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Development of an HPLC method for the determination of nifedipine in human plasma by solid-phase extraction☆

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Abstract

Nifedipine, a dihydropyridine calcium channel antagonist, is widely used in the treatment of hypertension and other cardiovascular disorders. A selective, sensitive and accurate high-performance liquid chromatographic method has been developed, validated and applied for determination of nifedipine in human plasma samples. A series of studies were conducted in order to investigate the effects of mobile phase composition, buffer concentration, mobile phase pH and concentration of organic modifiers, and to develop a convenient and easy-to-use method for quantitative analysis of nifedipine. The method involves solid-phase extraction on C_{18} cartridges. The chromatographic separation was accomplished on a Lichrocart Lichrospher 60 RP selectB column with a mobile phase composed of 0.020 mol/L KH_2PO_4 (pH 4.8) and acetonitrile (42:58, v/v). UV detection was set at 240 nm. The calibration curve was linear in the concentration range of 5.0–200.0 ng/mL for nifedipine in plasma and the limit of quantification was 5.0 ng/mL.

Keywords: Nifedipine; Solid-phase extraction; HPLC

1. Introduction

Nifedipine is a dihydropyridine calcium channel blocker and it is used in the management of hypertension; in the prophylaxis of angina pectoris and in the treatment of Raynaud's syndrome. Also, nifedipine has been tried in numerous non-vascular disorders [1,2].

There have been numerous publications describing the determination of nifedipine concentrations in plasma or serum by high-performance liquid chromatography (HPLC) after liquid–liquid extraction with various organic solvents such as dichloromethane [3], diethyl ether [4,5], a mixture of hexane and dichloromethane [6], mixture of isooctane and methyl-*tert*-butyl ether [7], ethyl acetate [8], mixture of *n*-pentane and dichloromethane [9], and chloroform [10].

On the other hand, Niopas and Daftsios [11] have performed the separation of nifedipine from plasma using solid-phase extraction on cyano cartridges. Other investigators [12,13] proposed an automated high-performance liquid chromatographic and column-switching technique for on-line clean up and analysis of nifedipine in human plasma. Also, some authors [14–16] have already proposed very sensitive HPLC methods for determination of nifedipine in human plasma using tandem mass spectrometry.

In this paper we are proposing a new method for determination of nifedipine in plasma samples using solid-phase extraction technique on C_{18} cartridges. In order to fulfil the aim, the method was first developed for the separation of and determination of nifedipine concentrations using internal standard method by optimising the experimental parameters and determining linearity for the investigated drug. Then we validated the method for the determination of nifedipine concentrations by evaluating

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recovery, selectivity, linearity, precision and accuracy. Finally, the method was used for determination of nifedipine in plasma samples obtained from healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Nifedipine was supplied by Sigma (Germany). Internal standard (I.S.), diazepam was purchased from Selectchemie AG (Switzerland). Acetonitrile and methanol were purchased from Across Organics (Belgium), potassium dyhydrogen phosphate and sodium carbonate were supplied from Merck (Germany). All reagents used were of analytical grade except acetonitrile and methanol which was HPLC grade. Oasis HLB columns for solid-phase extraction were purchased from Waters (USA); C_{18}, Lichrolut CN and RP select B cartridges were provided by Merck (Germany).

Stock solutions of nifedipine and diazepam were prepared at concentration 1000 μg/mL by dissolving appropriate amounts of nifedipine and diazepam in methanol. These solutions were stored at 4 °C. The working solutions were prepared freshly by diluting appropriate portions of these solutions with distilled water.

Human plasma was prepared from heparinized whole blood samples. Plasma samples were collected and stored at −20 °C. After thawing, samples were spiked daily with working solutions of nifedipine and diazepam. Plasma sampling, sample storage, extraction procedure and HPLC analysis were performed in a darkened room illuminated with artificial red light.

