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Liquid chromatographic assay of nifedipine in human plasma and its application to pharmacokinetic studies

Hisham S. Abou-Auda *, Tawfeeg A. Najjar, Khalil I. Al-Khamis, Badraddin M. Al-Hadiya, Nausha M. Ghilzai, Nasser F. Al-Fawzan

Department of Clinical Pharmacy, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Saudi Arabia

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Abstract

A highly sensitive, selective and reproducible reversed-phase high-performance liquid chromatographic method has been developed for the determination of nifedipine in human plasma with minimum sample preparation. The method is sensitive to 3 ng/ml in plasma, with acceptable within- and between-day reproducibilities and linearity ($r^2 > 0.99$) over a concentration range from 10–200 ng/ml. Acidified plasma samples were extracted using diethyether containing diazepam as internal standard and chromatographic separation was accomplished on C_{18} column using a mobile phase consisting of acetonitrile, methanol and water (35:17:48, v/v). The within-day precision ranged from 2.22 to 4.64% and accuracy ranged from 102.4–106.4%. The day-to-day precision ranged from 2.34–7.07% and accuracy from 95.1–100.1%. The relative recoveries of nifedipine from plasma ranged from 91.0–107.3% whereas extraction recoveries were 88.6–93.3%. Following eight 6-week freeze-thaw cycles, nifedipine in plasma samples proved to be stable with accuracy ranging from 0.64 to 3.0% and precision ranging from 3.6 to 4.15%. Nifedipine was also found to be photostable for at least 120 min in plasma, 30 min in blood and for 60 min in aqueous solutions after exposure to light. The method is sensitive and reliable for pharmacokinetic studies and therapeutic drug monitoring of nifedipine in humans after the oral administration of immediate-release capsules and sustained-release tablets to five healthy subjects.

Keywords: High-performance liquid chromatography; Nifedipine; Photostability; Freeze/thaw stability; Pharmacokinetics; Therapeutic drug monitoring

1. Introduction

Nifedipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester, is one of the most potent calcium-channel blockers belonging to the group of 1,4-dihydropyridines [1]. It is widely used in the treatment of vascular diseases such as hypertension, angina pectoris and Raynaud's phenomenon [2]. Nifedipine, a highly non-polar compound, is absorbed completely from the gastrointestinal tract, predominantly from the jejunum, but has a very low

* Corresponding author. Tel.: +966-1-467-7470; fax: +966-1-467-6789.
E-mail address: hisham@ksu.edu.sa (H.S. Abou-Auda)

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bioavailability mainly due to presystemic metabolism [3]. Following absorption, nifedipine is further metabolized in the small intestine and liver to more polar compounds which are primarily eliminated by the kidney [3]. Nifedipine is a photolabile compound, undergoing oxidative biotransformation in human body into pharmacologically inactive metabolites [4].

Early analytical methods for determining nifedipine levels in biological fluids were gas chromatographic (GC) alone [5–10] or combined with spectrometric analysis [11]. Although some of these methods required microliter volumes and their lower limit of detection could go as low as 2 ng/ml, they suffer from a lack of specificity and selectivity. Most of GC methods required laborious liquid-liquid and solid-phase extraction procedures to give adequate sample clean-up, thus increasing the complexity and time required for analysis.

In order to improve sensitivity and specificity, high performance liquid chromatographic (HPLC) methods [12–25] for the determination of nifedipine in plasma have been developed. Many of these methods involved very lengthy and time-consuming sample extraction. The major goal for the determination of nifedipine in plasma is to yield a reliable estimate of its pharmacokinetic parameters for therapeutic drug monitoring and bioavailability/bioequivalence purposes. These estimates depend on the ability of the analytical method to measure the drug level at the lower end of the plasma concentration range found in pharmacokinetic studies following the administration of therapeutic doses of the drug. One of the serious pitfalls in nifedipine determination by HPLC is that some of the previously published methods [14,20,21] codetermined nifedipine with its primary metabolite. This concomitant determination will lead to inaccurate pharmacokinetic data, especially for oral administration because of extensive first-pass metabolism of the drug [26]. In addition, many of the reported methods [12–25] lack full validation procedures recommended by the Food and Drug Administration (FDA), Health Canada and other related European agencies [27]. They also omitted accuracy especially for the lowest quantifiable concentration and have not conducted the photostability study of nifedipine.

In this study, we report a rapid, sensitive, selective, accurate and reproducible reversed-phase HPLC assay in human plasma. This method is suitable for processing large number of nifedipine plasma samples withdrawn during clinical pharmacokinetic studies.

