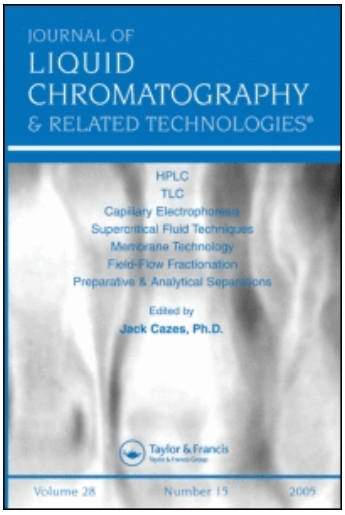

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DEVELOPMENT AND VALIDATION OF A RAPID AND SIMPLE STABILITY-INDICATING LC METHOD FOR NIFEDIPINE

Gislaine Kuminek[a]; Monika Piazzon Tagliari[a]; Andrea Granada[a]; Charise Dalazen Bertol[a]; Silvana Zucolotto Langassner[b]; Marcos Antonio Segatto Silva[a]; Hellen Karine Stulzer[ac]
[a] Laboratório de Controle de Qualidade, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Florianópolis, Brazil [b] Laboratório de Química Farmacêutica, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Campus Universitário Trindade, Florianópolis, Brazil [c] Laboratório de Controle de Qualidade, Departamento de Ciências Farmacêuticas, Universidade Estadual de Ponta Grossa, Ponta Grossa, Brazil

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DEVELOPMENT AND VALIDATION OF A RAPID AND SIMPLE STABILITY-INDICATING LC METHOD FOR NIFEDIPINE

Gislaine Kuminek,[1] Monika Piazzon Tagliari,[1] Andrea Granada,[1] Charise Dalazen Bertol,[1] Silvana Zucolotto Langassner,[2] Marcos Antonio Segatto Silva,[1] and Hellen Karine Stulzer[1,3]

[1]*Laboratório de Controle de Qualidade, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Florianópolis, Brazil*
[2]*Laboratório de Química Farmacêutica, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Campus Universitário Trindade, Florianópolis, Brazil*
[3]*Laboratório de Controle de Qualidade, Departamento de Ciências Farmacêuticas, Universidade Estadual de Ponta Grossa, Ponta Grossa, Brazil*

□ *A stability-indicating reversed-phase liquid chromatography method was developed for the determination of nifedipine in raw material. The analyses were performed on a reversed-phase Phenomenex Luna® C_{18} column (250 mm × 4.6 mm), maintained at 40 ± 1° C. The mobile phase was composed of methanol:water (70:30; v/v) and was eluted isocratically at a 1.2 mL min^{-1} flow rate. The method was validated in terms of specificity, linearity, quantification limit, detection limit, accuracy, precision, and robustness. The response was linear in the range of 0.2–1.0 mg mL^{-1} ($r^2 = 0.9992$). The relative standard deviation values for inter- and intra-day precision were 0.70% and 0.73%, respectively. Recoveries ranged between 97.5% to 101.6%. The method was successfully applied for the determination of nifedipine in raw material.*

Keywords liquid chromatography, nifedipine, stability-indicating method, validation

INTRODUCTION

Nifedipine (NFP) (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-5 pyridine dicarboxilic acid dimethyl ester) (Figure 1A) is a calcium channel blocker that has been widely used in the treatment of hypertension, angina, and myocardial infarction. NFP has a very low bioavailability, and it is photosensitive and thermally unstable. This compound, when exposed to daylight

Address correspondence to Hellen Karine Stulzer, Laboratório de Controle de Qualidade, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina. E-mail: hellen.stulzer@gmail.com

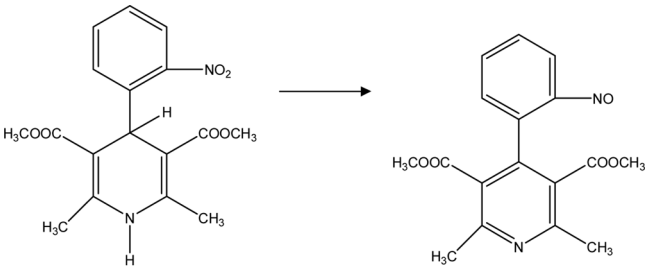

FIGURE 1 Nifedipine chemical structure (A) and nitrophenylpyridine (B).

