ELSEVIER

Journal of Chromatography B, 729 (1999) 265–270

JOURNAL OF CHROMATOGRAPHY B

Sensitive high-performance liquid chromatographic determination of nifedipine in cat plasma following improved sample treatment

Ikuko Nakamura[a,*], Masanobu Takahashi[b], Hiroshi Izumi[a]

[a]*Departments of Hospital Pharmacy and Physiology, Tohoku University School of Dentistry, Sendai 980-8575, Japan*
[b]*Department of Hospital Pharmacy, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan*

Received 9 November 1998; received in revised form 6 April 1999; accepted 7 April 1999

Abstract

A simple, easy and accurate reversed-phase high-performance liquid chromatographic method is described for the determination of nifedipine in cat plasma. The procedure involves extraction of nifedipine from plasma using a Sep-Pak C_{18} cartridge and ultraviolet detection at 350 nm. The present method provides the required reproducibility and sensitivity for the determination of low concentrations of nifedipine without interference from plasma components or photodegradation products. The method was validated over the range 1–50 ng/ml nifedipine. Accuracy and precision were, respectively, 97% or more and 5% or less over the concentration range examined. The minimum quantifiable concentration of nifedipine was found to be 1 ng/ml.

Keywords: Nifedipine

1. Introduction

Nifedipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester, is a Ca^{2+}-channel blocker that inhibits the transmembrane influx of Ca^{2+} into cardiac muscle cells and vascular smooth muscle cells through specific ion channels. It induces relaxation of smooth muscle and decreases peripheral vascular resistance [1–3]. It is widely used for the treatment of hypertension, angina pectoris and other cardiovascular disorders, but unexpected side effects, such as headache, flushing, dizziness, peripheral oedema and sexual dysfunction, have been reported [4–9]. These effects vary from person to person, and there is still a question as to whether there is a causal relationship between the occurrence of side effects and the plasma level of this drug (or indeed of other Ca^{2+}-channel blockers). An accurate method of measuring its plasma concentration is needed to help us answer this question and to allow us to determine the optimal dose for each individual patient.

Various methods for the determination of nifedipine in biological fluids have been reported, involving mainly gas-chromatography (GC) [10,11] or high-performance liquid chromatography (HPLC) with either UV detection [12–17] or electrochemical detection [18,19]. However, GC has serious drawbacks, such as thermal degradation of nifedipine during analysis at high temperatures. Consequently, HPLC is widely employed, with such purification procedures as extraction with organic solvents

*Corresponding author. Tel.: +81-22-717-8423; fax: +81-22-717-8423.
E-mail address: i-nakam@mail.cc.tohoku.ac.jp (I. Nakamura)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00167-X

[12,13,16–20] or column-switching [21,22] being used prior to injection in order to exclude interfering peaks due to endogenous components. A very simple and reproducible procedure is needed for the determination of the plasma nifedipine concentration, since nifedipine is highly sensitive to light [13] and since the sample size obtainable from animals or patients is limited.

In this paper, we report a way of measuring the plasma concentration of nifedipine simply, easily and accurately using a HPLC method, with a Sep-Pak C_{18} cartridge being used for extraction and with UV detection at 350 nm.

2. Experimental

2.1. Materials

Nifedipine was purchased from Sigma (St. Louis, MO, USA) and nisoldipine (internal standard, I.S.) was kindly supplied by Bayer (Wuppertal, Germany). All other chemicals were of reagent grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan). Deionized water was made in the laboratory using a Milli-Q system (Milli-Q Synthesis; Millipore, Tokyo, Japan).

2.2. Chromatography

The HPLC system (Tosoh, Tokyo, Japan) consisted of a UV-8020 UV–Vis absorbance detector, a CCPM-II multi-functional pump, a PX-8020 pump controller and a Chromatocorder 21 integrator. A 250×4.6 mm I.D. ODS-80Ts column (Tosoh, Tokyo, Japan) was used at room temperature. When electrochemical detection was employed (see experiment described in Discussion), a BAS LC-4C amperometric detector equipped with micro-HPLC systems (Bioanalytical Systems, West Lafayette, IN, USA) was used. The mobile phase consisted of methanol and 10 m*M* acetate buffer, pH 4 (75:25, v/v). The flow-rate was 1.0 ml/min and the UV detector was operated at 350 nm.

