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Short communication

HPLC method for analysis of a new 1,4-dihydropyridine: Application to pharmacokinetic study in rabbit

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Abstract

A high sensitive HPLC assay for plasma analysis of a new 1,4-dihydropyridine (nitrimidodipine) was developed to support the subsequent preclinical development of the compound. To 1 ml of rabbit plasma was added internal standard (3-(4-nitrooxy butyl)-5-ethyl-1,4-dihydro-2,6-dimethyl-4-(1-methyl-5-nitro-2-imidazolyl)-3,5-pyridine dicarboxylate) and 0.5 ml of 1 M HCl. The plasma was extracted using 5 ml ethyl acetate which evaporated under gentle stream of nitrogen. The residue was reconstituted in 200 μl mobile phase and 100 μl of aliquots were injected to HPLC system. Chromatographic separation was accomplished on octadecyl column (250 mm × 4.6 mm) using a mobile phase consisting of acetonitrile–water (45:55, v/v). The method was sensitive to 2.5 ng/ml in plasma (LOD), acceptable within- and between day reproducibility and a linearity ($r^2 > 0.9957$) over a concentration range from 5 to 400 ng/ml. The mean extraction efficacy was 90.6% and no interfering peaks of the blank plasma chromatograms were observed. By using the above procedure, a simple, sensitive and convenient HPLC assay for determination, stability evaluation and pharmacokinetic study of nitrimidodipine was developed.

Keywords: New dihydropyridine derivative; HPLC

1. Introduction

1,4-Dihydropyridine derivatives endowed with calcium antagonistic action have attracted much attention from the synthesis point of view over the past 20 years. In order to reach more selective and longer-acting drugs with less side effects, structural modifications have been made in nifedipine (Fig. 1A) as the leading compound of this class.

Changes in substituent pattern of the C_3, C_4 and C_5 position of nifedipine alter activity and tissue selectivity [1,2]. On the other hand, organic nitrate compounds such as nitroglycerine activate guanylate cyclase to increase the level of cyclic guanosine monophosphate (cGMP) and promote relaxation in various vascular smooth muscle tissues [3]. We have suggested that simultaneous uses of calcium antagonists and nitrate compounds could enhance the antihypertensive effect with little side effects. So the combination of nitrate and calcium blocking action in a single molecule was expected to have a potential vasodilating activity superior to that of the known [2].

Synthesis and investigation of calcium antagonistic activities in GPILSM (Guinea-Pig Ileal Longitudinal Smooth Muscle) of a number of nitroimidazolyl dihydropyridine derivatives containing nitrooxy alkyl substituents were reported by Miri et al. [2]. 3-(3-Nitrooxy propyl)-5-methyl-1,4-dihydro-2,6-dimethyl-4-(1-methyl-5-nitro-2-imidazolyl)-3,5-pyridine dicarboxylate (nitrimidodipine, **I**) is a new calcium channel blocker which has shown a remarkable IC_{50} upon pharmacological evaluations and now is in clinical evaluation [2]. As a consequence, it's measurement in biological fluids is required for further studies. Literature survey reports several methods for assay of 1,4-dihydropyridine derivatives [4–8]. We have developed a method for analyze **I** in plasma by high performance liquid chromatography. The stability of the molecule exposed to

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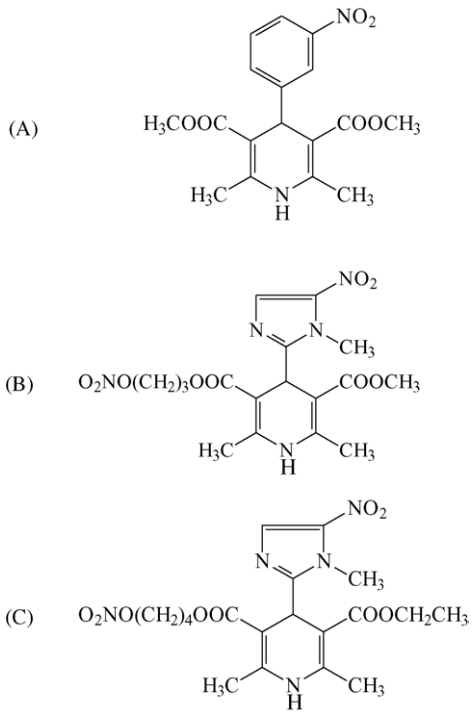

Fig. 1. Chemical structure of (A) nifedipine, (B) nitrimidodipine and (C) internal standard.

light has been also investigated because photodegradation of 1,4-dihydropyridines produce inactive oxidized compounds [9–11]. A preliminary pharmacokinetic study was conducted to demonstrate the usefulness of this method.

