The role of High performance liquid chromatography to Identification of rat liver microsomes responsible for the in-vitro metabolite formation of Dipyrone

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Dipyrone is a non-steroidal anti-inflammatory drug (NSAID), commonly used in the past as a powerful painkiller and fever reducer. It is better known under the brand names *Conmel®, Neo-Melubrina®, Magnopyrol®, Prodolina F®, Analgin®*, and *Novalgin®*.

Because of the risk of serious adverse effects, its use is justified only in serious situations where no alternative is available or suitable.

Objective. Dipyrone is a widely used well tolerated analgesic drug which is how-ever compromised by agranulocytosis as adverse effect. Subsequent to nonenzymatic hydrolysis, primary metabolic step is N-demethylation of 4-meth-ylaminoantipyrine (4-MAA) to 4-aminoantipyrine (4-AA). The aim of the present study was to identify the human cytochrome P-450 enzyme (CYP) mediating this reaction.

.***Methods.*** We identified the relevant CYP using virus expressed isolated human CYP, human liver microsomes and rat liver microsomes with chemical inhibition studies. The substrate of 4-methylaminantipyrine was employed at six different concentrations (25, 50, 100, 400, 800 and 1200 µmol/l) with varying concentrations of selective inhibitors of CYP1A2 (furafylline, fluvoxamine), CYP3A4 (ketoconazole), CYP2A6 (coumarin), CYP2D6 (Quinidine), CYP2C19 (omeprazole, fluvoxamine, tranylcypromine), CYP2C9 (sulphaphenazole) and CYP1A1 (alpha naphthoflavone). 4-MAA and 4-AA were analyzed by HPLC, and enzyme kinetic parameters (K_m and V_{max}) were determined by regression (Sig-ma plot 9.0.

Results. The N-demethylation of 4-MAA by microsomes prepared from baculovirus-expressing human CYP was pronounced with CYP2C19. Intrinsic clearance of the most active enzymes were 0.092, 0.027, and 0.026 for the CYP enzymes 2C19, 2D6 and 1A2 respectively. Metabolism by human liver microsomes was strongly inhibited by fluvoxamin, omeprazole and tranylcypromine (IC50 of 0.07, 0.07 and 0.18), but with, coumarin, sulphaphenazole, ketoconazole, Moclobemid, Quinidine alpha-naphthoflavone , and furafylline were (0.79, 1.20 , 1.36 , 1.44 , 3.46, 4.68 and 8.41) respectively.

Conclusion. The enzyme CYP2C19 apparently has an important role in N-demethyl-ation of 4-methylaminoantipyrine which should be further analyzed in clinical studies and which may also be interesting concerning the agranulocytosis.

KEY WORDS: Metamizole, 4-methylaminoantipyrine (4-MAA), 4- aminoantipyrine (4-AA), metabolism, Human CYP2C19.

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1 INTRODUCTION

1.1 Drug metabolism

The majority of drugs undergo a variety of chemical reactions in the liver and, to a much lesser extent, in other organs (e.g., intestinal wall, kidney, lungs). Such reactions include oxidation, reduction, hydrolysis, and conjugation (with glucuronic acid, amino acids, acetate, sulphate, and methyl groups) and are directed towards the production of metabolites that are more ionized, more water-soluble, and less capable of penetrating cell membranes and being sequestrated in tissues. The more polar or water-soluble a compound becomes, the more readily it is excreted through the kidney and hepato-biliary system. This biotransformation is extremely important because most drugs are lipid-soluble weak electrolytes so that they would be readily reabsorbed through the renal tubule or intestine and remain in the body. The rate of metabolism may be influenced by many factors among which the genetic make-up of the individual and drug interactions are the most important. Metabolism of some drugs, the acetylation of isoniazid being the best example, can proceed at a rapid rate in one subgroup of the population and at a slow rate in another genetically defined subgroup of the population. A slow rate may be due to the deficiency of a specific enzyme because of some genetic defect and results in an increased sensitivity to drugs. For example, in subjects with acetyltransferase deficiency, the speed of acetylation and inactivation of isoniazid is decreased and consequently the usual doses of the drug will produce toxic effects.

