Method development challenges and regulatory expectation in nifedipine

Aim: A novel and accurate high-throughput tandem mass spectroscopic method has been developed and validated for determination of nifedipine, a calcium-channel blocker. **Materials & methods:** Nifedipine was detected on a triple quadrupole tandem mass spectrometer by multiple reactions monitoring mode via ESI source. **Results:** The selective and sensitive method was linear in the concentration range of 0.501 to 360.100 ng/ml and reported no matrix effect. The percent accuracy of the method was found to be between 99.7 and 102.80%, while percentage of RSD was in range of 1.77 to 4.73%. **Conclusion:** Validated method was used for bioavailability studies under fasting and fed conditions.

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Clinical pharmacokinetics of noncommunicable disease such as hypertension has interested the academicians and practitioners alike due to intricacies and limited knowledge of labile metabolites. Calcium-channel blocker drug slows down the progression of coronary artery calcification. Nifedipine (1,4-dihydro-2, 6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester), belonging to the group of 1,4-dihydropyridines is one of the highly prescribed calcium-channel blockers that selectively dilates peripheral arteries [1]. It is widely used in the treatment of cardiovascular diseases such as hypertension, angina pectoris and Raynaud's phenomenon [2–4]. It inhibits the influx of extracellular calcium through myocardial and vascular membrane pores as well as restricts contractile processes of smooth muscle cells thereby decreasing total peripheral resistance and systemic blood pressure [5,6]. However, developing a robust method for analysis of Nifedipine in plasma is highly challenging due to multiple

reasons. Nifedipine is a photo-labile compound, undergoing oxidative biotransformation in human body into pharmacologically inactive metabolites, dehydronifedipine and hydroxydehydro nifedipine carboxylate [7].

In recent past, several analytical methods were reported for the determination of nifedipine in biological matrix. These methods include HPLC [8–14] coupled with UV detectors or electrochemical detector, but tedious sample processing technique, lengthy analysis time and lack of sensitivity were major limitations in these methods. Diverse detection techniques like electron-capture detection [15–20], nitrogen phosphorus detection [21] and mass spectrometric (MS) detection [22,23] were reported. However, scope of thermal decomposition of nifedipine under gas chromatography conditions was a concern and MS methods are usually preferred.

Nifedipine is metabolized by cytochrome P-450 IIIA4 enzymatic activity, *in vivo*. Nifedipine metabolism involves oxidative dehydrogenation to dehydronifedipine, followed

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by (oxidative) ester cleavage to the pyridine carboxylic acid metabolite and hydroxylation to the hydroxy-methyl metabolite, which is in pH- and temperature-dependent equilibrium with its lactone [24]. The *ex vivo* inter-conversion of nifedipine and its metabolites are challenging in terms of bioanalysis. Although Roosmalen *et al.* reported a method on estimation of polar metabolites of nifedipine, arresting *ex vivo* inter-conversion among metabolites and analyte [22] remained unaddressed. Reported LC–MS/MS methods showed marked limitations as these methods are not tuned to labile metabolite separation and arresting *ex vivo* degradation of nifedipine [25–28]. Role of dwell time in MS, special analytical precautions, use of appropriate anticoagulant along with detailed stability in diverse type of matrices remained unmet (Table 1). It is therefore imperative to develop a method where stabilization of nifedipine ester moiety, during blood sample collection as well as during sample preparation, is achieved. Additionally, appropriate measures were taken during sample preparation to avoid degradation, as nifedipine decomposed to different photo-products upon exposure to normal laboratory light conditions.

The main objective of this study was to develop a simple, reliable and accurate method for analysis of nifedipine in human plasma. Method development was challenging due to interfering metabolites/degradants and these metabolites were separated to make the method selective to nifedipine. The dynamic linearity ranges, high extraction recovery, along with sustained

reproducibility are secondary objectives of our method development and validation in conformance with current regulatory guidelines. The validated method was used for bioavailability studies in fast and fed condition.

Experimental methods

Materials

Nifedipine (purity: 99.8%) was procured from USP and Nifedipine- d_4 (purity: 96.3%) was supplied by CDN ISOTOPES INC., (Quebec, Canada). Dehydro nifedipine (purity: 98%) and hydroxydehydro nifedipine carboxylate (purity: 97%) were procured from Toronto Research Chemicals (Ontario, Canada). Ammonium acetate and acetone were of LC-MS grade and were procured from FLUKA (Sigma-Aldrich, MO, USA). Formic acid (AR grade), methanol (HPLC-grade) and acetonitrile (HPLC-grade) were supplied by S.D. fine chemicals Ltd, Mumbai, India. All aqueous solutions were prepared using water that was purified using Milli-Q® Gradient A10® (Millipore, Mosheim Cedex, France). Different individual lots of human plasma containing ethylene diamine tetra acetic acid tri-potassium salt (K_3 EDTA) as anticoagulant, used for preparation of calibration standards and quality control (QC) samples, were obtained from Clinical unit of Ranbaxy Research Laboratories (Noida, India). Hemolyzed and lipemic K_3 EDTA plasma were collected from the Drug Monitoring Research Institute Private Limited (Mumbai, India). Amber colored glass shell vials (1.2 ml) were procured from Chromatopak (Mumbai, India).

Table 1. Comparison of reported methods for nifedipine estimation.

Reference	Roosmalen <i>et al.</i> [22]	Streel <i>et al.</i> [25]	Wang <i>et al.</i> [26]	Wang <i>et al.</i> [27]	Patel <i>et al.</i> [28]
Plasma volume (ml)	0.100	1.200	0.500	0.500	0.200
Method LLOQ (ng/ml)	NAV	0.500	0.500	0.104	0.050
Recovery; analysis run time	NAV	95%	84.3%	87.0%	95.6%
	NAV	16 min	2.5 min	1.4 min	1.2 min
Matrix effect in hemolyzed and lipemic plasma	NAV	NAV	NAV	NAV	Yes
Labile metabolites separation/analysis	Dehydro nifedipine and hydroxydehydro nifedipine carboxylate	Dehydro nifedipine	Dehydro nifedipine	NAV	NAV
Analytical conditions	NAV	Nonactinic light	Darkened room	Sodium lamp illumination	Yellow light (570–580 nm)
Blood stability	NAV	NAV	NAV	NAV	NAV
Stability of nifedipine in plasma in the presence of dehydronifedipine and hydroxydehydro nifedipine carboxylate	NAV	NAV	NAV	NAV	NAV
Dwell time	NAV	NAV	0.3 s	NAV	100 ms

NAV: Data not available.