and certain wavelengths or artificial light, readily converts to a nitrophenylpyridine derivate (NFPD) (Figure 1B).[1–3]

The literature has reported some methods for NFP determination in biological fluids, which included gas-chromatography, high performance liquid chromatography with either UV detection or electrochemical detection, fluorescence procedures, first-derivative spectroscopy, voltammetric method, and LC–MS combining a simple liquid–liquid extraction.[4–15] Additionally, the methods reported to quantify NFP in bulk and in pharmaceuticals formulations involved a variety of analytical techniques such as high performance thin layer chromatographic, liquid chromatographic, gas chromatography, polarographic, micellar electrokinetic chromatography, electroanalytical, and spectrophotometric methods.[16–23]

Stability testing is an important part of a process of drug substance development. The purpose of the stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under influence of a variety of environmental factors such as temperature, humidity, and light and enables recommendations of storage conditions, retest periods, and shelf life to be established. The main aspects of the drug product that play an important role in shelf life determinations are the assay of active drug and degradants generated during the stability studies.[24,25]

In this way, the present paper describes a simple, rapid, precise, specific, and stability-indicating LC method for quantification of NFP in the presence of its main degrading product NFPD.

EXPERIMENTAL

Chemical and Reagents

The NFP reference standard was obtained from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water was provided by a Milli-Q® purification system (Millipore, Bedford, USA). Methanol of HPLC grade was

purchased from Vetec® (Rio de Janeiro, Brazil). All chemicals used were of pharmaceutical or special analytical grade.

Instruments and Analytical Conditions

The HPLC analysis was performed on a Shimadzu LC-10A system (Kyoto, Japan) equipped with a LC-10AD pump, SPD-10AV$_{VP}$ UV detector, SPD-M10A$_{VP}$ photodiode array (PDA) detector, SCL-10A$_{VP}$ system controller, DGU-14A degasser, CTO-10AS$_{VP}$ column oven, and the sample injection was performed via a Rheodyne 7125 valve with a 20 μL loop. The detector was set at 262 nm and peak areas were integrated automatically by computer using a Shimadzu Class VP® V 6.14 software program. The experiments were carried out on a reversed-phase Phenomenex (Torrance, USA) Luna® C_{18} column (250 mm × 4.6 mm I.D., with a particle size of 5 μm and pore size of 100 Å), maintained at 40 ± 1°C. A security guard holder (4.0 mm × 3.0 mm I.D.) was used to protect the analytical column. The mobile phase consisted of methanol:water (70:30; *v/v)* and was eluted isocratically at a 1.2 mL min^{-1} flow rate.

Standard and Sample Solution

A stock standard and a sample solution of 1.0 mg mL^{-1} were prepared by dissolving 50 mg of NFP reference standard and raw material, respectively, in mobile phase in a 50 mL volumetric flask. A standard solution of NFPD was also diluted in mobile phase to a final concentration of 0.8 μg mL^{-1}.

Method Validation

Analytical method development and validation play a major role in the discovery, development, and manufacture of pharmaceuticals. The International Conference on Harmonization (ICH) requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance. An ideal stability-indicating method is one that quantifies the drug and also resolves its degradation products. In this way, the parameters used to validate the presented method were required for the assay of a dosage form: specificity, linearity and range, quantification and detection limits, accuracy, precision, and robustness.[26]

System Suitability

The system suitability was carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed using six

replicate analyses of the drug at a concentration of 0.6 mg mL^{-1}, evaluating the following parameters: peak area, retention time, asymmetry, and theoretical plates.

Specificity

The specificity was determined according to ICH[26] by subjecting a sample solution (1.0 mg mL^{-1}) to accelerated degradation by acid, alkaline, neutral, oxidative, photolytic, and thermal stress conditions to evaluate the interference of degradation products in the quantitation of NFP.

Acid, alkaline, and neutral degradation of the drug were carried out with sample solutions in 0.1 N HCl, 0.1 N NaOH, and phosphate buffer pH 6.8, respectively. The oxidative degradation was induced by storing the sample in 1% hydrogen peroxide, at ambient temperature. Photolytic studies were performed after exposition of NFP in solid state to natural light. To investigate the drug thermal stability, bulk drug was spread in a thin layer on a Petri plate and subjected to dry heat at 80°C and 120°C. After the procedures, the samples were diluted in mobile phase to a final concentration of 0.6 mg mL^{-1}. All the samples were maintained protected from light, except in the photolytic studies and the experiments were conducted until 24 hr of exposition.

