DETERMINATION OF NIFEDIPINE IN RAT PLASMA USING HPLC-UV DETECTOR: A SIMPLE METHOD FOR PHARMACOKINETICS AND ORAL BIOAVAILABILITY STUDIES

MOSAB ARAFAT1*, ZAHAA AHMED1, MOMIR MIKOV2

1College of Pharmacy, Al Ain University of Science and Technology (6414, Al Ain, Abu Dhabi, UAE), 2Department of Pharmacology, Toxicology and Clinical Pharmacology, Medical Faculty, University of Novi Sad, Serbia (21000, Novi Sad, Serbia)

Email: mosab.arafat@aau.ac.ae

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ABSTRACT

Objective: To develop and validate a high-performance liquid chromatographic method (HPLC) for the determination of nifedipine (NFD) concentration in rat plasma.

Methods: 1.5 mol of sodium hydroxide solution was added to each plasma sample, followed by the addition of an extraction solvent based on n-hexane and dichloromethane (70: 30, v/v). The organic layer was transferred and evaporated to dryness under nitrogen flow. The residue was reconstituted with 0.5 mol of acetic acid, followed by the addition of n-hexane. After centrifuging the mixture, the supernatant organic layer of n-hexane was discarded, and the aqueous solution was injected onto the HPLC using a Phenomenex Luna-C18 reversed phase analytical column (250 x 4.6 mm, 5 µm). The mobile phase consisted of 0.01 mol aqueous ammonium formate: methanol: acetonitrile (55: 43: 2, v/v) with pH adjusted to 4.9 using formic acid. The flow rate was 0.8 ml/min; UV detector set at 235 nm and the samples were quantified using the peak area.

Results: A well-resolved NFD peak was achieved free of interference from endogenous compounds in rat plasma. Recovery of NFD was more than 93 % over concentrations ranged from 5.00 to 200 ng/ml. The limit of quantification (LOQ) of this assay was 6 ng/ml and, intra-and inter-day coefficient of variation (CV) were 5.75 % and 7.93 %, respectively. NFD was found to be stable in rat plasma after being stored at -30 °C over 90 d.

Conclusion: The stability, sensitivity, specificity and reproducibility of this method make it suitable for the determination of NFD plasma concentration in pharmacokinetics and oral bioavailability studies.

Keywords: Nifedipine, HPLC-UV assay, Validation, Stability, Plasma concentration

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Phenomenex Luna-C18 reversed phase analytical column (250 x 4.6 mm, 5 µm) was fitted with a Refillable Phenomenex guard column (10 x 2 mm, 5 µm) was used. The mobile phase (MP) was a mixture of 0.01 mol aqueous ammonium formate: methanol: acetonitrile (55: 43: 2, v/v) adjusted to pH 4.9 using formic acid. The flow rate of MP was running at 0.8 ml/min and the column temperature was set at 37 °C. Data were collected and processed using Shimadzu Class VP version 7.4 software and samples were quantified using the peak area.

Pharmacokinetic study design

NFD preparation (1 mg/ml) for oral administration was prepared by dissolving a 50 mg aliquot of NFD in 50 ml of PEG 400 (co-solvent) and milli-Q water (1:1, v/v) and stirred thoroughly at room temperature for 1 h. The drug solution was protected from light at preparation time and stored immediately after the fridge to be used within 12 h of preparation. NFD was administered to the rats orally by gavage in the dose of 5 mg/kg. Five male Wistar rats (age 2-3 mo, weight 290±30 g) were used in the experiment. They were kept in the experimental animal facility and given standard diet and water ad libitum. Temperature and light were controlled mimicking the natural habitat. The study was approved by Animal Ethics Committee. Blood samples were collected into heparinized tubes from the tip of the tail pre-dose and at 15, 30, 60, 90, 120, 150, 180, 240, 300 and 360 min after the dose. Blood samples were centrifuged at 3,000 rpm for 15 min to collect the plasma, which was stored at-30 °C prior to HPLC analysis. Immediately after the last blood sample, rats were sacrificed by carbon dioxide asphyxiation. Samples were analyzed within 6 of collections.

Sample preparation

Prior to analysis, NFD was extracted from plasma samples as follow: 80 µl of plasma aliquot was transferred into a micro-ependorf tube followed by the addition of 15 µl of internal standard (diazepam) and 20 µl of 1.5 mol sodium hydroxide solution. The mixture was vortex-mixed for 30 s and 400 µl of n-hexane: dichloromethane: methanol:sodium hydroxide (60:30:10:0.5, v/v/v/v) adjusted to pH 4.9 using formic acid. The flow rate of MP was running at 0.8 ml/min and the column temperature was set at 37 °C. Data were collected and processed using Shimadzu Class VP version 7.4 software and samples were quantified using the peak area.

