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Quantitation of nifedipine in human plasma by on-line solid-phase extraction and high-performance liquid chromatography

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Abstract

An analytical methodology for nifedipine quantitation in plasma by on-line solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) is described. The SPE cartridges contain C_2 and the analytes nifedipine and nitrendipine (internal standard) are separated on a C_{18} column with a mobile phase consisting of acetonitrile–13 m*M* phosphate buffer pH 7 (65:35, v/v) followed by UV detection at 338 nm. Validation of the method demonstrated good recoveries (>90%), sensitivity (limit of quantification, 2 ng/ml), based on a 500 μl sample volume, accuracy and precision (<5.5% in concentrations greater than the limit of quantitation). This methodology has been used for bioequivalence studies.

Keywords: Validation; Nifedipine; Nitrendipine

1. Introduction

Nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) (Fig. 1A) is the parent compound of the hydropyridine class of calcium-channel antagonists. It is widely used for the treatment of vascular disease, angina pectoris and hypertension, due to the selective dilatation of arteries [1]. It is quickly metabolised by oxidative mechanisms.

Nifedipine (NIF) (Fig. 1A) is one of the most sensitive dihydropyridines when exposed to daylight [2], and is quickly photodegraded in your nitroso analogue, dihydronifedipine [1,3]. Several methods have been described for the determination and quantitation of nifedipine in plasma. These methods involve liquid–liquid extraction with HPLC–UV [1–12] and electrochemical detection [13–16], GC with various detection methods [17,18], and on-line solid-phase extraction (SPE) with a column switching and different methods of detection [12,19]. These methods imply that the drugs are concentrated on an extraction pre-column; after washing, the pre-column is automatically back-flushed with the mobile phase to transfer the analytes to the analytical column. Repeated use of the pre-column for sample concentration and clean-up may result in deterioration due to precipitation of protein and the risk of blocking [20,21]. In order to avoid these problems the pre-column must be change often. The use of SPE with the ASPEC system [22] has been described in which the final extract is transferred manually to the autosampler. Most recently, two papers have described the used of LC–MS–MS [22,23].

The present paper describes a fully automated method for the determination of nifedipine and

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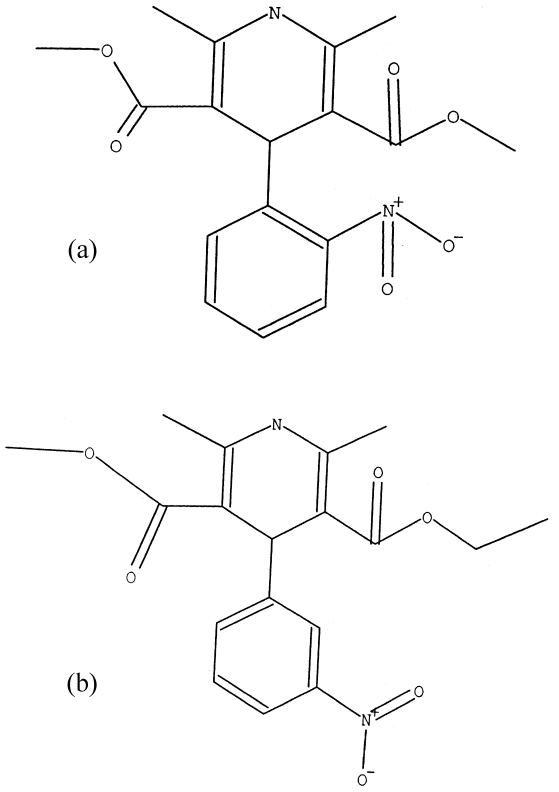

Fig. 1. (A) Nifedipine (NIF); (B) nitrendipine (NIT).

nitrendipine (internal standard, Fig. 1B) in human plasma using on-line solid-phase extraction with exchangeable cartridges for sample concentration and clean-up in order to prevent memory effects [21]. The method is suitable for the quantification of drug levels in pharmacokinetic studies.

2. Experimental

2.1. Reagents and materials

NIF was purchased from Cinfa Lab. (Pamplona, Spain) and nitrendipine (NIT) (internal standard) was obtained from Sigma. Acetonitrile and methanol (HPLC grade), disodium hydrogenphosphate and orthophosphoric acid were purchased from Scharlau (Barcelona, Spain), and potassium dihydrogenphosphate from Merck (Darmstadt, Germany); all analytical grade. Water was deionized and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Chromatography

Analysis by HPLC was performed using two 510 pumps, a variable-wavelength UV detector 484, operating at 238 nm, and Millenium 2010 was the acquisition and processing software, all from Waters (Milford, MA, USA). The autosampler was a Triathlon (Spark-Holland, Netherlands).

The analytical column was a Symmetry C_{18}, 5 μ, 150 mm×4 mm I.D. and the guard column with the same phase (Waters, Spain). The mobile phase was acetonitrile–phosphate buffer (KH_2PO_4/Na_2HPO_4) 13 m*M* pH 7 (65:35 v/v), delivered at a flow-rate of 0.8 ml/min.

The analytical column was washed after each injection with a linear gradient of acetonitrile–mobile phase of 100 to 25% mobile phase in 6 min and returned to initial conditions in 5 min.