2.3. Standard solutions

Stock solutions, 1 mg/ml, of both nifedipine and I.S. were prepared in methanol. Both solutions were kept protected from light with an aluminium foil covering, and they were stored at 4°C. Diluted solutions of nifedipine and I.S. in methanol were prepared daily from the stock solutions.

2.4. Animal experiments and collection of plasma samples

We used adult cats (2.8 to 4.1 kg body weight) for preparation of plasma. Cats were initially sedated with ketamine hydrochloride (30 mg/kg, i.m.) and then anaesthetized with a mixture of α-chloralose (50 mg/kg, i.v.) and urethane (100 mg/kg, i.v.), and artificially ventilated via a tracheal cannula with a mixture of 50 % air–50% O_2, after paralyzing by intravenous injection of pancuronium bromide (Mioblock, Organon, Teknika, The Netherlands; 0.4 mg/kg initially, supplemented with 0.2 mg/kg every hour). Blood pH, PaO_2 and $PaCO_2$ data were obtained at intervals of 90 min using a blood-gas analyzer (Model 148; Ciba-Corning, Medfield, MA, USA) and ventilation was adjusted to keep these parameters within normal limits. Rectal temperature was maintained at 37–38°C using a heating pad. Arterial blood was collected in plastic tubes containing sodium EDTA. Plasma was obtained by centrifugation in a refrigerated centrifuge at 10 000 *g* for 10 min.

2.5. Sample preparation

A small reversed-phase Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) was used to separate nifedipine from the plasma components prior to injection into the HPLC system. A Sep-Pak C_{18} was attached to a 50-ml shaded glass syringe and washed with 30 ml of methanol followed by 30 ml of water. For quality control samples, aliquots of the diluted stock solution of nifedipine were added to 0.5 ml of drug-free plasma to yield final concentrations of 1, 5, 10 and 20 ng/ml. These samples were stored at −70°C. A set of quality control samples was removed from storage at specific times (for the inter-day test, on ten different days), then thawed at room temperature and used to determine the accuracy and precision of the method. Calibration samples of nifedipine (from 1 to 50 ng/ml) were prepared in the same manner as the quality control samples (except for the freezing). I.S. was pipetted into the plasma

(quality control and calibration samples) to yield a final concentration of 200 ng/ml. After adding plasma containing nifedipine and I.S. to 10 ml of 10 m*M* HCl in test tubes and mixing, the samples were passed through the Sep-Pak C_{18} cartridge. The cartridge was washed with 50 ml of water and then eluted with 2 ml of methanol. The methanol fraction, which contained nifedipine and I.S., was freeze-dried using a centrifugal freeze dryer (Model RC-11; Yamato, Tokyo, Japan). Samples (50 μl), after reconstitution in the mobile phase (200 μl) and centrifugation at 10 000 *g* for 20 min, were injected onto the HPLC column. The whole analysis was carried out in a darkened room to avoid light-induced decomposition of the samples.

2.6. Method of validation

2.6.1. Calibration graphs

Calibration curves were constructed by plotting the peak-height ratio (nifedipine/I.S.) against the corresponding added plasma concentration. Linearity was examined by analyzing plasma samples spiked with known amounts of nifedipine (1, 2.5, 5, 10, 20, 30 and 50 ng/ml) on each of ten different days. The least-squares method was used to calculate regression lines and to evaluate the linearity of the calibration curve using a Macintosh Computer with StatView 5.0 (Abacus Concepts, CA, USA).

2.6.2. Precision and accuracy

The determination of inter-day precision and accuracy was accomplished by replicating the measurements of plasma samples on ten different days. These samples contained added nifedipine at four concentrations (1, 5, 10 and 20 ng/ml). The intra-day precision and accuracy of the analytical method were established by making ten replicate measurements of plasma samples containing added nifedipine at four different concentrations (1, 5, 10 and 20 ng/ml).

