concentration which should be at least 5 times the response compared to blank response.[30] The validation of LLOQ was conducted in at least six different batches of blank plasma. It was validated using an LLOQ sample for which an acceptable accuracy (RE) within ±20% and a precision (R.S.D.) below 20% were obtained.

Precision and accuracy

For determining the intra-day accuracy and precision, a replicate analysis of QC plasma samples of nifedipine was performed on the same day. The run consisted of a calibration curve and five replicates of each low, mid, and high concentration quality control samples. The inter-day accuracy and precision were assessed by analysis of three batches on different days. The precision was expressed as the relative standard deviation (R.S.D.) and the accuracy as the relative error (RE). The results were shown in Table 1.

Extraction recovery and matrix effect

The extraction recovery of nifedipine was determined by calculating the peak areas obtained from blank plasma samples spiked with analyte before extraction with those from blank plasma samples, to which analyte was added after extraction. Per the guidance of USFDA,[31] recovery experiments should be performed at three concentrations (low, mid, and high). So, this procedure was repeated for five replicates at three concentrations of 1.5, 50, 85 ng/mL. To evaluate the matrix effect on the ionization of analyte, i.e. the potential ion suppression or enhancement, nifedipine at three concentration levels were added to the extract of 300 µL of blank plasma, evaporated and reconstituted with 500 µL of mobile phase. The corresponding

peak areas (A) were compared with those of the nifedipine standard solutions evaporated directly and reconstituted with the same mobile phase (B). The ratio (A/B×100) % was used to evaluate the matrix effect. The matrix effect of internal standard was also evaluated using the same method.

Stability[32]

Freeze and thaw stability

The effect of freeze and thaw cycles on the stability of plasma samples containing nifedipine was determined by subjecting six aliquots of QC samples at low and high concentration unextracted quality control samples to four freeze–thaw cycles. After completion of four cycles, the samples were analyzed and the experimental concentrations were compared with the nominal values.

Long-term stability

Six aliquots of QC samples at low and high concentration un-extracted QC samples were stored at -70^0C for 30 days. Then, the samples were processed and analyzed and the concentrations obtained were compared with the nominal values.

Bench top stability

Six aliquots of QC samples at low and high concentration unextracted QC samples were kept at ambient temperature (25°C) for 24 h to determine the bench top stability of nifedipine in human plasma. Then the samples were processed and analyzed and the concentrations obtained were compared with the nominal values.

Post-preparation stability

To estimate the stability of nifedipine in the prepared sample, six aliquots of QC samples at low and high concentration were kept in an autosampler maintained at 10°C for about 24 h. Then, the samples were analyzed and the concentrations obtained were compared with the nominal values.

Stock solution stability

To test the stock solution stability of nifedipine and the I.S., five aliquots of stock standard (1 mg/mL for nifedipine) and the I.S. (1 mg/mL) solution were left at 2 to 10°C for 30 days. Then, the concentrations were analyzed and compared with the fresh stock solution.

RESULTS AND DISCUSSION

Optimization of the chromatographic separation and MS/MS working conditions

The separation and ionization of nifedipine and I.S. were affected by the composition of mobile phase. Therefore, the selection of mobile phase components was critical. In experiment, different ratio (50:50, 40:60, 30:70 and 20:80) of water/acetonitrile was used to mobile phase and 30% water and 70% acetonitrile (v/v) in mobile phase was believed suitable in view of retention time and peak shape of drug. Formic acid was employed to supply the ionic strength. It was found that a mixture of 0.1% formic acid buffer-water/acetonitrile could preferably improve peak shape and was finally adopted as the mobile phase. The selection of MRM transitions and associated acquisition parameters (collision energy) were evaluated for best response under positive mode by infusing a standard solution, via a syringe pump, into the mobile phase. The corresponding full-scan MS/MS spectra for nifedipine and nimodipine are shown in Fig. 2. As per the reports MRM technique provides inherent selectivity and sensitivity, hence we applied for the present study. The very narrow chromatographic peaks produced by HPLC indicated an increase in the chromatographic efficiency which produced a fast separation. Both nifedipine and I.S. were rapidly eluted with retention times less than 2.5 min. The shorter analysis time may meet the requirement for high sample throughput in bioanalysis.

Selection of IS

The best internal standard in LC–MS assay is a deuterated form of the analyte. In our laboratory, no deuterated compound was available, therefore, a compound being structurally or chemically like the analyte was considered. In LC–MS/MS the I.S. should also have similar chromatographic and mass spectrometric behaviors to the analyte, and mimic the analyte in any sample preparation steps. Nimodipine was chosen as the internal standard for the assay because of its similarity of structure, retention time and ionization to nifedipine. The results showed it was suitable in retention time and ionization of nifedipine.

Method validation

Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. As shown in Fig. 2, No significant response (≤20% for the analyte response and ≤5% of the internal standard response) was

observed at the retention time of the analytes and internal standard in the blank plasma as compared to the LLOQ standard.