Instruments

Sample processing was done using cleanert PEP-3 SPE, 30 mg/1 ml, solid-phase cartridges (Agela technologies, Tianjin, China). Processed samples were dried using Zymark Turbovap LV concentrator (Caliper Life Sciences, MA, USA). Liquid chromatography of Shimadzu scientific instruments (Shimadzu Corporation, Kyoto, Japan) was used for analysis along with mass spectrometer (MDS Sciex API 4000 Applied Biosystems, Toronto, Canada). Separation was done using ZORBAX SB-C18 column (150 × 4.6 mm, 5 µm) (Agilent technologies, CA and IL, USA).

Stock solutions preparation

Working standards of nifedipine and nifedipine-d₄ were dissolved in methanol to make a primary stock solution of concentration 1 mg/ml. Calibration curve (CC) standards and QC samples were prepared using different stock solutions. The prepared stock solutions were protected from light and stored in refrigerator between 1 and 10°C until use. Internal standard (IS) dilution of concentration 40,000 ng/ml was prepared everyday by taking appropriate dilution of the IS stock solution in acetonitrile:water (50:50, v/v). CC and QC working solutions for nifedipine were prepared by serially diluting the primary stock solution with acetonitrile:water (50:50, v/v) under red light condition.

CC standards & QC samples preparation

Spiked samples of CC standards and QC samples were prepared by spiking 2%, v/v of the aqueous working solutions in plasma. Final concentration of CCs standards was prepared in range of 0.501–360.100 ng/ml for nifedipine. Final spiked concentration of nifedipine was 0.502 ng/ml for lower limit of quantification (LLOQ); 1.450 ng/ml for low level (LQC); 106.33 ng/ml for medium level (MQC) and 266.582 ng/ml at higher limit of quantification (HQC). All samples were prepared under low light and chilled condition. All samples were stored in the freezer at below -50°C until analysis.

MS detection & chromatographic conditions

The HPLC system from Shimadzu scientific instruments (Shimadzu Corporation, Kyoto, Japan) used in this study comprised an intelligent pump (LC-10AD) with an on-line DGU-20A3 prominence solvent degasser, a SIL-HTc Shimadzu auto-sampler and a CI3M-20A prominence column oven. In the study all separations were achieved on ZORBAX SB-C18; 150 × 4.6 mm, 5 µm column. Precursor-product ion transitions with 400 ms dwell time, at m/z 347.1/315.1 for nifedipine and m/z 351.2/319.2 for nifedipine-d₄ was used in multiple reactions monitoring (MRM).

Analyte and IS were detected using MDS, Sciex API 4000 mass spectrometer having turbo ion spray in the positive ion mode. Auto sampler temperature of 5°C ± 1°C and column oven temperature of 35°C ± 1°C were as maintained during analysis.

Mass parameters were optimized for both nifedipine and nifedipine-d₄. Optimized spray source temperature was 400°C while that for curtain (CUR) gas was 20°C. Optimized value for nebulizer gas (GS1) was 60 psi. Optimized heater gas (GS2) was 45 psi while that of ions spray voltage (IS) was 5000 V. Optimum value of collision-activated dissociation was found to be 6 V while that of declustering potential was found to be 55 V. Optimized value of entrance potential (EP) was found to be 5 V while that of collision energy was found to be 12 V. Optimum collision cell exit potential was found to be 10 V. Ultra-high pure nitrogen was used as GS1, GS2, collision-activated dissociation and curtain gas. Quadrupole 1 and 3 were maintained at unit mass resolution.

Analyte to IS peak area ratio (y) against analyte concentrations (x) was used for constructing CCs. Analyst version 1.4.2 software (Applied Biosystems, CA, USA) was used for data acquisition and processing.

Sample preparation

Samples were thawed in an ice cold water bath and extracted employing SPE technique. 50 µl of IS (40,000 ng/ml) were added to 0.100 ml of plasma samples. After mixing, 500 µl of 2% formic acid in HPLC Grade was added to each sample.

Cleanert PEP-3 SPE cartridges were conditioned with 0.5 ml of methanol followed by 0.5 ml of HPLC-grade water. Then samples were loaded on the cartridges using an Eppendorf pipette. After loading cartridges were washed with 1 ml of HPLC-grade water twice. Total 1 ml of acetone was used for elution, 0.5 ml twice. Samples were extracted using centrifuge at 4000 r.p.m. for 1 min during each step. Stream of dry nitrogen was used for drying the samples at temperature of 40°C and pressure of 20 psi using Zymark Turbovap LV concentrator. 500 µl of mobile phase were used for samples reconstitution. Reconstituted samples were transferred into amber colored glass vials. An injection volume of 10 µl of each sample was used for analysis. All the steps till samples were loaded into auto sampler were carried out in red light condition.

Method validation

Method validation was conducted according to the US FDA guidance document on validation of bioanalytical methods [29] and had been detailed as per defined internal procedures [30].

Various validation exercises such as matrix effect evaluation, evaluation of linearity of the method, precision and accuracy studies, selectivity, recovery determination and stability studies under different conditions were carried out.

Sensitivity & selectivity

Selectivity testing was carried out to show that the presence of biological matrix, known metabolites, degradation products or co-administered drugs do not affect the analysis of the analyte of interest. A method is deemed highly selective if the method distinguishes and quantifies the analyte in the presence of endogenous and/or exogenous substances. The extent to which endogenous substances may contribute to the interferences for analyte and IS was determined by processing and analyzing six normal, one lipemic and one hemolyzed lots of K₃EDTA blank human plasma by the same extraction method. The blank plasma with minimum interference was taken and 2% of the aqueous dilution corresponding to concentration equivalent to lower limit of quantification LLOQ was spiked, processed and analyzed [29]. The aim of performing the selectivity check with these plasma samples is to ensure the quality of the results of study sample analysis. Acceptance criteria for selectivity included: the peak area of interfering peak at the retention time (RT) of analyte and IS should be less than 20% of analyte peak area and less than 5% of mean area of IS concentration at LLOQ level, respectively [29]. Signal and noise of LLOQ was used to demonstrate sensitivity. Acceptance criteria were ratio of S/N of four replicates of LLOQ and mean S/N of blank should be less than 5.

