The effect of genetic polymorphism of cytochrome enzymes on metamizole metabolism

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Abstract : Two formerly very frequently used analgesic drugs or active metabolites of analgesic drugs, namely 4-methylaminoantipyrine (derived from metamizole) and 4-dimethylaminoantipyrine (also termed antipyrine) were studied here concerning the specific enzymes involved in their oxidative biotransformation by genetically polymorphic and non-polymorphic enzymes. The main conclusions from this investigation are:In vitro metabolism of methylaminoantipyrine to 4-aminoantipyrine was characterized by a KM of 20.9 µmol/l and a Vmax of 58.5 pmol/mg/min in rat liver microsomes (1.25 mg/ml protein). Strongest inhibition of methylaminoantipyrine demethylation in rat liver microsomes was achieved with omeprazole with an IC_{50} of 0.05 mMIn human liver microsomes, mean KM and Vmax were 251 µmol/l and 144 pmol/mg/min, respectively (2.5 mg/ml protein). Strong inhibition of these reactions was observed after co-incubation with omeprazole (CYP2C19), fluvoxamine (CYP2C19 and CYP1A2), and tranylcypromine (CYP2C19) with IC_{50} values of 0.07, 0.07 and 0.18 mmol/l, respectively.Some formation of 4-aminoantipyrine from methylaminoantipyrine was observed in the incubations with recombinant CYP2C19, CYP2D6, CYP1A2, CYP2C8, CYP2A6, CYP1A1, CYP1B1, CYP3A4, CYP3A5, CYP3A7, CYP2C9 and CYP2E1 but the highest formation observed only with CYP2C19 with intrinsic clearances of 0.092 (CYP2C19), 0.027 (CYP2D6), 0.026 (CYP1A2), 0.017 (CYP1A1) and 0.016 (CYP2C8) µl/pmol CYP/min, respectively.With concluded that CYP2C19 is the most important cytochrome P450 enzyme

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

Introduction

The cytochromes P450 are a superfamily of enzymes which are found in all forms of living organisms. They are responsible for the metabolism of many endogenous compounds, participate in the activation/deactivation of many carcinogens and detoxify many xenobiotic. In particular, in humans they metabolize many drugs and hence are of great interest to pharmacologists and toxicologists. Its is readily identified by a pronounced absorbance band at 450 nm in the sort region of the visible spectrum when the carbon monoxide adduct of the reduced hem protein is formed (Pohl et al., 1984) hence the name P450. Human cytochrome P450 (P450) enzymes catalyze the metabolism of a wide variety of clinically, physiologically, and toxicologically important compounds. The concept of a

familial inherited component modulating drug response was described in the 1950s, often in connection with case- reports of unexpected drug response (Hughes et al., 1954; Kalow et al., 1956; Evans et al., 1960). The variation in drug metabolism was ascribed to different metabolic rates in the enzymes either activating or inactivating the drug. The term pharmacogenetic (the study of heritability of drug response) was coined prior to the current knowledge in molecular biology The current explosion of interest for this field stems from technological advance, such as the mapping of the human genome and SNP (single nucleotide

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polymorphism) maps constituting the basis for our understanding of individual genetic diversity, and the fact that the results of these efforts are publicly accessible. Pharmacogenetics has traditionally focused on polymorphic drug metabolism, even though hereditary differences in drug receptors and drug transportation systems are included in the concept.The genetic polymorphisms in drug metabolism and disposition were typically discovered on the basis of phenotypic differences among individuals in the poplation (Mahgoub et al., 1977), but the framework for discovery of pharmacogenetic traits is rapidly changing. Adverse drug reactions are common; they are responsible for a number of debilitating side effects and are a significant cause of death following drug therapy (Lazarou et al., 1998). It is now clear that a significant proportion of these adverse drug reactions, as well as therapeutic failures, are caused by genetic polymorphisms, genetically based interindividual differences in drug absorption, disposition, metabolism, or excretion. Most of the commercially available drugs are metabolized by the phase- I cytochrome P450 superfamily of DMEs. The clinical relevance is best characterized for the genetic polymorphisms in CYP2D6, CYP2C19 and CYP2C9 (Stormer et al., 2000a). CYP2D6 play important roles in the metabolism of beta-blockers, tricyclic antidepressants, antiarrythmic agents, antipsychotic agents and opioids. CYP2C19 is involved in the metabolism of proton-pump inhibitors whereas CYP2C9 metabolizes antidiabetics and anticoagulants.

Aims of study:

This study aimed to confirm that genetic knowledge regarding the genetic polymorphism of cytochrome enzymes has been developed so that it can be used in drug development and clinical response to drug treatments customized for each race that differs in its genes from another race.Thus, we guarantee greater drug efficacy at lower treatment costs These goals, with their important results, have increased the desire to conduct more studies to discover the relationship of genetic response to different cytochrome enzymes with drug metabolism.As these differences changed the sensitivity of the receptors (targets) to the particular drug molecule, and thus contributed to the emergence of a very important effect on the effectiveness and toxicity of the drug, especially in drugs with a narrow therapeutic margin.