2. Experimental

2.1. Chemicals and reagents

3-(3-Nitrooxy propyl)-5-methyl-1, 4-dihydro-2,6-dimethyl-4-(1-methyl-5-nitro-2-imidazolyl)-3,5-pyridine dicarboxylate (nitrimidodipine, **I**), and internal standard: 3-(4-nitrooxy butyl)-5-ethyl-1,4-dihydro-2,6-dimethyl-4-(1-methyl-5-nitro-2-imidazolyl)-3,5-pyridine dicarboxylate (IS) were synthesized by Miri et al. [2]. (Department of Medicinal Chemistry, Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.) The structure of nifedipine, nitrimidodipine (**I**) and internal standard (IS) are shown in Fig. 1. Acetonitrile, ethanol, ethyl acetate, hydrochloric acid, PEG 400, potassium oxalate (Merck, Darmstadt, Germany) were all HPLC grade or extrapure. The mobile phase was filtered and degassed by pressing through a 0.45 μm membrane filter (Millipore, Ont., Canada).

2.2. Chromatographic conditions

A liquid chromatographic pump (model Shimadzu, LC-10AD$_{vp}$) was equipped with a UV detector (Shimadzu, SPD-10AD$_{vp}$). The mobile phase consisted of acetonitrile–water (45:55, v/v) and was run at a flow rate of 1.5 ml/min (under a pressure of 1200 psi). The stationary phase was a reversed-phase μ-Bondapack ODS column (250 mm × 4.6 mm), and detection of samples was carried out at 320 nm. The LC system was operated isocratic at ambient temperature.

2.3. Standard solutions

Stock solutions of the compound **I** and internal standard were prepared by dissolving 10 mg of the material in 10 ml acetonitrile. The working solutions of **I** and IS were prepared by diluting stock solutions with acetonitrile. All stock and working solutions were stored at 4 °C in the dark or under sodium lamp. Different plasma standard samples containing 2.5, 5, 10, 25, 50, 75, 100 and 200 ng/ml were prepared by spiking drug free rabbit plasma with the working solution of the compound mentioned above. Before analysis a constant amount of working solution of internal standard was added to each plasma sample to a final concentration of 400 ng/ml.

2.4. Sample preparation

Forty microliters of standard solution (10 μg/ml) and 0.5 ml of HCl (1 M) were added to 1 ml of plasma, and the solution was mixed for a few seconds. Five millilitres of ethyl acetate was added to the solution which was subsequently shaken on a horizontal shaker (Labtron, LC-100, Iran) for 10 min, followed by centrifugation at 1400 × *g* for 10 min. The top organic layer was transferred to another clean, dry glass tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C (5 ml of ethyl acetate could be divided into two 2.5 ml fractions and the extraction procedure could be repeated twice in order to reach a better recovery yield). The resultant residue was reconstituted in 200 μl of the mobile phase and 100 μl was injected to the HPLC system.

2.5. Validation studies

For the determination of intra-day and inter-day accuracy and precision of the assay, aliquots of 1 ml of rabbit plasma were spiked with constant amount of internal standard and various quantities of **I** to yield 10, 50, 100 and 200 ng/ml. Accuracy was expressed as the mean (%) = [(mean measured concentration)/(expected concentration)] × 100. Precision was calculated as intra- and inter-day coefficient of variation [CV% = (S.D./mean) × 100]. Determinations were performed with three replicates on the same day, as well as on separate days.

2.6. Stability study

2.6.1. Stability in plasma at room temperature

Samples were prepared by adding 20 μl of stock solution of the compound **I** (10 μg/ml) to 1 ml of drug-free plasma and

