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DETERMINATION OF NIFEDIPINE AND ITS THREE PRINCIPAL METABOLITES IN PLASMA AND URINE BY AUTOMATED ELECTRON-CAPTURE CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

A sensitive, efficient, linear and reproducible capillary gas chromatographic method with electron-capture detection was developed for the quantitation of nifedipine and its primary metabolite M-I in plasma together with the urinary and principal metabolites M-II and M-III. On-column, rather than split–splitless, injection was employed to obviate oxidative degradation of nifedipine to M-I. The photosensitivity of nifedipine was re-examined under laboratory conditions and nifedipine was found to have a half-life in excess of two days when amber glassware and darkroom manipulations under red light were used. The method can determine nifedipine and its metabolites in plasma and urine after a single oral dose of 5 mg and can be applied to measure M-I production by human liver microsomes.

INTRODUCTION

Nifedipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (Adalat®, I in Fig. 1), is the archetype of an expanding class of drugs referred to as the dihydropyridine calcium antagonists. Nifedipine finds widespread clinical use in treatment of disorders of both coronary and peripheral blood vessels, including angina pectoris and hypertension. Furthermore, a considerable number of homologous and analogous dihydropyridines is under current clinical trial evaluation. This is a potent group of drugs which exerts its desired effects in vivo at concentrations of 10^{-9}–10^{-7} M [1]. Not surprisingly therefore sensitive techniques are required to detect and quantitate the levels of these drugs and their metabolic products in body fluids.

Several authors have addressed specifically the determination of nifedipine in plasma. Of the earliest assays the most common technique employed was packed-column gas chromatography (GC) with electron-capture detection (ECD) [2–7].

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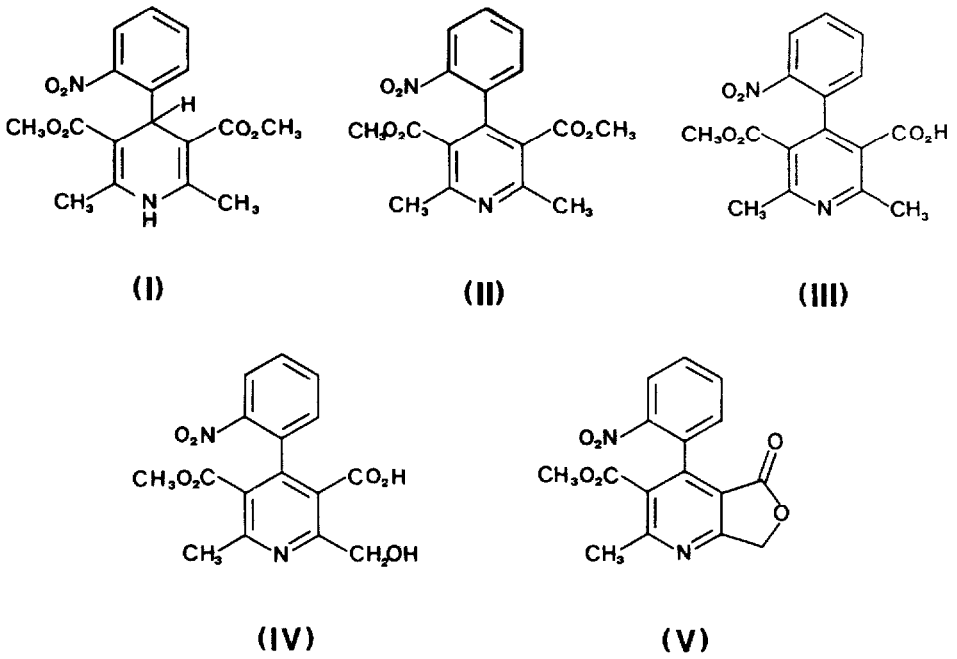

Fig. 1. Nifedipine (I) and its three principal metabolites M-I (II), M-II (III) and M-III that exists as ring-opened metabolite M-IIIa (IV) and a ring-closed lactone form M-IIIb (V).

Perusal of the chromatograms published in these studies reveals two principal problems: firstly, the characteristic low efficiency (ca. 500–2000 theoretical plates) of these methods and secondly and in one prominent case [6], unacceptable peak tailing. Where packed columns were combined with single-ion monitoring by mass spectrometry [8], however, the specificity of the assay compensated for the very low number of chromatographic plates obtained. Many workers have utilised reversed-phase high-performance liquid chromatography (HPLC) to separate and determine nifedipine in plasma. With the exception of one electrochemical HPLC method [9], the application of UV detection appears to be universal [9–18]. Some improvement in column efficiency (ca. 1000–10 000 plates) is achieved with these HPLC methods.