Linearity and LLOQ

The standard calibration curves for nifedipine were linear over the concentration range of 0.5–100 ng/mL ($r^2 > 0.99$) by using weighted least square linear regression analysis with a weigh factor of $1/x^2$. The lower limit of quantification for nifedipine was 0.5 ng/mL (S/N≥5) with 5µL injected into the HPLC column with RE within ±20% and R.S.D. lower than 20%. The representative calibration curve was shown in Fig. 3.

Precision and accuracy

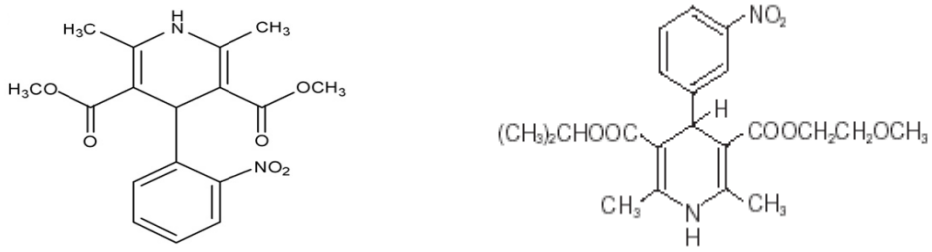
The data of intra-day and inter-day precision and accuracy for the method are listed in Table 1. The intra-day precision for low, mid and high QC levels of nifedipine were 9.0%, 3.8% and 5.8%, and that of inter-day analysis were 14.9%, 11.8%, 11.2%, respectively. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples per the guidance of USFDA, where the precision (R.S.D.) determined at each concentration level is required not exceeding 15%.

Extraction recovery and matrix effect

The extraction recoveries of nifedipine from human plasma were 100.8±8.1%, 92.2±2.7%, and 105.0±3.0% at concentration levels of 1.5, 50 and 85 ng/mL, respectively, and the mean extraction recovery of I.S. was 112.3±8.2%. In terms of matrix effect, all the ratios (A/B×100) % defined as in section 2 were between 85% and 115%, which means no matrix effect for nifedipine and I.S. in this method.

Stability

The nifedipine in plasma was found to be stable at room temperature for 24 h, at 10°C for 24 h, at the −70°C for 30 days, for four freeze and thaw cycles (Table 2). The stock solutions of nifedipine and I.S. were stable for at least 1 month with there being less than 5% difference in the measured concentrations of the stored and the freshly prepared solutions. The results from all stability tests presented in demonstrated a good stability of nifedipine over all steps of the determination.



Nifedipine **Nimodipine (IS)**

Figure 1: Chemical structure of nifedipine and nimodipine.