Linearity

Area ratio of analyte/IS was plotted against the theoretical concentration of analyte in order to determine linearity. Three batches consisting of one complete set of calibration standards and six replicates of QC samples at LLOQ, LQC, MQC and HQC levels were used to determine linearity. X² weighted linear least squares regression analysis was applied to slope, y-intercept, correlation coefficient (r) and the back calculated concentrations.

Within- & between- (intra- & inter)-batch precision & accuracy

Six replicates of QC samples at four different concentration levels, that is, LLOQ, LQC, MQC and HQC were used to determine within- and between (intra- and inter)-batch precision and accuracy. Within-batch precision and accuracy was analyzed by running two precision and accuracy batches on the same day. The third batch was analyzed on another day. Between-

batch (inter-batch) precision and accuracy were determined using the data of the three batches. Precision of the assay was measured by the percent (%) coefficient of variation (CV) over different concentration levels.

Percent CV should be within $\pm 20\%$ at LLOQ and $\pm 15\%$ for other QCs. Percent accuracy should be within $100 \pm 20\%$ at LLOQ and $100 \pm 15\%$ for other QCs.

Extraction recovery & dilution integrity

Recovery was determined by comparing the peak area of drug at spiked LQC, MQC and HQC in plasma (six replicate at each level) with the aqueous LQC, MQC and HQC having concentration equivalent to spiked QC samples. Recovery of the IS was determined similarly. Acceptance criteria for recovery were percent CV at all QC levels and IS should be $\leq 20\%$.

A concentration of approximately two-times the concentration of 90% ULOQ was used for determination of dilution integrity. Samples were diluted two-times and four-times with blank matrix to get the concentration within the CC range. These samples were then analyzed with a fresh CC standards. The acceptance criterion for QC samples was the same as for the precision and accuracy analytical run.

Matrix effect & matrix factor

Two different methods were used to determine matrix effect. In the first method, regions of ion suppression or enhancement were determined by infusing mixture of analyte and IS at high concentration at the rate of 10 $\mu\text{l}/\text{min}$ using a postcolumn (T-joint) infusion pump, while injecting a blank extracted plasma. In the second method, four normal, one lipemic and one hemolyzed K₃EDTA blank plasma lots selected during selectivity exercise, that is, free of any significant interference at the RT of analyte and IS were spiked with working solutions of LOQQC and HQC sample. Samples were prepared in duplicate and analyzed with a fresh set of CC standard. Acceptance criteria were % accuracy should be within $\pm 15\%$ and precision should be $\leq 15\%$ at the LOQQC and HQC concentrations. Matrix factor experiment helps to determine quantitative effect of matrix.

Peak response in the presence of matrix ions versus the peak response in the absence of matrix ions was used to determine absolute matrix effect (AME). Four normal, one lipemic and one hemolyzed lots of K₃EDTA blank plasma were used for determination of AME. Reference samples consisted of QC samples at LQC, MQC and HQC level. The control sample was reference solution prepared at appropriate concentration in reconstitution solution. Peak area response and peak area ratio (analyte/IS) methods were used

for determination of AME. AME was estimated using Formula 1. When, AME is equal to 1 it shows that there is no matrix effect, AME is less than 1, it shows ion-suppression and AME is more than 1 it shows that there is ion-enhancement.

$$\%AME = \frac{\text{Mean peak area response in post extracted samples}}{\text{Mean peak area response of analyte in neat solutions}} \times 100$$

Stability

Stability experiments of nifedipine and nifedipine- d_4 in stock solution and plasma were performed extensively. Stability studies such as freeze–thaw stability, room temperature stability of drug in plasma (bench-top stability), stock solution stability of both the drugs and IS, postpreparative stability at autosampler temperature at 5°C (auto sampler stability) and long-term stability below -50°C were carried out.

For sample collection and handling stability, fresh human K_3 EDTA whole blood was spiked with the nifedipine at the low and high QC concentrations (1.450 and 266.582 ng/ml) and with dehydro nifedipine and hydroxydehydro nifedipine carboxylate. The spiked blood samples were kept in ice cold water bath, under red light condition for 115 min (stability sample). After the storage period, freshly spiked blood samples (comparison samples) were prepared in an identical manner at low and high QC concentrations. Comparison and stability blood samples were centrifuged at 4000 r.p.m. for 15 min at 4°C to separate plasma from the blood and the resulting plasma was analyzed as per the extraction protocol. The stability was assessed by analyzing the four aliquots of low and high QC concentration samples from stability and comparison samples. Blood stability was determined using Formula 2.

$$\%stability = \frac{\text{Mean area ratio of stability samples}}{\text{Mean area ratio of comparison samples}} \times 100$$

The stability of nifedipine in plasma was assessed by analyzing four replicates of bulk-spiked QC samples at low and high concentrations during the sample storage and processing procedures. Bulk-spiked QC samples (stability QC) were spiked with nifedipine, dehydronifedipine and hydroxydehydro nifedipine carboxylate. Bench-top stability was assessed in ice cold water bath for approximately 7 h, which exceeds the residence time of the sample processing procedures. The freeze–thaw stability was evaluated after undergoing three freeze (below -50°C)–thaw (ice cold water bath) cycles. Long term stability was assessed after storage of the test samples below -50°C for 51 days. The auto-sampler storage stability was determined by storing the reconstituted QC samples for approximately 76 h under auto-sampler condi-

tion (maintained at 5°C) before being analyzed. The working solutions and stock solutions of nifedipine and the IS were also evaluated for stability at room temperature for 12 h and at refrigerator temperature (between 1–10°C) for 12 days, respectively. All stability exercises were performed against freshly spiked CC and the results were compared with freshly prepared QCs (comparison QCs). The results for all stability exercises were deemed acceptable if the mean concentration at each level is within $\pm 15\%$ and percent CV is $\leq 15\%$ at low and high QC concentrations.