Capillary GC has been applied in comparatively few cases and with either direct on-column or a splitless mode of injection in addition to either nitrogen-selective detection [19] or ECD [20, 21]. Noteworthy is the report of Tucker et al. [20] which combines high column efficiency (approximately $5 \cdot 10^5$ plates calculated from their published chromatograms) with good sensitivity (limit of detection 0.5 ng ml^{-1}), in contrast to some of the HPLC methods [10, 17] which are suitable only for the analysis at μg ml^{-1} concentrations of bulk drug substance in pharmaceutical formulations.

In the main, the foregoing methods embrace one of a number of serious and additional limitations. Bizarre internal standards have been employed including 11-oxoprogesterone [11], 4-dimethylaminobenzaldehyde [10], diazepam [2, 3, 7], methylclonazepam [5] and methylnitrazepam [6] but in many cases appro-

priate dihydropyridine homologues were used [4, 9, 12, 19–21]. Whilst most authors recognised the problem of the photolability of nifedipine, none has considered the thermal stability of the drug, particularly under GC conditions. Our preliminary experiences with nifedipine demonstrated that the primary metabolite formed by oxidation of the dihydropyridine moiety was also readily formed as an artefact in contact with hot metal such as is found in a GC injection port [22]. This was not the case if on-column injection was made.

In addition to inadequate attention to chromatographic artefacts, peak shape, suitability of internal standards and sensitivity, the final limitation of all previous methodology is the inability to separate, detect and quantitate the principal metabolites of nifedipine in plasma and urine. Most methods measure the parent drug only, a minority can determine one metabolite. To satisfy this demand we have developed an on-column GC–ECD method which can readily determine the concentration of nifedipine and its three principal metabolites (II–V, Fig. 1) in plasma and urine. This method can be automated to deal with a large number of samples and for which the chromatographic problems referred to above have been minimised.

EXPERIMENTAL

Materials

The substances to be analysed are shown in Fig. 1. There is much confusion in the literature as to both their correct and trivial nomenclature. In the former case we have used the nomenclature that appears in Chemical Abstracts and in the latter the system used by Raemsch and Ziegler [21], based upon which the metabolic scheme for nifedipine in man [23] is given in Fig. 2. The following compounds (CAS Registry number in square brackets) were used and were the gift of Bayer U.K.: 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester [21829-25-4] (nifedipine, I in Fig. 1); 2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester [67035-22-7] (M-

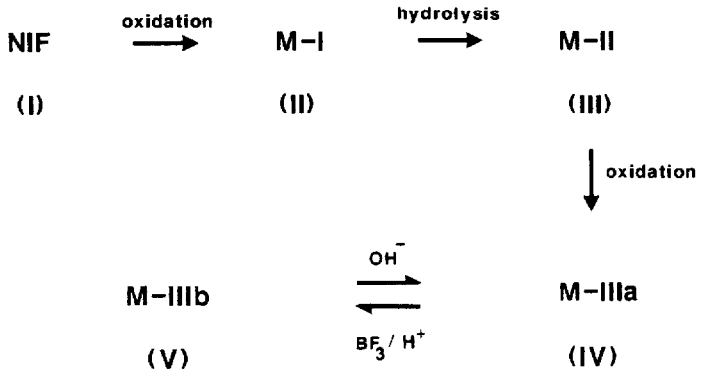

Fig. 2. Metabolic scheme for nifedipine (I) involving both oxidation and hydrolysis to yield the urinary excretion products M-II and M-III that exists as M-IIIa (IV) in the urine but can be converted to M-IIIb (V) with either H^+ of boron trifluoride.

I, II in Fig. 1); 2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid monomethyl ester [73372-63-1] (M-II, III in Fig. 1); 5,7-dihydro-2-methyl-4-(2-nitrophenyl)-5-oxofuro[3,4-*b*]pyridine-3-carboxylic acid methyl ester [34785-00-7] (M-IIIb, V in Fig. 1); 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid ethyl methyl ester [39562-70-4] (nitrendipine, internal standard for the plasma assay); 2,6-diethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid monomethyl ester (diethyl homologue of M-II, DEP, internal standard for the urine assay).

Inorganic reagents were Analar grade and solvents were HPLC grade. Iodoethane and tetrabutylammonium hydroxide (1 *M* in methanol) were obtained from Aldrich (Gillingham, U.K.) and boron trifluoride (14% in methanol) from Pierce U.K. (Cambridge, U.K.).

Preparation of solutions

Unless otherwise stated, all solid dihydropyridines, stock solutions, standards in plasma and unknown plasmas were handled in the darkroom under red light to avoid the photodegradation of nifedipine [12, 20]. The stability of nifedipine under various conditions of illumination is described later.

