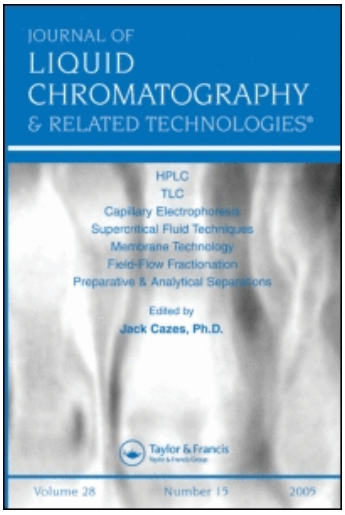

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MEASUREMENT OF NIMODIPINE METABOLISM IN RAT LIVER MICROSOMES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An isocratic, rapid, sensitive and selective reversed phase high-performance liquid chromatographic (HPLC) assay with ultraviolet detection has been used to quantify the in vitro nimodipine metabolism in rat liver microsomes. (±)Nimodipine and its metabolites were separated on a C18 HPLC column maintained at 40°C using a mobile phase consisting of ammonium acetate 0.05M pH 6.6 and methanol (40:60 v/v). The within- and between-run coefficients of variation (CVs) were < 4.2 % in the concentration range of 0.5-50 μM. The limit of detection for nimodipine and ist metabolites was in the range of 30 - 80 nM. The formation of nimodipine metabolites may be described by a sigmoid Vmax model according to Hill equation corresponding to enzyme kinetics associated with positive allosteric effect. The assay was accurate, selective and may be used in studies investigating the interaction of drugs, and substances found in daily food e.g. flavonoids, with nimodipine metabolism.

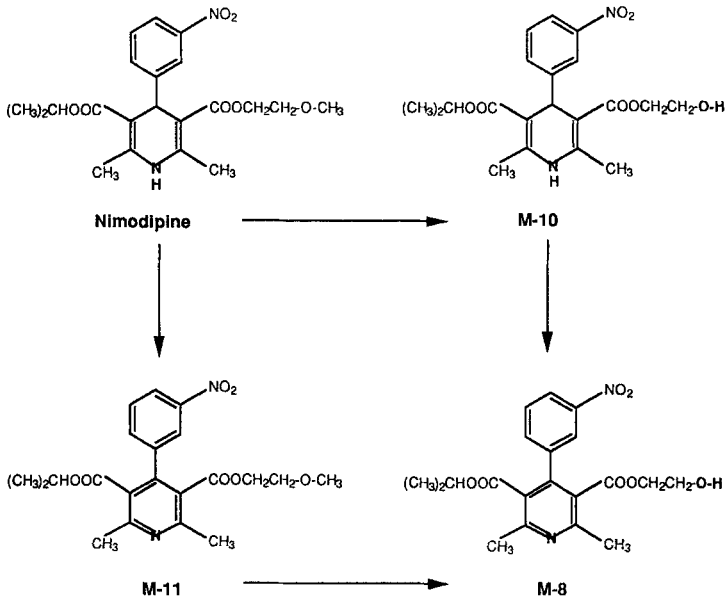

FIGURE 1. Metabolism of nimodipine.

INTRODUCTION

Nimodipine [Bay e 9736, (±)3-isopropyl 5-(2-methoxyethyl) 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylate] is a potent calcium antagonist of the class of dihydropyridines (1). It has been shown in man that nimodipine may dilate the cerebral arterioles and increase cerebral blood flow. The drug is effective in the treatment of a range of cerebrosvascular disorders. However, the actual interest in the drug focuses on its use in the prevention and treatment of the delayed ischaemic neurological deficits that frequently occur in patients with subarachnoidal haemorrhages resulting from sustained cerebral vasospasm (2). In mammals, the biotransformation of nimodipine is complex (1,3). Similar to other dihydropyridine derivatives, nimodipine is primarily biotransformed by the microsomal oxidase system in the liver by dehydrogenation of the 1,4-dihydropyridine nucleus, which leads to the formation of metabolite M11, and/or by O-demethylation, ester-cleavage producing metabolites M10, M9, respectively (1- 6). The metabolites M10 and M11 may be further biotransformed to the common metabolite M8 (Fig.1) by ring- oxidation and side chain-demethylation, respectively.

