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Note

Occurrence and measurement of nifedipine and its nitropyridine derivative in human blood plasma

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Nifedipine, [dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinecarboxylate] is a potent coronary vasodilator [1]. For pharmacokinetic studies in which nanogram amounts of nifedipine in blood plasma have been measured, the literature [1—4] invariably describes the use of gas—liquid chromatography (GLC) with either electron-capture detection or selective ion monitoring.

Two basic analytical approaches have been used in these studies. In the procedure reported by Higuchi and Shiobara [1], as well as that by Kondo et al. [2], nifedipine is oxidized to its more stable nitropyridine derivative [dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylate] II, prior to GLC analysis. Despite the loss of specificity in this approach, both groups of authors have found it necessary as, under the chromatographic conditions employed, nifedipine was found to partially degrade to the nitropyridine derivative II.

Conversely, Jakobsen et al. [3] and Testa et al. [4], utilizing similar chromatographic conditions, have found nifedipine to be stable and have measured the drug directly.

In the present work, the GLC methodology of Jakobsen et al. [3] has essentially been employed with similar results: no degradation to nitropyridine derivative II during chromatography was observed. This was shown by repeated injections of nifedipine extracted from blank plasma samples to which known nifedipine amounts had been added prior to analysis.

The procedure has been used to quantitate nifedipine in the plasma of subjects from a pharmacokinetic study. In addition to nifedipine which was measured directly, the nitropyridine derivative II was also quantitated. Positive

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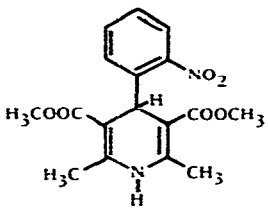
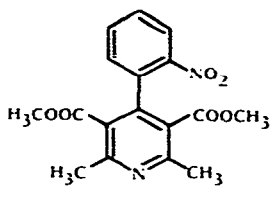


(I)



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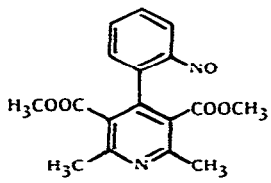

(III)

identification of the last-named substance as well as final proof that the nitropyridine derivative is a metabolite, since it is not formed during the plasma preparation or in contact with blood in vitro, contribute to confirm Jakobsen's observation [3] of the presence of a tentatively identified metabolite in plasma of patients treated with nifedipine. Prior to this time, the nitropyridine substance II was postulated as a precursor of known nifedipine metabolites [2,5], but had not been unequivocally found in plasma. The identity of the nitropyridine derivative II was confirmed by high-performance liquid chromatographic (HPLC) analysis.

EXPERIMENTAL

All sample handling and extraction steps were carried out under gold fluorescent lighting (e.g. General Electric, F40G0) to prevent light degradation of nifedipine.

Materials

Toluene, methanol, hexane, ethyl acetate, acetonitrile, were all glass distilled and supplied by Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Nifedipine, nitropyridine derivative and nitrosopyridine derivative were obtained from Bayer (Leverkusen, G.F.R.). Diazepam was supplied by U.S.P.C. (Rockville, MD, U.S.A.). Water was purified by a water conditioning system purchased from Continental Water Systems Corporation (El Paso, TX, U.S.A.).

In addition, the reagents included methanol treated with *n*-hexane and *n*-hexane treated with methanol. (Reagents were prepared by transferring 500 ml methanol and 400 ml *n*-hexane to a 1-l separatory funnel, shaking the mixture and allowing the phases to separate, the bottom layer being methanol treated with *n*-hexane, the upper layer, *n*-hexane treated with methanol.)

All glassware used during the extraction procedure was cleaned with chromic

acid, washed with distilled water, air-dried and rinsed with methanol.

Instruments

Gas—liquid chromatograph. The Hewlett-Packard 5750 Research gas chromatograph was equipped with a ^{63}Ni electron-capture detector (attenuated at 10 × 16) and a glass 1.83 m × 2.0 mm I.D. column packed with 2% OV-17 on Gas-Chrom Q (100—120 mesh). Argon—methane (95:5) was used as a carrier gas at a flow-rate of 32 ml/min. Injection port temperature was maintained at 245°C, column temperature at 235°C, detector temperature at 280°C. Injection volume corresponded to 3 μl, recorded chart speed to 0.64 cm/min.

A vortex evaporator (Buchler) was used at 40°C to remove the solvents from samples.

High-performance liquid chromatograph. The HPLC system consisted of a solvent pump (Waters Assoc., Model 6000A); injection valve, loop type (Valco, Model 6V6UHPA); a column, 200 × 6 mm O.D. × 4 mm I.D., Nucleosil® 5 C_{18}, particle size 5 ± 1.5 μm, Macherey, Nagel and Co., Düren, G.F.R., No. 715260; UV detector at 235 nm, range 0.04 (Schoeffel, Model 770); recorder 1 mV (Varian, Model 25). Mobile phase consisting of water—methanol—acetonitrile (55:36:9) was degassed and used at a flow-rate of 1.0 ml/min.