Stock solutions. For calibrating the plasma assay, nifedipine, M-I and nitrendipine were each dissolved (12.50 mg) in methanol (250.00 ml) in amber glass volumetric flasks, and subsequent dilutions (1:100) with distilled water gave stock solutions with concentrations of 500 ng ml^{-1} containing 1% methanol. For calibrating the urine assay, M-II (12.50 mg) and its diethyl homologue (5.00 mg) were each dissolved in methanol (2.5 ml) and diluted to 250.00 ml with water in amber glass volumetric flasks to yield stock solutions of concentration 50 and 20 $\mu g\ ml^{-1}$, respectively, containing 1% methanol. Where necessary the M-II stock solution was diluted 1:10 with water. Additionally and according to Kuhlmann et al. [24], M-IIIb (12.50 mg) was dissolved in methanol (2 ml) and left at 37°C for 1 h after the addition of 1 *M* sodium hydroxide (1 ml). The solution was neutralised with 1 *M* hydrochloric acid (1 ml), 1 *M* phosphate buffer (pH 7.0, 10 ml) was added, and the solution was diluted to 250.00 ml with water in an amber glass volumetric flask. Subsequent dilution with water (1:10) gave a stock solution of the ring-opened M-IIIa equivalent to 5 $\mu g\ ml^{-1}$ M-IIIb lactone. All the aforementioned stock solutions were frozen and kept as aliquots at −20°C.

Calibration of the plasma assay. Fresh human plasma (500 μl) was spiked with nifedipine, M-I (0.5–100 ng each) and nitrendipine (50 ng as internal standard). For reasons of precision, stock solutions were pipetted using graduated 10- and 100-μl glass syringes which could not easily be handled in the darkroom. Therefore both stock and calibration solutions were put into 4-ml amber glass screw-topped septum vials (Chromacol, London, U.K.), and solutions were transferred in daylight from one to the other with the minimum of light exposure (always < 10 s) by piercing of the septa with the graduated glass syringes.

Calibration of the urine assay. Fresh human urine (100 μl) was spiked with M-II (50–2000 ng) and M-IIIa prepared from M-IIIb stock solution as described above (5–200 ng) together with DEP (500 ng as internal standard).

Glassware. Amber screw-topped septum vials (4 ml) were used throughout both

the plasma and urine assays. All vials had been cleaned with dilute hydrochloric acid, then chromic acid solution, washed with water and finally sonicated in methanol.

Analysis of samples

Plasma. Whole heparinised blood from patients or volunteers was centrifuged in the darkroom and the resultant plasma transferred to a plastic container under red light and then stored at −20°C. To plasma (unknown or standard, 500 μl) to which had been added nitrendipine (50 ng as internal standard) were added TRIS buffer (20 m*M*, pH 9.0, 200 μl) and toluene (1.0 ml), and the vials were shaken mechanically for 1–2 min. After centrifugation, upper toluene layers (400 μl) were transferred to 2-ml amber glass autosampler vials (Chromacol vials with Hewlett-Packard septa and tops) which were loaded onto an HP7673A robotic autosampler. Samples (1 μl) were injected in the on-column mode into a Hewlett-Packard HP5890 chromatograph equipped with a ^{63}Ni electron-capture detector (15 mCi). Injection was achieved directly into a wide-bore OV-1 (HP-1) wall-coated open tubular (WCOT) capillary column (30 cm × 0.53 mm I.D., 2.65 μm film thickness) which was connected via a dead volume butt connector (Hewlett-Packard, Winnersh, U.K.) to an OV-1 (HP-1) WCOT capillary column (25 m × 0.32 mm I.D., 0.52 μm film thickness). The injection port was unheated, the detector temperature was 300°C and the following temperature programme was employed: initial temperature, 210°C for 1 min; rate, 15°C min^{-1} to 270°C; final time, 12 min with an equilibration time after cooling of 1 min. Carrier gas was helium (1.0 ml min^{-1}) with a nitrogen make-up flow-rate of 60 ml min^{-1}. A suitably programmed HP3392A computing integrator was used both to process chromatographic signals and to drive the autosampler. The HP5890 chromatograph permits a "column compensation" procedure to be used whereby a blank run without injection is performed and the rising baseline signal due to the temperature programme is stored electronically and then subtracted in real time from subsequent chromatograms. In this way a flat baseline can be obtained when using ECD with a temperature programme. Under the described conditions the retention times obtained were 5.2, 7.2 and 8.6 min for M-I, nifedipine and nitrendipine, respectively. Due to an interfering negative peak eluting at approximately 15 min the chromatograms were run for 17 min.