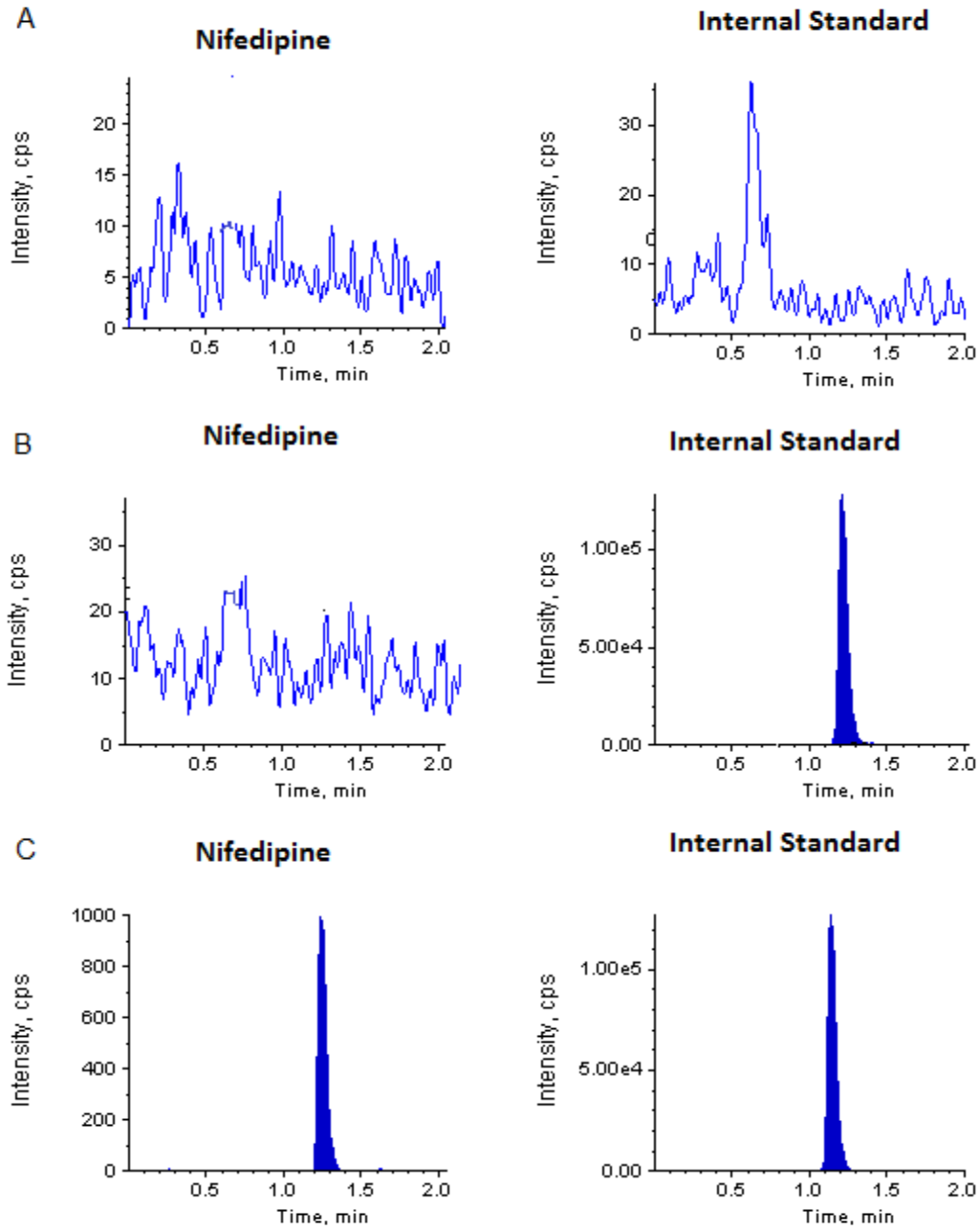


Figure 2: Representative chromatogram of [A] blank plasma; [B] blank plasma with internal standard; [C] LLOQ sample.

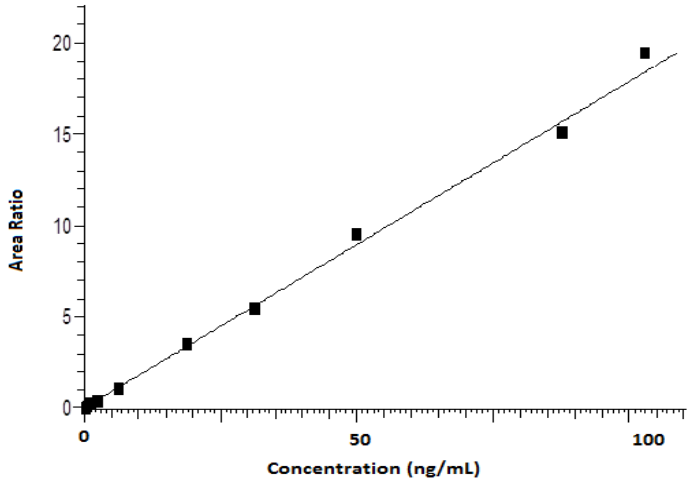


Figure 3: Representative calibration curve.

Table 1: Precision and accuracy data for nifedipine.

Quality control Run	Concentration found Mean ± SD (ng/mL)	RSD (%)	RE
Intra- day (*n*=6)			
LLOQ	0.50 ± 0.09	18.2	1.0
LQC	1.54 ± 0.14	9.0	2.4
MQC	46.31 ± 5.49	11.9	7.4
HQC	83.01 ± 4.81	5.8	2.3
Inter- day (*n*=18)			
LLOQ	0.45 ± 0.08	16.8	9.8
LQC	1.28 ± 0.18	13.9	14.9
MQC	935.8 ± 27.8	2.97	10.09
HQC	80.38 ± 8.96	11.2	5.4

Nominal concentrations of LLOQ, LQC, MQC and HQC are 0.5, 1.5, 50 and 85 ng/mL respectively.

Table 2: Stability data for nifedipine in plasma (*n*=6).

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Auto-sampler (24 h)	1.5	1.42 ± 0.19	13.3	5.6
	85	73.16 ± 2.47	3.4	13.9
Bench top (24 h)	1.5	1.32 ± 0.05	3.5	11.9
	85	75.96 ± 2.80	3.7	10.6
Freeze and thaw (4 cycles)	1.5	1.46 ± 0.19	13.2	2.5
	85	89.33± 4.58	5.1	5.1
Long term (-70°C) (30 days)	1.5	1.52 ± 0.13	8.8	1.5
	85	93.88 ± 2.69	2.9	10.4

CONCLUSION

The recently developed high-performance liquid chromatography–tandem mass spectrometry (HPLC -MS/MS) method for the determination of nifedipine in human plasma has been established. Compared with the published methods, reduced ion suppression/enhancement and offering superior sensitivity, satisfactory resolution and selectivity. The method can also be applied to a pharmacokinetic study of nifedipine given in tablet form to healthy volunteers. Therefore, this analytical HPLC-MS/MS method can be considered as a promising technique that has obvious advantages compared with conventional analytic techniques in this field of application.

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