Journal of Chromatography, 353 (1986) 175–180
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 748

QUANTITATIVE ANALYSIS OF THE DIHYDROPYRIDINES, 3-(2-FUROYL)-5-METHOXYCARBONYL-2,6-DIMETHYL-4-(2-NITROPHENYL)-1,4-DIHYDROPYRIDINE AND NIFEDIPINE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

N. D. HUEBERT*
Department of Clinical Research, Merrell Dow Research Institute, 16, rue d'Ankara, 67084 Strasbourg Cedex (France)
M. SPEDDING
Department of Pharmacology, Merrell Dow Research Institute, 16, rue d'Ankara, 67084 Strasbourg Cedex (France)
and
K. D. HAEGELE
Department of Clinical Research, Merrell Dow Research Institute, 16, rue d'Ankara, 67084 Strasbourg Cedex (France)

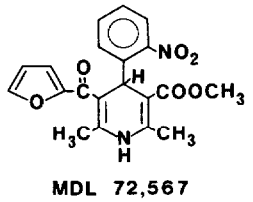
SUMMARY

An analytical method based on solvent extraction and reversed-phase high-performance liquid chromatographic separation with electrochemical detection has been developed for the dihydropyridines, 3-(2-furoyl)-5-methoxycarbonyl-2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine (MDL 72.567) and nifedipine. The analysis includes an internal standard of similar light sensitivity to correct for possible photodegradation during the procedure. The specificity of electrochemical detection precludes interference from oxidized metabolites. Total analysis time was 35 min per sample, and the detection limit for quantification was 1–2 ng/ml. Linear regression analysis gave calibration curves with coefficients or correlation of 0.9992 for MDL 72.567 (1–100 ng) and 0.997 for nifedipine (1–50 ng). Assays for within-run and day-to-day reproducibility gave coefficients of variation of 3.9% and 6.0%, respectively, at concentrations of 50 ng/ml. The method has been applied to the analysis of plasma levels of nifedipine and MDL 72.567 in dogs.

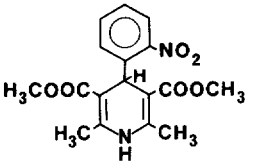
INTRODUCTION

The 4-aryl-1,4-dihydropyridine calcium antagonists act by blocking calcium channels in vascular smooth muscle. The resulting reductions in contractility lead to systemic vasodilatation and falls in peripheral resistance. The analysis of the dihydropyridines is complicated by their sensitivity to light and heat. Previously published methods seemed inappropriate because of their non-specificity[1], use of high temperatures[1–6], lack of sensitivity[1,4,7,8] or lack of a suitable internal standard[2–5,7–10]. The present specific and sensitive method, based on high-performance liquid chromato-

0021-9673/86/$03.50



MDL 72,567



NIFEDIPINE

Fig. 1. Structural formulae of MDL 72.567 and nifedipine.

graphy with electrochemical detection (ED), has been developed for the analysis in plasma of 3-(2-furoyl)-5-methoxycarbonyl-2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine (MDL 72.567), a novel calcium antagonist, and nifedipine (Fig. 1).

EXPERIMENTAL

Chemicals

Ethyl acetate (Uvasol, Merck, Darmstadt, F.R.G.) was used for all extractions as other spectroscopic brands were found to contain interfering contaminants. *n*-Hexane was purchased from Baker (Deventer, The Netherlands). Spectroscopy-grade methanol (Merck), acetonitrile (Burdick & Jackson, Muskegon, MI, U.S.A.)

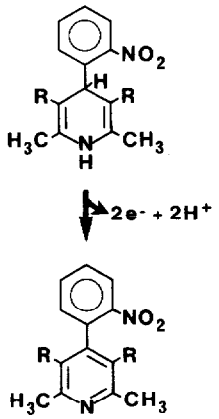


Fig. 2. Oxidation of the 2-nitrophenyldihydropyridines at the detector surface.

and water with a resistance greater than 10 MΩ cm^{-1} (Millipore Milli Q system) were used in the preparation of the HPLC solvent.

