Original research

Role of human liver microsomes in *in vitro* metabolism of metamizole

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Received: 10-02-2022, **Revised:** 06-03-2022, **Accepted:** 12-03-2022, **Published:** 31-03-2022

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HOW TO CITE THIS: *Abdalla SOA (2022) Role of human microsomes in in vitro metabolism of metamizole. Mediterr J Pharm Pharm Sci. 2 (1):109-114. https://doi.org/10.5281/zenodo.6399950*

Abstract: Metamizole or Novalgin® is a widely used well tolerated analgesic drug which is however compromised by agranulocytosis as adverse effect. Subsequent to nonenzymatic hydrolysis, the primary metabolic step is N-demethylation of 4-methylaminoantipyrine (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. This study 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 per 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 (Sigma plot 9.0). 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 (IC_{50} of 0.07, 0.07 and 0.18, respectively) 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. 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.

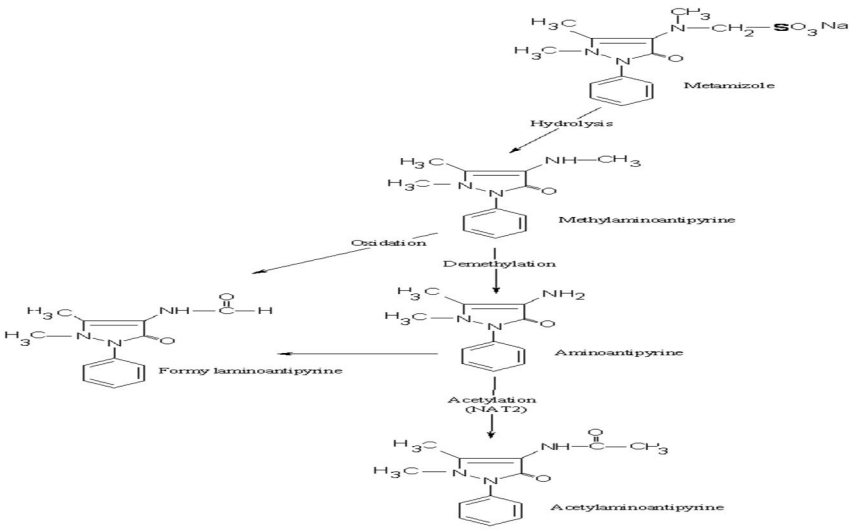
Keywords: 4-aminoantipyrine (4AA), 4-methylaminoantipyrine (4-MAA), human CYP2C19, metabolism, metamizole

Introduction

Metamizole (sodium [N-(1, 5-dimethyl-3-oxo-2-phenylpyrazolin-4-yl)-Nmethyl-amine] methane-sulphonate monohydrate) is a pyrazoline derivative available in oral and parenteral dosage forms acting as inhibitor of cyclooxygenase enyzmes. It has been used as a weak non-steroidal anti-inflammatory agent and as a potent analgesic and anti-pyretic drug in many countries for more than 60 years [1]. Oral doses of 0.5 to 1.0 gm have been effective in treating fever [2]. Repeated doses (up

to four times per day) can be administered, the maximum recommended dose is 3 to 4 gm per day [3]. Metamizole has been associated with fatal agranulocytosis and has been withdrawn from the United States market in 1979 [1]. The complex metabolism of metamizole has been the subject of many *in vivo* studies [4]. However, the specific CYP catalyzing the formation of the active metabolite 4-AA is still not known. The biotransformation pathway of metamizole is shown in **Figure 1** and it is well established [5]. It is non-enzymatically hydrolysed in the gastric juice to the active moiety 4-methylaminoantipyrine 4-omes: CYP, cytochrome P-450 by high performance liquid chromatographic.

Figure 1: Structure and biotransformation of metamizole and its main metabolites in man [6]



Material and methods

Chemicals: All chemicals and reagents were of analytical or pharmaceutical grade.

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).

Preparation of microsomes: Human hepatic microsomes were prepared by fractionation as described previously [7]. Approximately 8 gm of liver per experiment was allowed to thaw at room temperature in homogenization buffer (Tris 20 mM, Na-EDTA 5 mM, sucrose 254 mM pH 7.4 in ice bath). The suspension was centrifuged at 9 000 g for 30 minutes and the resulting supernatant was further centrifuged at 105 000 g for 60 minutes in an ultracentrifuge. The microsomal pellets were suspended in 250 mM sodium/potassium phosphate buffer (pH 7.4) containing 5 mM EDTA and 30% glycerol (v/v). They were stored in aliquots at - 80 until use. Microsomal protein concentration was determined as described previously with bovine serum albumin as a reference standard [8]. The rat liver microsomes were prepared following the same procedures described for human liver microsomes and protein concentration was determined using the BCA method (Pierce chemical. Rocford, IL), see ref. [9].