2.3. Solutions

Two 1 mg/ml NIF stock solutions (A and B) were prepared in methanol: one for calibration curve spiked samples and chromatographic standards (A) and the other for quality control (B). The stock standard solution of 1 mg/ml NIT was prepared in methanol. Chromatographic standards and the standard for the recovery study were prepared by dilution of stock solution A with mobile phase, until concentrations of 100, 10 and 1 ng/μl NIF. The internal standard solution used was 1 ng/μl of NIT.

Spiked samples of plasma were prepared with blank plasma and dilutions of stock solution A until the desired concentration.

All solutions were stored in the dark at 4°C where they were stable for at least 2 months.

2.4. Sample preparation

The apparatus consisted of a solvent delivery unit (SDU) with a purge pump and six-port solvent selection valve, an autosampler and a programmable on-line SPE unit (Prospekt), all purchased from Spark-Holland.

The exchangeable cartridges were 10 mm×2 mm I.D. and were filled with C_2 (Analytichem, 40 μm particle size).

Brown vials containing 800 μl of plasma with

internal standard (30 ng/ml NIF) were placed in the autosampler, which was programmed to apply 500 μl of plasma to every cartridge; the temperature of the carousel was 6°C. One millilitre of acetonitrile–water (5:95, v/v) was used for the needle wash.

Before application of plasma, the cartridge was activated with a methanol flow-rate of 2.5 ml/min for 1 min, and with water at the same flow-rate for 2 min. The flow-rate was then reduced to 0.5 ml/min and the cartridge flushed for 1 min with acetonitrile–water (30:70, v/v); at that time, the flow was directed through the loop of the autosampler and the plasma was transported to the cartridge. The cartridge was rinsed with acetonitrile–water (5:95, v/v) at 0.5 ml/min for 3 min and water for 2.30 min. The analytes were eluted with mobile phase for 2 min.

The Prospekt cannot be programmed to use the cartridge more than once in a cycle; therefore, in order to be able to reuse it (up to four times) in a new series of the same study, it was purged with 4 ml of water and 4 ml of methanol. The cartridge was automatically changed for a new one, and a new cycle started.

3. Results and discussion

3.1. Chromatographic separation

Fig. 2A shows typical chromatograms of chromatographic standards dissolved in mobile phase, Fig. 2B those of blank plasma, free of interfering peaks, and Fig. 2C those for spiked samples with 10 ng/ml NIF and 30 ng/ml NIT (I.S.). The retention times for chromatographic standards were 3.12±0.04 and 4.38±0.07 for nifedipine and nitrendipine, respectively.

3.2. Recovery and linearity

The nifedipine and internal standard peak areas were measured after injection of nifedipine and nitrendipine solution in mobile phase. These were compared with those obtained with blank plasma spiked with known amounts of the two compounds. The recovery results were 91.02±1.86% and 89.24±2.76% for, respectively, 10 and 100 ng/ml of NIF, and 83.82±1.98% for 30 ng/ml of NIT. The

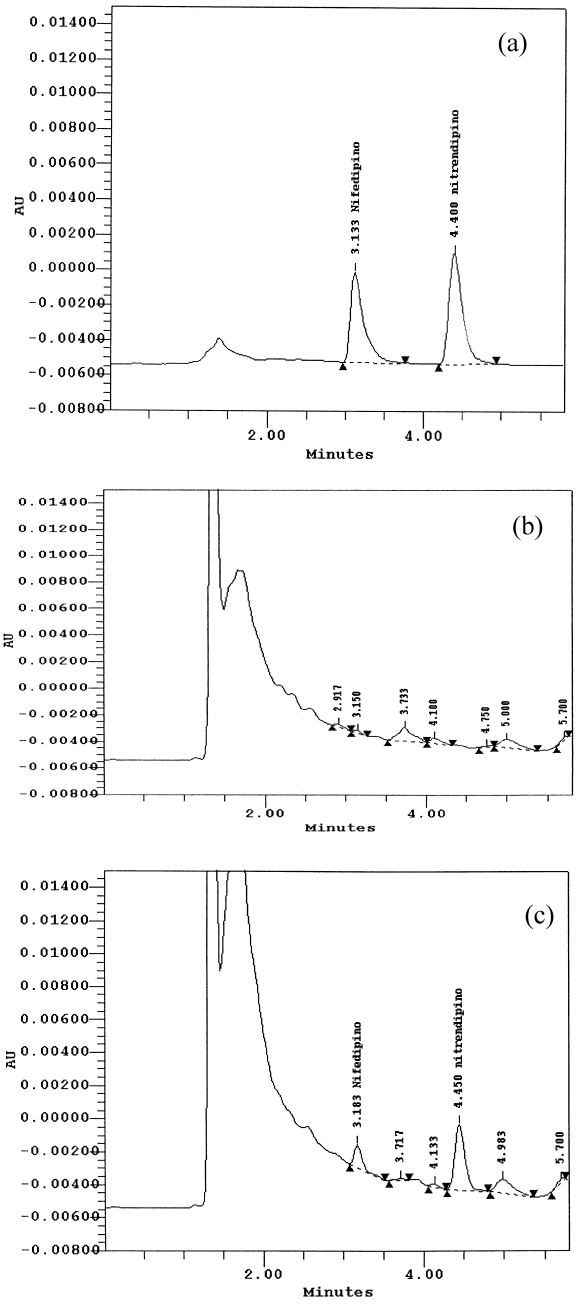

Fig. 2. (A) Chromatographic standands, 18 ng NIF and NTD in mobile phase; (B) blank plasma; (C) spiked plasma sample with 10 ng/ml NIF and 30 ng/ml NIT.