2. Materials and methods

2.1. Apparatus

A Waters HPLC system (Milford, MA) was used, consisting of model M-45 solvent delivery pump, an autosampler (model WISP-712), model 481 UV/VIS variable wavelength detector set at 240 nm, in combination with a data module integrator (model 746). Chromatographic separations were accomplished using a supelcosil LC-18, 5 μm, 15 cm × 4.6 mm stainless steel column (Supelco, Bellefonte, PA) with a guard precolumn of same packing material.

2.2. Reagents

Nifedipine hydrochloride was kindly supplied by Bayer (Wuppertal, Germany). Diazepam (as internal standard) was obtained from Sigma Chemicals (St Louis, MO). Methanol and acetonitrile were of HPLC grades (BDH Chemicals, Poole, UK). All other chemicals and reagents were of analytical grade.

2.3. Standard solutions

Nifedipine hydrochloride (10 mg) was dissolved in 100 ml of methanol in dark under a sodium lamp. The solution was further diluted with methanol 10-fold to give a working standard solution of 10 μg/ml concentration. The solution was protected from light with an aluminium foil wrapping and stored at −70°C. The solution was stable for at least 3 months. The internal standard, diazepam (10 mg) was dissolved in 100 ml of methanol to give a 100 μg/ml stock solution, which is further diluted to give 1 μg/ml of work-

ing standard solution and stored at −70°C (stable for at least 1 month).

2.4. Chromatographic conditions

The mobile phase consisted of acetonitrile-methanol-water (35:17: 48, v/v). The pH of the mixed solvent system was adjusted to pH 3.8 with phosphoric acid. The mixture was filtered through a 0.22 μm membrane (Millipore, Bedford, MA) under vacuum, then degassed by flushing with nitrogen for 5 min. The mobile phase was pumped isocratically at a flow rate of 1.2 ml/min during analysis, at ambient temperature. The chromatograms were recorded and integrated at a chart speed of 0.25 cm/min. The effluent was monitored at 240 nm at sensitivity of 0.02 AUFS.

2.5. Analytical procedure

Sample preparation and analysis were conducted at room temperature under a sodium lamp. Nifedipine working standard (10 μg/ml) was added to 15-ml graduated centrifuge tubes in volumes of 0, 10, 25, 50, 75, 100, 125, 150, and 200 μl. Drug-free human plasma was added to complete volume to 10 ml, vortex-mixed for 30 s to yield final calibration standard concentrations of 0.0 (no nifedipine added), 10, 25, 50, 75, 100, 125, 150 and 200 ng/ml. Each of these standard solutions were distributed in disposable polypropylene microcentrifuge tubes (1.5-ml, Eppendorf, Hamburg, Germany) in volumes of 1.2 ml and stored to protect them from light at −70°C pending analysis. For preparation of samples for injection onto HPLC system, a 100 μl aliquot of the internal standard (diazepam, 1 μg/ml) was added to 1 ml of plasma sample in a 10-ml glass stoppered tube, and vortex-mixed for 15 s. The sample was then alkalinized by addition of 100 μl of 1 N NaOH, vortex-mixed for 30 s, and 5 ml of diethylether was added. This mixture was vortex-mixed for 1 min and centrifuged at $2400 \times g$ for 10 min. The supernatant organic layer was quantitatively transferred to another 10-ml glass centrifuge tube and the contents were evaporated to dryness at room temperature under a stream of pure nitrogen. The residue was reconstituted in 250 μl of mobile phase, vortex-mixed for 30 s, transferred to 1.5 ml eppendorf tubes and centrifuged at $8500 \times g$ for 5 min to precipitate any particulate matter. Aliquots of 80 μl were injected onto the column.

2.6. Clinical study

Five healthy volunteers participated in this study. Four volunteers were administered a single dose (2×10 mg) of nifedipine capsules (Adalat®) and one volunteer was given a single dose (20 mg) of nifedipine slow release tablets (Adalat® Retard). An indwelling venous cannula was inserted into the antebrachial vein and blood samples were drawn before drug administration and at 0.167, 0.333, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 7, 8, 10 and 12 h after drug administration. The samples were collected into heparinized tubes and centrifuged immediately and the plasma samples were frozen at −70°C pending analysis. The sampling was carried out under a sodium lamp or extremely subdued light and all tubes and syringes were wrapped in aluminum foil because of the photolability of nifedipine.