Bioavailability studies

Open-label, balanced, randomized three-treatment, three-period, three-sequence, single-dose, crossover bioavailability studies in healthy adult human subjects under both fasting and fed conditions were carried out. Both the studies were carried out separately. Both studies consisted of 21 healthy adult male volunteers who had given written informed consent. The study was approved by Ethics Committee of Institutional Review Board at Majeedia Hospital (New Delhi, India).

Condition for fasting study: Subjects received a standard dinner after admission. Subjects were required to fast at least 10 h prior to dosing till 4 h postdose. Dose was administered with 240 ml of drinking water at an ambient temperature. No food was allowed for at least 4 h postdose. Lunch, snacks, dinner, breakfast, lunch, snacks and dinner at 4, 9, 13, 24, 28, 33 and 36 h postdose, respectively, were allowed. Water was restricted to 1 h predose to 2 h postdose.

Condition for fed study: Subjects received a standard dinner after admission. After an overnight fast of at least 10 h, subjects were served high-fat high-calorie breakfast. Dose was administered 30 min after the start of a high-fat high-calorie breakfast (nonvegetarian breakfast, comprising of 800–1000 kcal). Subjects were asked to consume this breakfast within 30 min of serving in each period.

Thereafter, subject received lunch, snacks, dinner, breakfast, lunch, snacks, dinner, breakfast, lunch, snacks and dinner at approximately 4, 9, 13, 24, 28, 33, 37, 48, 52, 57 and 36 h postdose, respectively. Subjects were restricted to drinking water 1 h predose to 2 h postdose (except for 240 ml of drinking water administration during dosing). Thereafter, it was allowed at all times.

For both bioavailability studies, all the subjects were given a single oral dose of Procardia XL extended release tablets 90 mg of Pfizer Labs, Division of Pfizer Inc, USA (Reference, R) and two in-house manufactured-formulations of nifedipine extended release tablets 90 mg of Sun Pharmaceutical Private Limited, India (Test [A] and Test [B]).

The blood samples were collected predose (duplicate) and at 1.000, 2.000, 3.000, 4.000, 4.500, 5.000, 5.500, 6.000, 6.500, 7.000, 8.000, 9.000, 10.000, 11.000, 12.000, 13.000, 14.000, 15.000, 16.000, 18.000, 20.000, 22.000, 24.000, 28.000, 32.000, 36.000, 42.000, 48.000 and 60.000 h postdose in each period of the study. After separation of plasma from blood by centrifugation, plasma samples were stored at a temperature below -15°C until they are analyzed.

Incurring sample reanalysis

A total of 120 samples each selected from single dosed biostudies in fasting conditions and fed condition were used to determine incurred sample reanalysis. 80% of samples consisted of study samples from a $C_{\max}$ time point and elimination phase (at least three-times of LLOQ concentration) while the remaining 20% were selected from random sample time points.

Results & discussion

Method development

During method development, both ESI and atmospheric pressure chemical ionization sources were investigated. ESI offered much higher signal intensities for both analyte and IS than the latter and was thus chosen as the ionization source. Scanning solution consisted of 50 ng/ml of both the analytes and IS in mobile phase. The scanning solution of analyte and IS was infused separately to determine optimum mass spectrometer parameters. Both the compounds have the ability to accept protons and give protonated species $[\text{M}+\text{H}]^{+}$ ions in Q1 mode so tuning was done in positive mode. Protonated parent ions for nifedipine, nifedipine- d_4 , hydroxydehydro nifedipine carboxylate and dehydronifedipine were observed at m/z 347.1, 351.2, 347.5 and 345.1 (Figure 1), respectively. The $[\text{M}+\text{H}]^{+}$ ions from nifedipine, nifedipine- d_4 , hydroxydehydro nifedipine carboxylate and dehydronifedipine were selected as the precursor ion and subsequently fragmented in MS/MS mode to obtain the product ion. The fragment ion at m/z 315.1, 319.2, 315.4 and 284.1 (Figure 1) was produced as the prominent product ion for nifedipine, nifedipine- d_4 , hydroxydehydro nifedipine carboxylate and dehydronifedipine, respectively. Precursor and the product ions of nifedipine were monitored using MRM due to the high sensitivity of MRM data acquisition. Cross talk between nifedipine and its metabolite was avoided by selecting dwell time appropriately. At dwell time 100 ms cross talk in MS/MS was observed while monitoring nifedipine and hydroxydehydro nifedipine carboxylate in MRM mode. Finally 400 ms dwell time was found appropriate and no cross talk was observed for nifedipine, dehydronifedipine and hydroxydehydro nifedipine carboxylate between their MRMs and peak

shapes also improved significantly. Curtain gas having arbitrary value of 15 was found suitable. Efficient mobilization took place at arbitrary value 60 of Gas 1 (nebulizer gas) and 45 of Gas 2 (heater gas). Capillary temperature of ion source and ion spray voltage significantly controlled charge competition between nifedipine and other analytes (i.e., deuterated IS and nifedipine metabolites). Therefore, ion source temperature was optimized at 400°C and ion spray voltage set at 5000 V.

In the preliminary study, peak properties and response function were optimized by changing type of organic modifier, organic to aqueous ratio, buffer type, buffer strength.

Buffers such as ammonium acetate, ammonium formate and formic acid were tried to identify the optimal mobile phase. Ten micromolars of ammonium acetate gave highest response along with good peak shape. Acetonitrile and methanol were tried in different compositions with buffers as mobile phase.