Assay validation

Based on a number of previous reported methods [21-26, 31-33], the HPLC assay validation method was developed as following: A stock solution of an aliquot NFD (25 µg/ml) was prepared by dissolving 2.5 mg aliquot of NFD in 100 ml of a mixture containing mili-Q water and methanol (20: 80, v/v) and then NFD (25 µg/ml) was further diluted to 250 µl with mill-Q water and methanol to obtain a 100 ng/ml of NFD calibration curve solution. Three different batches of six standard curves each were prepared by spiking rat plasma with NFD calibration curve solution, spiking MP with NFD calibration curve solution and spiking PEG with NFD calibration curve solution to give concentrations in the range 5 to 100 ng/ml. QC samples (6, 12, 24 and 48 ng/ml of NFD) were prepared by spiking blank rat plasma with NFD calibration curve solution. The calibration curve solutions were stored at-30 °C until used for assay validation and sample analysis. Linearity was assessed by least-square regression and lack-of-fit analysis. Accuracy was expressed as the percentage of the spiked concentration and precision as the coefficient of variation (CV). LOQ is defined as the concentration with signal-to-noise ratios of 10: 1. For inter-day, accuracy and precision determination analyses of each concentration were carried out daily for 6 consecutive days whereas for intra-day accuracy and precision evaluations analyses were carried out 6 times on the same day. Recovery was estimated by comparing peak areas in QC samples (n = 6) with drug solutions at corresponding concentrations. The stability of NFD was tested in plasma at 4 different concentrations (12.5, 25, 50 and 100 ng/ml NFD). Short term stability at -30 °C was tested for freshly prepared samples and over 2 d, while long term stability was tested at-30 °C over 30 and 90 d. Another stability test was carried out after 5 freeze-thawing cycles for each sample at-30 and 25 °C.

Statistical analysis

Analysis of variance (one-way ANOVA) was used to compare mean values of variables determined for different calibration curves spiked with different materials. Differences were considered statistically significant when *p<0.05. The analysis was performed using the Minitab program (Version 1.5; Minitab Inc, USA).

RESULTS

The NFD peak with a retention time of 4.8 min was well-resolved and free from the interference of endogenous compounds in rat plasma. The total run time for each sample was 6 min. At least six calibration curve standards of rat plasma were tested. Thus, blank plasma was used in the preparation of the calibration curves. NFD was found to be stable in plasma after being protected from light for at least 24 h at room temperature with no noticeable changes in NFD concentrations in plasma.

The calibration curve of NFD spiked with rat plasma, PEG and MP (n = 6) reported in table 1 were linear over the concentration range from 5.00 to 100 ng/ml (r² 0.9997±0.0016) with a mean intercept of 0.15±0.0509. Similarly, the calibration curve of NFD spiked with PEG and MP (table 1) was linear over the same concentration range (r² 0.9996±0.0004) with a mean intercept of 0.0904±0.0281 and 0.0176±0.0371, respectively. It is evident that a linear correlation is existed between the peak area and the concentrations of NFD spiked with rat plasma, PEG and MP. This result also demonstrates that no considerable interference exists as no significant difference was found (p>0.05).

Measured NFD, inter-and intra-day accuracy and precision values for different NFD concentrations are presented in table 2 and table 3, respectively. The lowest concentration was used in the construction of QC samples was 6 ng/ml at which intra-and inter-day CV was 5.75 % and 7.93 % with a recovery of more than 93 %. This verifies that the present method is very sensitive. Furthermore, the present experimental setup was testing the NFD stability in plasma before extraction. NFD was found to be stable in rat plasma after 5 freeze-thawing cycles for each sample at 30 and 25 °C and after storage at -30 °C over 90 d (table 4). These results proof that the assay is stable, selective and sufficiently sensitive for oral bioavailability and pharmacokinetic studies in rats.

For verification, this developed method was used to analyze plasma samples from five rats after an oral administration of 5 mg/kg NFD solution. Fig. 2 shows the plasma concentration-time profiles for NFD over 6 h after the oral dose (5 mg/kg) of NFD solution. It is apparent that the presented HPLC method is sensitive enough to detect the plasma concentration-time profiles for a drug with well-known low oral bioavailability.

Table 1: Comparison between r², slopes and intercepts of different standards curves (NFD–HPLC assay)

<table>
<thead>
<tr>
<th>St Curves</th>
<th>Regression (r²)</th>
<th>Slope (mean±SD)</th>
<th>Intercept (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma spiked with NFD</td>
<td>0.9997±0.0016</td>
<td>1.0029±0.32</td>
<td>+0.0152±0.0509</td>
</tr>
<tr>
<td>PEG spiked with NFD</td>
<td>0.9998±0.0023</td>
<td>1.0371±0.14</td>
<td>-0.0904±0.0291</td>
</tr>
<tr>
<td>MP spiked with NFD</td>
<td>0.9996±0.0064</td>
<td>1.0082±0.52</td>
<td>+0.0176±0.0371</td>
</tr>
</tbody>
</table>

Data are means±SD, n=6.