Urine. Urine (unknowns or standards; 100 μl) to which had been added DEP (500 ng as internal standard), 1 *M* hydrochloric acid (200 μl) and ethyl acetate (3 ml) in 4-ml amber glass screw-topped septum vials was shaken mechanically for 1–2 min, centrifuged, and the upper organic layers were transferred to a set of similar vials. Iodoethane and tetrabutylammonium hydroxide (20 μl each) were added, and the mixture was heated at 60°C in a heating block for 1 h. Samples were then evaporated in vacuo using a Savant evaporating centrifuge (V.A. Howe, London, U.K.) at 40°C. To the dry residues 1 *M* hydrochloric acid (500 μl) and toluene (2 ml) were added, the vials were shaken mechanically for 1–2 min, centrifuged, and the upper toluene layers were transferred to a third series of clean vials within which the samples were again evaporated in vacuo. Methanolic boron trifluoride (200 μl) was then added, and the samples were heated again for 1 h at

60 °C. Finally, water saturated with potassium chloride (500 μl) was used to precipitate the boron trifluoride, toluene was added (3 ml), and the samples were shaken mechanically and centrifuged. The toluene upper phases, now containing the lactone M-IIIb, formed by the action of boron trifluoride on the urinary metabolite M-IIIa, together with the ethyl esters of the urinary metabolite M-II and the internal standard DEP, were tansferred to a fourth series of clean vials containing 10% aqueous sodium thiosulphate (500 μl) to remove any iodine, shaken mechanically for 1–2 min, centrifuged, and the toluene layers (500 μl) were transferred to autosampler vials as described above. This final clean-up step, added to the basic derivatisation methodology of Kuhlmann et al. [24], was found to reduce dramatically the chromatographic background. As before, samples (1 μl) were injected automatically on-column using the HP7673A autosampler. A different OV-1 column of the same dimensions was used for the metabolite assay, through which the helium flow-rate was 0.33 ml min^{-1}, and the following temperature programme was employed: initial temperature, 250 °C for 3 min; rate, 10 °C min^{-1} to 270 °C; final time, 5 min with a post-cooling equilibration time of 1 min. An additional increase of the temperature to 300 °C for 6 min was required with one batch of tetrabutylammonium hydroxide as there was a late peak eluting from the column. The column compensation routine was again used to give a flat baseline. Under these conditions retention times of 6.4, 7.1 and 7.9 min were observed for M-II, DEP (internal standard) and M-IIIb, respectively. Chromatograms were run up to 16 min to elute late interfering peaks.

Investigation of nifedipine photosensitivity

Nifedipine is known to be extremely light sensitive, decaying to the 2-nitroso derivative in daylight [10] with a half-life of around 1 h, depending upon the solvent [12, 20]. In order to assess the stability of nifedipine under our analytical procedures, we carried out a number of investigations under normal laboratory lighting and in the darkroom. Aliquots (2 ml) of water saturated with nifedipine (approximately 8 μg ml^{-1}, made up in the darkroom) were subjected to one of three treatments. Solutions were left in clear glass septum vials under normal laboratory lighting (daylight plus fluorescent light) (treatment A), in amber glass septum vials in normal laboratory lighting (treatment B) or in clear glass vials in the darkroom kept a distance of 1 m from the red darkroom light as their only source of illumination (treatment C). Aliquots (10 μl) were taken for GC assay for nifedipine content at intervals up to 13 h.

Investigation of nifedipine chromatographic thermal stability

Four chromatographic configurations were employed to assess the thermal stability of nifedipine. In the first set of experiments aliquots (1.0 ml) of either nifedipine or M-I solutions (100 ng ml^{-1} extracted into toluene as described) were injected manually into a split–splitless injector operating in the splitless mode and mounted on the HP5890 gas chromatograph and fitted with, on one occasion, a WCOT SE-30 column (25 m × 0.31 mm I.D.) with a temperature programme of 90 °C for 5 min and 30 °C min^{-1} to 260 °C and, on another occasion, a WCOT OV-101 column of the same dimensions and with the same temperature

programme. The helium carrier gas flow-rate for both was 2 ml min^{-1}. In the second set of experiments, aliquots (100 μl) of either nifedipine or M-I solutions (10 $\mu g\ ml^{-1}$ extracted into toluene as described) were injected manually on-column via an on-column injector and using a silica injection needle. The same columns and temperature programmes were used as for the splitless injection, but with a helium flow-rate of 6 ml min^{-1}.