When nimodipine is administered to rats or humans the metabolites M8, M10 and M11 are detectable in the plasma. The areas under the plasma concentration curves (AUC) of M8

and M10 are higher than that of M11 (4-6). Assuming that the clearance and subsequent reaction rates of these metabolites are similar, these data suggest that the O-demethylation of nimodipine is an important pathway for the elimination of the drug. In man, it has been well documented that the nucleus oxidation of dihydropyridine drugs is mainly catalyzed by a specific enzyme cytochrome P-450 3A (CYP3A) (7). In the rat, this oxidation is similar to that in man but the participation of cytochromes UT-A (cyp 2C11) and PCN-E (cyp 3A) was also reported (8). In contrast to the nucleus dehydrogenation, little attention was given to the oxidative cleavage of side-chain carboxylic esters and of ethers bound to the dihydropyridine ring. The identity and number of CYP(s) involved in these reactions are still unknown (7, 9). In order to investigate the metabolism of nimodipine in detail a rapid and selective analytical assay is needed. A number of chromatographic assays have already been developed to measure nimodipine and its metabolites in human and animal body fluids (1-6, 9-11). In these assays, a clean-up step, e.g. liquid-liquid extraction with diethyl ether-hexane (1:1) or ethyl acetate, was necessary. This manipulation is time consuming and requires the use of ambre glass ware to prevent the light catalyzed dehydrogenation of the the dihydropyridine nucleus. A one step analytical assay with direct injection would be an advantage. Therefore, we have developed a HPLC method with direct injection to investigate nimodipine biotransformation in *in vitro*.

MATERIALS AND METHODS

Reference Compounds

Reference substances (±)nimodipine (Bay e 9376) and its metabolites M8 (Bay o 1762), M10 (Bay m 5397) and M11(Bay m 8922) were supplied by Bayer AG (Wuppertal, F.R.Germany).

Chemicals

Methanol (E. Merck, Darmstadt, F.R.G.) was HPLC gradient grade and ammonium acetate solution 5 M (Fluka, Buchs, Switzerland) was analytical grade. Other chemicals such as acetone, D,L-isocitric acid trisodium salt dihydrate and magnesium chloride were of the highest commercially available purity (E. Merck, Darmstadt, F.R.G.). Isocitrate dehydrogenase from pig heart and NADP disodium salt were purchased from Boehringer (Mannheim, F.R.G.). Naringenin was obtained from Roth GmbH (Karlsruhe, F.R.G.), α-naphthoflavone and quinidine base 97% from Aldrich Chemie (Buchs, Switzerland), (±) verapamil hydrochloride,

troleandomycine, quinine and erythromycine from Sigma Chemie (Buchs, Switzerland), midazolam and its metabolites α-hydroxy- and 4-hydroxy-midazolam from Hoffman-La Roche (Basel, Switzerland) and cyclosporine A from Sandoz (Basel, Switzerland). Rat liver microsomes were prepared from male Sprague Dawley rats according to a method described previously (12). The microsomal protein concentration was assayed by a modification of Lowry's method (13).

Standard Samples

For daily calibration of chromatography 10 µmol of each reference substance were dissolved in 1 ml of acetone. The stock solution was diluted with bidistilled water to the desired concentrations. The content of the organic solvent was 50% (v/v) in the final solutions. Standard samples were then treated in the same manner as the unknown samples.

Instrument Parameters

A HPLC system (Waters 625, Milford, MA, USA) consisting of a solvent delivery pump model 625, a photo diode array detector model Waters 991 set at 218 and 238 nm, an autosampler model WISP 700 and a column oven operated at 40°C were used. Signals were processed and recorded by a data management system (Waters software version 6.22, Milford, MA, USA). Nimodipine and its related compounds were separated on a Spherisorb S3 ODS II (100 x 4 i.d.mm) column (Knauer AG, Berlin, F.R.G.).

Mobile Phase

The mobile phase was a mixture of 400 ml of diluted ammonium acetate solution (0.05 M, pH 6.6) and 600 ml of methanol and saturated with helium for 10 min at a flow rate of 100 ml/min before use. For the routine analysis a flow rate of 0.5 ml through the column was maintained at a pressure of 85-95 bar.

Stability Test

The stability of nimodipine (1.0 - 50 µM) and its metabolites (0.5 - 20 µM) in phosphate buffer 0.1M pH 7.4 was assayed in 1.5 ml polypropylene test tubes (Eppendorf, Vaudaux Co., Schönbuch, Switzerland) by exposing the aqueous solutions to the laboratory light for 0, 2, 4, 8, 16 and 32 hours at room temperature. A further test was performed by exposing the

spiked samples containing nimodipine without microsomal protein in ambre polypropylene test tubes (Eppendorf, Vaudaux Co., Schönbuch, Switzerland) at 37°C to the laboratory light for 0-120 minutes.