Five calibrators of nifedipine were prepared by making serial dilutions from stock solution and spiking them into drug-free human plasma in the concentration range from 5 to 200 ng/mL. The standard samples were prepared according to the procedures as unknown samples. The calibration curves were obtained by plotting the peak height ratio of nifedipine to internal standard versus concentration of nifedipine in ng/mL. Quality control (QC) samples were prepared at low, medium and high levels (7, 30 and 70 ng/mL) in the same way as plasma samples for calibration.

2.2. Chromatographic system

Chromatographic analysis was carried out on a Perkin-Elmer liquid chromatography system (USA) consisted of a pump PE LC series 200, autosampler PE LC ISS Series 200, diode array detector PE LC 235 C and column oven PE model 101. The chromatographic system is controlled by software package Turbochrom Version 4.1. plus and UV-spectrometric data are produced by program TurboScan Version 2.0.

A series of studies were conducted in our laboratory in order to develop a convenient and easy-to-use method for quantitative analysis of nifedipine in plasma. Several HPLC method variables with respect to their effect on the separation of nifedipine and diazepam from the matrix were investigated. With respect to the location and shape of the peaks of investigated drug and internal standard (diazepam) in the corresponding chromatograms, a set of column packing including C8, C18 and RP-select B with different lengths and particle sizes were tested. The final choice of the stationary phase giving satisfying peak shape, resolution and run time was a Lichrocart Lichrospher 60 RP-select B, 250 mm × 4 mm I.D. (5 μm, particle size), protected by a guard column Lichrospher RP-select B 4 mm × 4 mm (5 μm).

3. Results and discussion

3.1. Chromatographic analysis

In our extensive preliminary experiments a series of aqueous mobile phases containing buffer solutions with different pH values in combination with different modifiers including acetonitrile, methanol and triethylamine with different volume fractions were tested.

The amount of organic modifier present in the mobile phase will influence on analytes that are retained predominantly by adsorption onto the stationary phase. The results that were obtained over an acetonitrile range of 50–65% in the mobile phase 0.020 mol/L KH_2PO_4 were used to determine an optimal amount of organic modifier that should be used for the separation of nifedipine and I.S. The best results (good separation between two peaks, short time of analysis) were obtained when the percentage of acetonitrile in the mobile phase was 58%.

Additional studies were also done to check the effect that mobile phase pH and buffer concentration had on analytes retention and resolution. Results from this investigation show that there were no significant changes in retention and resolution of investigated drugs when the concentration of buffer increased from 0.01 to 0.05 mol/L and the concentration of 0.020 mol/L was chosen to minimize the equilibration time of column after each analytical batch of experiment. The effect of pH on the retention and separation was observed over the range 2.5–7.0 using phosphate as buffer salt. Nifedipine has a low p*K*a (p*K*a < 1) so almost all of the drug is uncharged in the investigated pH range and therefore the retention of nifedipine was largely unaffected by changes in pH. On the other hand, the retention of I.S. decreased with decreasing mobile phase pH. Additionally, a mobile phase pH of 4.8 was also chosen since it provides good retention and resolution between the two peaks and to avoid pH extremes and thus prolong column life.

From these data it was determined that mobile phase consisted of 0.020 mol/L KH_2PO_4, pH 4.8 and 58% acetonitrile would provide good retention for nifedipine and diazepam as well as an acceptable run time of less than 7 min for the separation.

Typical chromatogram of standard solutions of nifedipine and diazepam produced by the developed HPLC method are shown in Fig. 1(a).