RESULTS

Chromatographic thermal stability of nifedipine

The impact of injection mode upon nifedipine stability is shown in Fig. 3. When nifedipine is introduced into a hot metal injection port in the splitless mode (Fig. 3A), the method used by Tucker et al. [20], considerable decomposition to M-I occurs. When M-I itself is injected a single peak is observed (Fig. 3B). The same phenomenon was observed whether SE-30 or OV-101 WCOT columns were employed. Presumably the hot metal injector surface provides a substrate for the

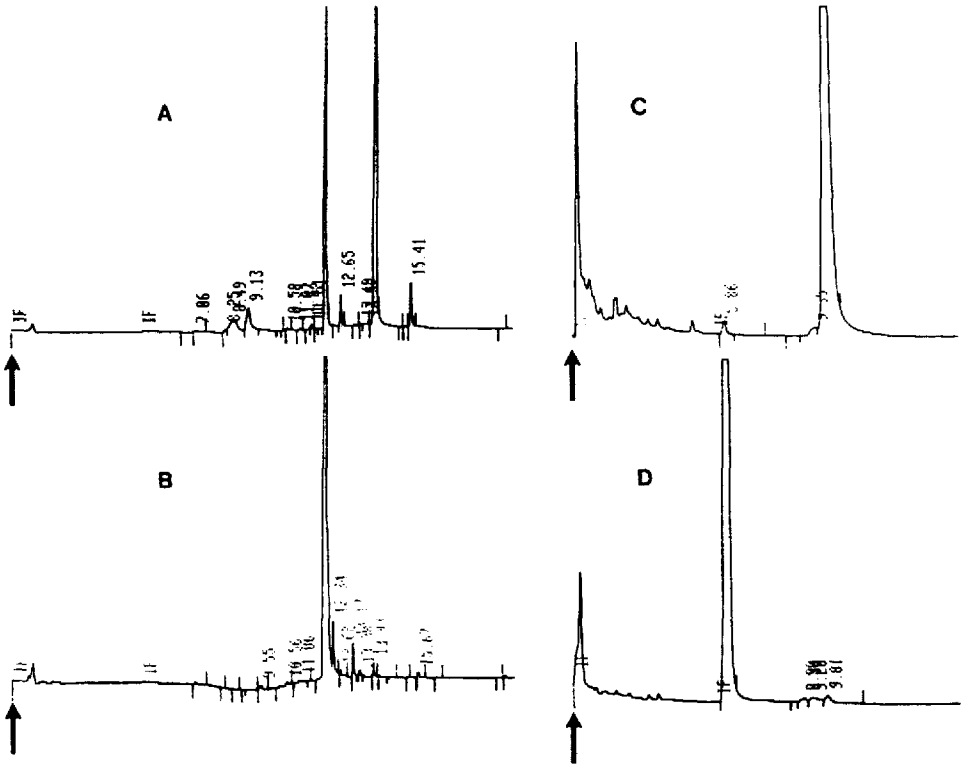

Fig. 3. (A) Injection of nifedipine (100 pg) via a split-splitless injector in the splitless mode into an SE-30 WCOT capillary column (see text). Arrows indicate time of injection. Earlier eluting peak is M-I, later eluting peak is nifedipine. (B) Injection of M-I (100 pg) under same conditions as Fig. 3A. (C) Injection of nifedipine (1 ng) via an on-column injector into an SE-30 WCOT capillary column (see text). (D) Injection of M-I (1 ng) under same conditions as Fig. 3C.

oxidation of the picogram quantities of nifedipine injected. However, when manual on-column injection into the same columns and using the same temperature programme is effected no significant degree of nifedipine decomposition to an M-I artefact is observed (Fig. 3C, D). This common initial experience of ours [22] militated against the use of split–splitless injection methods for nifedipine analysis. We chose not to investigate this problem in any further detail but rather elected to use an on-column injection technique with an autosampler as described above. This phenomenon was probably not observed in the previous packed-column methods [2–7] which almost invariably and fortuitously employ the earliest type of on-column injection.

Photosensitivity of nifedipine

Analysis of twelve consecutive samples taken during treatment A over 12 h of normal laboratory lighting showed a linear monophasic decay in nifedipine concentration ($r^2=0.997$) with a first-order rate constant of $-0.191\ h^{-1}$ which permitted calculation of a half-life of 3.6 h. Treatment B (seven consecutive samples over 13 h) also showed a monophasic decay ($r^2=0.984$) with a half-life for photodecomposition of 50.4 h. Five samples taken over 8 h in the darkroom (treatment C) showed no significant decay of nifedipine concentration with time ($r^2=0.086$) and thus a half-life was not estimated from the infinitessimal negative slope. It must be concluded that nifedipine is highly photolabile in aqueous solution in clear glass under normal laboratory illumination and our estimate of a 3.6 h half-life is of the same order as values previously reported in toluene solution [12, 20]. Our experiments were carried out in winter months and additionally our laboratories do not have a south-facing vista. In other laboratories daylight sensitivity of nifedipine might be even greater. That notwithstanding, the precaution of using amber glass vials appears justified extending the half-life to over two days.