3. Results and discussion

Since the pharmacological activity of nifedipine seems to correlate best with its plasma concentration, a method that reliably measure levels of nifedipine is highly desirable. The chromatographic conditions described here were arrived at after investigating several mobile phases and internal standards. Being acidic, the mobile phase provided optimum sensitivity and adequate separation and sharp peaks. Typical chromatograms for blank plasma, plasma spiked with internal standard, and plasma containing internal standard and 25 ng/ml of nifedipine are depicted in Fig. 1. The retention times of nifedipine and internal standard were 6.60 and 14.40 min, respectively. The blank chromatogram showed that no interference would occur with endogenous substances.

Quantitation of nifedipine in plasma was carried out by determining the slope of the calibra-

tion curve, constructed using peak-area ratio for nifedipine and the internal standard (diazepam) obtained for the calibration standard. The calibration curves of nifedipine were typically described by $Y = 0.0119(\pm 0.0165) + 0.0052(\pm 0.0002)X$, ($r^2 = 0.994 \pm 0.005$; $n = 7$), where Y corresponds to the peak-area ratio of nifedipine to the internal standard and X to the concentration of nifedipine added over a concentration range of 10–200 ng/ml. The results indicate linearity throughout the range of nifedipine concentration studied. There was minor day-to-day variability in slopes and intercepts where acceptable linearity ($r = 0.997$) was achieved.

Replicate samples ($n = 8$) spiked at three control concentrations (15, 80 and 175 ng/ml) were used to assess intraday (within-day) precision as well as accuracy of nifedipine assay in plasma. Selection of concentrations for analysis was made to allow for definition of precision at low, medium and high concentrations of the linear range. Precision is expressed as the percent coefficient of variation (CV%) for the drug/internal standard ratios. Accuracy is expressed as a percent (100 × observed conc./theoretical conc.). The intraday (within-day) precision ranged from 2.22 to 4.64%, and accuracy ranged from 102.4 to 106.4% (Table 1). The day-to-day (or inter-day) precision in plasma samples were similarly evaluated over a period of 6 weeks (Table 1). Precision ranged from 2.34 to 7.06% and accuracy from 95.1% to 100.1%.

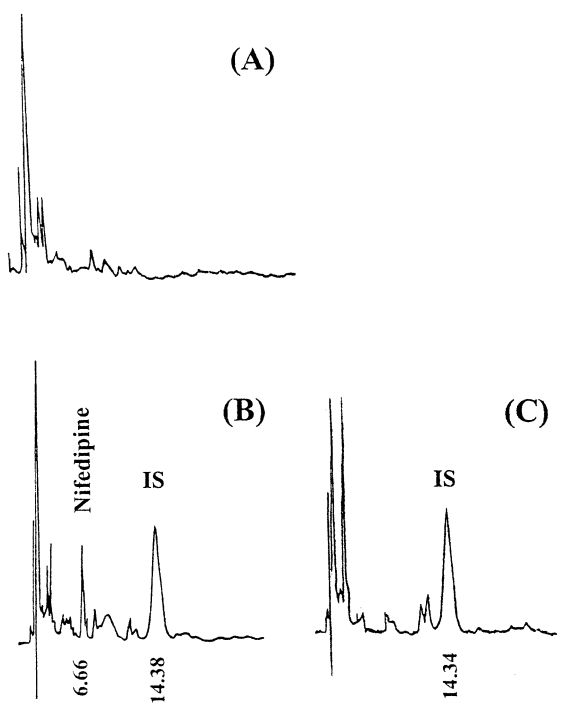

Fig. 1. Liquid chromatograms of plasma samples: A, blank plasma; B, plasma extract from healthy subject following oral administration of nifedipine capsule (nifedipine concentration, 25 ng/ml); C, blank plasma spiked with the internal standard.

Heparinized plasma spiked with 0.0 (blank), 5.0, 7.5, 10 and 20 ng/ml of nifedipine were used to assess the sensitivity of the method, based upon both signal intensity and variability. Peak heights of both drug and noise (at the drug retention time in the blank) were measured manually from the chromatograms. The average signal-to-noise ratio was then calculated at each of the concentrations. The lowest measurable concentration (limit of quantitation) was found to be 3 ng/ml with a CV% of 13%.