2.6.3. Extraction recovery

The analytical recovery of nifedipine from plasma was determined as follows. Drug-free plasma with added nifedipine (25 and 100 ng/ml) was passed through the Sep-Pak C_{18} cartridge using the method described above. Recovery was calculated by comparing the peak heights so obtained with those obtained by direct injection of the diluted standard solution of nifedipine.

2.7. Measurement of systemic arterial blood pressure

For this part of the study, we used 13 anaesthetized cats given nifedipine at 0.1 or 1 μg/kg/min (i.v.). The conditions of anaesthesia were similar to those previously described in Section 2.4 and as described before [23,24]. Systemic arterial blood pressure was recorded from the femoral catheter via a Statham pressure transducer.

3. Results

Fig. 1 shows typical chromatograms obtained from extracted plasma with only I.S. (200 ng/ml) added (Fig. 1A) and from plasma spiked with 25 ng/ml of nifedipine as well as 200 ng/ml of I.S. (Fig. 1B). In spite of the occurrence of many peaks in the blank plasma (Fig. 1A), the peaks for nifedipine and I.S. never overlapped with those of endogenous substances in any test, indicating that nifedipine and I.S. were both clearly separated from the plasma components. Using the method reported here, the re-

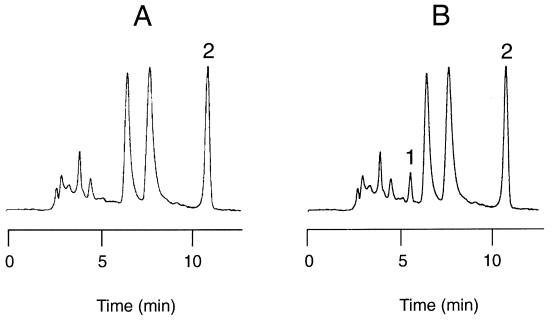

Fig. 1. Chromatograms obtained for (A) cat plasma containing 200 ng/ml of nisoldipine (internal standard, I.S.) and (B) cat plasma spiked with 25 ng/ml of nifedipine and 200 ng/ml of I.S. Drug-free plasma (0.5 ml) containing nifedipine and I.S. was added to 10 ml of 10 m*M* HCl in tubes, mixed and passed through a Sep-Pak C_{18} cartridge. Aliquots (50 μl) of sample in the mobile phase were injected onto the HPLC column after elution with methanol, evaporation and centrifugation. The mobile phase consisted of methanol and 10 m*M* acetate buffer, pH 4 (75:25, v/v). The flow-rate was 1.0 ml/min and the UV detector was operated at 350 nm. Peak identification: (1) nifedipine and (2) nisoldipine.

tention times for nifedipine and I.S. were approximately 5.7 and 10.8 min, respectively (Fig. 1B). The calibration curve for nifedipine (from 1 to 50 ng/ml) was typically described by $y=0.023x+0.009$, where y corresponds to the peak-height ratio for nifedipine with respect to I.S. and x corresponds to the added nifedipine concentration (Fig. 2). Excellent linearity was observed for all calibration curves over the range 1–50 ng/ml ($r^2=0.975$, $p<0.0001$).

Using the quality control samples, the inter-day precision and accuracy as well as the intra-day precision and accuracy were also calculated for the present method (Tables 1 and 2). The inter-day precision and accuracy of the method throughout the entire working range were <5% and 97.2–102.0%, respectively (Table 1). The intra-day precision and accuracy (determined using four different nifedipine concentrations) were <4% and 99.0–101.0%, respectively (Table 2).

The values obtained for the absolute recovery of nifedipine at 25 and 100 ng/ml were 92 and 89%, respectively.