Preparation of samples

It was considered important to include in the procedure a dihydropyridine of similar light sensitivity to correct for potential losses due to photodegradation. Thus the procedure was developed so that nifedipine could be used as an internal standard for MDL 72.567 and *vice versa*. Preliminary photodegradation experiments showed that, under conditions of mixed fluorescent light and daylight, MDL 72.567 and nifedipine had half-lives of 54 and 78 min, respectively. In addition, all procedures were carried out under conditions of minimal indirect light exposure, and sample tubes were covered with aluminium foil throughout the procedure. To 1 ml of plasma was added the appropriate amount of internal standard (50 ng of MDL 72.567 for nifedipine analysis or 15.4 ng of nifedipine for MDL 72.567 analysis), dissolved in methanol. The solution was made basic by the addition of 1 ml of 1 *M* sodium bicarbonate, and the aqueous phase was extracted twice with 2 ml of ethyl acetate–*n*-hexane (75:25). The organic phases were pooled and evaporated under nitrogen. The residue was redissolved in 100 μl of methanol, and 25 μl of this solution was injected into the HPLC system.

Chromatographic system

The chromatographic system consisted of a WISP 710A automatic injector (Waters, Milford, MA, U.S.A.), a Waters Model 590 pump, a Waters μBondapak C_{18} reversed-phase column, an LC-4 electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and a Houston Instruments Omniscribe Series D5000 strip-chart recorder (Bausch & Lomb, Austin, Texas, U.S.A.). The mobile phase consisted of 0.07 *M* phosphate buffer (pH 7.0)–methanol–acetonitrile (52:36:12). The organic solvent content of the mobile phase may be reduced with time to compensate for losses in column efficiency and to maintain adequate separation and constant retention times. The detector potential was maintained at +1.00 V *vs.* a Ag/AgCl reference electrode. The dihydropyridines are oxidized to their corresponding pyridines at the electrode surface (Fig. 2).

Calibration

Quantification was by peak-height measurement of both the compound to be analysed and the internal standard. For calibration purposes, 1-ml blank samples of plasma were spiked with known amounts of MDL 72.567 (between 1 and 100 ng) or nifedipine (between 1 and 50 ng) and the appropriate internal standard. Calibration curves were constructed by plotting the ratios of the peak heights of the compound to be analysed and the internal standard *vs.* the amount of compound added.

Animal experiments

Normotensive mongrel dogs (9.9–10 kg) were anaesthetized with sodium pentobarbitone (20–25 mg/kg, i.v.) after premedication with levomepromazine (0.5 mg/kg, s.c.). The femoral vein and artery were cannulated. Blood pressure in the abdominal aorta was measured by use of an indwelling catheter connected to a Statham pressure transducer; this catheter was also used for taking blood samples. MDL

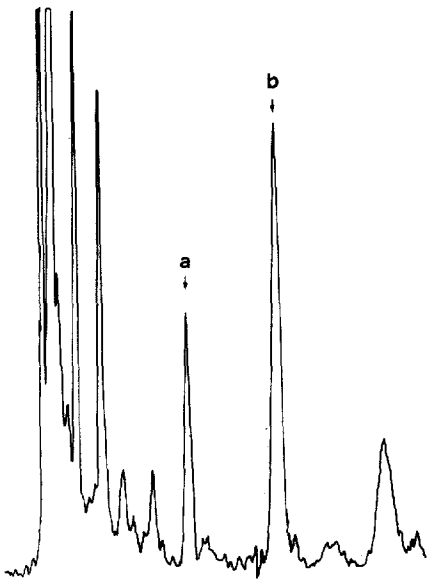


Fig. 3. Chromatographic separation of (a) MDL 72.567 and (b) nifedipine in dog plasma extract.

72.567 or nifedipine was administered intravenously in a dose of 500 ng/kg. Blood samples were collected just before and 1, 5, 10, 20, 30 and 45 min and 1, 1.5 and 2 h after drug administration. The plasma obtained by centrifugation was kept frozen at −20°C until analysed.