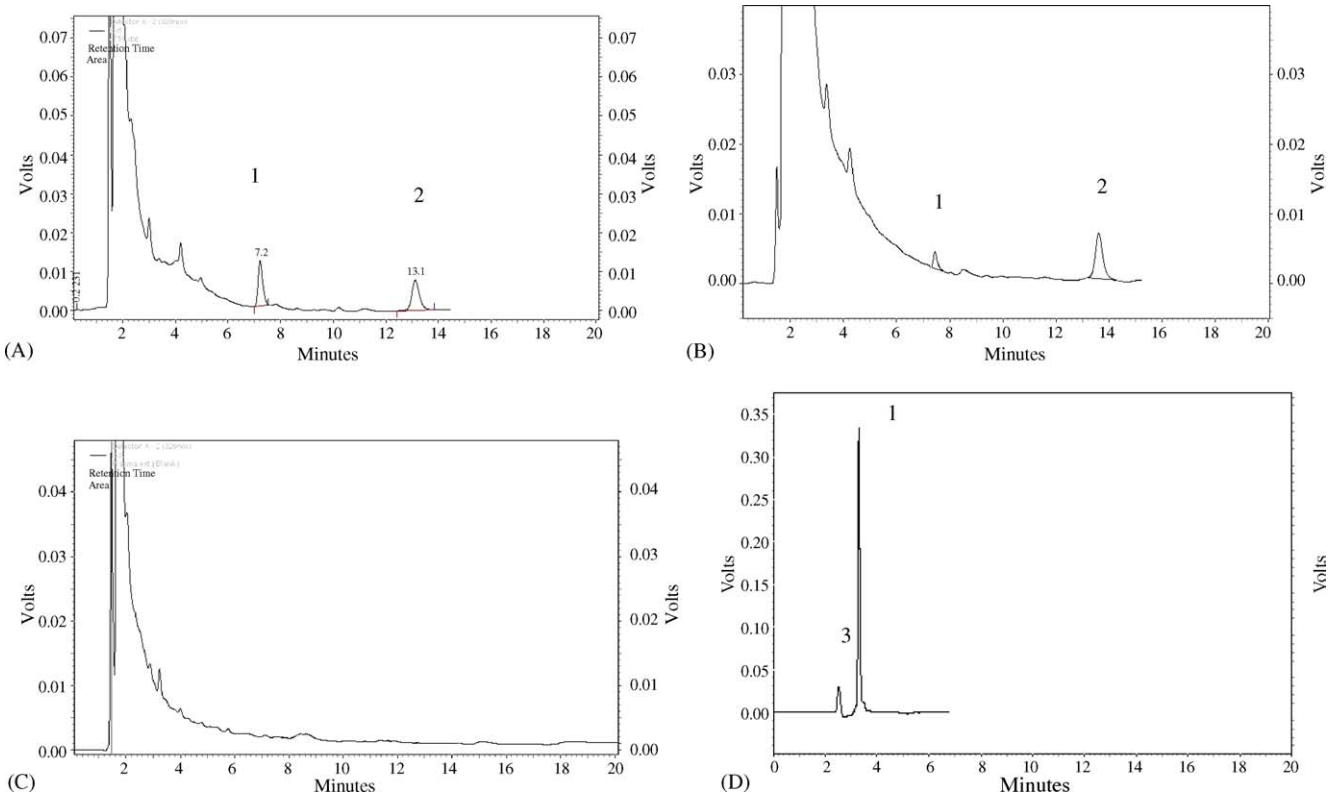

Fig. 2. Chromatograms of nitrimidodipine (**I**) in rabbit plasma: (A) sample obtained 5 min after an IV dose of 1.5 mg/kg of the compound; (B) sample spiked with 5 ng/ml nitrimidodipine and 400 ng internal standard; (C) blank rabbit plasma; peak 1: nitrimidodipine; peak 2: internal standard; (D) chromatogram of the compound after 10 h exposure to light. Peak 1: the compoumd; peak 3: photodecomposed derivative.

kept at room temperature while being protected from light. Remaining for 0, 2, 4, 6, 12, 24 and 48 h under the above mentioned conditions, constant amounts of internal standard were added to each sample prior to analysis. The concentration of intact compound in plasma samples was calculated and plotted versus time.

2.6.2. Stability in plasma at −20 °C

All above procedure were repeated after 0, 4, 12, 24 and 48 h, 1, 2, 3 and 4 weeks, and 3 months remaining at −20 °C.

2.6.3. Stability in acetonitrile solution

Standard solutions of compound **I** having 10 μg/ml was prepared in acetonitrile. Aliquots were removed for assay after 0, 2, 4, 6, 12, 24 and 48 h, 1 and 2 weeks, 1 and 3 months. Twenty microliters (200 ng) aliquots were assayed in triplicate for each solution at each period.