Determination of nifedipine and M-I in plasma

Fig. 4 displays various aspects of the plasma assay: a blank plasma (Fig. 4A) showing a peak after 6.8 min; this peak was found in various amounts and became prominent when calibration samples were made up from plasma previously stored in a soft plastic container suggesting that the compound might be a halogenated (electron-capturing) plasticiser; blank plasma spiked with nifedipine (20 ng; Fig. 4B) and with nifedipine, M-I (20 ng each) and nitrendipine (50 ng; Fig. 4C); plasma taken from a healthy volunteer 30 min after an oral dose of 5 mg nifedipine (Fig. 4D). As with SE-30 and OV-101 phases employed with on-column injection (vide supra), it can be seen that OV-1 gives insignificant degradation of nifedipine to M-I.

The number of theoretical plates calculated from the retention time and peak width at half height [25] for nifedipine and M-I was consistently $5 \cdot 10^4$–$7 \cdot 10^4$; with one exception [20], this is the best chromatographic efficiency reported to date for these compounds.

Calibration curves for nifedipine and M-I were linear up to 200 and 100 ng ml^{-1}, respectively, with intercepts not significantly different from zero and cor-

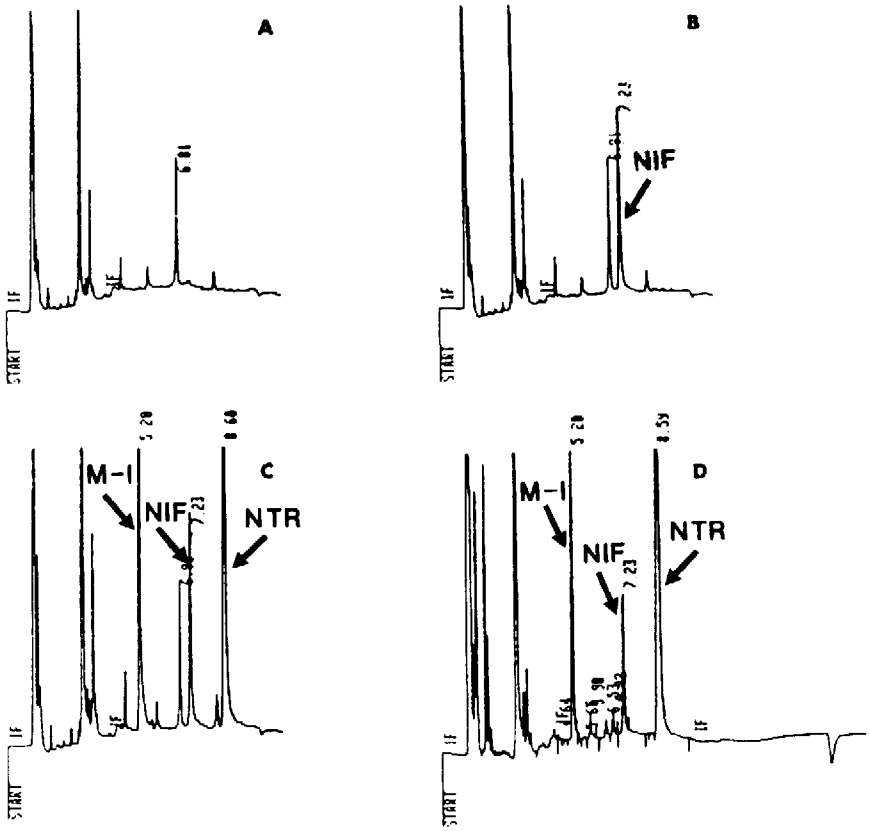

Fig. 4. Chromatograms of (A) human plasma (500 μl), (B) plasma spiked with 20 ng nifedipine, (C) plasma spiked with 20 ng M-I, 20 ng nifedipine and 50 ng nitrendipine and (D) plasma 30 min after the administration of a 5-mg capsule of nifedipine (nifedipine and M-I levels were 21 and 22 ng ml^{-1}, respectively). Peaks: NIF = nifedipine; NTR = nitrendipine.

relation coefficients between 0.992 and 1.000. Limits of detection for nifedipine and M-I were 1 and 0.5 ng ml^{-1}, respectively.

Analysis of pooled correlation data from duplicates collected over a period of three months for both nifedipine ($n=91$) and M-I ($n=94$) gave coefficients of variance of 0.9% for nifedipine (range 1–73 ng ml^{-1}) and 0.7% for M-I (range 0.5–60 ng ml^{-1}). Intra-assay coefficients of variation of 7.8% for nifedipine (mean value 36 ng ml^{-1}, $n=30$) and 10.2% for M-I (mean value 39 ng ml^{-1}, $n=30$) were calculated. Comparison of expected and found concentrations for spiked samples in the same concentration range gave an accuracy of determination of both nifedipine and M-I of >95%. Recovery of both nifedipine and M-I in this assay was quantitative (98–101%).