Material

The samples of rat liver from male Wistar rats weighing between 180 to 220 g, aged 3 months were kindly provided by the Department of Toxicology at the University of Göttingen.

RLM (5- 10 mg protein /ml), 2 mM NADPH and 25 to 1200 µmol/l 4-methylaminoantipyrine in a final volume of 100 µl.

The reactions were stopped by adding ice-cold acetonitrile (100 µl). The resulting mixture was centrifuged at 13000 rpm for 5 min. 100 µl of the supernatant were used for HPLC analysis. The formation of 4-aminoantipyrine was linear with time between 6 and 10 min.

1.2 Investigations of the metabolism of metamizole by RLM

HPLC Analysis. The substances 4-aminoantipyrine (AA) (peak 1, Fig. 5) and 4-methylaminoantipyrine (MAA) (peak 2) have been measured with HPLC as illustrated in a chromatogram obtained from injection of 20 µl of standard solution with 25 µmol/l from AA and MAA. As shown in the figure, the separation was completed within 10 min. The retention times were 5.08 min for AA and 6.72 min for MAA (Fig. 6).

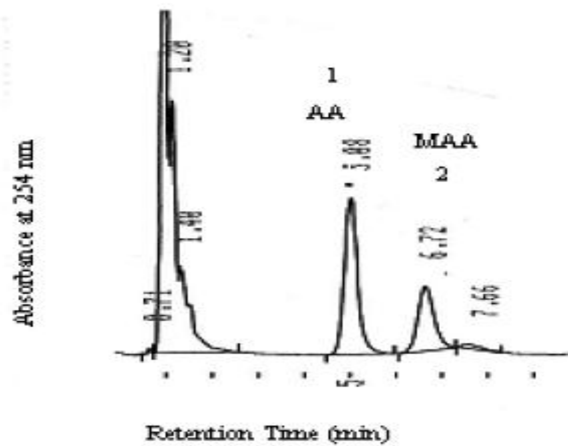


Fig. 1.Typical chromatograms of the main metabolites of metamizole obtained from standard samples of reference substance. Peaks: AA (5. 08 min), MAA (6.72 min). The mobile phase consisted of 80 % of 50 mM sodium phosphate buffer (pH 6. 0), 15 % acetonitrile and 5% methanol degassed before use, and the flow rate was 1. 0 ml/min.

Incubation of 4-methylaminoantipyrine with all microsomal preparations resulted in the formation of the 4-aminoantipyrine as the only detectable metabolite of 4-methyl-aminoantipyrine are shown in (Fig. 7).

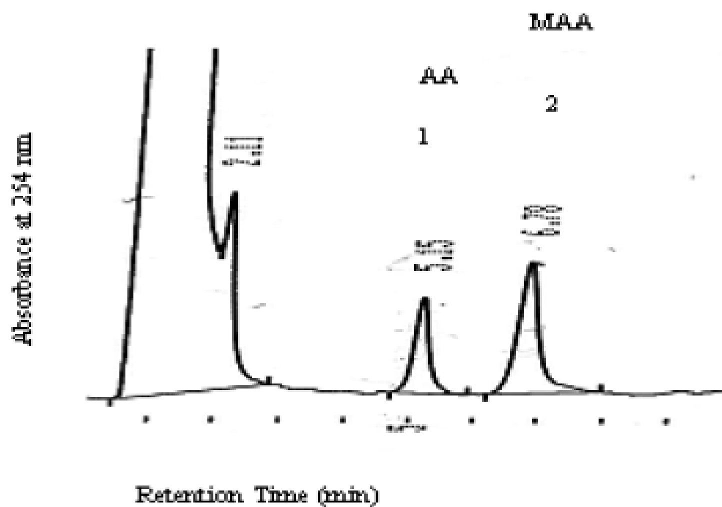


Fig. 2 Elution profiles of 4-methylaminoantipyrine and its metabolites by HPLC with a RP-8 endcapped (5 µm particale size, 125 x 4 mm internal dimensions) column equipped with a pre-column (100 nm pore size, diol-coated, 5 µm particle size). The mobile phase consisted of 80 % (v/v) of 25 mM sodium phosphate buffer (pH 6.0),

15% acetonitrile and 5% (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with a detector linked to a computer data system. The chromatogram shows 4-methylaminoantipyrine and its metabolite formed by microsomes. A reaction mixture (200 µl) with 1.25 mg/ml of microsomal protein of male Wistar rats, 1.0 mg/ml of NADPH and 50 µmol of 4-methylaminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer, (pH 7.4). 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC. Peaks: AA (5.15 min), MAA (6.78 min).