Incubation condition: In Eppendorf reaction vessels 250 µl of methylaminoantipyrine solution in methanol representing one of five concentration levels (HPLC, Instrumentation and chroma-

tographic conditions). The HPLC is very suitable to study the formation of metamizole metabolites in human liver microsomes [6, 10]. The HPLC system consisted of a L-600A pump and 655A-40 auto-sampler. The system was equipped with LiChrospher 100 RP-8e select column five µm preceded by a pre-column (100 Diol, 5 µM). The mobile phase consisted of 80% of 50 mM sodium phosphate buffer (pH 6.0), 15% acetonitrile and 5% methanol. The flow rate was 1.0 ml per min. The absorbance was measured at 254 nm, linked to computer data system with an ultraviolet (UV) detector. The injection volume in these analyses was 40 µl and the retention times of 4 methyl-aminoantipyrine (4-MAA), 4-aminoantipyrine (4-AA) and 4-dimethylaminoantipyrine (4-DMAA) were 7.05, 5.37 and 9.38 minutes, respectively.

HPLC-method: Sensitivity, precision, selectivity (separation of inhibitors) incubation of 4-methylaminoantipyrine with microsomes resulted in the formation of the 4-aminoantipyrine as the only metabolite of 4-methylaminantipyrine.

Results

Inhibition metabolism of 4-methylamino-antipyrine: (HLM with inhibitors) an inhibitory effect on 4-methylaminoantipyrine N-demethylation was seen for tranylcypromine, fluvoxamine emprazole (**Table 1** and **Figure 1**), no relevant inhibition was observed in incubations with ketoconazole, furafylline, sulphaphenazole, alpha-naphthoflavone and coumarin and moclobromine, metabolism of 4-methylamino-antipyrine was inhibited 42.569, 40.4346d 354 by 100 the CYP2C19 inhibitors omeprazole, tranylcypromine and omeprazole (0.159.28, 0.061289 and 0.85 mM) reactively with IC_{50} values of 0.2168, 0.48 0.0895.2 and 1.1711808, respectively. The IC values seen with alpha-naphthoflavone, quinidine, sluaphaph-enazole, ketoconazole, furafylline, coumarin and moclobemid are 4.307380, 16.927939, 562.87, 0.24561, 0.149 and 5 269.14 02881.00, 1.13, 7.01, 14380 and 06 which suggested that CYP2C19 was responsible, primary, for the metabolism of 4-methylaminoantipyrine. Metabolism of metyl-aminoantipyrine by virus expressed human P450 isoforms. 4-methylaminoantipyrine was converted into 4-aminoantipyrine by all specimens tested especially at more elevated substrate concentration, however, considerable formation rates above 75 pmol per min were seen (**Table 2** and **Figure 1**). The formation of 4-aminantipyrine AA was monitored by HPLC analysis with UV detection. Results are as average of duplicate incubation.

Table 1: Inhibition effects of inhibitors on 4-aminoantipyrine formation from methylaminoantipyrine.

Inhibitors	% of inhibition	K_i	IC_{50} (mM)
Omeprayole	51.09	0.06	0.07
Fluvoxamine	57.12	0.o6	0.07
Trancylcpromine	50.32	0.15	0.18
Coumarin	31.86	0.66	0.79
Sulphphenayole	25.06	1.oo	1.20
Ketocobayole	27.95	1.13	1.36
Moclobemide	43.24	1.20	1.44
Quinidine	11.35	2.88	3.46
Alpha-Naphthoflavone	18.65	3.90	4.68
Furafyline	16.09	7.01	8.41

Extract from human liver microsomes obtained 20 min after incubation with 4-MAA 50 µmol/l with 50 µM from chemical inhibitors was monitored by HPLC analysis.

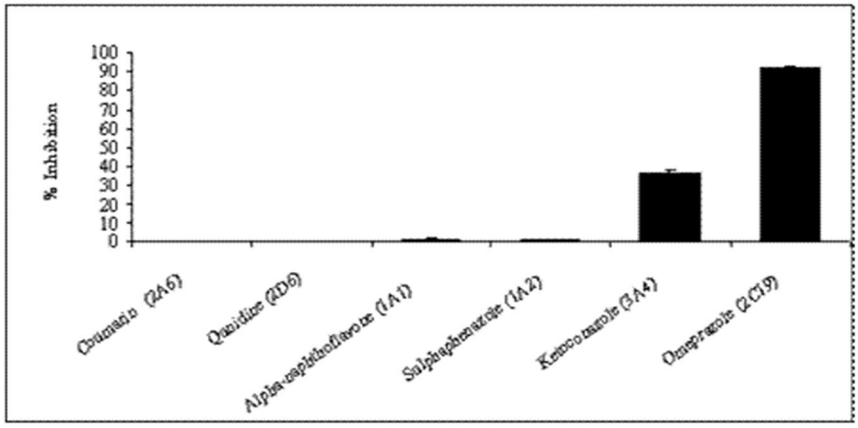


Figure 1: Inhibition of the metabolism of 4-dimethylaminoantipyrine by characteristic inhibitors of specific CYP enzyme

