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Research Article

HPLC method for the determination of nifedipine in rat plasma: development, validation, and application to pharmacokinetic drug-herb interaction study

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Abstract

A simple and rapid high-performance liquid chromatography (HPLC) was developed to determine the plasma level of nifedipine in rats after its single administration and combination with herbs. Nifedipine was extracted with acetonitrile to precipitate protein from plasma samples. The separation was implemented on a C_{18} column with a mobile phase of acetonitrile: water (63:37, v/v). The calibration curve displayed good linearity in the 30–1000 ng/mL range. The lower limit of quantification (LLOQ) was 30 ng/mL. The intraday and interday assay accuracy and precision met the criteria of validation and study sample analysis. The recovery was found to be 101.89%. Stability studies showed that nifedipine was stable after 12 h at room temperature and 21 days at -20 °C. No significant difference was examined between the pharmacokinetic parameters of nifedipine with or without *Gynura procumbens* leaf extract. The proposed method was helpful for the pharmacokinetic interaction study of nifedipine combined with herbal in rats.

Keywords

Nifedipine, drug-herb interaction, pharmacokinetic, HPLC

Introduction

Hypertension is the leading cause of cardiovascular disease and premature death worldwide (Mills et al. 2020; Al-Makki et al. 2022). According to previous studies, 47.5% of patients affected with this condition use an antihypertensive drug along with herbal medicine (Liwa et al. 2014). In addition, the combination of these medications causes herb-drug interactions (HDIs), raising significant concern among healthcare professionals, scientists, regulatory authorities, and consumers (World Health Organization 2021). HDIs can become clinically significant when considerable changes occur to the pharmacokinetic parameters of a drug. These interactions also have the potential to interfere with the absorption, distribution, metabolism, and excretion of co-administered drug (Zhou et al. 2004; Yang et al. 2006; Tarirai et al. 2010).

Nifedipine is a calcium channel blocker that belongs to the dihydropyridine subclass. It is primarily used as an antihypertensive and antianginal medication. Nifedipine

undergoes 95% metabolism in the liver through the CYP3A4 pathway (Khan et al. 2022). Herbal medicines are mixtures of several compounds with the potential to cause interactions, including induction or inhibition of metabolizing enzymes and drug efflux proteins. The inhibitory effect of herbs on efflux proteins and CYP3A4 can lead to enhanced plasma and tissue concentrations, causing toxicity. Meanwhile, the inductive effect can cause reduced drug concentrations, decreased drug efficacy, and treatment failure (Pal and Mitra 2006).

In response to the occurrence of HDIs, several bioanalytical methods of drug quantitative evaluation biological matrix have been developed, which are essential for pharmacokinetic studies. Therefore, bioanalytical methods developed must be well-designed and adequately validated to a satisfactory standard, ensuring their reliability when applied to drug analysis. A particularly versatile analytical tool in this context is high-performance liquid chromatography (HPLC). This tool is often used in the identification and quantitative estimation of a drug in the biological matrix (Pushpa Latha and Sailaja 2014).

Previous studies have used HPLC as an analytical tool to detect nifedipine in the biological matrix (Shahriyar and Lau-Cam 2000; Yritia et al. 2000; Niopas and Daftsios 2003; Rajesham et al. 2008; Arafat et al. 2016), but it was only to determine nifedipine alone or simultaneously with other conventional drugs. Therefore, this study aims to develop and validate a simple, rapid, and sensitive method to detect nifedipine plasma levels when given with or without herbal medicines. In this study, *Gynura procumbens* leaf extract was used as a herbal medicine, which was often used in hypertension therapy. The validation parameters were based on criteria of US FDA guidance for industry bioanalytical method validation and study sample analysis (2022).

Experimental

Chemicals and materials

Nifedipine micronized, manufactured by CHEMLINE Healthcare (Lugano, Switzerland), was gifted by Research Center Kimia Farma Ltd. (Indonesia). *Gynura procumbens* leaf extract was purchased from Herbal Indo Utama (Indonesia). HPLC grade acetonitrile (Merck, Germany) and double-distilled water (Ipha Laboratories, Indonesia) were used as the mobile phase. Blank plasma was obtained from the Indonesian Red Cross Society.

Chromatographic conditions

HPLC with a UV detector (Jasco) and Enduro C18G column (250 mm × 4.6 mm i.d., 5 µm) were used in this study. The UV detector was used at a wavelength of 235 nm. The mobile phase used was a mixture of acetonitrile and water phase in a ratio of 63:37 (v/v). Elution was carried out at a flow rate of 1 mL/min for 7 min.