Two different preparations of rat liver microsomes were used. Mean Vmax (arithmetic mean of 5 incubations) was 58.5 pmol/min (standard deviation, SD, 32.2 pmol/min) and mean KM was 20.9 µmol/l (SD 3.8) as shown in fig. 7. The corresponding intrinsic clearance was 2.8 µl/min (SD 1.9). The quantity of microsomal protein was 0.250 mg protein in each assay. HPLC chromatograms from incubations of the metamizole metabolite 4-methylaminoantipyrine with hepatic microsomes of untreated male Wistar rats weighing between 180 to 220 g, aged 3 months and two control incubation samples without NADPH and zero incubation time are shown in (Fig. 9) right and left respectively.

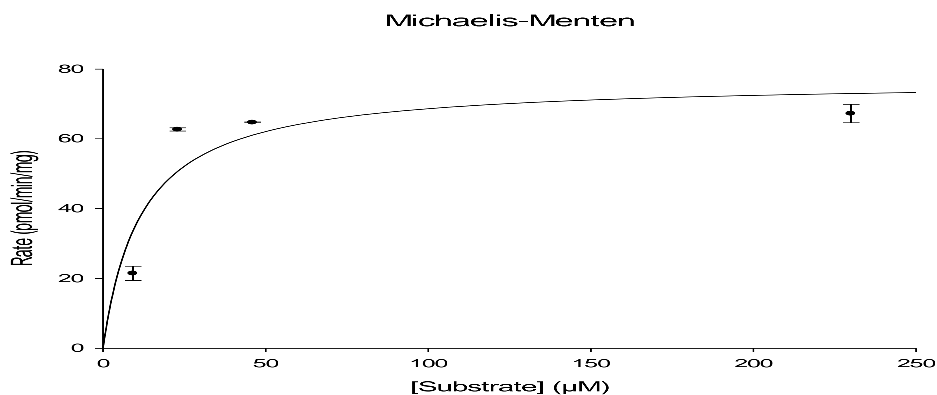


Fig. 3.Kinetic plot of demethylation of 4-methylaminoantipyrine

by RLM. The reaction mixture (200 µl), 1.25 mg/ml of microsomal protein of male Wistar rats, 1.0 mg/ml of NADPH. 4-methyl-aminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer, (pH 7.4). 4-methyaminoantipyrine metabolites were extracted and analyzed by HPLC.

RLM			
Inhibitors	% Inhibition	K_i mM	IC_{50}
Omeprazole	91.90	0.04	0.05
Ketoconazole	36.60	0.14	0.77
Sulphaphenazole	(no inhibition)	0	0
Coumarin	(no inhibition)	0	0
Quinidine	(no inhibition)	0	0
Alpha-naphthoflavone	(no inhibition)	0	0

Fig. 1 (Right) Extract from rat liver microsomes obtained 20 min after incubation with MAA without NADPH. Peaks: MAA (6.76 min). (Left) Extract from rat liver microsomes obtained

0 min after incubation with MAA. The metabolite was not seen in the right and left by HPLC with a RP-8 endcapped (5 µm partical size; 125 x 4 mm internal dimensions) column equipped with a pre-column (100 Å pore size, diol-coated, 5 µM particle size). The mobile phase consisted of 80 % (v/v) of 50 mM sodium phosphate buffer (pH 6.0), 15% acetonitrile and 5% (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with a UV detector linked to computer data system. chromatogram of 4-methylaminoantipyrine (4-MAA) metabolites by microsomes. A reaction mixture (200 µl) 0.71 mg/ml of microsomal protein of male wistar rats, and 50 µmol of 4-methylaminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer, (pH 7.4). 4-methylaminontipyrine metabolites were extracted and analyzed by HPLC.