High peak response and improved peak shape was found with Acetonitrile compared to methanol, used in mobile phase [14]. Moreover methanol in mobile phase likely affected stability of nifedipine in clinical sample analysis, probably due to nifedipine labile metabolite. It was found that in the combination of acetonitrile and 10 mM ammonium acetate solution in the ratio of 85:15, v/v was best suited composition for mobile phase to achieve optimum system response. A number of reversed-phase C18 columns, such as Discovery-C18, Poroshell 120 EC-C18, Novapack-C18, LiChrospher® 60 RPSelect B and Zorbax SB-C18 were tested to obtain optimal response, suitable retention time and good peak shapes for analyte and IS. Although retention of analytes was found in most of the columns, interference from endogenous compounds could not be eliminated completely. Moreover separation of polar metabolites could be achieved using Zorbax SB-C18 column as it was essential to separate nifedipine from hydroxydehydro nifedipine carboxylate, chromatographically (Figure 2). Moreover, desired separation of nifedipine from rest of allied metabolites could only be achieved by using ZORBAX SB-C18 (150 $\times$ 4.6 mm ID, 5 μm) and above mentioned mobile phase composition. The flow rate, optimized at 0.600 ml/min, restricted analysis run time within 3.8 min.

Diverse methods were evaluated in order to optimize sample extraction. Protein precipitation with methanol and acetonitrile was evaluated. Also liquid–liquid extraction different extracting solvents such as ethyl acetate, dichloromethane and different combination dichloromethane and isopropyl alcohol were evaluated.

Low extraction recovery was observed for both protein precipitation and liquid–liquid extraction

Figure 1. Product ion mass spectra. (A) Nifedipine (NIF) (m/z 347.1 $\rightarrow$ 315.1), **(B)** NIF- d_4 (m/z 351.2 $\rightarrow$ 319.2), **(C)** hydroxydehydro nifedipine carboxylate (HNC) (m/z 347.5 $\rightarrow$ 315.4) and **(D)** dehydro nifedipine (DNIF) (m/z 345.1 $\rightarrow$ 284.1) in positive ionization mode.

Figure 2. Representative chromatograms of spiked samples. (A–E) The peaks of the internal standards are shown in the right hand side panel; representative chromatograms for nifedipine (RT 2.87 min) and nifedipine- d_4 (IS) (RT 2.86 min) in human plasma of (A) blank; (B) zero sample; (C) LLOQ; (D) ULOQ and (E) incurred sample along with hydroxydehydro nifedipine carboxylate (RT 1.84) and dehydro nifedipine (RT 3.09); unshaded peaks were not observed in spiked calibration standard and quality control samples, being nifedipine metabolites. (B) Matrix effect: postcolumn infusion (T-Joint) method for nifedipine and nifedipine- d_4 (RT 2.88) to demonstrate absence of any ion suppression/enhancement. RT: Retention time.

mechanism. Additionally, cluster of components was co-eluting with nifedipine suggesting potential of phospholipid-based matrix effects. Therefore SPE was evaluated in order to minimize co-elution of interfering components. Cartridges such as HLB,

Bond Elut Plexa, PEP-3 SPE and Strata-X were tried. Cleanert PEP-3 SPE cartridges showed improved extraction recovery. Hydrophilic vinyl pyrrolidone and hydrophobic polydivinyl benzene moieties of these cartridges provided an excellent capacity to

retain analytes of wider polarity spectrum. Formic acid solution was added to the samples in order to break drug-protein binding and maximize analyte/s retention on hydrophilic /hydrophobic balanced stationary phases. Pre-treatment with 2% of 500 μ l was found to be optimum. Washing of cartridges was done with HPLC Grade water. Methanol, acetonitrile and acetone were tried as elution solvents to remove unwanted co-eluting matrix components. Acetone gave best results as it nullified the matrix effect. Therefore, for the first time, we found that acetone proved to be best eluting solvent during plasma sample extraction phase of 1,4-dihydropyridines class of compound. Matrix factor provided convincing proof of least matrix interference of nifedipine and its metabolite components for the first time as delineated in Figure 3. Furthermore, nifedipine oxidation to dehydronifedipine has been restricted by using both chilled condition during sample preparation and K_3 EDTA anticoagulated plasma.

LLOQ concentration of 0.501 ng/ml was sufficient to characterize pharmacokinetic parameter with 90 mg extended-release tablet of nifedipine. However, employing 100 μ l of aliquot volume, 500 μ l of dried sample reconstitution volume and 10 μ l sample-

injection volume, there is an immense possibility of reducing LLOQ (i.e., increasing sensitivity) to 50 pg/ml. The labeled isotope (nifedipine- d_4) was used as an IS since it effectively compensated for variability during the analyte extraction phase or ionization in a mass spectrometer.

Method validation

Selectivity

All the lots of blank plasma used for selectivity studies met the acceptance criteria. Figure 2 shows the typical chromatograms of a blank plasma, blank plasma spiked with IS (Zero sample), a spiked plasma sample with nifedipine at LLOQ and ULOQ level. It can be seen no interfering peaks from endogenous compounds were observed at the retention times of the analyte and IS. Selectivity of nifedipine in presence of both the metabolites (dehydronifedipine and hydroxydehydro nifedipine carboxylate) was found acceptable. The chromatograms presented in Figure 2 indicated that the method is selective. The SPE method employed gave a very good selectivity for the analysis of nifedipine and IS in the blank human plasma. All the chromatograms were Gaussian in shape and deemed acceptable.

Figure 3. Matrix effect: postcolumn infusion (T-Joint) method for nifedipine and nifedipine- d_4 (RT 2.88) to demonstrate absence of any ion suppression/enhancement.

Linearity

Weighted least square regression analysis with weighting factor of x^2 was used for determining linearity of Nifedipine. Standard plot using eight-point CC was used. The CC was linear from 0.501 to 360.100 ng/ml. Chromatographic response versus concentrations was determined by weighting factor of x^2 . The correlation Coefficient (r) was consistently 0.99 or greater during the course of method validation for nifedipine.

Within- & between- (intra- and inter)-batch precision & accuracy

Table 2 shows a summary of within and between batch precision and accuracy data for QC samples containing nifedipine. Both intra and inter batch percent CV values ranged from 1.75 to 4.73% for assay method of nifedipine. The accuracy for assay method of nifedipine ranged from 99.70 to 102.80%.