Preparation of working and calibration standard solutions

A stock solution of nifedipine was prepared at a concentration of 1000 µg/mL in acetonitrile: double-distilled water (50:50, v/v). Subsequently, the stock solution was diluted to a final concentration of 100 µg/mL. Working solutions of nifedipine were prepared from diluting stock solution (100 µg/mL) in the range of 0.3–10 µg/mL. Calibration standards were prepared by diluting working solutions with blank plasma to obtain a range of concentrations from 30 to 1000 ng/mL.

Preparation of plasma samples

Plasma samples were prepared through protein precipitation with acetonitrile. Plasma samples were thawed and vortexed for 10 sec. Plasma samples (50 µL) were transferred into microcentrifuge tubes. Acetonitrile (100 µL) was added to precipitate the plasma protein, followed by vortexing for 30 sec and centrifugation at 12000 rpm for 10 min. The supernatant (50 µL) was injected for HPLC analysis.

HPLC method validation

Selectivity and Specificity

The selectivity of the method was determined by blank plasma samples from 6 different lots to test potential interference substances. The specificity was evaluated by the spiked substance (a marker of herbal medicine) to plasma samples to detect and differentiate nifedipine from other substances. Furthermore, responses detected must not be more than 20% of the lower limit of quantification (LLOQ) of nifedipine.

Linearity

Linearity was evaluated by analyzing calibration standards (in the range of 30 to 1000 ng/mL) at 6 different concentrations in 5 replicates. Calibration curves were obtained over linear regression by plotting the nifedipine concentrations (x) as abscissa versus the peak area (y) as ordinate.

Accuracy and precision

Accuracy and precision were determined by analyzing the quality control (QC) in intraday (within-run) and interday (between-run). Intraday and interday were determined using single-day and five-day analysis, respectively. QC samples consist of LLOQ, low (LQC), medium (MQC), and high (HQC) concentrations. The concentrations were as follows: 30, 90, 400, and 800 ng/mL. The formula assessed accuracy: %error = [(mean measured concentrations – actual concentrations)/actual concentrations] × 100%, and precision: % coefficient of variation

(CV) = (SD/mean measured concentrations) × 100%. The accuracy and precision at each concentration level must be in ±15%, and the LLOQ must be in ±20%.

Extraction recovery

The recovery (extraction efficiency) was assessed at the QC samples. Recovery was evaluated as a percentage by comparing the peak area of the analyte after the extraction procedure with the peak obtained from directly injecting the analyte at the same concentration.

Stability

Stability evaluations were carried out to examine the short-term and long-term stability. The samples used included LQC and HQC with 3 replicates for each concentration. Storage for short-term and long-term stability was carried out at room temperature for 12 hours and -20 °C for 21 days, respectively. QC samples were analyzed with freshly spiked calibration standards. The accuracy and precision of LQC and HQC samples were determined.

Application of the method to pharmacokinetic study

The pharmacokinetic study was approved by the Animal Ethics Committee (Approval No.: KEP/I/2023/VIII/H310723DN/FKSN) of Institut Teknologi Bandung (Bandung, Indonesia). A total of 6 male Wistar rats were randomly divided into 2 groups. Group 1 was given a single nifedipine 1 mg/kg. Group 2 was given nifedipine 1 mg/kg concomitant with *Gynura procumbens* leaf extract 154 mg/kg. The route of administration of each group was intragastric oral. Blood samples were taken through the femoral artery at 0, 0.05, 0.1, 0.25, 0.5, 1, 1.5, 2, 3, 4.5, 6, and 8 hours and kept in heparin-filled microcentrifuge tubes. The plasma was collected and stored at -20 °C until analysis.

Pharmacokinetic analysis

Pharmacokinetic parameters were determined by following a two-compartment model described by an equation (Gibaldi and Perrier 1982):

$$C = Ne^{-ka.t} + Le^{-\alpha.t} + Me^{-\beta.t}$$

where: C is the plasma concentration, α and β are apparent first-order distribution and elimination rate constants, respectively, ka is the absorption rate constant, t is the time after administration of drug, and L, M, and N are coefficients.

Data were obtained using the residual method. The curve was generated from a plot of the natural logarithm of the plasma concentration versus time. The slope of distribution and elimination phases were α and β, respectively. Ka was the slope created from the plot of the natural logarithm of a second series of residual concentration – time.