Linearity, Quantification Limit, and Detection Limit

The linearity response was assessed in the range of 0.2–1.0 mg mL^{-1}. Appropriate amounts of the stock solution were diluted with mobile phase, yielding concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg mL^{-1}. Triplicate injections of each concentration were carried out on three different days. Peak area ratios of standard compounds were plotted against theoretical concentrations of standards. The linearity was expressed as a correlation coefficient by linear regression analysis. The quantification limit (QL) and detection limit (DL) were calculated from the slope and the standard deviation of the response of the mean of three calibration curves.

Precision

The precision assay was determined by repeatability (intra-day) and intermediate precision (inter-day). The repeatability of the analytical method was evaluated by assaying six samples solution of NFP 0.6 mg mL^{-1}, during the same day, under the same experimental conditions. Intermediate precision was evaluated by assaying solutions on 3 different days. Peak areas were

determined and compared. Precision was expressed as percentage of relative standard deviation (R.S.D).

Accuracy

The accuracy of the developed method was evaluated by a recovering test. NFP sample solutions of 0.2 mg mL^{-1} were fortified with 3 known concentrations of reference standards at 3 different levels lower, medium, and upper concentration. The recovery of added standard was determined in triplicate analysis and calculated taking by the formula: $R\% = \left(\frac{Fs-St}{Ss}\right) \times 100$, in which *R* is the recovery, *Fs* is the fortified solution, *Ss* is the sample solution, and *St* is the standard solution.

Robustness

The robustness was established by introducing small changes in the chromatographic system, such as flow rate (1.0, 1.2, and 1.4 mL min^{-1}) wavelength (260, 262, and 264 nm), column temperature (35, 40, and 45°C) and percent of methanol in the mobile phase (65, 70, and 75%). Robustness of the method was carried out in triplicate at NFP concentration of 0.6 mg mL^{-1}.

Nuclear Magnetic Resonance (NMR) Spectroscopy

In addition to the validation parameters, it was necessary to investigate and confirm the degrading product, NFPD. In this way, the NFP sample was analyzed by NMR analysis (Varian AS 400) before and after exposed to natural light from 48 hours. Spectra were recorded at 400 MHz with deuterated chloroform ($CDCl_3$) as solvent and tetramethylsilane as the internal standard. NFP powder sample was dissolved in $CDCl_3$ and protected to light, and the spectrum was rapidly recorded. Additionally, a spectrum was taken after exposure of the same NFP solution to natural light.

RESULTS AND DISCUSSION

Method Development

Different chromatographic conditions were tested to develop the stability-indicating LC method. The mobile phase was optimized through the evaluation of different buffers (phosphate and acetate) and organic solvents (acetonitrile and methanol). The use of methanol and water (70:30, *v*/*v*) as mobile phase resulted in better peak symmetry and short

NFP retention time. Moreover, this simple mobile phase, without salts (buffers), is an advantage over the methods previously cited in the literature. For the selection of the best wavelength detection, a PDA detector was used.

The system suitability results showed that the parameters were within the suitable range. The mean theoretical plates, retention time (min), peak area, and peak asymmetry ±RSD (%) found were 3840.77 ± 1.663, 5.30 ± 0.25, 11617313 ± 0.456, and 0.80 ± 0.11, respectively.

The forced degradation was performed to validate the stability-indicating capability of the developed method and to identify the key factors which will impact the stability of the drug product.

Method Validation

Specificity

Photodiode array detection was used as an evidence of the specificity of the method, which showed a single peak for pure drug in 262 nm. The NFP and NFDP samples were first identified through their respective standards by comparing the retention time. The percentages of NFP degraded in acid, alkaline, neutral, thermal 80, and 120°C conditions were 20.01%; 30.13%; 10.14%; 23.8%, and 25.9%, respectively, after 24 hr of exposure. The natural light conditions showed more significant degradation, with 71.44% of NFP degraded after 24 hr, followed by oxidative condition with 43.7% of degradation after the same exposure time. After the stress assay, one additional peak was observed at 4.5 min, related to the degradation product NFPD. However, it did not interfere with the drug detection; it is well separated from the NFP peak. To confirm the separation between NFPD and NFP peaks, a co-injection of both was realized and showed different retention time of each compound. The more evident chromatograms of NFP degradation extend under various stress conditions is represented in Figure 2.