3.2. Liquid–liquid and solid-phase extraction procedures

In order to obtain satisfactory values for recovery of nifedipine and diazepam and to investigate the effect of plasma matrix different liquid–liquid and solid-phase extraction procedures were tested. Recovery studies were performed by analysing

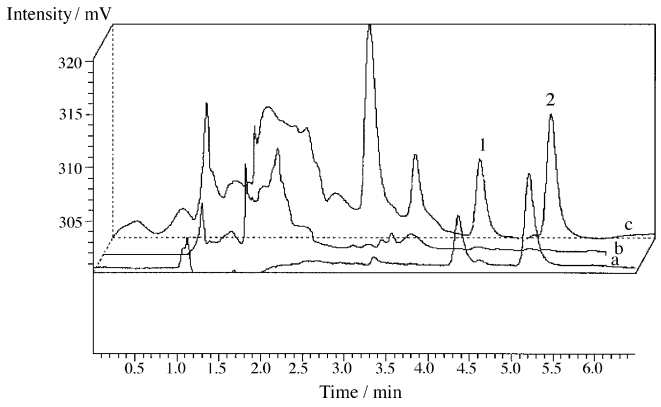

Fig. 1. Chromatograms of standard solution (a) of nifedipine and diazepam (350 ng/mL), blank (b) and spiked plasma sample (c) 1-nifedipine (200 ng/mL), 2-diazepam (196 ng/mL).

plasma samples spiked with nifedipine at three concentration levels. Three replicate samples for each concentration were extracted and chromatographed. The extraction recoveries were calculated by comparing the peak height of nifedipine and internal standard obtained for three concentration levels ($n = 3$ for each level for nifedipine, $n = 9$ for internal standard) and those resulting from the direct injection ($n = 3$, working solutions) of the theoretical amount of either nifedipine or internal standard (=100% recovery).

Various liquid–liquid extraction procedures that have been previously used from other investigators [4–7] for the purpose to obtain satisfactory values for recovery of nifedipine and internal standard also were tested by means of different organic solvents.

Liquid–liquid extraction procedures using diethyl ether, ethyl acetate, a mixture of MTBE: *iso*-octane (75:25, v/v) and hexane: dichloromethane (70:30, v/v) were tested.

The results from this investigation show that relatively low recovery for nifedipine (approximately 60–70%) can be obtained when mixture of hexane and dichloromethane and mixture of isooctane and methyl-*tert*-butyl ether was used. In other hand, satisfactory values for recovery of nifedipine can be achieved when diethyl ether or ethyl acetate was used (90–95%), but these liquid–liquid extraction yielding poor separation from the plasma endogenous interferences. However, the disadvantage of these methods employing liquid–liquid extraction of nifedipine from plasma samples is that they involve several steps which can produced highly variable values for recovery, takes time and great reagent consumption.

To develop solid-phase extraction method that would provide efficient clean up of the complex plasma matrix different cartridges for solid-phase extraction (Oasis HLB, C_{18}, Lichrolut CN and RP-select B) were tested. A solid-phase extraction vacuum manifold (Merck) was used for sample preparation.

3.2.1. Oasis HLB

The cartridge was conditioned sequentially by 1 mL methanol and 1 mL water. Spiked sample was introduced into the cartridge under vacuum at 5 psi. A solution of 5% methanol in water was used to rinse the cartridge and investigated drugs were eluted with 1 mL methanol. The eluate was evaporated using Centrivap concentrator at 40 °C. After reconstitution of the residue with 300 μL methanol, 100 μL volume was injected into the HPLC system.

3.2.2. Lichrolut CN

The solid-phase extraction procedure using CN cartridge has been proposed by Niopas and Daftsios [11] and we used this procedure for comparison with other solid-phase extraction procedures in order to obtain good separation of investigated drugs from the matrix interferences and satisfactory values for recovery.

3.2.3. C_{18} and RP select B

Cartridges were conditioned sequentially by 2 mL methanol, 2 mL water, followed by 0.1 mL of 0.1 mol/L sodium carbonate solutions. Spiked plasma sample was introduced into the cartridge under vacuum at 5 psi. Water (4 mL) was used to rinse the cartridge. The clean up was accomplished with additional 0.05 mL of methanol through the cartridge. Elution was then performed in two steps: the first one with 0.2 mL of methanol and this eluate was collected in a clean tube without vacuum, and the second step with additional 0.1 mL methanol. An aliquot of 100 μL was injected into the HPLC system.