The constant k_{10} is the apparent first-order elimination rate constant from the central compartment, and k_{12} and k_{21} are the transfer rate constants between the central and the peripheral compartments.

k_{21}, k_{12}, and k_{10} were calculated as (Gibaldi and Perrier 1982; Geisz and Bourin 1986):

$$k_{21} = (L{\cdot}\beta{\cdot}ka + M{\cdot}\alpha{\cdot}ka + N{\cdot}\alpha{\cdot}\beta)/(L(ka - \alpha) + M(ka - \beta)),$$

$$k_{10} = \alpha{\cdot}\beta/k_{21},$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10}$$

The half-life of distribution ($t_{1/2\alpha}$) and elimination ($t_{1/2\beta}$) were determined using the relationship $t_{1/2} = 0.693/k$. The area under concentration plasma-time curve from time zero to the last time point (AUC_{0-t}) was obtained by linear trapezoidal summation. The AUC_{0-t} was extrapolated to infinity ($AUC_{0-\infty}$) using the last measured concentration divided by β. The volume of distribution (VD) was calculated from the dose divided by $AUC_{0-\infty}$ times β. The clearance (Cl) was resulted using the equation Cl = β·VD.

Statistical analysis

The difference in pharmacokinetic parameters between the two groups was performed by Minitab (version 21; Minitab, Inc., State College, Pennsylvania) using the 2-sample *t*-test. In this study, P values less than 0.05 were considered significant.

Results and discussion

Finding pharmacokinetic interactions of drugs used with herbs requires an analytical method. The analysis method was very important in determining the quantitative levels of drugs in biological samples. Therefore, the HPLC method was developed in this study to handle large numbers of samples easily, sensitively, and cost-effectively. In addition to HPLC, a frequently used analytical method was liquid chromatography-tandem mass spectrometry (LC-MS/MS). Several studies showed that LC-MS/MS could separate and more accurately quantify nifedipine compared to others (Kallem et al. 2013; Ezzeldin et al. 2014; Logoyda 2020). However, the major disadvantage of LC-MS/MS was the high cost for most studies and clinical laboratories (Heath et al. 2014).

Chromatographic separation was performed on an Enduro C18G column (250 mm × 4.6 mm i.d., 5 μm) using the isocratic elution method with a 1 mL/min flow rate. Several mobile phase mixtures had previously been used for the analysis of nifedipine in rat plasma such as a mixture of acetonitrile: methanol: 0.01 M phosphate buffer pH 5.2 (55:15:30, v/v) (Shahriyar and Lau-Cam 2000), 0.01 mol aqueous ammonium formate: methanol: acetonitrile (55:43:2, v/v) (Arafat et al. 2016), 0.085% of phosphoric acid: acetonitrile (55:45, v/v) (Mohri and Uesawa 2001).

Table 1. Intra- and inter-day Accuracy (% error) and Precision (% CV) of QC samples (n = 5).

Actual concentration (ng/mL)	Intraday			Interday		
	Measured concentration (ng/mL)	Error (%)	CV (%)	Measured concentration (ng/mL)	Error (%)	CV (%)
30	35.42 ± 0.40	18.08	1.13	32.97 ± 2.67	9.92	8.10
90	91.62 ± 3.11	1.80	3.39	88.03 ± 2.61	-2.19	2.96
400	421.44 ± 9.37	5.36	2.22	403.75 ± 7.81	0.94	1.93
800	820.57 ± 21.73	2.57	2.65	811.49 ± 32.27	1.44	3.98

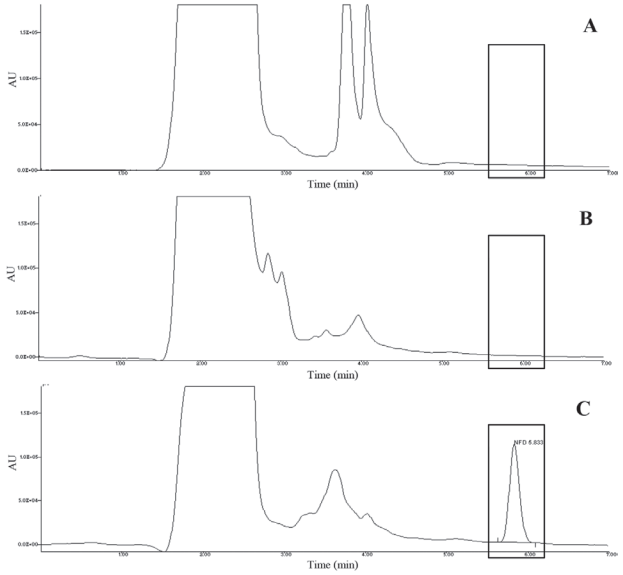

Figure 1. HPLC chromatogram of nifedipine. **A.** Plasma sample spiked with 5 µL/mL of kaempferol; **B**. Nifedipine-free plasma; **C.** Plasma sample spiked with 1000 ng/mL of nifedipine.