The process of photodecomposition of the NFP can also be observed by ^{1}H NMR spectroscopy. Figure 3A shows a typical ^{1}H-NMR spectra of NFP before light exposure and the NFPD spectra after natural light exposure is presented in Figure 3B. The comparison between the spectra of NFP solution before and after exposure to natural light suggests the formation of a degradation product. The main evidence observed in Figure 3 is the absence of the singlet signal in δ 5.72 corresponding to N-H indicated the NFP aromatization in the dihydropyridine moiety (turning it into a pyridine ring). The obtained spectrums were also in accordance with literature.[27]

Linearity, Quantification Limit, and Detection Limit

The linearity of detector response was assessed for various solution standards over the range of 0.2–1.0 mg mL^{-1}. The value of the determination

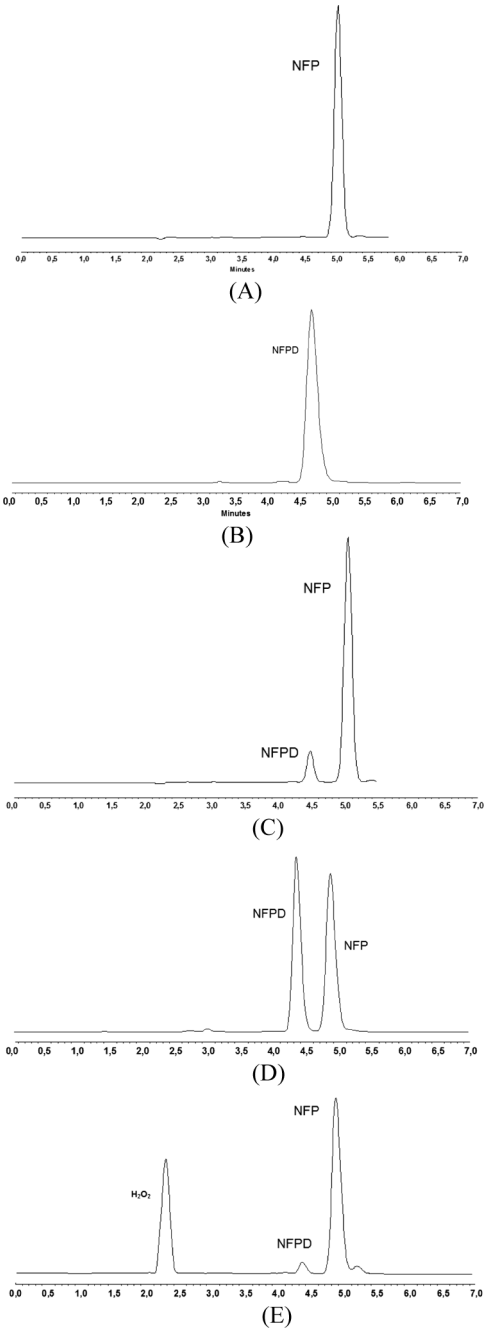

FIGURE 2 Chromatograms of NFP standard (A), NFPD standard (B), co-injections of NFP and NFPD standards (C), chromatograms obtained under stress studies: natural light (D), and oxidative (E).