In thirteen cats, mean blood pressure was decreased by 12.6 or 27.7 mmHg (basal arterial blood pressure, 94±6.2 mmHg) 15 min after the administration of nifedipine at 0.1 or 1 μg/kg/min, respectively. The levels of nifedipine in the plasma were 2.28±0.10 and 20.78±1.11 ng/ml, respectively. Fig. 3 shows typical chromatograms obtained using cat plasma collected 15 min after the administration of nifedipine (1.0 μg/ml/min, i.v.; Fig. 3A) and cat plasma spiked with 50 ng/ml of nifedipine (Fig. 3B).

Table 1
Inter-day precision and accuracy of ten parallel measurements of nifedipine at various concentrations

Added (ng/ml)	Found[a] (ng/ml)	Precision (%)	Accuracy (%)
1	1.02±0.04	3.92	102.0
5	4.86±0.21	4.32	97.2
10	10.08±0.30	4.84	101.2
20	20.00±0.79	2.58	99.0

[a] Reported as mean±SD.

Table 2
Intra-day precision and accuracy of ten parallel measurements of nifedipine at various concentrations

Added (ng/ml)	Found[a] (ng/ml)	Precision (%)	Accuracy (%)
1	1.01±0.03	2.97	101.0
5	4.97±0.15	3.02	99.4
10	9.90±0.12	1.21	99.0
20	19.95±0.13	0.65	99.8

[a] Reported as mean±SD.

4. Discussion

There is considerable variation among the values reported in the literature for the lower limit of quantification obtained for the determination of nifedipine in plasma by HPLC regardless of whether

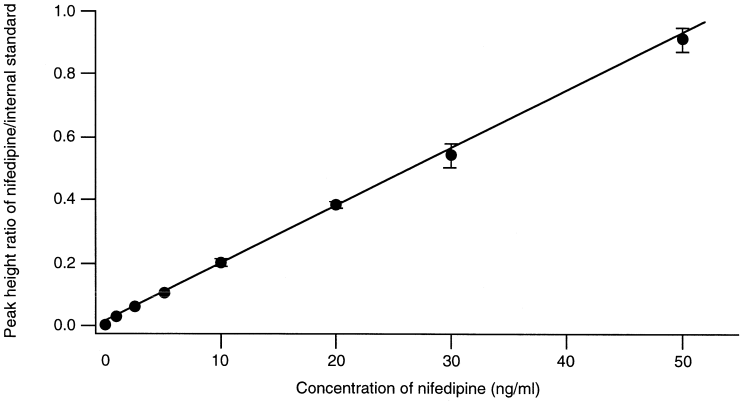

Fig. 2. Typical calibration curve for nifedipine concentrations from 1 to 50 ng/ml. Calibration curves were constructed by plotting the peak-height ratio (nifedipine/I.S.) against the corresponding added plasma concentration.

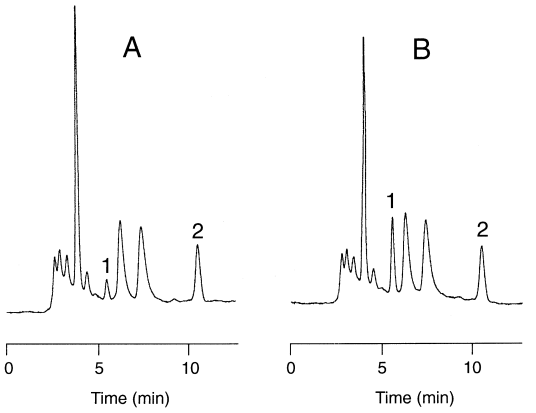

Fig. 3. Chromatograms obtained for (A) cat plasma containing 100 ng/ml of I.S. and (B) cat plasma spiked with 50 ng/ml of nifedipine and 100 ng/ml of I.S. Plasma was prepared from cat blood collected 15 min after the administration of nifedipine (1.0 μg/ml/min, i.v.). Sample preparation procedure was the same as in Fig. 1. Peak identification: (1) nifedipine and (2) nisoldipine.