Recovery & dilution integrity

The recovery found at LQC, MQC and HQC level was 88.24, 89.79 and 87.64% for nifedipine, respectively. The % mean recovery of all three QC levels was 88.56% whereas the % mean recovery of IS was 88.90% with a %CV of 1.25 and 1.81%, respectively. The percent CV for all the levels was below 20%. Recoveries were consistent, precise and reproducible with this proposed extraction method.

Dilution integrity samples were within the acceptance criteria for accuracy ($100 \pm 15\%$) and %CV ($<15\%$). % accuracy was 98.46 and 96.18% with %CV of 2.16 and 1.81% after two- and four-fold dilution with blank plasma, respectively. Hence the incurred samples can be diluted, if required during clinical samples analysis.

Matrix effect & matrix factor

Chromatography of analyte or IS as well as accuracy of the method may be affected by Matrix effect, ion suppression or enhancement, due to co-eluting endogenous component. Therefore to ensure the selectivity of method, matrix effect as well as matrix factor studies were carried out. Results of these studies (shown in Table 3) indicate that different lots of plasma did not show variations in plasma concentration.

Matrix effect studies show that there is no ion suppression/enhancement at RT of nifedipine and IS (Figure 3).

Matrix components, for example, lyso-phosphatidyl choline (m/z 496/184), phosphatidyl choline (m/z 758/184) may show suppression or enhancement of analyte and IS response. This was assessed by estimating the matrix factor across all QC concentration levels. The mean absolute matrix factor at the low, medium and high QC concentration from six lots of plasma samples was 0.960, 0.957 and 0.965, respectively (Table 3). The CVs of absolute MF and IS normalized MF from six lots of plasma samples were $<2\%$. These results showed that ion suppression or enhancement from the plasma matrix components were nullified for the first time to the best of the knowledge of authors.

Stability

The results of all the stability exercises obtained were well within the acceptable limits ($CV \leq 15\%$ and accuracy within $\pm 15\%$). The freeze–thaw stability calculated at the end of third freeze and thaw cycle for nifedipine ranged from 98.93 to 102.14% (Table 4). The bench top stability carried out for 6.44 h for nifedipine in matrix was 97.50 to 99.33% (Table 4). The stocks solution study carried out by keeping stock solution of

Table 2. Intra- and inter-batch precision and accuracy of nifedipine.

QC samples	LLOQ	LQC	MQC	HQC
Concentration (ng/ml)	0.502	1.450	106.633	266.582
Intrabatch*	0.5083	1.4456	109.6183	270.4198
Intrabatch $\pm$ SD	0.00900	0.03280	5.18658	6.20860
Intrabatch %CV	1.77	2.27	4.73	2.30
Intrabatch % nominal	101.26	99.70	102.80	101.44
Interbatch mean†	0.5068	1.4538	109.3439	272.1976
Interbatch $\pm$ SD	0.00887	0.03041	5.09992	7.65803
Interbatch %CV	1.75	2.09	4.66	2.81
Interbatch percent nominal	100.95	100.26	102.54	102.11

*Mean concentration of 12 replicates – two precision and accuracy analytical run.
†Mean concentration of 18 replicates – three precision and accuracy analytical run, global mean.

Table 3. Matrix effect and matrix factor for nifedipine in six different lots of human plasma.

Lots of human plasma	Matrix effect				Matrix factor			
	LOQQC 0.502 ng/ml*		HQC 266.582 ng/ml*		LQC		MQC	
	Calculated concentration (ng/ml)	Calculated concentration (ng/ml)	Using peak area response	Using analyte/IS peak area ratio	Using peak area response	Using analyte/IS peak area ratio	Using peak area response	Using analyte/IS peak area ratio
Matrix Lot#1	0.492	258.716	0.95	1.01	0.98	1.00	0.94	1.00
	0.491	264.938						
Matrix Lot#2	0.501	258.047	0.97	1.00	0.94	0.99	0.99	1.01
	0.495	259.923						
Matrix Lot#3	0.509	262.401	0.98	1.00	0.95	1.00	0.96	1.00
	0.466	259.385						
Matrix Lot#4	0.493	269.252	0.96	1.01	0.96	1.00	0.95	1.00
	0.511	264.238						
Matrix Lot#5†	0.506	256.412	0.94	1.00	0.97	1.00	0.97	1.01
	0.491	268.615						
Matrix Lot#6‡	0.496	269.863	0.96	1.01	0.94	0.99	0.98	1.00
	0.511	262.141						
Mean	0.4968	262.8276	0.960	1.006	0.957	0.996	0.965	1.004
SD(±)	0.01247	4.59810	0.0141	0.0058	0.0163	0.0040	0.0187	0.0029
%CV	2.51	1.75	1.47	0.58	1.71	0.40	1.94	0.29
N	12	12	6	6	6	6	6	6
% Nominal	98.97	98.59						

*Nominal concentration (ng/ml).

†Hemolyzed plasma lot.

‡Lipemic plasma lot.

nifedipine in refrigerator for 12 days between 1 and 10°C showed that nifedipine is stable in solution form for 12 days when stored in refrigerator. The percent stability of stock solutions of Nifedipine was 98.57% while that for nifedipine- d_4 was found to be 101.76%. Auto sampler stability at 5°C was found to be 76.08 h. The plasma samples were found to be stable when stored below -50°C for a period of 51 days (Table 4). Long-term stability of nifedipine ranged from 99.40 to 100.27% (Table 4). The working solutions of nifedipine and IS were found stable for 8.46 h at room temperature. The blood stability for 115 min in ice cold water bath was found to be acceptable for nifedipine. The % stabilities at LQC and HQC concentration were 101.87 and 99.56%, respectively (Table 5).