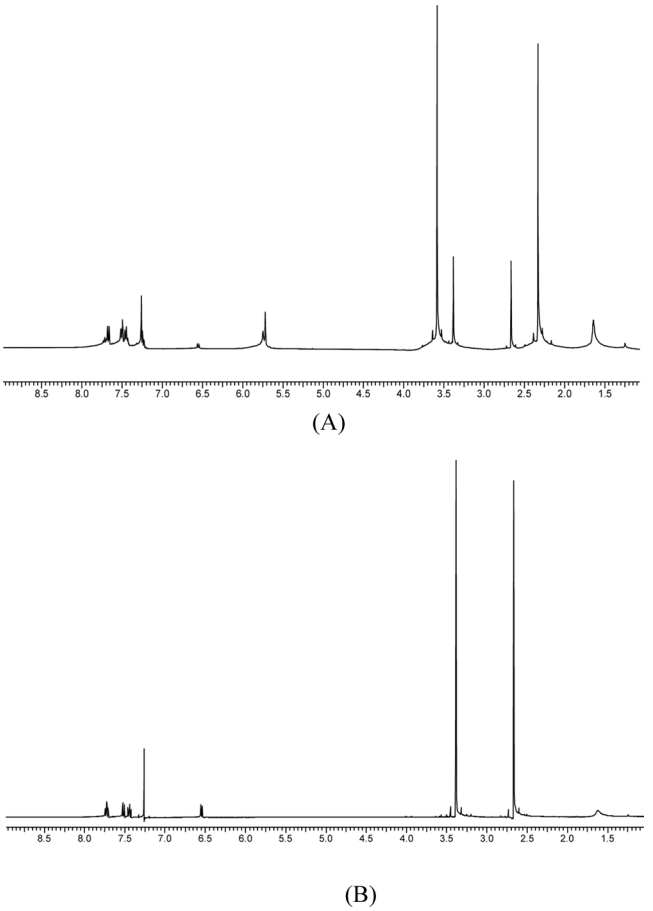

FIGURE 3 ^{1}H NMR spectrum of NFP (A) and NFP after photo-decomposition (B), 400 MHZ, solvent $CDCl_3$.

coefficient calculated ($r^2 = 0.9992$, $y = 6000000 \pm 1.98x - 366601 \pm 2.31$; where, x is concentration and y is the peak absolute area), indicated the linearity of the calibration curve for the method. The quantification and

TABLE 1 Results from the Precision

Sample Solution ($0.6\,mg\,mL^{-1}$)	(%) Recovered[a] ± RSD[b]	Sample Solution ($0.6\,mg\,mL^{-1}$)	(%) Recovered[a] ± RSD[b]
Day 1	99.81 ± 0.75	Morning	99.66 ± 0.55
Day 2	98.98 ± 0.66	Afternoon	101.03 ± 0.67
Day 3	99.56 ± 0.71	Night	98.85 ± 0.99
Mean inter-day	99.45 ± 0.70	Mean same-day	99.84 ± 0.73

[a]Mean of three replicates.
[b]RSD = Relative standard deviation.

TABLE 2 Results from the Recovery Test

Fortified Solution (mg mL^{-1})	Mean Concentration found (mg mL^{-1})	(%) Recovery ± RSD
0.4	0.39	97.5 ± 0.76
0.6	0.61	101.6 ± 0.44
1.0	0.99	99.0 ± 0.63

[a]RSD = Relative standard deviation.

the detection limits calculated were 0.09 mg mL^{-1} and 0.01 mg mL^{-1} respectively, which indicate the adequate sensitivity of the method.

Precision

The repeatability and intermediate precision of the method were based on intra-day (morning, afternoon, and night) and three different days. It was performed, by replicate injections (n = 6) of 0.6 mg mL^{-1} NFP sample solutions (Table 1). The amounts of NFP found were equivalent ($P < 0.05$), and the relative standard deviation values were within the acceptance criteria of 2%.[26]

Accuracy

The accuracy was assessed from three replicate determinations of three different fortified solutions (Table 2). No significant differences were observed between amounts of NFP added and the amounts found

TABLE 3 Chromatographic Conditions and Range Investigated During Robustness Testing

Variable	Range investigated	NFP[a] (%)
Flow rate (mL min^{-1})	1.0	100.16
	1.2	100.01
	1.4	101.07
Mobile phase pH	2.5	99.91
	3.0	99.66
	3.5	99.78
Wavelength (nm)	260	99.89
	262	100.02
	264	100.03
Column temperature (°C)	35	100.08
	40	100.21
	45	100.33
Percent of methanol	65	99.97
	70	99.85
	75	101.04

[a]Mean of three replicates.

($P < 0.05$). The obtained values were within the range of 97.5–101.6%, satisfying the acceptance criteria for the study, which was ±3%.

Robustness

The results and experimental range of the variables evaluated in the robustness assessment are given in Table 3. The analysis performed, demonstrating that changes of chromatographic conditions, did not influence significantly with the analytical results.

CONCLUSIONS

The proposed liquid chromatographic method was validated in terms of specificity, linearity, accuracy, precision, and robustness and proved to be capable of separating NFP and its major degradation product, which demonstrated its suitability for use as a stability-indicating method during stability studies. The short analytical run time of less than 5.0 min leads to a cost effective and rapid chromatographic procedure.

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