Determination of M-II and M-III in urine

Both of these metabolites together with the internal standard DEP require derivatisation prior to chromatography. In the case of M-III, which exists in the urine in the ring-opened form M-IIIa, this was accomplished by chemical con-

version to the ring-closed lactone M-IIIb using boron trifluoride. In a prior step the carboxylic acid metabolite M-II together with its diethyl homologue as internal standard were ethylated using iodoethane and tetrabutylammonium hydroxide coupled with a final clean-up step using sodium thiosulphate. Whilst this is a somewhat lengthy procedure, it nevertheless gives good chromatographic separation, is sensitive and reports on both nifedipine urinary metabolites simultaneously. There is no necessity to measure nifedipine or M-I since they do not appear in the urine by virtue of their lipophilicity [26].

Fig. 5 displays aspects of the urinary metabolites as follows: blank urine (Fig.

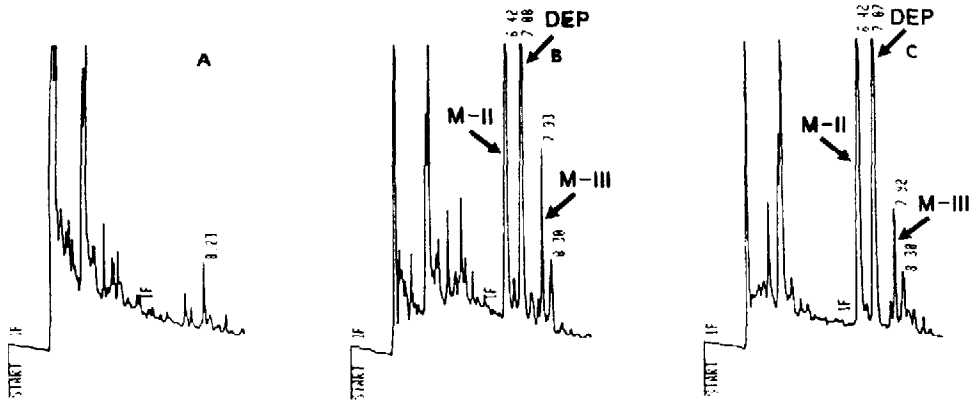

Fig. 5. Chromatograms of (A) human blank urine (100 μl), (B) urine spiked with 1000 ng M-II, 500 ng DEP and 100 ng M-III and (C) M-II and M-III levels (10.8 and 0.92 μg ml^{-1}, respectively) in a 0–2 h urinary collection after the administration of a 5-mg capsule of nifedipine (M-II and M-III levels were 10.8 and 0.92 μg ml^{-1}, respectively).

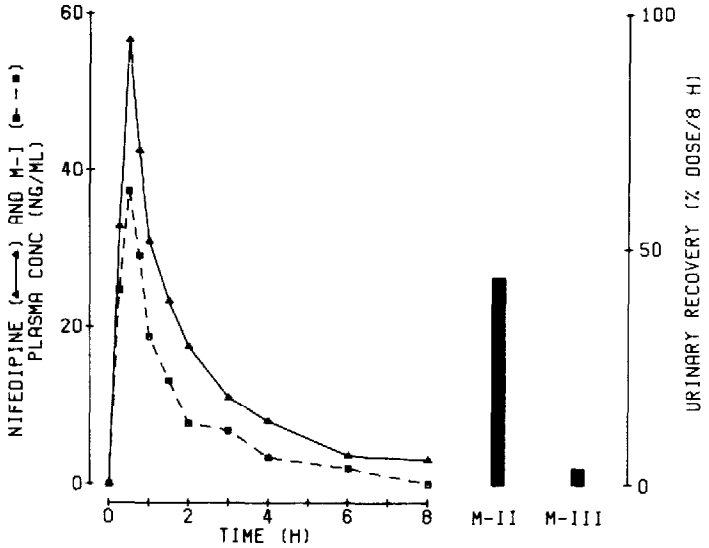

Fig. 6. Nifedipine and M-I plasma concentration-time profile and urinary recovery of M-II and M-III in a healthy volunteer after the administration of a 5-mg capsule of nifedipine.

5A) showing a peak after 8.2 min, blank urine spiked with M-II, M-IIIa and DEP (1000, 100 and 500 ng ml^{-1}, respectively; Fig. 5B) and a bulked urine collected from a healthy volunteer up to 2 h after an oral dose of 5 mg nifedipine.

