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Note

High-performance liquid chromatography of the metabolites of nitrendipine and investigation into the metabolic pathways of this dihydropyridine[a]

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The dihydropyridine calcium entry blocker nitrendipine [(±)-ethylmethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate] (VIII, Fig. 1) undergoes rapid first-pass metabolism producing metabolites that are inactive as antihypertensive agents [1,2]. Up to now it has generally been accepted that dihydropyridine compounds are oxidized initially by cytochrome P-450 enzymes to the corresponding pyridines [3–6]. Further oxidative biotransformation pathways

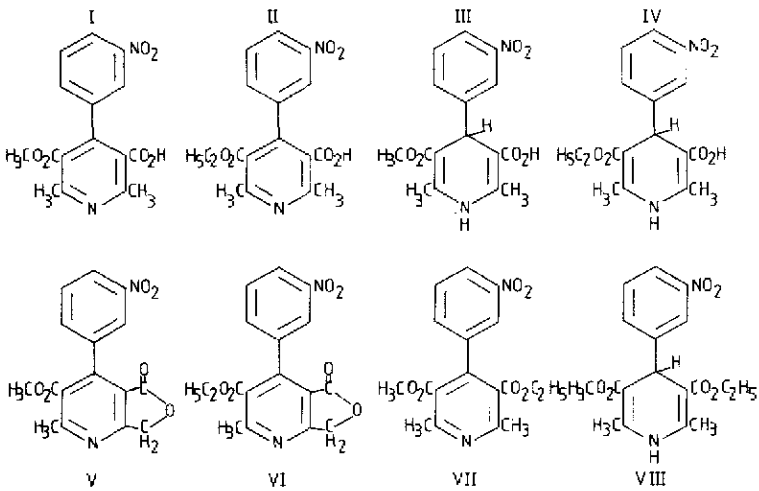

Fig. 1. Structures of nitrendipine (VIII) and seven of its metabolites; the numbering refers to the elution order in the HPLC system described in this paper (see also Fig. 2).

[a] Dedicated to Professor Dr. C. J. Estler on the occasion of his 60th birthday.

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involve ester cleavage or methylhydroxylation (position 2 or 6) with consecutive rearrangement of the molecule and the formation of two different lactones [methyl-2-4-(3-nitrophenyl)-5-oxo-5,7-dihydrofuro-(3,4-*b*)-3-pyridine carboxylate (V) and the corresponding ethyl compound (VI, Fig. 1) are formed from nitrendipine-pyridine] [3,7–11]. It has been demonstrated for only two other dihydropyridine compounds, nimodipine and amlodipine, that ester cleavage of the dihydropyridine structure might be possible [12,13].

To investigate the possibility of this metabolic pathway for the therapeutically used nitrendipine, a method was required by which all the possible metabolites of nitrendipine could be analysed. Monocarboxylic esters of model compounds with an unsubstitued aryl moiety can be separated from the parent compounds by ion-pair chromatography [14], but monocarboxylic esters of nitroaryl-substituted dihydropyridines cannot be separated from the parent compounds by the same analytical system.

This paper describes a method by which the monocarboxylic esters of nitrendipine and of its pyridine can be analysed together with the parent compounds, and by which it can be demonstrated that formation of the pyridine is also not exclusively the first step of the oxidative biotransformation of nitrendipine. Additionally this method might serve as a tool to investigate the activity of at least three different isoenzymes of the microsomal cytochrome P-450s, which are involved in the biotransformation of dihydropyridine compounds.

EXPERIMENTAL

(±)-Nitrendipine, nitrendipine-pyridine, the corresponding monocarboxylic esters of both compounds, and the two lactone compounds derived from nitrendipine-pyridine (Fig. 1) were gifts of Bayer (Wuppertal, F.R.G.).

Acetonitrile and water were of HPLC quality (Chromasolv™, Riedel-de Haen, Seelze, F.R.G.). All other chemicals were commercial products of the highest purity available.

Microsomes from rat liver (male Wistar control rats, weighing 200–250 g) were prepared using a method described in the literature [15]. The cytochrome P-450 content of the microsomal preparation was determined according to Omura and Sato [16]. All other enzymes and cosubstrates were from Bochringer Mannheim (Mannheim, F.R.G.).