2.6.4. Photostability

Standard solutions of compound **I** at 10 μg/ml were prepared in acetonitrile. Using the standard solutions prepared as described before, aliquots of 100 μl were removed for assay after 0.083, 0.167, 0.25, 0.5, 1, 4, 6, 12 and 24 h after exposure to a 100 W Shahab tungsten lamp (as an artificial light). Because it was only necessary to chromatograph the two pure substances (compound **I** and it's photodecomposed derivative), for this portion of the experiment, the assay was done using a modified mobile phase containing methanol–acetonitrile–water (70:25:5, v/v) with a flow rate of 1 ml/min. Under this condition the retention times of the compound and photodecomposed derivative were 3.6 and 2.9 min, respectively. Concentration of the intact compound was measured at the end of each time period.

2.7. Pharmacokinetic protocol

Four adult male albino rabbits weighing 2.9–3.7 kg were used in this study. On the day of experiment, a 20 G butterfly heparinized catheter was inserted into the central artery of the ear of each rabbit. A single bolus intravenous dose of 1.5 mg/kg of the compound dissolved in a mixture of PEG 400-ethanol-water (5:65:30, v/v/v) was administered to each rabbit over a 1 min period. Blood samples were collected from marginal ear vein at 2, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 120, 180, 240 and 360 min after administration. Plasma was separated by immediate centrifugation (3700 × *g*, 10 min) at room temperature and was kept at −20 °C until analysis. Pharmacokinetic parameters were calculated by a non-compartmental method using WinNonlin 1.1 software program.

Table 1
Intra-day and inter-day accuracy and precision for spiked compound **I** in rabbit plasma

Added concentrations (ng/ml)	Concentration found (ng/ml) (mean, $n = 3$)		Intra-day		Inter-day	
	Intra-day	Inter-day	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)
10	9.7	9.0	97	13.6	90.3	15.0
50	45.7	45.0	91.4	3.1	90.1	4.6
100	100.7	103.4	100.7	4.5	103.4	4.0
200	202.7	196.7	101.4	4.2	98.4	5.3

3. Results and discussion

3.1. Method setup

Various organic solvents, with different pH conditions were tested for extraction procedure and ethyl acetate proved to be the best choice because of smaller interference of endogenous components and also acidic pH of plasma gave up the highest extraction recovery ($90.6 \pm 2.5\%$). After testing different ratios of mixtures of solvents such as methanol/acetate buffer (pH 4.0), acetonitrile/double distilled water at different flow rates, under the assay conditions described, the compound was clearly separated from endogenous compounds of plasma and internal standard with retention times of 7.2 and 13.1 min. The total HPLC run time was about 20 min (separation factor $\alpha = 1.23$). Typical chromatograms of blank plasma, spiked plasma, actual sample obtained from the pharmacokinetic study and photodecomposition of the compound are shown in Fig. 2. The limit of detection using 1 ml of plasma was about 2.5 ng/ml. Standard curves prepared for the compound in rabbit plasma was linear over 5–400 ng/ml. The mean ($n = 3$) calibration curve for the compound was $Y = 0.005X + 0.016$, $r^2 = 0.9957$ where Y and X are the peak area ratios of analyte/internal standard and concentration (ng/ml), respectively. The compound concentration as low as 5 ng/ml could be quantities (LOQ). The results for the intra- and inter-day accuracy and precision are shown in Table 1.

The accuracy of the assay was more than 90.06% and coefficient of variation did not exceed 15%. The degree of deviation from the theoretical values showed a trend which could be inversely related to the concentration of the analyte.

3.2. Stability

3.2.1. Stability in plasma at room temperature

Under the described experimental conditions, concentration of the intact compound remained in plasma after each period was plotted versus time as it is shown in Fig. 3.

Data analysis shows that degradation of the compound in plasma at ambient temperature matches to a first order kinetic, with correlation coefficient factor of 0.75. 91.5% of compound **I** remain intact after 24 h (Fig. 3), so it can be suggested not to keep the compound in plasma at room temperature for long periods.

3.2.2. Stability in plasma at −20 °C

Data analysis shows that degradation of the compound in plasma at −20 °C matches a first order kinetic with correlation coefficient of 0.8. As it can be concluded from Fig. 3, the half-life of the compound is about 62.7 days, so it is suggested to analyze the plasma samples containing the compound as soon as possible.

3.2.3. Stability in acetonitrile solution

The intact concentration of the compound in acetonitrile solution was plotted versus time and is shown in Fig. 3. As it is shown by the graph the compound proved to have a remarkable stability in the solution and remain almost all intact after 3 months.