The results from this investigation show that the satisfactory values for recovery of nifedipine and internal standard were obtained when solid-phase extraction is performed on C_{18} and RP-select B cartridges. Also, in the case when extraction was performed on the Lichrolut CN cartridges [11], good results for the recovery were obtained, but in this method the analytes are not concentrated and therefore good sensitivity cannot be achieved. In order to improve the extraction procedure, C_{18} cartridges for solid-phase extraction were conditioned with 0.1 mol/L sodium carbonate solutions before introducing the spiked samples. During the initial development of the procedure lower eluent volume was advantageous to avoid the need for evaporation at low plasma drug levels. It was found that the first 0.2 mL eluent removed 70–80% of nifedipine from the column, and the additional 0.1 mL of methanol was sufficient to achieve almost complete recovery. The minimum volume required achieving recovery higher than 90% was 0.3 mL, and best results were obtained with the addition of 0.2 mL of methanol followed by another portion of 0.1 mL.

Typical chromatograms of the blank human plasma (b) and plasma sample spiked with nifedipine and internal standard (c) are shown in Fig. 1. No interfering peaks were found at the retention times of nifedipine and internal standard.

The developed HPLC method was used for analysis of patient plasma samples after oral administration of nifedipine. Typical chromatograms of plasma samples of patients prepared according procedures for sample preparation before (a) and after (b) a single 40 mg oral dose of nifedipine are shown in Fig. 2.

3.3. Assay validation

Linear detector response for the peak-height ratios of nifedipine to internal standard was observed in concentration range between 5.0 and 200.0 ng/mL with a correlation

Table 1
Intra- and inter-day precision and accuracy

Nifedipine nominal concentration (ng/mL)	Intra-day			Inter-day		
	Mean ($n=6$) observed concentration (ng/mL)	R.S.D. (%)	Relative error (%)	Mean ($n=15$) observed concentration (ng/mL)	R.S.D. (%)	Relative error (%)
7.0	6.9	7.2	−1.4	7.1	5.3	1.4
30.0	30.6	6.2	2.0	30.1	4.4	0.3
70.0	70.1	4.4	0.1	69.5	4.2	−0.7

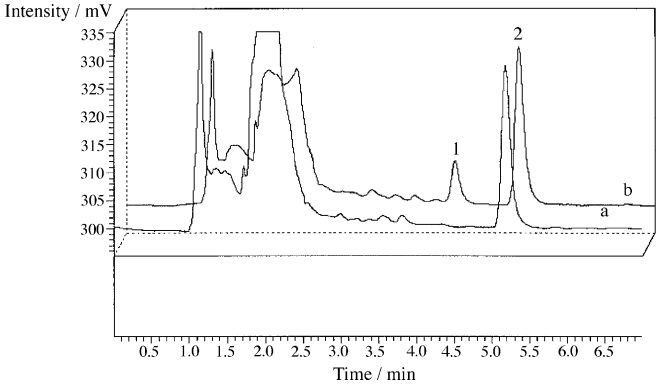

Fig. 2. Chromatograms of plasma samples from healthy volunteers before (a) and after (b) administration of 40 mg nifedipine [c(nifedipine) = 65.7 ng/mL, c(diazepam) = 196 ng/mL].

coefficient of 0.999 ± 0.003. Respective regression equation calculated by the least-squares method for nifedipine obtained after preparation of samples using solid-phase extraction was: $y = 0.004(\pm 0.0013) \times x - 0.0033(\pm 0.0002)$ ($n = 5$ days).

Under the experimental conditions used, the lower limit of detection was 2 ng/mL at a signal-to-noise ratio of 3. The limit of quantification was defined as the lowest amount detectable with a precision of less than 15% ($n = 5$) and an accuracy of ±15% ($n = 5$). The limit of quantification was found to be 5 ng/mL.

Intra- and inter-day precision and accuracy were determined in five different days by measuring plasma quality control samples at low, middle and high concentration levels of nifedipine.