Material and methods

Chemicals. All chemicals and reagents were of analytical grade unless stated otherwise. HPLC-grade acetonitrile and methanol were obtained from J.T.Baker (Mallinckrodt Baker, Holland), the other chemicals and reagents were obtained from following sources: Aminoantipyrine, ketoconazole, alpha-naphthoflavone, omeprazole and sulphaphenazole were purchased from Sigma chemical (Steinheim, Germany), while coumarin and quinidine were obtained from (Fluka Steinheim, Germany). NADPH was purchased from Roche (Mannheim, Germany) and the internal standard (4-dimethylaminoantipyrine, 4-DMAA) was ordered from Sigma chemical (Steinheim, Germany). Potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany).

Microsomes and Human P-450 isoforms. Baculovirus-derived microsomes expressing human P-450 CYP3A4/OR (Cat. No. P207, Lot 67), CYP3A5/OR (Cat. No. P235, Lot 21), CYP3A7/OR (Cat. No. P237, Lot 9), CYP1A1/OR (Cat. No. P211, Lot 22), CYP1A2/OR (Cat. No. P203, Lot 28), CYP2C9/OR (Cat. No. P242, Lot 3), CYP2C8/OR (Cat. No. P252, Lot 10), CYP2C19/OR (Cat. No. P219, Lot 19), CYP2D6/OR (Cat. No. P217, Lot 43), CYP2E1/OR (Cat. No. P206, Lot 19), CYP2A6/OR (Cat. No. P254, Lot 7) were all obtained from Gentest (Frankfurt, Main, Germany).

Results discussion

Investigations of the metabolism of metamizole by HLM:

HPLC Analysis , Typical chromatograms obtained from reference substances of 4-aminoantipyrine (AA, peak 1) and methylaminoantipyrine (MAA, peak 2) obtained after injection of 10 µmol from AA and MAA into the HPLC system are illustrated in Fig.1. The

separation was complete within 20 min. The retention time of AA was 7.70 min, the retention time of MAA was 11.30 min and that of the internal standard (I.S., dimethylaminoantipyrine) was 16.75 min (Fig.4).

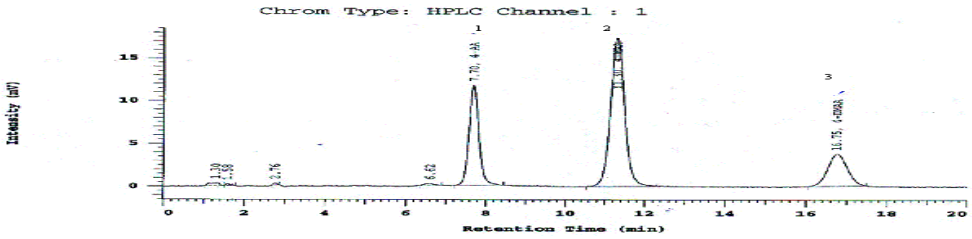

Fig 1 : Typical chromatograms of the main metabolites of metamizole obtained from standard samples. Peaks 1: AA (7.70 min), 2 MAA (11.30 min), peak 3 I.S (16.75 min) the concentration of MAA and AA =10 µmol/l. The mobile phase consisted of 75 % (v/v) of 25 mM sodium phosphate buffer (pH 6.0) and 25% (v/v) methanol. The flow rate was 1.0 ml/min.

The formation of 4-aminoantipyrine from 4-methyaminoantipyrine in the human liver microsomes increased in a protein-concentration and time-dependent manner. The metabolism of 4-methyaminoantipyrine was analyzed in six preparations of human liver microsomes (HLM) with an incubation time of 20 min, and two control incubation samples, one without NADPH and one with zero incubation time.

4-Methylaminopyrine (200 µmol) was incubated with human liver microsomes (10 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-methylaminoantipyrine and microsomes were incubated without NADPH and also not if incubated with NADPH but with an incubation time of zero. The

formation was linear at to 10 mg/ml microsomal protein and 20-min incubation time Unless specified, the standard incubation mixture containing 10 mg/ml microsomal protein was incubated at 37°C for 20 min. The formation of 4-aminoantipyrine from 4-methylaminoantipyrine by one preparation of human liver microsomes is illustrated in a chromatogram . and in dependence from substrate concentration .