RESULTS

The chromatographic separation of MDL 72.567 and nifedipine in dog plasma is shown in Fig. 3. The total HPLC analysis time was 35 min. The retention times of MDL 72.567 and nifedipine were 15 and 21 min, respectively. Calibration curves yielded straight-line relationships with correlation coefficients of 0.9992 over a range of 1–100 ng for MDL 72.567 and 0.997 over a range of 1–50 ng for nifedipine. The analysis of blank samples to which both MDL 72.567 and nifedipine had been added yielded within-run coefficients of variation of 3.9% (n = 5) and day-to-day reproducibility with coefficients of variation of 6.0% (n = 15) at concentrations of 50 ng/ml. The detection limit for quantification was 1–2 ng/ml. Plasma decay curves for MDL 72.567 and nifedipine and the corresponding blood pressure effects following i.v. administration to anaesthetized dogs are shown in Figs. 4 and 5. The plasma levels in dogs declined over a 2-h period following intravenous administration of a 500 ng/kg dose but were still measurable, and the hypotensive effect was still evident at the end of that period.

DISCUSSION

Several gas chromatographic (GC) methods for the analysis of nifedipine have

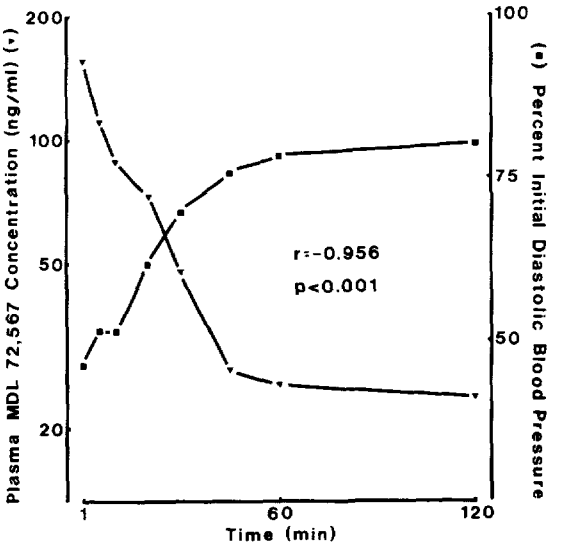


Fig. 4. Plasma decay curve for MDL 72.567 following i.v. administration of 500 ng/kg to an anaesthetised dog, and the resultant blood pressure reduction expressed as a percentage of the initial diastolic blood pressure; the latter (100%) was determined just prior to drug administration. The first data points graphically presented correspond to blood pressure measurement and MDL 72.567 concentration 1 min post-dose.

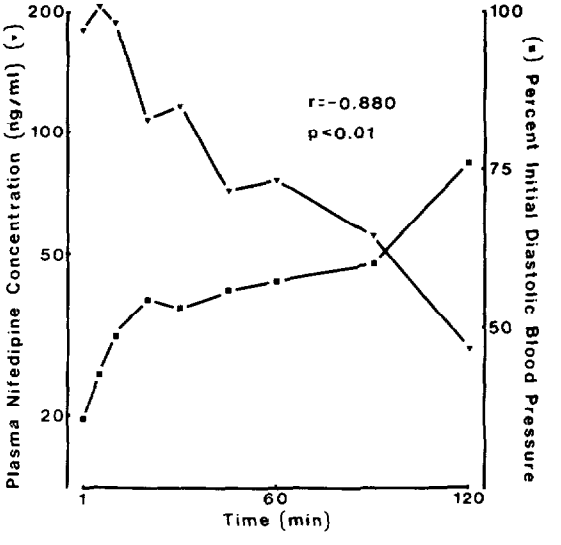


Fig. 5. Plasma decay curve for nifedipine following i.v. administration of 500 ng/kg to an anaesthetised dog, and the resultant blood pressure reduction expressed as a percentage of the initial distolic blood pressure; the latter (100%) was determined just prior to drug administration. The first data points graphically presented correspond to blood pressure measurement and nifedipine concentration 1 min post-dose.