4-methylaminoantipyrine (50 µM) was incubated with rat liver microsomes (5 mg/ml of microsomal protein) at 37 °C for 20 min and the metabolites were analyzed by HPLC after extraction.

The metabolites were not seen when 4-methyaminoantipyrine and microsomes were incubated without NADPH and with NADPH but without any incubation time (0 min). In rat liver microsomes, the metabolism of 4-methyaminoantipyrine was strongly inhibited by a concentration of 50 µM omeprazole as shown in Fig. 10, the inhibition was (92 % inhibition) and to a lesser degree by ketoconazole (37 % inhibition) and but no inhibition was detected with alpha-naphthoflavone, coumarin, quinidine and sulphaphenazole as shown in Table 12.

Table 1 Estimated % inhibition of the formation of 4-aminoantipyrine by selective chemical inhibitors added at a concentration of 50 µM. A reaction mixture (200 µl), 5

mg/ml of microsomal protein of male Wistar rats, and 50 µmol of 4-methylaminoantipyrine was incubated for 20 min at 37°C in 25 mM potassium phosphate buffer, (pH 7.4) 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC.

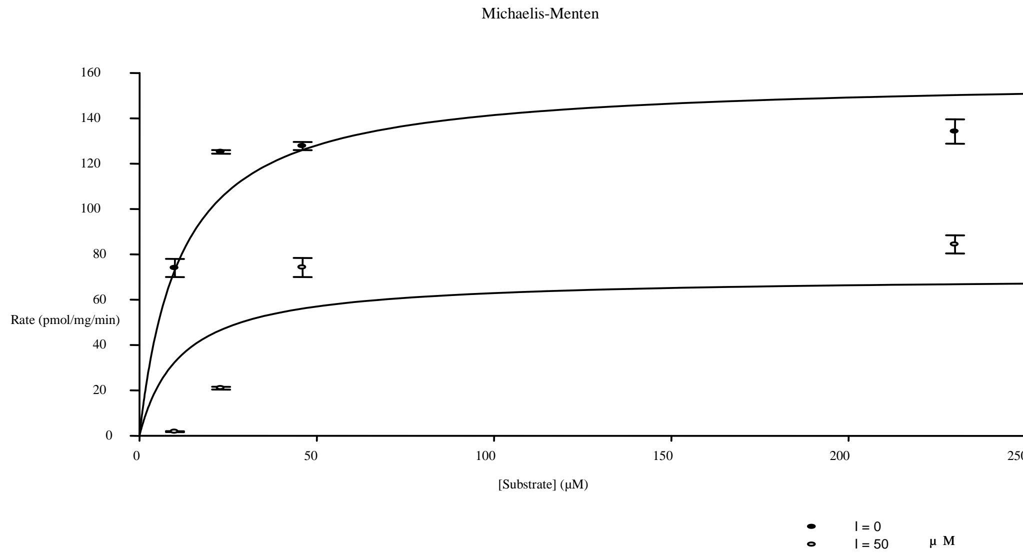
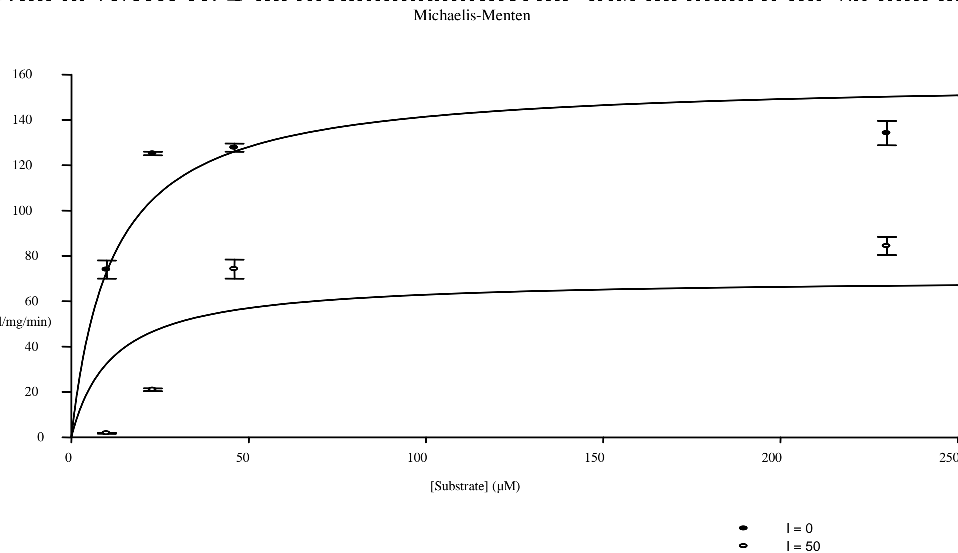