Recovery Test

For recovery experiments, 10 µmol of each standard were dissolved in 1 ml of methanol. The stock solution was diluted with water to the desired concentrations. The content of the organic solvent was 50% (v/v) in the final solutions. The analytical recoveries of three nimodipine concentrations (0.5, 5.0, 50 µM) and three metabolites concentrations (0.5, 2.5, 5.0 µM) were determined in three differents occasions by comparing the peak heights of spiked and protein precipitated samples with those of corresponding amounts dissolved in methanol:bidistilled water (1:1).

Selectivity and Limit of Detection Test

In order to investigate the possible interference by other drugs, each test compound was dissolved in phosphate buffer and 2 nmol were injected separately onto the column. The signals were recorded for 1 hour. The limit of detection was determined by diluting a sample of 1 µM nimodipine and its metabolites with phosphate buffer. The diluted samples were then treated and analyzed in the same manner as the unknown samples. The detection limit of the assay was defined as a signal-base line ratio 3:1.

In vitro Nimodipine Metabolism Assay

In a 1.5 ml amber polypropylene test tube (Eppendorf, 2000 Hamburg 65, F.R.G.), nimodipine (concentration range 1.0 - 50 µM) was added as a 25-fold concentrated solution in 50% acetone:bidistilled water and incubated in 0.1 M sodium phosphate buffer, pH 7.4, in the presence of a NADPH generating system (0.5 IU isocitrate dehydrogenase, 1 mM NADP, 5 mM sodium isocitrate and 5 mM magnesium chloride). After a preincubation of 2 minutes at 37°C, reactions were started by the addition of microsomal protein previously kept in ice. The final volume was 0.5 ml. After incubating at 37°C for 5 minutes under an air atmosphere, reactions were stopped by the addition of 0.5 ml of ice-cold methanol, followed by vortexing for 15 seconds and placing in ice. After a brief centrifugation at 10'000 x g for 5 minutes (0 °C), 100 µl of the upper phase were placed into a conical vial, and a volume of 40 µl was injected onto the HPLC column. The reproducibility of the assay was investigated 7 times on the same day by using the same rat liver microsomes and 15 µM substrate.

In order to investigate the formation kinetics of the metabolite M8 nimodipine was replaced in the metabolism reaction by M10 and M11 in two separated experiments. The other conditions were unchanged.

Data Analysis

The calibration graphs of nimodipine (0.2 - 50 μM) and its metabolites (0.2 - 20 μM) were constructed daily using triplicates of 6 different concentrations. The concentration of substances in the biotransformation reaction was quantified by comparing the peak-heights of the corresponding signals with those of the calibration graph. The metabolite concentrations were then transformed to reaction rates by taking into account the incubation time and the amount of microsomal protein. Reaction rates against corresponding substrate concentrations were fitted by nonlinear regression and using the allosteric Hill equation $V = V_{max} * C^N / (C^N_{50} + C^N)$ (14), where V is the reaction rate at the substrate concentration C, V_{max} is the maximal velocity, C_{50} is the substrate concentration at half V_{max} and N is the parameter describing the sigmoidicity of the curve. The apparent constants C_{50}, N and V_{max} were directly estimated by using the Profit software package (QuantumSoft, 8023 Zurich, Switzerland). Results were reported as mean ± standard deviation (SD) of at least three experiments performed in duplicate.

RESULTS

The typical HPLC chromatograms for reference substances dissolved in mobile phase (A) and for *in vitro* nimodipine metabolism reactions before (B) and after (C) incubation are shown in Fig.2. The retention times of M8, M10, M11 and nimodipine were 6.8, 5.9, 14.2 and 11.8 minutes, repectively. Their corresponding retention capacity factors (k') were 3.9 , 3.3, 9.1 and 7.4, respectively. In comparison with Fig. 2A, the metabolites M10 and M8 produced by *in vitro* reactions (Fig.2C) were not separated with base line resolution. The spectrum analysis revealed that the signal corresponding to M10 was pure and that of M8 was contaminated by unknown substance(s). The purity of the M8 signal was 89 ± 6% (n=5). In the metabolism assay investigating the formation of M8 from M10 the respective signals were separated with base line resolution.