been described in the literature[1–6], but the dihydropyridines are not stable at the high temperatures necessary for GC analysis. On-column degradation of MDL 72.567 in GC–mass spectrometry (MS) has been observed[11]. Kondo *et al.*[1] have reported the on-column oxidation of nifedipine in GC with electron capture detection. They attempted to circumvent this problem by oxidizing nifedipine and analysing it as its pyridine analogue. Since the latter represents an important intermediate in nifedipine metabolism[12], such a procuedure inherently lacks specificity. For these reasons, a liquid chromatographic procedure was investigated.

The dihydropyridines are very sensitive to light, decomposing to their 2-nitrophenylpyridine and 2-nitrosophenylpyridine derivatives on exposure to UV or visible light[2,7,8]. In the present method, such losses were corrected for by including an internal standard of similar light sensitivity in the work-up procedure. In addition, all manipulations were carried out in minimal indirect light and the sample tubes were covered throughout the procedure with aluminium foil to minimize exposure to light.

The detection and quantification of the dihydropyridines by our method takes advantage of the ease of oxidation of the dihydropyridine ring at an appropriate voltage (Fig. 2). The limit of detection is similar to that for various other published methods[2–4,7,9,11]. The specificity of our method precludes the interference by the oxidized metabolites in the quantification of MDL 72.567 and nifedipine. Bratin and Kissinger[8] have described a dual-electrode electrochemical assay involving reduction and oxidation of the aromatic nitro or nitroso group. This allows the analysis of nifedipine and its confirmed metabolites but the detection limit of this method is 20 ng/ml, which is not low enough for pharmacokinetic investigations.

Our methods has been applied to the analysis of MDL 72.567 and nifedipine in dog plasma following i.v. administration of 500 ng/kg of drug to anaesthetized dogs, and we observed a correlation of diastolic blood pressure reduction with the plasma level of MDL 72.567 or nifedipine. The dihydropyridines are very potent compounds and their threshold effects on blood pressure are normally subthreshold for existing assay procedures. Our improved assay increases the sensitivity of detection of the dihydropyridines so that plasma levels can now be measured when only small effects on blood pressure are evident. Our method has also been applied to the measurement of nifedipine plasma levels and dihydropyridine concentrations in tissues and incubation medium in *in vitro* tissue incubation experiments.

REFERENCES

1 S. Kondo, A. Kuchiki, K. Yamamoto, K. Akimoto, K. Takahashi, N. Awata and I. Sugimoto, *Chem. Pharm. Bull.*, 28 (1980) 1.
2 P. Jakobsen, O. Lederballe Pedersen and E. Mikkelsen, *J. Chromatogr.*, 162 (1979) 81.
3 J. Dokladalova, J. A. Tykal, S. J. Coco, P. E. Durkee, G. T. Quercia and J. J. Korst, *J. Chromatogr.*, 231 (1982) 451.
4 S. R. Hamann and R. G. McAllister, Jr., *Clin. Chem.*, 29 (1983) 158.
5 L. J. Lesko, A. K. Miller, R. L. Yeager and D. C. Chatterji, *J. Chromatogr. Sci.*, 21 (1983) 415.
6 K. D. Rämsch and J. Sommer, *Hypertension*, 5 (1983), Supp II-18.
7 P. Pietta, A. Rava and P. Biondi, *J. Chromatogr.*, 210 (1981) 516.
8 K. Bratin and P. T. Kissinger, *Curr. Separ.*, 4 (1982) 4.
9 T. Sadanaga, K. Hikida, K. Tameto, Y. Matsushima and Y. Ohkura, *Chem. Pharm. Bull.*, 30 (1982) 3807.
10 P. R. Bach, *Clin. Chem.*, 29 (1983) 1344.
11 K. D. Haegele, unpublished results.
12 C. H. Kleinbloesem, J. Van Harten, P. Van Brummelen and D. D. Breimer, *J. Chromatogr.*, 308 (1984) 209.