Fig. 4.Inhibition kinetic plot of metabolism of MAA with RLM by omeprazole. The reaction mixture (200 µl), contained 1.25 mg/ml of microsomal protein of male Wistar rats and 1. 0 mg/ml of NADPH. 4-methylaminoantipyrine was incubated for 20 min at 37 °C with
methyaminc



1.3 Investi

Human liver ze the role of c ial centrifugatio ıre homogenizec ei, peroxisomes, is then centrifu is resuspended of metamizole l 50 enzymes inv lly identified.

The aim of the present study, therefore, was to validate a HPLC method suitable to study the formation of metamizole metabolites in RLM and HLM and to get insights into the CYP enzymes involved. This first study of aminopyrine using in-vitro rat liver microsomes was by

(Imaoka et al., 1988) but with metamizole our investigations may be according to our best knowledge the in-vitro investigations of the metabolism of metamizole by RLM, HLM and heterologonsly expressed CYP enzymes.

The establishment of appropriate HPLC analytical methods started with the quantification of the reference substances of 4-aminoantipyrine and 4- methylaminoantipyrine. The separation was completed with in 13 min. The relative order of retention times was AA at 5.08 min and MAA at 6.72 min, as shown in Fig. 6 in results. Similar results were observed in the analysis of metamizole metabolites to study their formation in human liver microsomes (Geisslinger et al., 1996). For the incubations of metamizole with rat liver microsomes we used two different preparations of rat liver microsomes. Mean Vmax (arithmetic mean of 5 incubations) was 58.5 (standard deviation, SD, 32.2 pmol/mg protein/min) and mean KM was 20.9 (SD 3.8) µmol/l are shown in Fig. 8. The corresponding intrinsic clearance was 2.8 µl/mg protein/min (SD 1.9). The quantity of microsomes protein was 1.25 mg protein. Analysis of metamizole metabolism was performed with hepatic microsomes of untreated male Wistar rats weighing between 180 to 220 g, aged 3 months, because the female rats had lower N-demethylation activity of 4-methylaminoantipyrine than male rats (Imaoka et al., 1988). The two control incubation samples without NADPH and zero incubation time are shown in Fig. 9 at the right and left part, respectively. 4-methylaminopyrine (50 µmol/l) was incubated with rat liver microsomes 5 mg/ml of microsomal protein at 37°C for 20 min and the metabolites were analyzed by HPLC after extraction. The metabolite was not seen when 4-methylaminoantipyrine and microsomes were incubated without NADPH and with NADPH but incubation time zero. This metabolite was almost not seen in an assay system with NADPH but its formation inhibited by omeprazole as shown in (Fig. 10). Omeprazole is a strong inhibitor of CYP2C19 (Imaoka et al., 1988). The formation of 4-aminoantipyrine from 4-methylaminoantipyrine was strongly inhibited by a concentration of 50 µM omeprazole (92 % inhibition) and to a lesser degree by ketoconazole (37% inhibition) but no inhibition was detected with alpha-naphthoflavone, coumarin, quinidine and sulphaphenazole (Table 12). However, our results indicated that CYP2C19 re

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presents the contributor to the main of metabolism of metamizole in rat liver.