linearity for NIF was checked with a calibration curve made with blank plasma spiked with 2, 10, 25, 50, 100 and 200 ng/ml of nifedipine, with six

determinations for each concentration. In the following 4 days, each level of the calibration curve was analysed in triplicate.

The calibration curve equation is $y = bx \pm a$; the linear regression analysis was performed by plotting the relative peak area NIF/NIT (y) versus analyte concentration (x) in ng/ml, with a weighting factor $1/C^2$. The mean equation of the calibration curve obtained from six points was $y = 0.034x + 0.005$ ($n = 4$ days) with a determination coefficient of $r^2 = 0.996$. On average, the slope of the calibration graph obtained on four different days did not vary by more than 2.93%; the intercept was not significantly different from the theoretical zero value, demonstrating good stability of the measuring system.

3.3. Precision and accuracy

The intra-assay ($n = 6$) and inter-assay ($n = 4$) precision and accuracy were evaluated from the calibration curve results. The precision and accuracy were given in terms of relative standard deviations (RSD) and percent relative error (RE), respectively.

The precision and accuracy results are given in Table 1. In the concentration interval investigated, all variation coefficients and relative errors, intra- and inter-assay, were $<5.5\%$, except for the limit of quantitation (LOQ), which had a precision of 10.09%.

3.4. Sensitivity

Based on a 500 μl sample volume, the lower NIF limit of quantification (LOQ) (RSD and percent bias $\leq 20\%$ for six replicate extractions) was 2 ng/ml. This LOQ was sufficient to determine the NIF levels in human plasma with bioequivalence assays.

3.5. Stability

Stability studies of spiked samples were carried out.

3.5.1. Autosampler stability

The stability of NIF and NIT in the autosampler was checked for 12 and 24 h at carousel temperature for 10 and 100 ng/ml spiked samples of nifedipine and 30 ng/ml nitrendipine concentration. No significant differences were found between $t = 0$, $t = 12$ h and $t = 24$ h.

3.5.2. Freeze and thaw stability

Spiked samples with 10 and 100 ng/ml of NIF were stored at -20°C and were subjected to three freeze–thaw cycles. The recovery decreased to $<6.5\%$ in the third cycle, but this is not a significant degradation.

3.5.3. Long-term stability

The quality control, plasmatic standard and volunteer samples were stored at -20°C. The samples were stable at -20°C for at least 1 month.

Table 1
Accuracy and precision of the validation

Amount added (ng/ml)	Intra-day ($n = 6$)			Inter-day ($n = 4$)		
	Amount found[a] (ng/ml)	RSD[b] (%)	RE[c] (%)	Amount found[a] (ng/ml)	RSD[b] (%)	RE[c] (%)
2	1.977±0.20	10.087	7.584	2.009±0.02	1.085	5.115
10	10.501±0.51	4.826	5.473	9.814±0.49	5.016	4.847
25	24.057±0.68	2.845	4.304	24.205±0.31	1.295	3.459
50	49.114±1.60	3.254	3.962	49.981±1.34	2.679	3.550
100	101.427±3.68	3.631	2.239	102.400±1.37	1.340	3.036
200	193.999±9.30	4.795	4.171	204.481±10.73	5.248	4.560

[a] Reported mean±SD.
[b] RSD = $\sigma/\bar{X} \times 100$.
[c] %RE = (amount added − amount found)/amount found.

4. Conclusions

A sensitive procedure based on on-line SPE with HPLC has been developed for the determination of NIF and NIT (I.S.) in human plasma. The LOQ is 2 ng/ml, which is similar to other HPLC–UV methods [10–12]; only when the analysis is with LC–MS–MS [22,23] is the sensitivity greater (LOQ 0.5 ng/ml). But, in this case, the equipment is very expensive and is not available in all clinical laboratories. For the GC methods [17,18] that we have available, the sensitivity values given are LOQ 1–3 ng/ml for the analysis of NIF, but the dihydropyridines are not stable at the high temperatures necessary for GC analysis [11,13].

In order to prevent photodegradation of nifedipine, all sample preparations and analysis should be carried out under sodium light or in foil-wrapped containers [2,24].

On-line SPE allows working with minimum sample manipulation and exposure to daylight. The maximum exposure to laboratory light for each sample was approximately 30 s, always using amber glass vials. No photodegradation was observed, as shown in the stability studies.

It was possible to continue the assay during the night and at the weekend without human supervision. Another advantage was the low exposure to potential biohazardous materials.

This automatic HPLC method is very useful for the analysis of the large number of samples generated in clinical trials.

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