In the routine analysis, samples were injected every 16 minutes and in a run of 20 hours no interfering signals, that would hamper nimodipine metabolites quantification were observed. Calibration curves were linear in the concentration range 0.2-50 μM of nimodipine and 0.2-20 μM of its metabolites. The correlation coefficients were > 0.999. Least squares linear regression analysis of the standard calibration plots resulted in the following equations:

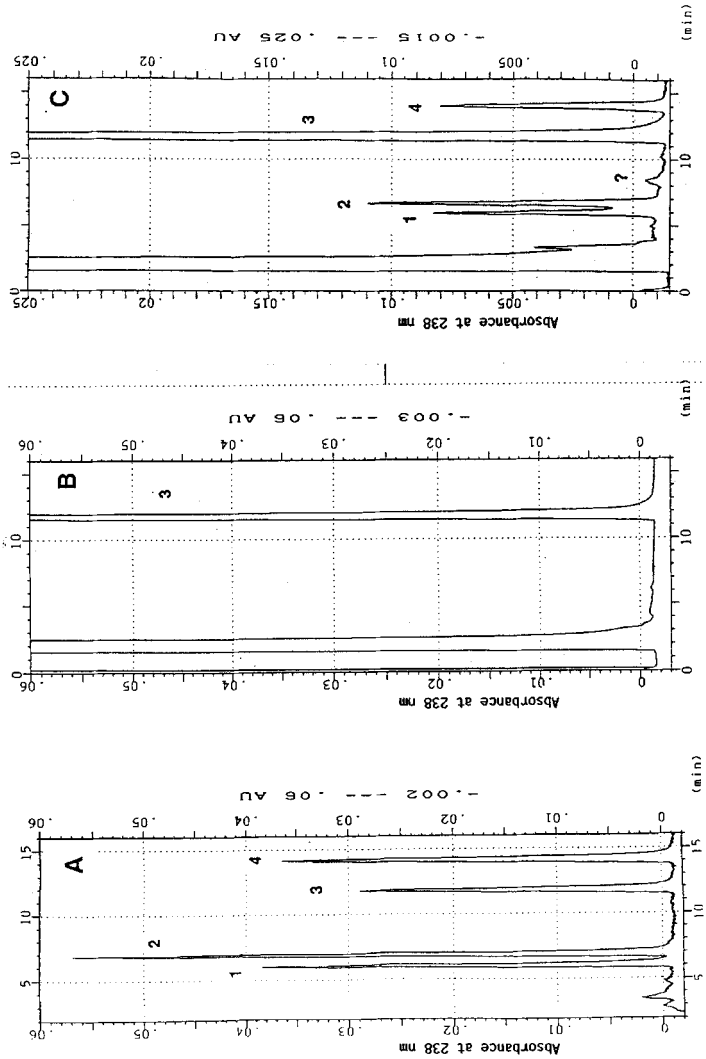

FIGURE 2. Separation of nimodipine and its metabolites by HPLC technique. (A) reference substances; (B) before incubation; (C) after incubation. Peak identification: (1) M10, (2) M8, (3) nimodipine; (4) M11; (?) unknown product.

TABLE 1

Precision and Accuracy of the Analysis of Nimodipine and its Metabolites.

		Within-day assay (n=3)			Between-day assay (n=5)		
compound	added (μM)	found (μM)	CV (%)	bias (%)	found (μM)	CV (%)	bias (%)
M8	0.65	0.64	3.77	-1.54	0.68	2.07	4.85
	1.62	1.62	2.50	0.10	1.72	1.92	6.13
	3.23	3.08	2.22	-4.64	3.25	2.64	0.73
M10	0.61	0.64	3.89	4.92	0.63	2.80	3.21
	1.53	1.53	2.77	0.00	1.56	2.45	2.22
	3.05	3.06	2.18	0.29	3.14	2.23	2.91
M11	0.59	0.58	3.78	-1.69	0.59	3.45	0.23
	1.48	1.55	2.99	4.66	1.46	2.54	-1.49
	2.97	2.92	2.31	-1.74	3.01	1.86	1.50
Nimod.	0.59	0.63	4.12	6.66	0.61	3.80	3.60
	1.48	1.58	3.66	6.76	1.47	3.21	-0.97
	2.95	2.83	2.71	-1.55	3.02	2.71	2.23
	14.8	14.5	2.21	-1.55	15.5	2.11	4.89
	44.4	44.6	1.97	0.49	45.3	2.35	2.09

Note: a) CV(%) = SD*100/added. b) bias(%) = (found – added)*100/added

M8: y = 5.361 x - 0.004; M10: y = 9.736 x + 0.002; M11: y = 3.958 x + 0.009; Nimodipine: y = 8.322 x - 0.077. The intercept with the y-axis was very close to zero. The within-day precision of the assay was determined for each metabolite by analyzing several spiked samples with 3-5 different concentrations 3 times a day, and reproducibility was evaluated by measuring the same samples on 5 different days. Data in Table 1 show that the between-day precision was somewhat better than that of within-day, this may be due to the too small number of repetition (n) used in the within-day investigation. Generally, for drug concentrations ranging from 0.6 to 40 μM the between-day and within-day coefficients of variation (CVs) of this assay were less than 4.2 %.