Preparation of samples for GLC analysis

Plasma (1 ml) was placed in a 15-ml centrifuge tube to which 50 ng diazepam (internal standard) had been added. Sample was extracted twice with 2 ml of toluene, each time centrifuging the sample (ca. 1000 *g* for 10 min). Each toluene extract was transferred to a clean 15-ml centrifuge tube using Pasteur disposable pipets. The combined toluene extracts were evaporated to dryness on an evaporator. The sample was reconstituted with 1.0 ml methanol treated with hexane. The methanol solution was washed with 3 ml hexane treated with methanol and centrifuged. The hexane layer was discarded using disposable pipets and the methanol evaporated to dryness. The sample was reconstituted with 200 μl of toluene before injection.

Preparation of standards for GLC analysis

Control plasma samples (1 ml) were spiked with 5, 10, 15, 25, and 50 ng nitropyridine derivative, with 10, 25, 50, 100 and 200 ng nifedipine and with 50 ng diazepam as internal standard. The samples were processed as described above. The ratios of nifedipine peak height to diazapam peak height and of nitropyridine derivative peak height to diazepam peak height were calculated and the calibration curves constructed.

Preparation of samples for HPLC analysis

After completing the GLC analysis, the sample was evaporated to dryness and redissolved in 100 μl of methanol before injection on the HPLC column.

RESULTS AND DISCUSSION

Nifedipine (I) in solution undergoes facile degradation. Daylight-induced

decomposition to the nitrosopyridine derivative III and to the nitropyridine derivative II under UV irradiation has been reported [4]. In addition, nifedipine is known to be thermally unstable. It is not surprising, therefore, that controversy exists in the literature concerning the detection and quantification of this substance in complex matrices (e.g., blood plasma). In particular, the GLC methodology used for quantitation is subject to question since higher temperatures (typically 230°C—250°C) are involved and the possibility of thermal degradation of nifedipine to its nitroderivative during chromatography cannot be discounted.

Jakobsen et al. [3] and Testa et al. [4] emphasize that they did not observe degradation of nifedipine during GLC analysis and therefore claim their assay being superior to the indirect method which involves oxidation of nifedipine to the more stable nitropyridine derivative II prior to analysis [1,2]. The last-mentioned approach may be used for nifedipine analysis only when the absence of the nitropyridine derivative in the original sample can be demonstrated.

The sensitivity reported by Jakobsen et al. [3], which is required for clinical studies, could not be achieved using the Hewlett-Packard 5750 research gas chromatograph. Therefore, the samples had to be concentrated prior to analysis. Five-fold concentration of the extract was accompanied by increase in the chromatographic background. Consequently, a more extensive clean-up procedure had to be developed. This procedure is applicable to the simultaneous quantification of nitropyridine derivative and nifedipine. Nitrosopyridine derivative III is separated from these two substances eluting first from the chromatographic column. Linearity of the procedure was established for 10—200 ng nifedipine/ml plasma and 10—50 ng nitropyridine derivative/ml plasma. Concentrations below 10 ng/ml can be reliably estimated for both substances. Amounts down to 1 ng can be detected. Reproducibility and accuracy of the procedure described in Table I will deteriorate when whole blood or fast-prepared lower quality plasma are analyzed.

Our results obtained during the analysis of 1480 clinical blood plasma samples and during additional studies of the effect of fresh whole blood and fresh plasma on nifedipine stability, lead us to conclude that the nitropyridine derivative is indeed present in the blood of subjects to whom nifedipine had been administered. The following experiments justify our conclusion. Daily calibration curves were constructed by analyzing a series of blank plasma samples spiked with known amounts of nifedipine and internal standard (diazepam). Concurrently, a five-month stability study (10 and 200 ng nifedipine/ml in frozen plasma) was conducted. In addition, nifedipine and internal standard were added to the subjects' control plasma (plasma from blood samples taken before administration of nifedipine) and analyzed. Only nifedipine was observed in the chromatograms obtained from these experiments (see Fig. 1A and B). However, in the chromatograms from subjects' plasma samples taken after nifedipine administration, the nitropyridine derivative II was observed in addition to nifedipine (Fig. 1C). The kinetic profile of the nitropyridine compound was similar to that of nifedipine for all subjects studied (for example, see Fig. 2).