The results of the method validation study are presented in Table 1. The intra- and inter-day repeatability's (Table 1) expressed as relative standard deviation (R.S.D.) was found to be 4.2–7.2%, indicating good precision of proposed method. An indication of accuracy was based on the calculation of the relative error of the mean observed concentration as compared to the nominal concentration. Accuracy data are presented in Table 1. Relative errors at all three concentrations studied are less than 2.0% and it is obvious that the method is remarkably accurate which ensures obtaining of reliable results.

Stability of nifedipine in plasma was investigated using spiked samples at two different concentration levels prepared in triplicate. Spiked samples were extracted and analysed after different storage conditions: immediately, after staying in an autosampler for 2, 12 and 24 h. Also, spiked sample were extracted after one and two freeze/thaw cycles and after one month stored at −20 °C and analysed by proposed HPLC method. Stability was determined by comparing the nominal concentration of nifedipine in samples analysed immediately and the test samples.

The results from the stability studies show that relative errors at two different concentrations studied are less than 6.2% and it is obvious that nifedipine added to plasma are stable in the different storage conditions.

Ruggedness was performed on the second HPLC column of the same type by injecting the standard solution of nifedipine and internal standard. Relative error was calculated by comparing the mean peak height for both substances to those obtained by changing pH value of the mobile phase from 4.8 to 5.0 and from 4.8 to 4.6 or to those obtained by changing the buffer concentration from 0.02 mol/L to 0.025 mol/L and from 0.02 mol/L to 0.015 mol/L. The values for the relative standard deviation are ranged from 0.5 to 2.1% and therefore can be concluded that this method is rugged enough.

References

[1] Martindale, The Extra Pharmacopeia, 31th ed., Royal Pharmaceutical Society, London, 1996, p. 940.
[2] Goodman, Gilman, The Pharmacological Basis of Therapeutics, 10th ed., McGrow Hill, New York, 2001, pp. 853.
[3] M. Zaater, E. Hasan, N. Najib, Pol. J. Pharmacol. 52 (2000) 307.
[4] H.S. Abou-Auda, T.A. Najjar, K.I. Al-Khamis, B.M. Al-Hadiya, N.M. Ghilzai, N.F. Al-Fawzan, J. Pharm. Biomed. Anal. 22 (2000) 241.
[5] A. Goldnik, M. Gajewska, D. Marszalek, M. Przyzycki, Acta Pol. Pharm. 53 (1996) 9.
[6] A. Jankowski, H. Lamparczyk, J. Chromatogr. A 668 (1994) 469.
[7] J.S. Grundy, R. Kherani, R.T. Foster, J. Chromatogr. B 654 (1994) 146.
[8] P. Thongnopnua, K. Viwatwongsa, J. Pharm. Biomed. Anal. 12 (1994) 119.
[9] P.A. Soons, J.H. Schellens, M.C. Roosemalen, D.D. Breimer, J. Pharm. Biomed. Anal. 9 (1991) 475.
[10] B.J. Gurley, R.G. Buice, P. Sidhu, Ther. Drug Monit. 7 (1985) 321.
[11] I. Niopas, A.C. Daftsios, J. Pharm. Biomed. Anal. 32 (2003) 1213.
[12] M. Yritia, P. Parra, E. Iglesias, J.M. Barbanoj, J. Chromatogr. A 870 (2000) 115.
[13] V. Nitsche, H. Schlutz, A. Eichinger, J. Chromatogr. 420 (1987) 207.
[14] J. Dankers, J. van den Elshout, G. Ahr, E. Brendel, C. van der Heiden, J. Chromatogr. B Biomed. Sci. Appl. 710 (1998) 115.
[15] B. Streel, C. Zimmer, R. Sibenaler, A. Ceccato, J. Chromatogr. B Biomed. Sci. Appl. 720 (1998) 119.
[16] A.B. Baranda, C.A. Mueller, R.M. Alonso, R.M. Jimenez, W. Weinmann, Ther. Drug Monit. 27 (2005) 44.