In this method, the mobile phase used was very simple consisting of acetonitrile and water (63:37, v/v) with a total elution time of 7 mins. UV detection was performed at a wavelength of 235 nm. These conditions led to separation with a retention time of approximately 5.8 min, good peak shape (symmetrical) (Fig. 1C), and no interference by other components in blank plasma samples on the retention time of nifedipine (Fig. 1B). The results indicated that the developed method was highly selective. The spiked kaempferol as a marker of *Gynura procumbens* leaf extract in plasma samples did not give any interfering peaks (Fig. 1A), indicating the specificity of the method in detecting nifedipine.

In line with previous studies, various methods had been used to extract nifedipine in plasma. The majority of the extraction process was performed from alkaline media with an immiscible organic solvent, evaporated, and reconstituted with a suitable mobile phase or organic solvent (Shahriyar and Lau-Cam 2000). Meanwhile, nifedipine was a photosensitive drug (Bayomi et al. 2002). For sample preparation, protein precipitation with acetonitrile was used in this method. Protein precipitation was the most rapid and inexpensive plasma sample preparation technique compared to liquid-liquid extraction, solid phase extraction, and the modifications (Margaryan et al. 2020). The final ratio was 2:1 (v/v) acetonitrile to the plasma samples. This method successfully obtained the lowest amount of analyte (LLOQ) of 30 ng/mL extracted from 50 µL of plasma samples.

The calibration curve was linear in the 30–1000 ng/mL range (Fig. 2). The regression equation generated from 5

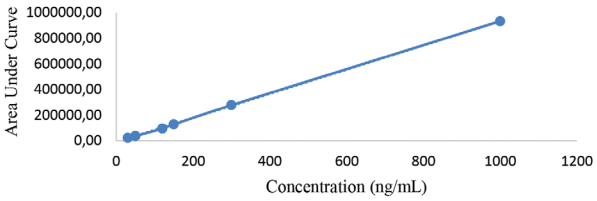

Figure 2. Calibration plot of nifedipine in plasma.

replicates of the calibration curve was y = 945.09x – 11445 with a correlation coefficient value of 0.9998. The accuracy obtained from intra and interday QC samples were 1.80–5.36% and -2.19–1.44%, respectively, except for LLOQ, which was 18.08% and 9.92%. Intraday and interday's precision was 2.22–3.39% and 1.93–3.98%, respectively, as shown in Table 1. Intraday and interday LLOQ precision were 1.13% and 8.10%, respectively. These results showed that the method was accurate and precise as well as meeting the criteria for bioanalytical assay.

Percent recovery was obtained when comparing the analyte peak after the extraction procedure with the peak obtained from directly injecting the analyte at the same concentration. The percent recovery of nifedipine in plasma was a range of 97.29–107.89%, as presented in Table 2.

Table 2. Extraction recovery of nifedipine in plasma (n = 4).

Actual concentration (ng/mL)	Recovery (%)	CV (%)
30	97.29 ± 7.01	7.20
90	101.94 ± 0.92	0.90
400	100.43 ± 1.46	1.45
800	107.89 ± 1.88	1.74

A stability evaluation was carried out to ensure that every step taken in handling, analysis, and storage conditions did not affect the analyte concentration. The validation storage period should be performed on QC that had been stored for a while equal to or longer than the storage period of the tested sample. The results of the stability evaluation are presented in Table 3. LQC and HQC samples of nifedipine were stable for 12 hours and after 21 days of storage at -20 °C. During preparation and storage procedures, samples were always kept in amber glass containers to avoid exposure to light.

Table 3. Long- and short-term stability of nifedipine in plasma (n = 3).