Incubation mixtures contained 50 μM of the different substrates, which were added in 10 μl of 50% methanol, and usually 0.2 nmol of cytochrome P-450 per ml. Incubation was carried out using a general procedure [6] in an NADPH-generating system (13.8 mM glucose-6-phosphate, 4 U per ml glucose-6-phosphate dehydrogenase, 0.85 mM NADP, in 0.1 M potassium phosphate buffer, pH 7.8). After incubation for 30 min at 37°C, the *in vitro* incubation mixtures were cleaned up on Bond Elut™ extraction columns (Analytichem, Harbor City, CA, U.S.A.) (C_{18}, 100 mg) which had been pretreated in sequence with 2 ml of

methanol and 1 ml of 1% aqueous formic acid. Then the columns were washed once with 1 ml of 1% aqueous formic acid, and the substrates and reaction products were eluted from the columns with 1 ml of acetonitrile. The organic layers were dried under a stream of nitrogen in the dark at ambient temperature. The residues were dissolved in 1 ml of 10% acetonitrile in 0.5% phosphoric acid.

Aliquots of 50 μl were analysed on a Nucleosil™ RP C_8 column (10 μm, 25 × 0.4 cm I.D.; Macherey & Nagel, Düren, F.R.G.). The HPLC system consisted of two solvent-delivery pumps (T 414), a Rheodyne injection valve, a dual-wavelength detector (Uvicon 430) and a controller–integrating device (data system 450) (HPLC apparatus from Kontron Eching, F.R.G.).

The monocarboxylic esters were separated from the dihydropyridine and from the pyridine by a gradient (40 to 60% acetonitrile in 0.5% phosphoric acid) during 12 min at a flow-rate of 1.4 ml/min (*ca.* 100 bar). The eluent was monitored simultaneously at 234 nm (where the dihydropyridine and the pyridine can be detected) and 345 nm (where only the dihydropyridine compounds can be detected). The compounds were quantitated by determination of the peak area after calibration with external standards.

For serial analyses and improved precision internal standardization would be useful. The dihydropyridine compound that is almost insensitive to light, diethyl-1,4-dihydro-2,6-dimethyl-4-phenyl-3,5-pyridine dicarboxylate [5], eluted less than 1 min after nitrendipine and would perhaps be suitable.

All enzymic experiments were done in five independent incubation mixtures, and recovery analyses were repeated ten-fold. Mean ± S.D. values were calculated using the one-way analysis of variance statistic program on an Hewlett-Packard HP 97 calculator.

RESULTS AND DISCUSSION

When the monocarboxylic esters of nitrendipine were analysed together with nitrendipine or its pyridine in the ion-pair HPLC system described in the literature [14] the nitroaryl-substituted dihydropyridine and its pyridine were eluted from the column as broad peaks over 3 min with the monocarboxylic esters as rider peaks (data not shown). Variations of the eluent or using gradient separation did not alter the chromatographic behaviour of the compounds. By replacing the C_{18} 3 μm (15 × 0.46 mm I.D.) column used in the ion-pair separation system by a C_{18} 10 μm (25 × 0.4 cm I.D.) column and using a gradient from 40 to 60% acetonitrile over 12 min all the compounds could be separated as distinct narrow peaks (Fig. 2A and B). Owing to the very low noise of the signal the detection limit of the four monoesters and of the two lactones was below 50 ng/ml. The recovery of the pyridine monocarboxylic esters was 60% and that the dihydropyridine monocarboxylic esters was 75%. The recovery of the lactones, the pyridine-nitrendipine, and nitrendipine was 85–90%. When (±)-nitrendipine was incubated with microsomal cytochrome P-450 in presence of an NADPH-