In order to confirm the identity of the nitropyridine derivative II in plasma

TABLE I

REPRODUCIBILITY AND ACCURACY OF NIFEDIPINE AND NITROPYRIDINE DETERMINATION IN BLOOD PLASMA

Compound added (ng/ml)	Number of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
*A. Nifedipine determination**			
10	5	96.0	4.5
25	9	105.3	9.0
50	9	98.9	4.8
100	9	100.9	3.7
200	8	98.6	1.1
*B. Nitropyridine derivative determination***			
5	5	72.0	11.9
10	9	90.0	7.5
15	5	105.8	8.2
25	10	104.0	6.4
50	5	91.6	1.9

*Amounts of nitropyridine derivative added varied from 5 to 50 ng per ml plasma.
**Amounts of nifedipine added varied from 10 to 100 ng per ml plasma.

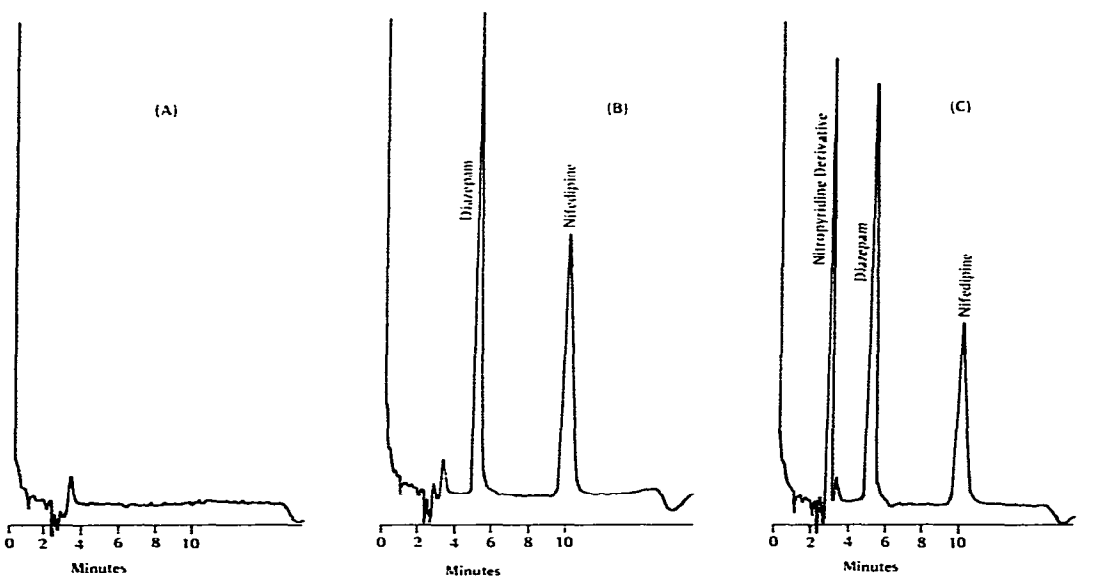

Fig. 1. GLC analysis of (A) control plasma before administration of nifedipine; (B) control plasma before administration of nifedipine spiked with 100 ng/ml nifedipine and 50 ng/ml diazepam; and (C) plasma, 20 min after administration of nifedipine.

from subjects to whom nifedipine had been administered, HPLC analysis of control plasma extract containing II and of a typical plasma extract exhibiting high nitropyridine content in the GLC analysis were carried out. In both instances a peak appeared at the retention time of the nitropyridine derivative (compare Fig. 3A, B and C). The peak identity was further confirmed by col-

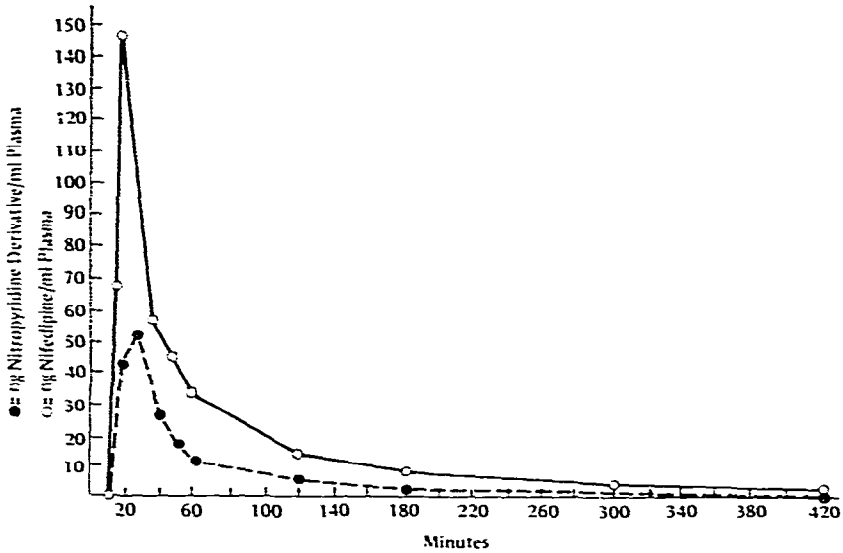

Fig. 2. Typical kinetic profile of nifedipine (·—o—) and nitropyridine derivative (- - • - -) for a subject administered the drug.