Conditions	Actual concentration (ng/mL)	Measured concentration (ng/mL)	Error (%)	CV (%)
-20 °C 21 days	90	89.51 ± 1.94	-0.54	2.16
	800	791.26 ± 12.37	-1.09	1.58
12 hours room temperature	90	90.70 ± 1.70	0.01	1.87
	800	751.76 ± 11.12	-6.0	1.48

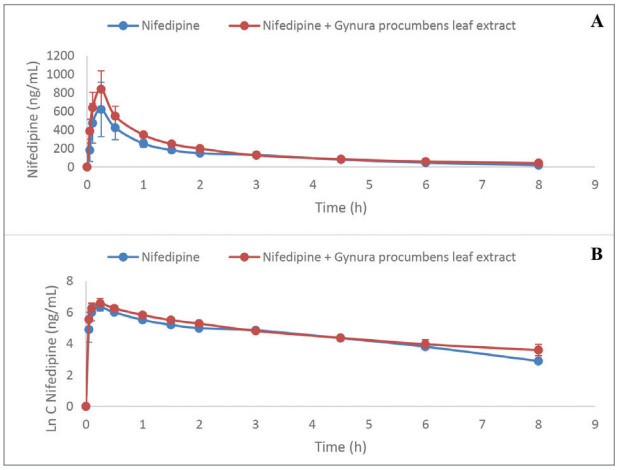

Figure 3. A. Concentration-time (mean ± SD) and **B.** Natural logarithm-time (mean ± SD) of nifedipine in plasma after a single dose of nifedipine 1 mg/kg (n = 3) and co-administration of nifedipine with *Gynura procumbens* leaf extract 154 mg/kg (n = 3) in male Wistar rats.

The pharmacokinetic profile of nifedipine alone and nifedipine co-administered with *Gynura procumbens* leaf extract showed a two-compartment oral model (Fig. 3). The concentration of nifedipine increased to the maximum concentration at 15 min (0.25 h) after administration. The results showed that the concentration of nifedipine decreased in a biphasic phase. The first phase was the distribution phase and the second was the elimination phase showing that nifedipine followed a two-compartment model. The residuals method was a frequently employed method for dividing a curve into its several exponential components (Gibaldi and Perrier 1982). This method was used to fit a curve into the experimental data when drugs did not fit into a one-compartment model (Ahmed 2015).

The co-administration of *Gynura procumbens* leaf extract with nifedipine did not alter nifedipine profile or pharmacokinetic parameters (Table 4). In this study, the compounds contained in *Gynura procumbens* leaf extract were not able to inhibit CYP3A4. Inhibition based on the CYP3A4 mechanism could lead to increased levels of the drug. For example, a well-known pharmacokinetic interaction was between

Table 4. Pharmacokinetic parameters of nifedipine in male Wistar rats.

Parameters	Nifedipine (n = 3)	Nifedipine + *Gynura procumbens* leaf extract (n = 3)
α (/h)	2.16 ± 0.52	1.33 ± 0.49
β (/h)	0.35 ± 0.04	0.21 ± 0.03
Ka (/h)	13.97 ± 10.55	14.86 ± 7.19
K_{21} (/h)	1.33 ± 0.10	0.55 ± 0.02
K_{10} (/h)	0.56 ± 0.11	0.51 ± 0.22
K_{12} (/h)	0.61 ± 0.46	0.47 ± 0.29
$t_{1/2\beta}$ (h)	1.99 ± 0.27	3.41 ± 0.49
$t_{1/2\alpha}$ (h)	0.34 ± 0.09	0.64 ± 0.32
AUC_{0-8} (ng.h/mL)	1047.98 ± 188.36	1166.30 ± 305.31
$AUC_{0-\infty}$ (ng.h/mL)	1103.37 ± 209.08	1322.97 ± 261.36
Cl (mL/h.Kg)	938.46 ± 171.00	789.89 ± 173.09
VD_{β} (L/Kg)	2.63 ± 0.21	3.99 ± 1.39

grapefruit and most conventional drugs. A previous report revealed that grapefruit juice significantly increased the bioavailability of more than 30 prescription drugs through co-inhibition of the efflux transporter P-glycoprotein (P-gp) and the enzyme CYP3A4 (Tarirai et al. 2010).

The present HPLC assay met the criteria for validation parameters and proved reproducible for the determination of nifedipine levels in rat plasma with a relatively simple sample preparation procedure. Nifedipine concentrations in rat plasma were successfully measured up to 8 hours after intragastric administration.

Conclusion

In conclusion, the developed and validated HPLC method with a protein precipitation procedure led to a simple and rapid measurement with a total elution time of 7 min to quantify nifedipine in this study. Furthermore, it provided satisfactory selectivity and specificity, good linearity (r = 0.9998) of 30–1000 ng/mL, accuracy, precision, and recovery of ~100%. The results showed that plasma samples of nifedipine were stable in storage under the intended conditions. This method was successfully applied to the pharmacokinetic study of nifedipine interaction with herbs.

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