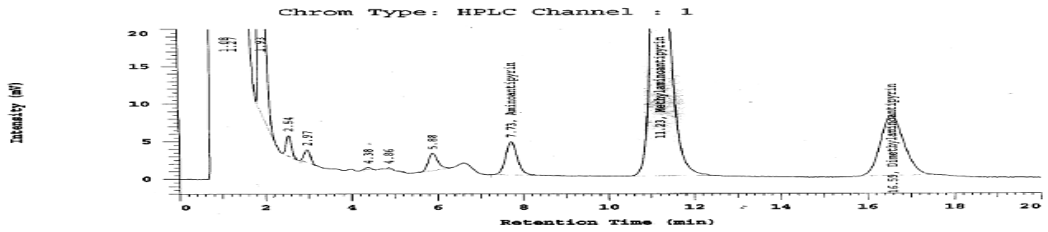

Fig 2 : HPLC-analysis of an extract of 4-methyaminoantipyrine and its metabolites after incubation with human liver microsomes. HPLC was performed with a RP-8 endcapped (5 µm) (125 x 4 mm) column equipped with a pre-column (100 Diol, 5 µM). The mobile phase consisted of 75 %(v/v) of 25 mM sodium phosphate buffer (pH 6.0) and 25%(v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with an UV detector linked to a computer data system. A reaction mixture (100 µl; 2.5 mg/ml of microsomal protein of HLM) with 1.0 mg/ml of NADPH, and 100 µmo/l of 4-methylamino-antipyrine was incubated for 20 min at 37°C in 50 mM potassium phosphate buffer (pH 7.4). 4-methylamino-antipyrine metabolites were extracted and analyzed by HPLC. Peaks: AA at7.73 min, MAA at 11.23 min, I.S. at 16.59 min.

A maximum biotransformation rate (V_{max}) of 144 (standard deviation 20) pmol/min/mg was measured. The quantity of microsomes protein in one sample was 2.5 mg protein.

The mean Michaelis-Menten constant (K_M) was 251 µmol/l with astandard deviation of 40 µmol/l as shown. The intrinsic clearance was 0.6 µl/min/mg. the in-vitro intrinsic clearance of 4-methylaminoantipyrine to 4-aminoantipyrine and from 4-dimethylaminoantipyrine to 4-methylaminoantipyrine. was calculated at the (CL int, in-vitro =V_{max}/ K_m) (Houston, 1994) and (Naritomi et al., 2003).

Table 1.Enzyme kinetic parameters for metamizole demethylation by human liver microsomes The metamizole concentration range varied from 25 to 800 µmol/l. The protein concentration of HLM protein was 10 mg/ml.

Preparation	K_M	V_{max}	Intrinsic clearance
HL 001	251	144	0.57
HL 009	201	163	0.81
HL 010	289	147	0.51
HL 003	152	312	2.05
HL 014	228	111	0.49
Liver withouth CYP2C19 (genotype *2/*2)			
HL 016	477	166	0.34

Investigations of the metabolism of metamizole by recombinant Human CYP450s

The enzymes kinetics of metabolism of 4-metylaminoantipyrine were finally studied by recombinant Human specific human CYP enzymes. 4-methyaminoantipyrine was converted into 4-aminoantipyrine by all specimens tested especially at more elevated substrate concentration. The enzyme kinetic To confirm the specific CYP enzyme involved in biotransformation of 4-methyl-aminoantipyrine, which appeared to be the enzyme CYP2C19 according to the inhibition study and according to one experiment with human liver microsomes from a CYP2C19 deficient subject, microsomes expressing individual recombinant human P450 isozymes (CYP1A1, CYP1B1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7) were incubated with different concentrations of 4-MAA from 25 to 800 µmol/l in the presence of an NADPH-regenerating system at 37°C for 20 min. Some formation of 4-AA was

observed in the incubations with (CYP1A1, CYP1B1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7) whereas the high formation and of 4-AA was observed only with CYP2C19 and with CYP1A2respectively.

Table 2 Enzyme kinetic parameters for metamizole demethylation by cytochrome P450. The metamizole concentration range varied from 25 to 800 µM. The concentration of baculovirus-expressed enzymes was 0.6 pmol/µl. All data represent the mean of minimally two experiments.

Enzyme	V_{max} (pmol/pmolCYP/min)	K_m (µmol/l)	Cl_{int} (µl/pmol CYP/min)	Cl extrapolated (l/min)
CYP2C19	11	120	0.092	2.253
CYP2D6	3.7	138	0.027	0.269
CYP1A2	8.1	317	0.026	1.199
CYP1A1	2.6	150	0.017	0.465
CYP2C8	4	245	0.016	0.413
CYP2A6	3.8	260	0.015	0.588
CYP2E1	2.3	169	0.014	0.510
CYP3A7	2.2	177	0.012	1.790
CYP2C9	2.2	216	0.010	0.031
CYP1B1	1.4	193	0.007	0.614
CYP3A5	1.5	263	0.006	0.012
CYP3A4	1.6	315	0.005	0.009

The average immunoquantified levels of the various specific P450s in human liver microsomal samples were 25, 42, 1.2, 1.43, 6.7, 16.85, 17.88, 31.29, 2, 26.82, 33.63, and 96 pmol/mg proteins in human liver for the CYP2C19, CYP1A2, CYP1B1, CYP1A1, CYP2D6, CYP2C8, CYP2A6, CYP2E1, CYP3A7, CYP2C9, CYP3A5, and CYP3A4 respectively.

The clearances extrapolated for a typical human liver based on the content of specific human CYP enzymes were 2.253, 0.269, 1.199, 0.465, 0.413, 0.588, 0.510, 1.790, 0.031, 0.614, 0.012 and 0.009 l/min given in the order of cytochrome P450 enzymes as above. In conclusion of our in vitro investigations, cytochrome P450 2C19 appeared as the primary enzyme metabolizing metamizole. The data presented here supported the results obtained from clinical studies that CYP2C19 is clinically important enzyme responsible of the metabolism of a number of therapeutic agents.

Conclusion.

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

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