The recoveries of nifedipine (relative and extraction) from plasma and that of the internal standard (extraction) were quantitated using the standards (15, 80 and 175 ng/ml) for the drug and 1 μg/ml for the internal standard, diazepam. The relative analytical recovery was measured by adding the drug and internal standard to drug-free plasma (eight replicates for each standard) to achieve the concentrations shown in Table 2. The spiked plasma was then analyzed by the developed method. The relative recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma with actual added amounts. As shown in Table 2, the relative recovery of nifedipine from plasma ranged from 91.0 to 107.3%. The extraction recoveries were calculated by comparing the observed concentrations obtained from the processed standard samples to direct injections of stock solutions prepared at concentrations which represented 100% recovery. The extraction recovery of nifedipine from plasma ranged from 88.6 to 93.3% (Table 2). As observed, the extraction recovery using diethylether was less than 100%. In spite of this fact, the choice of diethylether as the extract-

Table 1
Within-day and day-to-day accuracy and precision of nifedipine in plasma

Added concentration (ng/ml)	Within-day*			Day-to-day*		
	Measured concentration (ng/ml)	Accuracy (%)	Precision (%)	Measured concentration (ng/ml)	Accuracy (%)	Precision (%)
15	15.53 ± 0.35 ($n = 8$)	3.5	2.22	14.76 ± 1.04 ($n = 8$)	−1.58	7.06
80	85.09 ± 3.95 ($n = 8$)	6.36	4.64	80.04 ± 2.77 ($n = 8$)	0.05	3.46
175	179.13 ± 6.22 ($n = 8$)	2.36	3.47	166.34 ± 3.90 ($n = 8$)	−4.95	2.34

* Mean value represents different plasma samples for each concentration.

Table 2
Analytical recovery of nifedipine from plasma

Added concentration (ng/ml)	Measured concentration (ng/ml)		Extraction recovery (%)	Relative recovery (%)	
	Aqueous	Plasma		Mean	Range
15	16.3 ± 0.8	15.2 ± 0.7	93.3 ± 5.0	102.7	96.0–107.3
80	87.1 ± 3.0	78.4 ± 2.7	90.1 ± 3.9	98.0	94.0–103.3
175	196.0 ± 2.0	173.7 ± 6.5	88.6 ± 2.7	99.3	91.0–102.3

ing solvent provided adequate purity to plasma samples over several other organic solvents, such as hexane, dichloromethane and ethyl acetate. It also minimized the endogenous interfering peaks and noisy baseline. The internal standard extraction recovery was found to be consistent from all eight pools tested, averaging 92.0 ± 1.50% with a range of 90.0–94.3%.

The specificity of the method was examined by preparing and analyzing (in duplicate) heparinized blank plasma obtained from eight male volunteers, in addition to different commonly used drugs. A list of drugs tested for interference and their retention times is shown in Table 3. Any drug that had a retention time close to that of nifedipine or the internal standard was considered as having the potential for interference. No significant chromatographic interference was observed.

The stability of the drug was determined:

1. in processed and reconstituted sample;
2. through eight freeze-thaw cycles during a period of 6 weeks.

To evaluate stability in processed samples, two concentrations (80 and 175 ng/ml of nifedipine) reconstituted in mobile phase, plasma and whole blood, were used for photostability study. The nifedipine preparations were divided into two 50-ml volumetric flasks. One had been kept in dark and the other was kept under fluorescent lighting at room temp (20°C). The samples were analyzed at 0, 10, 20, 30, 40 and 60 min for nifedipine in whole blood and up to 120 min in aqueous and plasma samples. From Table 4, whole blood samples were shown to be stable (on light exposure) for 30 min, which is an optimum period of time for withdrawing blood from human subject, separating the serum (or plasma), then protected from light with an aluminum foil wrapping, and eventually kept in freezer pending analysis. On the other hand, plasma samples were stable for at least 120-min exposure to light. Those in aqueous solution were stable up to 60-min light exposure. Stability in heparinized plasma through eight

Table 3
Retention times for commonly used drugs using the current method[a]

Drug	Retention time (min)
Amiodarone	3
Benzamide	ND
Carbamazepine	ND
n-Cetylpyridinium	ND
Chloramphenicol	ND
Clomipramine	ND
Diltiazem	ND
Fluvoxamine	ND
Furosemide	3
Glibenclamide	12
S-guanidine	4.5
Haloperidol	ND
Hydrazoline	4
Meclofenamate	5
Metoclopramide	2
Nitrazepam	4.5
Phenacetin	2
Procainamide	ND
Propranolol	4.5
Propyl paraben	4
Quinidine	ND
Thiopentane	3
Thymol	3
Tolbutamide	4.25

[a] ND, not detected.

Table 4
Percentage of nifedipine remaining in aqueous solution, plasma and blood at two different concentrations under fluorescent lighting and in the dark (at room temperature)

	Dark		Light	
	80 ng/ml	175 ng/ml	80 ng/ml	175 ng/ml
Aqueous[a]	82.7	98.0	77.9	80.3
Plasma[a]	99.0	97.6	94.8	92.7
Blood[b]	99.3	99.4	90.6	86.2

[a] After 120 min.
[b] After 60 min.

freeze-thaw cycles (6-week period at -20 ± 5°C to room temperature), has been confirmed (Table 5). Samples were removed from freezer, allowed to stand on the bench top, under room lighting, for 30 min to thaw, and then assayed for the nifedipine content.