Table 2: Enzyme kinetic parameters for metamizole demethylation by cytochrome P450

Enzyme	V_{max} pmol/pmolCYP/min	K_m µ mol per l	Cl-$_{int}$ µl/min/pmol	Cl- extrapolate l per min
CYP2C19	11	293	0.038	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	04	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
CYP1B1	1.4	115	0.012	0.614
CYP2C9	2.2	216	0.010	0.031
CYP3A5	1.5	263	0.006	0.012
CYP3A4	1.6	315	0.005	0.009

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

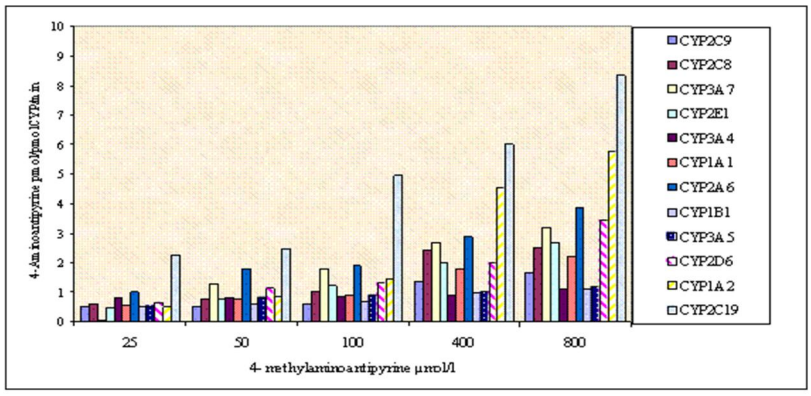


Figure 2: Cytochrome P450 isozymes in the in-vitro demethylation of methylaminoantipyrine.

Discussion

The major from being CYP2C9 followed by CYP2C19 and CYP2C8 [10, 11]. Our results from the inhibition study indicated the omeprazole, fluvoxamine and tranylcypromine are highly potent selective inhibitors of CYP2C19. Also, in other studies as ratio [12, 13] have been high affinity site of 4-metylaminoantipyrine demethylation and with coumarin (K_i = 46.18 µM) at the low affinity site of 4-metylaminoantipyrine demethylation, respectively. Quinidine had relatively small effect on the metabolite formation rate at the low affinity and very weak affinity was alpha-naphthoflavone (K_i = 3.90, **Table 2**). The overall results from human liver microsomes incubation experiments with inhibitors and without inhibitors. The V_{max} and K_m parameters studies indicated the CYP2C19 was responsible primarily for the metabolism of 4-methylaminantipyrine. This was evidenced by the ability of CYP2C19 to catalyze the reactions comprising the metabolic pathway of 4-methylaminatipyrine [14]. The strong Inhibition observed with human liver microsomes when tranylcypromine, omeprazole and fluvoxamine were used as inhibitors (**Figure 2**). To determine the CYP reaction phenotype of 4-methylaminoantipyrine microsomes expressing individual recombinant human CYP (CYP1A2, CYP1A1, CYP2C19, CYP2A6, CYP2D6, CYP3A4, CYP3A5, CYP3A7, CYP2C8, CYP2C9 and CYP2E1). These were incubated with different five concentrations from 4-methylaminoantipyrine. Under these condition the highest formation rate of 4-aminoantipyrine was observed with CYP2C19 (high affinity site with a K_m of (79.69 µml per l). CYP2C19 had the highest catalytic efficiency (intrinsic clearance, V_{max} / K_m) (± SD) of measurements (**Table 1**). CYP1A2 is the low affinity site and after repeated incubation with CYP1A1, CYP1A2 and CYP2C19 the CYP2C19 is high affinity site a similar effect was observed in the all incubation in the present studies [15].

Conclusion: This study supported the results obtained from clinical studies that CYP2C19 is clinically important enzyme responsible of the metabolism of a number of therapeutic agents and the effect of CYP2C19 in metabolism of methylaminoantipyrine.

Conflict of interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical issues: Including plagiarism, informed consent, data fabrication or falsification and double publication or submission have completely been observed by authors.

Author declaration: I confirm all relevant ethical guidelines have been followed and any necessary IRB and/or ethics committee approvals have been obtained.

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