Table 2: Measured NFD, Intra-day accuracy and precision of the HPLC method for the analysis of NFD in rat plasma samples
A number of analytical methods have been reported for determination of NFD in plasma [16-28]. The HPLC method developed here is based on a number of previously reported methods [21-26, 31-33], but with modifications to the organic modifier ratios and concentrations in the MP, the flow rate, the pH strength and the volume of the extraction agent. The ratio of organic solvent in the MP is essential to retain the drug molecule on the stationary phase of the column. Thus, the assessment of MP containing buffer solution with different pH strength in combination with several organic solvents such as acetonitrile, methanol and triethylamine with different volume were tested.

To determine an optimal amount of organic solvent, which can be used in the MP for the separation of the analyte, 35 to 65 % of the organic solvent mixture (methanol and acetonitrile) was used. The best peak separation with short analysis time was obtained, when the percentage of the organic solvent mixture (methanol and acetonitrile) in the MP was 45 %. The addition of 43% methanol and 2% acetonitrile were found to be necessary to produce a well-resolved peak, free of interference from adjacent endogenous compounds.

The effect of different pH strength and buffer concentration in MP on NFD retention time and resolution were assessed. Several concentrations of the aqueous ammonium formate in the buffer solution varying from 0.01 to 0.05 mol were tested. As a result of this assessment, no significant change in retention and resolution of NFD was observed, when the concentration of buffer has increased from 0.01 to 0.05 mol. For pH assessment, previous studies showed that NFD exhibits maximum stability in solutions with low pH value, as NFD is a weak acid drug molecule having low pKa value of 3.93 [34]. Therefore, different pH values with acidic range as mentioned earlier were assessed for NFD peak resolution and retention. The retention of NFD was significantly unchanged in the buffered solution over the tested pH ranges (3.5 to 6.5) with clear peak resolution appearance. However, a mobile phase with pH 4.9 was chosen to protect the used column from the low pH effect on column validity. As a result of this MP assessment, a good NFD retention with less than 6 min running time for analysis was obtained.

For normal light protection, a complete NFD degradation has been reported upon exposure to normal light for 2 h [11]. Based on these findings, an appropriate protection from light during sample preparation was taken into account to avoid any possibility of drug photo-degradation for the plasma sample during treatments. A number of organic solvents have been reported for NFD extraction,
such as dichloromethane [35], diethyl ether [13], a mixture of hexane and dichloromethane [36], a mixture of isooctane and methyl-tert-butyl ether [37], ethyl acetate [38], a mixture of n-pentane and dichloromethane [39] and chloroform [40]. Based on that, many organic solvent mixtures with different ratios were assessed such as a mixture of diethyl ether and ethyl acetate, a mixture of isooctane and methyl-tert-butyl ether and a mixture of n-hexane with dichloromethane. The result from this assessment study showed that less cloudy samples with an optimum recovery (up to 70 %) for NFD could be obtained by using a mixture of n-hexane and dichloromethane [70:30, v/v]. Therefore, the addition of 40 mL of 20% mol sodium hydrogen solution prior to the addition of organic solvent mixture was necessary for complete extraction to eliminate any sample turbidity and to increase the recovery of NFD up to satisfactory level. Thus, the used extraction solvents were found to be essential to give satisfactory recoveries for NFD and to provide distinctive chromatograms. Besides, a linear correlation existed between the peak area ratio of plasma, MP and PEG spiked NFD. Under the experimental conditions used, LOQ of this assay was found to be 6 ng/mL, whereas LOD was found to be 2 ng/mL. This points out that the present method was found to be very sensitive stable.

It is evident that the plasma concentration of NFD after the oral administered dose was low indicating low oral bioavailability of NFD when administered orally. However, the presently developed method is highly sensitive to detect the plasma concentration-time profiles for a drug with well-known low oral bioavailability (0.47–0.82%) [41]. HPLC method developed in this paper is applicable for the determination of NFD in vivo studies of different kind of NFD drug delivery dosage forms [42].

**CONCLUSION**

In conclusion, the HPLC method described here is simple, sensitive, selective, reproducible, linear, precise, accurate with short retention time and stable requiring only a small blood collection volume from rat plasma. The method was fully validated and is applicable for the determination of NFD in vivo as well as for the assessment of pharmacokinetic and oral bioavailability studies of the different type of drug dosage forms.

**CONFLICT OF INTERESTS**

Declared none.

**REFERENCES**


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