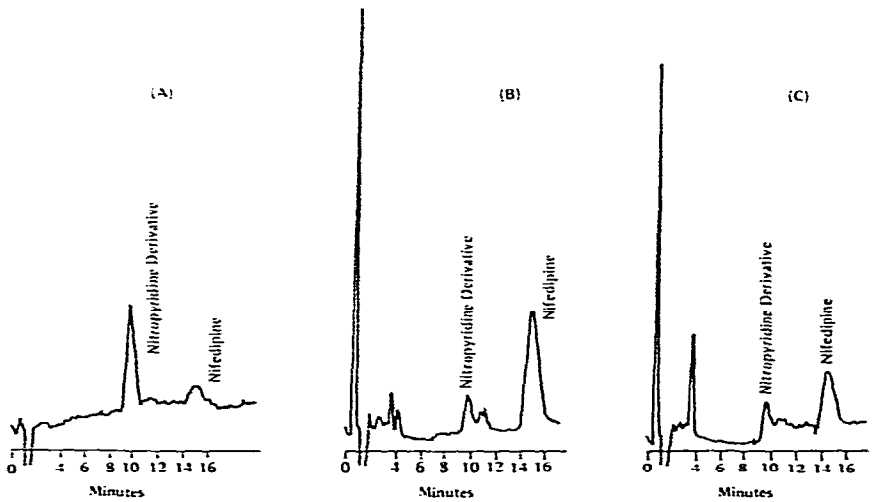

Fig. 3. HPLC chromatograms of (A) reference substances, nitropyridine derivative and nifedipine, demonstrating their retention times; (B) control plasma spiked with 100 ng nitropyridine derivative and 200 ng nifedipine per ml plasma; (C) subject's plasma taken 30 min after nifedipine administration.

lecting the material eluted under the HPLC peak and subsequently reanalyzing it by GLC. A chromatogram typical of the nitropyridine derivative was obtained, confirming the identity of the substance.

The possibility of nitropyridine derivative being formed from nifedipine during the plasma preparation or in contact with blood in vitro was investigated by adding known amounts of nifedipine (equivalent to 25 ng/ml and 200 ng/ml) to fresh whole blood and to fresh fast-prepared plasma. In total, sixteen experiments were carried out. Simultaneously, calibration curves for both the

TABLE II

OCCURRENCE OF NITROPYRIDINE DERIVATIVE IN FRESH PLASMA AND WHOLE BLOOD CONTAINING NIFEDIPINE

Nifedipine added (ng/ml)	Nitropyridine derivative determined (ng/ml)	Nifedipine determined (ng/ml)	Percentage recovery of nifedipine
Analysis of nifedipine added to fresh blood plasma			
25	< 2	25.5	102.0
	< 2	24.9	99.6
	< 2	28.4	113.6
	< 2	26.7	106.8
		Average	105.5
200	ca. 2	186.8	93.4
	ca. 2	188.1	94.1
	ca. 2	181.2	90.6
	ca. 2	176	88.0
		Average	91.5
Analysis of nifedipine added to fresh whole blood			
25	< 2	19.7	78.8
	< 2	23.0	92.0
	< 2	20.5	82.0
	< 2	24.3	97.2
		Average	87.5
200	ca. 2	158.4	79.2
	ca. 2	165.5	82.8
	ca. 2	124.3	62.2
	ca. 2	169.7	84.9
		Average	77.3

nitropyridine derivative and nifedipine were constructed. The results (Table II) clearly indicated that nifedipine in direct contact with either fresh human blood plasma or fresh whole blood is not significantly converted to the nitropyridine derivative during the processing of the samples prior to freezing and analysis. Lower nifedipine recoveries (Table II) obtained, in particular when whole blood was analyzed, were caused by lower extraction efficiency from this medium. However, they do not influence the conclusion on the stability of nifedipine under given experimental conditions.

Since the samples of plasma from subjects administrated nifedipine, prepared under conditions identical to those described above, invariably showed the presence of both nifedipine and its nitropyridine derivative (the amount of nitropyridine derivative expressed as percentage of nifedipine varied from subject to subject from 17 to 90%), it is concluded that the nitropyridine derivative, which has been postulated as a precursor of known nifedipine metabolites [2,5], is present in the blood of subjects who have been administered nifedipine. Consequently, a selective assay for nifedipine without preliminary oxidation to the nitropyridine derivative must be employed in order to accurately assess the content of nifedipine in plasma or serum samples.

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