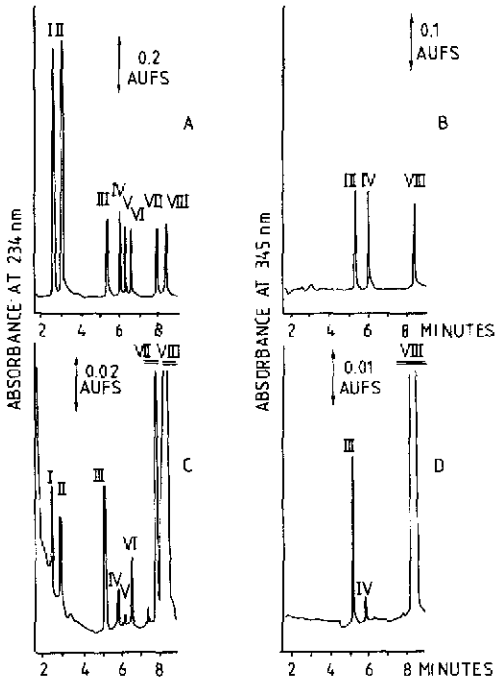

Fig. 2. HPLC of nitrendipine and its metabolites. A standard mixture of nitrendipine-pyridine monocarboxylic methyl ester (1 μg, I), nitrendipine-pyridine monocarboxylic ethyl ester (1 μg, II), nitrendipine monocarboxylic methyl ester (0.2 μg, III), nitrendipine monocarboxylic ethyl ester (0.2 μg, IV), nitrendipine-pyridine methyl lactone (0.2 μg, V), nitrendipine-pyridine ethyl lactone (0.2 μg, VI), nitrendipine-pyridine (0.2 μg, VII), nitrendipine (0.2 μg, VIII) was analysed on an RP C_8 column (25 × 0.4 cm I.D., 10 μm particle size) with gradient elution (40% to 60% acetonitrile in 0.5% phosphoric acid) at a flow-rate 1.4 ml/min (*ca.* 100 bar). (A) Detection at 234 nm; (B) detection at 345 nm.

The formation of the various metabolites of nitrendipine after *in vitro* incubation of 50 μ*M* nitrendipine for 30 min at 37°C on rat liver cytochrome P-450 (0.2 nmol/ml) in 0.1 *M* potassium phosphate buffer (pH 7.8) with an NADPH-generating system (glucose-6-phosphate 13.8 m*M*; glucose-6-phosphate dehydrogenase 4 U/ml; NADP 0.85 m*M*); the *in vitro* incubation mixtures were developed on Bond Elut extraction columns as described in Experimental. (C) Detection at 234 nm; (D) detection at 345 nm.

generating system, pyridine formation was the main reaction (Fig. 2C and D) with a rate of 1.5 nmol/nmol P-450 min. But in a competitive reaction the methyl monoester (III) and the ethyl monoester (IV) of nitrendipine were also formed. The rate of formation of the monocarboxylic methyl ester was 0.05 nmol/nmol P-450 min and that of the monocarboxylic ethyl ester was 0.01 nmol/nmol P-450/min, which is in good agreement with the literature report that an ethyl ester is cleaved faster than a methyl ester [14]. The oxidative ester cleavage proceeded much faster when the pyridine of nitrendipine was used as substrate: the methyl monoester (I) was formed at a rate of 0.5 nmol/nmol P-450/min compared with a rate of 0.1 nmol/nmol P-450/min for the formation of the ethyl monoester (II).

The ethyl lactone (VI) was formed from the pyridine at a rather similar rate of 0.3 nmol/nmol P-450/min in a competitive reaction. The corresponding methyl lactone (V) was formed slower, at *ca.* 0.02 nmol/nmol P-450/min, possibly owing to steric hindrance of methyl-hydroxylation (at the side where the ethyl ester is positioned). The lactones were not formed when the monocarboxylic esters of the pyridine were incubated in the *in vitro* system, which means that these compounds do not serve as a substrate for further oxidative metabolism in rat liver microsomes. On the other hand, the monocarboxylic esters of nitrendipine served as substrates for the formation of the corresponding pyridine. Both monoesters were oxidized at a rate of 0.2 nmol/nmol P-450/min, *i.e. ca.* 1/7 the rate of the conversion of nitrendipine into its pyridine.

In conclusion, the oxidative reaction of nitrendipine is predominantly the pyridine formation, followed by ester cleavage or a hydroxylation of the methyl group (position 2 or 6). Ester cleavage on the dihydropyridine can occur in competition with pyridine formation at a much lower rate than ester cleavage on the pyridine. However, the pyridine formation is not exclusively necessary for side-chain reactions on the molecule. These results underline the recent finding that the substituents of a dihydropyridine compound can influence the mechanisms and the metabolic pathways of biotransformation, as demonstrated for nifedipine, compared with the metabolism of model dihydropyridine compounds with an unsubstituted aryl moiety [5,17]. The method described in this paper can be used to determine the activity of at least three different isoenzymes of the microsomal cytochrome P-450 family that are mainly involved in the metabolic conversion of dihydropyridine compounds [5,6,11,14]: the pyridine formation is mainly catalysed by P450IIIA4, the ester cleavage by P450IIB1, and the side-chain hydroxylation by P450IA1 [18–21].

It should be noted that nitrendipine was used in this study as a racemic mixture, and that the enantiomers of nitrendipine might be metabolized at different turnover numbers at the enzymes indicated above. It is also possible that ester cleavage on the dihydropyridine structure can occur at different rates on the two nitrendipine enantiomers.

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