The absolute recovery of nimodipine and its metabolites was 97 ± 2 % (n=12). It was independent of the nature of compounds and their concentrations ranged between 0.5 and 50 μM. By injection of 40 μl of the incubation solution onto the HPLC column the limit of detection was 60, 30, 80 and 40 nM for M8, M10, M11 and nimodipine, respectively.

Unlike to other dihydropyridine derivatives, nimodipine and its metabolites are relatively stable for usual laboratory manipulations. Incubated in ambre polypropylene test tubes and in phosphate buffer 0.1 M, pH 7.4, nimodipine and its metabolites were stable for at least 120 min at 37° C. Therefore the metabolism reactions could be carried out in this device and under the laboratory light.

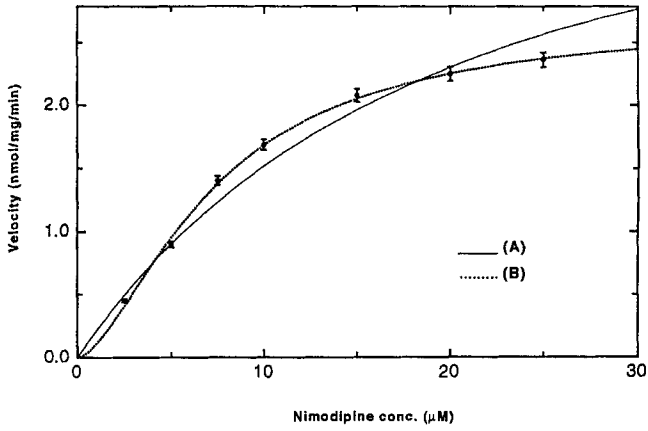

FIGURE 3. Substrate dependent enzyme kinetics of M11 was simulated by Michaelis-Menten (A) and Hill equation (B)

Under the described conditions rat liver microsomes metabolized nimodipine mainly by O-demethylation and by nucleus dehydrogenation leading to the formation of metabolites M8, M10, M11. During the first 15 minutes the concentration of metabolites in the incubation was in the range of nmole/ml and less than 1% of the total nimodipine was metabolized. The concentration of metabolites in the *in vitro* assay is ranked M11 > M8 > M10. The initial rate of the metabolites formation was linear with microsomal protein in the range of 100 - 700 μg per assay and for up to 6 minutes (M11) and 15 minutes (M8 and M10). Therefore 300 μg of rat microsomal protein and 5 min. incubation time were used in all experiments. No nimodipine metabolites were detected when one of the components, such as microsomal protein, substrate or NADP were omitted from the incubation.

The substrate dependent kinetics of nimodipine metabolites formation mediated by rat liver microsomes were studied in the substrate concentration range between 1.0 and 50 μM. The formation kinetics of the primary metabolites M10, M11 may be better described by a sigmoid V_{max} model equivalent to the Hill equation, $V = V_{max} * C^N / (C^{N}_{50} + C^N)$ (14), rather than by the classic Michaelis-Menten model (Fig. 3). Data analysis of different rat liver microsomes preparations revealed that the apparent C_{50} and V_{max} were in the range 9.1-37.1 μM and 0.5-2.6 nmol/min/mg protein, respectively. The value of N varied from 1.0 to 1.5. The formation of the secondary metabolite M8 from M10 and M11 obeyed Michaelis-Menten enzyme kinetics. The apparent kinetic parameters Km (Vmax) were 9.34 μM (1.03 nmol/mg/min) and 21.4 μM (1.83 nmol/mg/min) for M10 and M11, respectively. The enzyme kinetic parameters of primary

TABLE 2

Enzyme Kinetic Parameters of Nimodipine Metabolism in Rat Liver Microsomes .