Application

Bioavailability studies

The plasma mean concentration time profile of Reference (R) and Test products (A) and (B) under fasting condition is depicted in Figure 4. The estimated pharmacokinetic parameters derived from the plasma

concentration profiles are summarized in Table 6A. The C_{max} of Reference product in fasting condition was 111.20 ± 42.38 (ng/ml) whereas that for Test product (A) was found to be 124.00 ± 52.23 (ng/ml) and for Test Product (B) was 104.35 ± 52.23 (ng/ml). The plasma mean concentration time profile of Reference and Test products (A) and (B) under fasting condition is depicted in Figure 5. The C_{max} of Reference product in fed condition was 150.96 ± 66.73 (ng/ml) whereas that for Test product (A) was found to be 174.49 ± 97.73 (ng/ml) and for Test Product (B) was 149.37 ± 81.13 (ng/ml). Ratio of least square means under fasting condition (A/R)% for C_{max} was 111.93, for AUC_{0-t} was 97.91 and for $AUC_{0-\infty}$ was 97.43. Similarly (B/R)% for C_{max} was 127.67, for AUC_{0-t} was 110.10 and for $AUC_{0-\infty}$ was 111.14. Ratio of least square means under Fed condition for (A/R)% for C_{max} was 113.89, for AUC_{0-t} was 106.40 and for $AUC_{0-\infty}$ was 106.83. Similarly (B/R)% for C_{max} was 100.04, for AUC_{0-t} was 99.20 and for $AUC_{0-\infty}$ was 99.49. Statistical evaluation of pharmacokinetic parameters is shown in Table 7A and B.

Table 4. Different stability parameters of nifedipine in the presence of dehydronifedipine and hydroxydehydro nifedipine carboxylate.

		Stability QC samples		Comparison QC samples	
		LQC	HQC	LQC	HQC
Stability parameters	Nominal concentration (ng/ml)	1.450	266.582	1.451	268.671
Benchtop stability in matrix (6.44 h)	Mean	1.4138	264.7988	1.4233	272.2938
	SD(±)	0.05394	3.75139	0.06287	6.42414
	%CV	3.82	1.42	4.42	2.36
	%Nominal	97.50	99.33	98.09	101.35
	N	4	4	4	4
Freeze–thaw stability (3 freeze–thaw cycles, below -50°C)	Mean	1.4345	272.2910	1.4448	265.5438
	SD(±)	0.02530	5.05620	0.05076	4.41797
	%CV	1.76	1.86	3.51	1.66
	%Nominal	98.93	102.14	99.57	98.84
	N	4	4	4	4
Auto-sampler stability (76.08 h)	Mean	1.4333	264.4338	1.4403	264.9035
	SD(±)	0.01791	1.38508	0.01700	2.62424
	%CV	1.25	0.52	1.18	0.99
	%Nominal	98.84	99.19	99.26	98.60
	N	4	4	4	4
Long-term stability below -50°C (51 days)	Mean	1.4413	267.2988	1.4453	267.0438
	SD(±)	0.02112	2.58482	0.04160	3.68756
	%CV	1.47	0.97	2.88	1.38
	%Nominal	99.40	100.27	99.60	99.39
	N	4	4	4	4

Table 5. Blood stability of nifedipine in the presence of dehydronifedipine and hydroxydehydro nifedipine carboxylate.

	LQC		HQC	
	Stability samples	Comparison samples	Stability samples	Comparison samples
	Area ratio (analyte/ISTD)	Area ratio (analyte/ISTD)	Area ratio (analyte/ISTD)	Area ratio (analyte/ISTD)
	0.0661	0.0636	12.4800	12.4870
	0.0652	0.0649	12.5702	12.6309
	0.0656	0.0641	12.4695	12.4989
	0.0650	0.0645	12.5185	12.6404
Mean	0.06548	0.06428	12.50955	12.56430
SD(±)	0.000486	0.000556	0.045591	0.082622
%CV	0.74	0.87	0.36	0.66
N	4	4	4	4
%Stability	101.87		99.56	

Incurred sample reanalysis

The results of incurred sample reanalysis showed that 98.4% sample point concentrations for nifedipine were within $\pm 20\%$ of original concentration value for fast-ing study and 97.65% for fed study.

The % difference from the original analysis was calculated using formula 3.

$$\% \text{ difference} = \frac{\text{Reanalyzed concentration} - \text{original concentration}}{\text{Mean concentration}} \times 100$$

These results additionally supported our improved

method techniques and reproducibility of data for subject sample analysis as well.

This study compares two new formulations, developed in-house, to understand bioavailability performance against Procardia XL reference drug. Procardia XL is a round bi-convex rose-pink film coated tablet with dissolution profile determined as 13% in first 4 h and 64% at around 12 h (having assay as 99.78% mg/tablet). While Test A is a brown-colored round biconvex film coated, fast-release tablet with dissolution profile determined as 19% in first 4 h and 75% at around 12 h (having assay as 97.70% mg/tablet).

Figure 4. Plasma concentration–time profile of nifedipine in humans. (A, circles) After administration of reference and test product. (B, squares) Containing 90 mg extended-release tablets nifedipine in fasted condition. R (diamonds): Reference product..

Figure 5. Plasma concentration–time profile of nifedipine in humans. (A, circles) After administration of reference and test product, (B, squares) containing 90 mg extended release tablets nifedipine in fed condition. R (diamonds): Reference product.

On the other hand, Test B is a brown colored round biconvex film coated, slow-release tablet with dissolution profile determined as 11% in first 4 h and 59% at around 12 h (having assay as 99.73% mg/tablet).

With comparative content of nifedipine active pharmaceutical ingredients for all three types of formulations, dissolution profile of two test formations was targeted to have slow and fast release in *in vitro* assay. Since the release profile of two test proto-type (Test A and Test B) in-house manufactured-formulations was found to be predictive and comparable with Procardia XL, *in vivo* study in healthy volunteers was planned with these three formulations (Reference,

Test A and Test B) for evaluation of safety and efficacy of the two test proto-types. Finally, based on the bioavailability data interpretations, any one of the proto-type test formulation will be advanced for conducting bioequivalence studies towards regulatory submission. This study is path-breaking for clinical trials conduct as we have recently contributed in clinical drug development of anti-cancer drug [31]. The identification of novel metabolites, as well as their separation from parent drug, illuminates regulatory toxicology perspective and such novel approach can be employed for conduct of adaptive clinical trials towards regulatory submissions.