UV detection or electrochemical detection was used. Thus, values of 1 [18], 1–2 [20], 2 [13,17,19], 5 [16] and 15 ng/ml [22] have been reported. Using electrochemical detection, Horváth et al. [18] determined nifedipine in dog plasma over the concentration range 1–50 ng/ml. However, the inter-day precision of their method was approximately 18% at concentrations of 1 and 2.5 ng/ml, suggesting that the data for these lower concentrations varied considerably from assay to assay. Furthermore, a wavelength of 254 nm or less was commonly applied in most of the earlier studies with UV detection [13,20], and significant baseline noise was present in the published chromatograms. Recently, Grundy et al. [16] improved the signal-to-noise ratio for low nifedipine concentrations by employing UV detection at 350 nm instead of 254 nm.

Adequate sample purification prior to HPLC is needed for the determination of the plasma nifedipine concentration, since the analysis of biological samples by chromatography is affected by the presence of interferences. Two main methods, i.e., extraction with organic solvents (such as toluene [12,19], pentane–dichloromethane [13], *n*-hexane–dichloromethane [17,18], *n*-hexane–ethyl acetate [20] or methyl-*tert*.-butyl ether–isooctane [16]) or solid-phase extraction with special extraction columns (in an off-line [15] or on-line arrangement [22]), have been employed for plasma clean-up. In a preliminary experiment, we used the extraction procedure with organic solvents described by Horváth et al. [18]. However, we found that this method, when used with organic solvents such as hexane–dichloromethane (70:30, v/v), showed a low reproducibility regardless of whether UV detection or electrochemical detection was used, because the peak for nifedipine overlapped with those for the substances extracted, thus producing interfering peaks. This probably occurs due to contamination by the water layer in the organic layer, indicating that pretreatment by organic solvents may not be suitable for examinations carried out in laboratories using a limited amount of sample from animals or patients.

For these reasons, we decided to use a Sep-Pak C_{18} cartridge [25,26] for purification of the plasma samples obtained from cats, in combination with a detection wavelength of 350 nm in the present study. Following the use of the Sep-Pak C_{18} cartridge, chromatograms were obtained for methanol eluates of blank plasma samples and spiked plasma samples. While many peaks were observed for the blank plasma sample under the present conditions (Fig. 1A), no peaks for plasma components or endogenous compounds interfered with the measurement of nifedipine or I.S. over the ranges 1–50 and 100–200 ng/ml, respectively (Fig. 1B). These results suggest that the present method provided the required reproducibility and sensitivity for the determination of low concentrations of nifedipine without interference from plasma components or photodegradation products. Furthermore, our present HPLC study shows that making measurements with UV detection at 350 nm after extraction with a Sep-Pak C_{18} cartridge enables precision and accuracy to both be kept at an acceptable level (<5 and $>97\%$, respectively), even at a concentration of 1 ng/ml (Tables 1 and 2).

In an adjunct to the present study, the applicability of the analytical method described here was examined by testing the correlation between blood pressure reduction and the plasma concentration of nifedipine in 13 anaesthetized cats given the drug at 0.1 or 1 μg/kg/min (i.v.). In these 13 cats, mean blood pressure was decreased by 12.6 or 27.7 mmHg (basal arterial blood pressure, 94 ± 6.2 mmHg) 15 min after the administration of drug at 0.1 or 1 μg/kg/min, respectively. The levels of nifedipine in

the plasma were 2.28±0.10 and 20.78±1.11 ng/ml, respectively. These data show that the depressor effect of nifedipine on blood pressure is evident in the cat at a lower plasma concentration than that reported for the dog [18] and that the method presented here can be used in studies on those species that are affected by nifedipine at very low plasma concentrations.

In conclusion, the use of a Sep-Pak C_{18} cartridge for extraction and UV detection at 350 nm makes it possible to determine in a simple manner the concentration of nifedipine in plasma. The use of such a method should enable studies to be carried out on the relationship between the actions or side effects of nifedipine and its plasma concentration.