Calibration curves for M-II and M-III were linear up to 20 and 2 $\mu g\ ml^{-1}$, respectively, with intercepts not significantly different from zero and correlation coefficients between 0.994 and 0.999 for M-II and between 0.989 and 0.999 for M-III. Limits of detection for both M-II and M-III were 0.15 $\mu g\ ml^{-1}$.

The within-assay coefficients of variation fell in the range 1.1–7.0% for M-II and 6.3–11.2% for M-III (five sets were run each containing four to ten samples). Inter-assay coefficients of variation of 4.2% for M-II (mean value 7.4 $\mu g\ ml^{-1}$, $n=10$) and 17.6% for M-III (mean value 0.32 $\mu g\ ml^{-1}$, $n=10$) were calculated. This latter estimate of precision is low since it is estimated at only twice the limit of detection. When concentrations of M-III as high as M-II were used the precision of determination of M-III matched that of M-II. Accuracy of determination of both M-II and M-III calculated from spiked urine samples was >92%. M-II was determined as its ethyl ester for which no authentic material was available and thus no estimate of recovery could be made. However, experiments with toluene spiked with authentic M-IIIb showed that the extraction of M-IIIa and its subsequent lactonisation to M-IIIb by the method described herein was virtually complete with a recovery of >96%.

DISCUSSION

To gain a comprehensive insight into the disposition and metabolic fate of nifedipine, the principal plasma and urinary drug-related materials require quantitation. Both nifedipine and M-I are lipophilic enough to evade urinary excretion which is the final excretory fate of metabolites M-II and M-III (see refs. 1 and 24). The assays described to date for these compounds have either been of restricted applicability or of insufficient sensitivity and chromatographic efficiency. In this paper we describe analytical procedures for the quantitative determination of nifedipine, M-I, M-II and M-III which utilise capillary GC–ECD and automated on-column injection. The chromatographic efficiency of the method is high and the assay is sensitive, linear, accurate and reproducible. Our considerable experience with this assay investigating the pharmacokinetics and metabolism of nifedipine is the subject of supplementary reports. Fig. 6 shows a typical plasma concentration–time curve for nifedipine and M-I and the urinary recovery of M-II and M-III after a single oral 5-mg capsule of nifedipine.

With specific reference to published capillary GC methods for the determination of nifedipine [19–21], all of these have failed to address the problem of thermolability of nifedipine under conditions of split–splitless hot injection. Our early experiences with this drug led us to conclude that nifedipine is oxidised to considerable proportions of M-I in the split–splitless injection device [22], thus clouding the interpretation of any biological experiments. We have reported this phenomenon in greater detail here; employing OV-1, OV-101 and SE-30 phases it was possible to locate the artefact to the site of injection: no oxidation of nifedipine occurs when on-column injection is employed. For the purpose of our stud-

ies of nifedipine pharmacology therefore, we have elected to adopt this procedure as an invariable one.

The discovery of a human glucocorticoid-induced cytochrome P-450 isozyme [27], the gene for which has recently been cloned and sequenced in both rat [28, 29] and man [30] and that is identical in nucleotide sequence to the gene which codes for an isozyme which metabolises nifedipine to M-I [31, 32], has considerably enlivened the nifedipine metabolism field. Sadly, however, much of the biological experimentation is limited by the lack of an assay for M-I production which is sensitive enough to detect the very low levels produced in vitro by purified human enzyme fractions. Nifedipine is only sparingly soluble in water (ca. 8 $\mu g\ ml^{-1}$), and in order to generate detectable amounts of M-I from human liver microsomal cytochrome P-450 incubations, Guengerich et al. [32] used enzyme assays containing 10% acetone to assist in solubilising nifedipine at 70 $\mu g\ ml^{-1}$ and thereby promoting detectable conversion to metabolite (ca. 200 ng ml^{-1}) using their relatively insensitive HPLC procedure. The methods described in this paper permit purely aqueous solutions of nifedipine to be used in this type of in vitro experiment and thereby obviate the possible confounding effects of 10% acetone on enzyme kinetics and antibody binding. Not only therefore can 1–10 ng ml^{-1} M-I readily be detected with high precision in such assays, but the relatively precious resource of human liver microsomes can also be scaled down to 8 pmol P-450 per tube instead of the 100 pmol per tube previously consumed [32]. The GC assay here described is thus amenable to the study of nifedipine metabolism in vitro.

Finally, the question of nifedipine photosensitivity under laboratory conditions has been readdressed. It is clear that, by following the procedures which we describe using amber glassware and darkroom manipulations under red light, nifedipine is robust enough to tolerate laboratory analysis with a half-life in excess of two days.

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