The present method was successfully applied to the determination of nifedipine levels in several pharmacokinetic studies conducted in our institution. Fig. 2 shows the plasma concentration–time profiles of nifedipine after the oral administration of a 2×10 mg dose of immediate release capsules to four healthy male subjects and a 20 mg dose of slow release tablet to one healthy subject. The terminal phases of nifedipine in both studies were well characterized and the analytical assay was able to detect low concentrations at both ends of the plasma concentration–time profiles for both formulations. Table 6 shows the calculated pharmacokinetic parameters ($AUC_{0-\infty}$, C_{max}, T_{max}, K_{el}, $t_{\frac{1}{2}}$ and MRT) of nifedipine in these subjects.

Table 5
Stability of nifedipine in plasma through eight freeze (-20 ± 5°C)/thaw (room temperature) cycles during a period of 6 weeks

Added concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	Precision (%)
15	15.45 ± 0.64	3.00	4.15
80	80.68 ± 2.91	0.84	3.60
175	176.11 ± 6.44	0.64	3.66

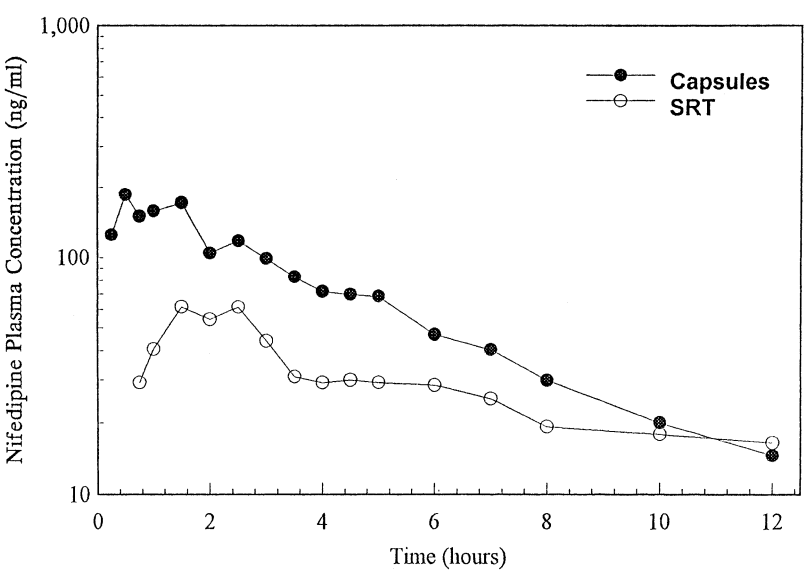

Fig. 2. Nifedipine plasma concentrations following oral administration of a capsule formulation of nifedipine (2×10 mg) to four healthy male adults (closed circles) and one sustained release tablet formulation to one male adult (open circles).

Table 6
Pharmacokinetic parameters of nifedipine in a healthy male adult after oral administration of a single dose (20 mg) of sustained-release tablet, and in four healthy male adults after the administration of a single dose (2×10 mg) of immediate-release capsules

Parameter[a]	Capsules[b]	SRT
$AUC_{0-\infty}$ (ng h/ml)	856.30 ± 412.55	552.93
C_{max} (ng/ml)	196.42 ± 33.63	61.35
T_{max} (h)	1.00 ± 0.58	1.50
K_{el} (1/h)	0.210 ± 0.028	0.0895
$t_{\frac{1}{2}}$ (h)	3.34 ± 0.41	7.74
MRT(h)	3.62 ± 1.74	10.80

[a] $AUC_{0-\infty}$, area under the plasma concentration–time curve from time 0 to infinity; C_{max}, peak plasma concentration; T_{max}, time to peak plasma concentration; K_{el}, elimination rate constant; $t_{\frac{1}{2}}$, elimination half life; MRT, mean residence time in the body.
[b] Mean $\pm$ SD.

4. Conclusions

The method was sensitive enough to detect a concentration as low as 3 ng/ml and the standard curve was linear up to at least 200 ng/ml. Based on problems encountered by several past assay publications and validation, the present study has served to develop a satisfactory sensitive, specific, accurate and fully validated assay method of nifedipine in human plasma. Both accuracy and precision values throughout the concentration range (10–200 ng/ml) were acceptable. The relative recovery results in Table 2 indicated the extraction yield ability of diethylether for nifedipine from plasma (91–107.3%). This method can be reliably applied to clinical pharmacokinetic studies.

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