References

[1] R.M. Robertson, D. Robertson, in: J.G. Hardman, L.E. Limbird (Eds.), Goodman and Gilman's the Pharmacological Basis of Therapeutics, 9th ed, Pergamon Press, New York, 1996, Ch. 32, p. 759.
[2] J.A. Oates, in: J.G. Hardman, L.E. Limbird (Eds.), Goodman and Gilman's the Pharmacological Basis of Therapeutics, 9th ed, Pergamon Press, New York, 1996, Ch. 33, p. 781.
[3] R.A. Kelly, T.W. Smith, in: J.G. Hardman, L.E. Limbird (Eds.), Goodman and Gilman's the Pharmacological Basis of Therapeutics, 9th ed, Pergamon Press, New York, 1996, Ch. 34, p. 809.
[4] H. Suzuki, T. Tominaga, H. Kumagai, T. Saruta, J. Hypertens. 6 (suppl) (1988) S649.
[5] V. Saano, O. Raatikainen, P. Paronen, H. Komulainen, Int. J. Clin. Pharmacol. Res. 9 (1989) 247.
[6] M.A. DeWood, R.A. Wolbach, Am. Heart J. 119 (1990) 468.
[7] R.J. Weiss, Am. Fam. Phys. 44 (1991) 2075.
[8] W.D. Hall, Am. J. Cardiol. 69 (1992) 33E.
[9] J. Hosie, A.D. Bremner, P.J. Fell, G.V. James, P.A. Saul, S.H. Taylor, J. Cardiovasc. Pharmacol. 22 (suppl A) (1993) S9.
[10] S. Kondo, A. Kuchiki, K. Yamamoto, K. Akimoto, K. Takahashi, N. Awata, I. Sugimoto, Chem. Pharm. Bull. 28 (1980) 1.
[11] S.R. Hamann, R.G. McAllister Jr., Clin. Chem. 29 (1983) 158.
[12] J. Dokladalova, J.A. Tykal, S.J. Coco, P.E. Durkee, G.T. Quercia, J.J. Korst, J. Chromatogr. 231 (1982) 451.
[13] C.H. Kleinbloesem, J. Van Harten, P. Van Brummelen, D.D. Breimer, J. Chromatogr. 308 (1984) 209.
[14] K. Miyazaki, N. Kohri, T. Arita, H. Shimono, K. Katoh, A. Nomura, H. Yasuda, J. Chromatogr. 310 (1984) 219.
[15] M.E. Sheridan, G.S. Clarke, M.L. Robinson, J. Pharm. Biomed. Anal. 7 (1989) 519.
[16] J.S. Grundy, R. Kherani, R.T. Foster, J. Chromatogr. B 654 (1994) 146.
[17] A. Jankowski, H. Lamparczyk, J. Chromatogr. A 668 (1994) 469.
[18] V. Horváth, A. Hrabeczy-Pall, Z. Niegreisz, E. Kocsi, G. Horvai, L. Godorhazy, A. Tolokan, I. Klebovich, K. Balogh-Nemes, J. Chromatogr. B 686 (1996) 211.
[19] H. Suzuki, S. Fujiwara, S. Kondo, I. Sugimoto, J. Chromatogr. 341 (1985) 341.
[20] N.D. Huebert, M. Spedding, K.D. Haegele, J. Chromatogr. 353 (1986) 175.
[21] T. Ohkubo, H. Noro, K. Sugawara, J. Pharm. Biomed. Anal. 10 (1992) 67.
[22] M. Telting-Diaz, M.T. Kelly, C. Hua, M.R. Smyth, J. Pharm. Biomed. Anal. 9 (1991) 889.
[23] H. Izumi, I. Nakamura, K. Karita, Am. J. Physiol. 268 (Regulatory Integrative Comp. Physiol. 37), (1995) R1196.
[24] H. Izumi, Y. Ito, J. Physiol. (Lond) 510 (1998) 915.
[25] R.C. George, Clin. Chem. 27 (1981) 198.
[26] P.M. Kabra, B.E. Stafford, L.J. Marton, Clin. Chem. 23 (1977) 1284.