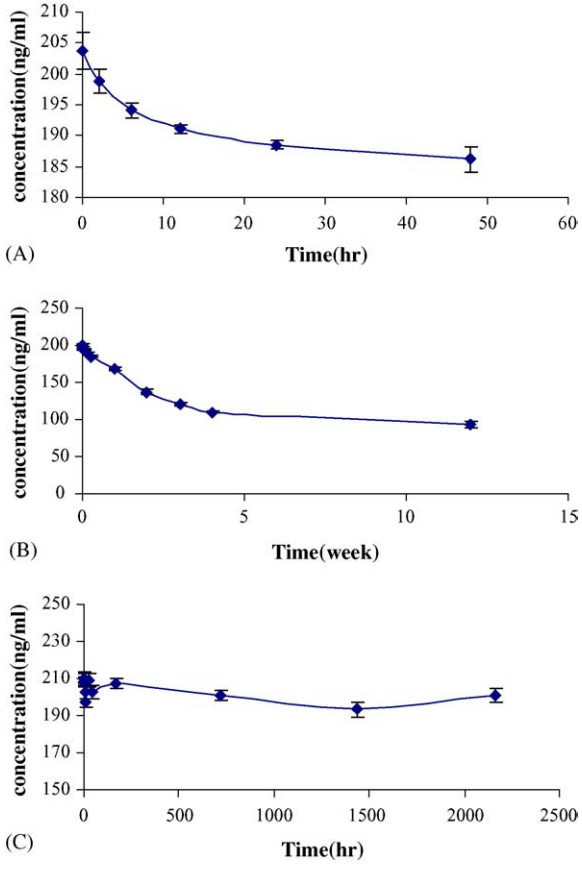

Fig. 3. Rate of degradation of nitrimidodipine: (A) in plasma at room temperature; (B) in plasma at −20 °C; (C) in acetonitrile solution. (Each point represents mean ± S.D. of three independent determinations.)

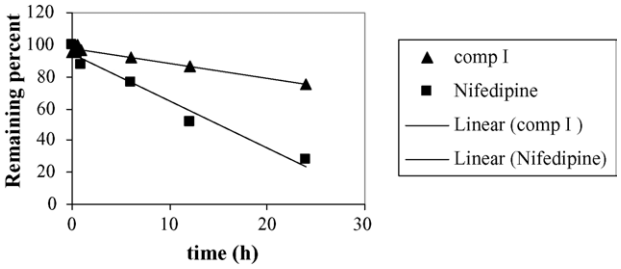

Fig. 4. Photodegradation of methanolic solution of nitrimidodipine (**I**) and nifedipine irradiated by artificial light ($n = 3$).

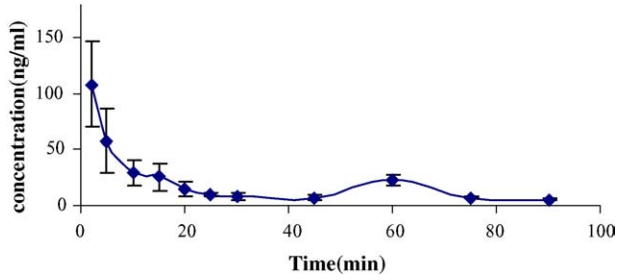

Fig. 5. Mean nitrimidodipine (**I**) plasma concentration–time profile in rabbits following an intravenous administration of 1.5 mg/kg of nitrimidodipine in four rabbits.

3.2.4. Photostability

Some 1,4-DHPs are well known to be unstable through photodecomposition especially nifedipine which is rapidly photodegraded. Under our experimental conditions the curve of break down of the concentration of compound **I** versus time was linear with correlation coefficient of 0.97 ($Y = -5.7X + 858.5$) and suggests a zero order photodecomposition kinetic with a photodecomposition half-life of 51.0 h. Fig. 4 demonstrates the photodegradation of the compound **I** and nifedipine. A comparison between photostability of the compound and nifedipine exposed to artificial light illustrates that 96.1% of the compound and 27.8% of nifedipine (with photodecomposition half-life of 8.3 h) (24) remains intact after 4 h exposure to light. The difference in photodecomposition values between compound **I** and nifedipine were statically significant (p value < 0.05, Student's t-test) which confirms that the compound is much more photostable than nifedipine.

3.2.5. Pharmacokinetic study

The mean plasma concentration–time profile plotted after IV administration of the compound is shown in Fig. 5. After a single i.v. dose of 1.5 mg/kg plasma concentration–time curves were biexponential.

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