Rat	M10		M11	
	C_{50}(N)	V_{max}	C_{50}(N)	V_{max}
A	9.13(1.48)	0.51	18.90(1.19)	1.72
B	9.75(1.24)	0.59	16.63(1.24)	1.23
C	19.80(0.96)	0.94	37.08(1.04)	2.61

Note: C_{50} (μM) V_{max} (nmol/mg/min) N (sigmoidicity)

TABLE 3

Specificity of Nimodipine Assay.

Compound	Retention time (min)
M8	6.8
M10	5.9
M11	14.2
Nimodipine	11.8
Cyclosporine A	-----(*)
Erythromycine	-----(*)
Midazolam	12.5
α-OH-Midazolam	6.8
4-OH-Midazolam	5.5
α-Naphthoflavone	-----(*)
Naringenin	-----(*)
Quinidine	12.0
Quinine	14.5
Troleandomycine	-----(*)
Verapamil	13.0

Note: (*) no signal was detected after 60 minutes.

metabolites M10 and M11 investigated with three different rat liver microsomes samples are summarized in Table 2. Reproducibility of the reaction was investigated 7 times by using the same rat liver microsomes sample and 15 μM substrate. Under our standard conditions the precision of Michaelis-Menten parameters for M8, M10 and M11 formation reaction was between 3.3% and 4.4 %.

The assay is specific for the measurement of nimodipine metabolites in drug interaction studies. In fact, compounds such as cyclosporine A, erythromycine, midazolam, α-naphthoflavone, naringenin, quinidin, quinine, troleandomycine and (±)verapamil added to the nimodipine metabolism reactions did not interfere with the detection of metabolites M8, M10 and M11. However, the midazolam metabolites α-OH-midazolam and 4-OH-midazolam have shown chromatogaphical interferences with M8 and M10, respectively. Data are summarized in Table 3 .

DISCUSSION

The present HPLC assay is suitable for *in vitro* metabolism studies of nimodipine. Any inexpensive HPLC system can be used. However, the use of a photo diode array (PDA) detector set to detect simultaneously at two wavelengths has some advantages over the variable single wavelength detector, because the absorption maxima of dihydropyridine derivatives are at 238 nm, whereas their pyridine oxydative forms absorb maximally at 218nm. Thus, the assay allows to quantify nimodipine and its metabolites more accurately and in parallel. Another advantage is that the purity of the signals (e.g. M8) can be assessed. In our method the nature of the substance(s) which interfered with the signal of M8 was not clear. However in the metabolism assay investigating the formation of M8 from M10 these signals were resolved with base line separation (data not shown). It is therefore possible that other nimodipine metabolite(s) e.g. M9, are formed and coeluated with M8. The use of peak height for the quantitation may already minimize the error of the M8 concentration. Moreover, by using a short column filled with 3 μm ODS material and a low flow rate, we have further improved the detection limit of nimodipine and its metabolites. The major advantage of the present method is that the assay does not require a clean-up procedure. After protein precipitation by methanol, followed by a brief centrifugation, the sample is injected directly onto the HPLC column by an autosampler. The assay shows a stable baseline, allowing the overnight analysis of a large number of samples. However, in order to maintain good stability in routine analysis, the analytical column must be washed after about 80 injections (by back flush).

The present assay has been successfully used in the investigation of nimodipine metabolism in rat liver microsomes. Under the described conditions, nimodipine is biotransformed rapidly to metabolites M8, M10 and M11. The substrate dependent kinetics of

nimodipine metabolites formation may be described by enzyme kinetics with positive allosteric effect (14). In this model, the K_m of Michaelis-Menten kinetics is replaced by C_{50} and N may indicate the number of substrate molecules binding to isoenzyme(s) involved in the reaction. When N=1, the equation returns to that of Michaelis-Menten enzyme kinetics. In our data, N varies from 1.0 to 1.5 indicating that nimodipine may bind to an allosteric site and then potentiate the activity of CYP isoform(s) involved in the nimodipine metabolism. The apparent constant C_{50} was in the range of 9.1-37.1 μM and the V_{max} was between 0.5 and 2.6 nmol/min/mg protein. The formation of the secondary metabolite M8 from the primary metabolites M10 and M11 was also rapid. In a detailed kinetic analysis it was found that M10 competitively inhibits the formation of M8 from M11 and vice versa.

In conclusion, we developed a reliable, sensitive and technically simple method for the *in vitro* determination of nimodipine and its metabolites. The present assay does not only allow to investigate nimodipine metabolism, but also to study the interference of drugs or substances found in daily food, e.g. flavonoids, known as enzyme inhibitors in the biotransformation of dihydropyridine drugs (15), as well.

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