Table 6. Estimated pharmacokinetic parameters derived from the plasma concentration profiles.			
Parameters	Test (A)		Test (B)
Summary of the pharmacokinetic parameters of nifedipine in fasting condition			
C _{max} (ng/ml)*	111.20 ± 42.38	124.00 ± 52.23	104.35 ± 52.23
AUC _{0-t}	2433.62 ± 1251	2379.94 ± 1126.46	2235.80 ± 1204.98
AUC _{0-∞}	2467.15 ± 1334.56	2308.04 ± 1039.56	2203.79 ± 1243.48
Summary of the pharmacokinetic parameters of nifedipine in fed condition			
C _{max} (ng/ml)*	150.96 ± 66.73	174.49 ± 97.73	149.37 ± 81.13
AUC _{0-t}	2459.28 ± 1621.66	2641.61 ± 1616.22	2475.32 ± 2105.19
AUC _{0-∞}	2476.46 ± 1643.13	2673.61 ± 1619.10	2472.33 ± 2154.67
*Log-transformed parameters, the antilog of the mean (i.e., the geometric mean) is reported. C _{max} is maximum concentration. AUC _{0-t} is area under curve from initial point to time t. AUC _{0-∞} is area under curve from initial point to infinity.			

Table 7. Statistical evaluation of pharmacokinetic parameters.

Test/reference	Parameter	C _{max}	AUC _{0-t}	AUC _{0-∞}
Summary of the statistical evaluation of pharmacokinetic parameters of nifedipine in fasting condition				
(A/R)	Ratio of least square means	111.93	97.91	97.43
	90% confidence interval	83.41–108.51	82.72–104.61	82.14–107.3
	Power	79.57	87.73	78.31
	Intrasubject variability	24.29	21.61	22.86
(B/R)	Ratio of least square means	95.14	93.02	93.88
	90% confidence interval	83.41–108.51	82.72–104.61	82.14–107.3
	Power (%)	79.57	87.73	78.31
	Intrasubject variability(%)	24.29	21.61	22.86
Summary of the statistical evaluation of pharmacokinetic parameters of nifedipine in fed condition				
(A/R)	Ratio of least square means	113.89	106.4	106.83
	90% confidence interval	100.88–128.58	92.11–122.9	92.01–124.04
	Power	85.56	71.83	68.73
	Intrasubject variability	20.21	24.12	25.01
(B/R)	Ratio of least square means	100.04	99.2	99.49
	90% confidence interval	88.61–112.94	85.88–114.59	85.69–115.51
	Power (%)	85.56	71.83	68.73
	Intrasubject variability (%)	20.21	24.12	25.01

(A/R) is ratio of test product A and reference product R.

(B/R) is ratio of test product B and reference product R.

Conclusion

Sensitive, selective and robust LC–MS/MS method for the quantification of nifedipine in human plasma was developed and validated. SPE gave high recoveries for nifedipine and IS from human plasma, without compromising matrix interference/ion suppression though matrices range. Sample preparation and analysis was found to be less time consuming. Method was found to be linear from 0.501 to 360.100 ng/ml. % Accuracy and percent CV (Precision) was within acceptable limits.

Labile metabolites were well separated resulting in improved selectivity and stability performance of the method in biological matrix. *In situ* inter-conversion between nifedipine and its metabolites in presence of matrix enzymes was brought to negligible level due to use of chilled environment, antioxidant anticoagulant (K₃EDTA) and sample handling under red light condition. Additionally, monitoring the critical parameters of MS like increase in dwell time to 400 ms and decrease of ion source temperature to 400°C provided accurate and precise assay of nifedipine from human plasma.

SPE methodology, embedded with unique steps, improved recovery (>88%) and was adopted in plasma sample preparation that provides consistent extraction recovery with minimal interference from metabolites or matrix components. Nifedipine, in presence of its

metabolites, was found to be stable in human plasma during sample preparation, analysis and storage phases. The assay performance results indicate the method to be sensitive, selective, accurate and reproducible for the routine determination of nifedipine in human plasma. The method is therefore applicable for pharmacokinetic determination of wide range of formulations such as 20, 30, 60 and 90 mg as it provides direction for analyzing maximum plasma concentration for nifedipine and its metabolites.

The validated method was used for bioavailability studies under fasting and fed condition. The C_{max} of reference product in fasting condition was 111.20 ± 42.38 (ng/ml) whereas that for Test product (A) was found to be 124.00 ± 52.23 (ng/ml) and for Test Product (B) was 104.35 ± 52.23 (ng/ml). The plasma mean concentration time profile of Reference and Test products (A) and (B) under fasting condition is depicted in Figure 5. The C_{max} of Reference product in fed condition was 150.96 ± 66.73 (ng/ml) whereas that for Test product (A) was found to be 174.49 ± 97.73 (ng/ml) and for Test Product (B) was 149.37 ± 81.13 (ng/ml). The effect of food on nifedipine for C_{max} was noted when concentration profile for Reference drug was compared. For the first time, the challenges in arresting labile metabolite inter-conversion in photo-labile drug, nifedipine estimation

from plasma has been achieved using MS/MS. This method has been successfully applied to pilot studies at fasting and fed condition with test formulations as developed in-house. Based on results for predictive bioequivalence, power for conduct of pivotal study as well as formulation modification was executed. The validated method can also be applied reliably in future exploratory studies.

Future perspective

The study has been done on LC–MS/MS; in future we can do this study using ultra high pressure liquid chromatography along with mass spectrometer in order to reduce the analysis time further. In coming years one can see the sample collection method changing significantly. Methods using minimal amount of plasma will be given much importance. New methodologies of sample preparation will be developed. Due importance shall be given to sensitivity of method.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Nifedipine, a crucial drug for hypertension management, is frequently used in patients as extended or prolonged release dosage form for which the drug accumulation/dose dumping effect needs to be critically focused.
- This study highlights the effect of fasting and fed diets that impact on bioavailability of nifedipine.

Results

- Method development was challenging due to interfering metabolites/degradants, and these metabolites were separated to make the method sensitive to nifedipine.
- The dynamic linearity ranges, high extraction recovery, along with sustained reproducibility are secondary objectives of our method development and validation in conformance with current regulatory guidelines.

Conclusion

- The validated method for estimation of nifedipine was found to be simple, reliable, accurate and highly selective for estimation of nifedipine and can be applied to pharmacokinetic studies.

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