ORIGINAL ARTICLE

Evaluation of the activity of P450 enzymes in rats: use of the single marker or combined drug administration

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Abstract

OBJECTIVES: A "cocktail" of several substrates is frequently used to assess metabolic activity of multiple cytochrome P450 enzymes in one session. Some interactions among substrates can appear and may influence the rate of biotransformation of other ones. Our current work was aimed on the influence of tolbutamide on cytochrome P450-mediated metabolism of phenacetin and vice versa.
DESIGN: In the presented work, the biotransformation rates of phenacetin and tolbutamide (markers of rat CYP1A2 and CYP2C6/11 metabolic activities, respectively) administered either separately or both simultaneously were compared. The model of isolated perfused rat liver was used.
RESULTS: Phenacetin had no significant effect on tolbutamide hydroxylation. Tolbutamide addition to the perfusion medium significantly increased the rate of O-deethylation of phenacetin.
CONCLUSION: Some differences in the rate of P450-mediated metabolism can be observed when comparing assessment using combination of two model substrates with the common way (single marker administration). Due to these differences, results obtained by the mentioned methodologies might not be fully comparable.

INTRODUCTION

Cytochrome P450 enzyme (CYP) metabolic activity may be influenced by many endogenous and exogenous factors, especially by co-administered drugs (Krizkova *et al.* 2008). Metabolic activity of various CYP's is most often measured using selective substrate of distinct P450 enzyme. In early phase of drug discovery, there are utilized so called "high-throughput" methods increasing the efficiency and effectiveness of assay to assess metabolic activity of many P450 enzymes in short time (Smith *et al.* 2007; Testino & Patonay, 2003; Zlokarnik *et al.* 2005). There are often applied several substrates simultaneously, so as the activity of multiple P450 enzymes could be measured (so called "cocktail approach") (Petsalo *et al.* 2008; Scott *et al.* 1999; Sharma *et al.* 2004; Smith *et al.* 2007; Tanaka *et al.* 2003; Testino & Patonay, 2003; Yao *et al.* 2007; Zhang *et al.* 2008; Zlokarnik *et al.* 2005). Some pharmacodynamic-based drug-drug interactions and adverse effects can appear in combination of the substrates *in vivo*. A "cocktail" approach can open a further question - can one marker influence the biotransformation rate of the other one? The aim of the present work was to compare biotransformation rate of phenacetin (PHE) and tolbutamide (TB) (markers of CYP1A2

Abbreviations

CYP	– cytochrome P450
HTP	– hydroxytolbutamide
PAR	– paracetamol
PHE	– phenacetin
TB	– tolbutamide
S.E.M.	– standard error of the mean
ANOVA	– analysis of variance
MR	– metabolic ratio

and CYP2C6/11 metabolic activities, respectively) administered either separately or simultaneously.

METHODS

The experiment was carried out on 24 male Wistar albino rats weighing 200 ± 20 g (Biotest, Czech Republic). After 10 days of adaptation to controlled laboratory conditions (21–22 °C; humidity 50–60%; light from 6:00 to 18:00, diet and water *ad libitum*), rats were randomly allocated into 3 groups (A, B, C) of 8 animals. The model of isolated perfused rat liver described elsewhere was used (Zendulka *et al.* 2008). Briefly, the animal was anesthetised, *vena portae* was cannulated and liver was isolated from abdominal cavity. Liver was perfused in modified recirculating apparatus described by Miller (Miller *et al.* 1951) with tempered, oxygenated William's medium E. Liver viability was maintained by constant perfusion rate and pressure and pH of perfusate, pO_2 was monitored. Liver was weighted and visually examined after finishing the perfusion. Bile flow rate was not monitored, but bile production was checked. In the Group A, the rate of PHE *O*-deethylation was examined to assess CYP1A2 activity. In the Group B, TB hydroxylation rate was examined to assess CYP2C6/11 activity. In the Group C, both substrates as a bolus (TB and PHE) were given simultaneously, so as to assess activity of both CYP1A2 and CYP2C6/11. TB and PHE are so-called model substrates (in pharmacokinetic studies also called as "markers") widely used for assessment of metabolic activities of cytochrome P450 enzymes (CYP2C6/11 and CYP1A2, respectively). TB is clinically used antidiabetic agent. PHE is an analgesic drug, in some countries it is still used as a low-dose component of analgesic mixtures. Also the combined TB+PHE treatment may by prescribed. The rate of metabolism

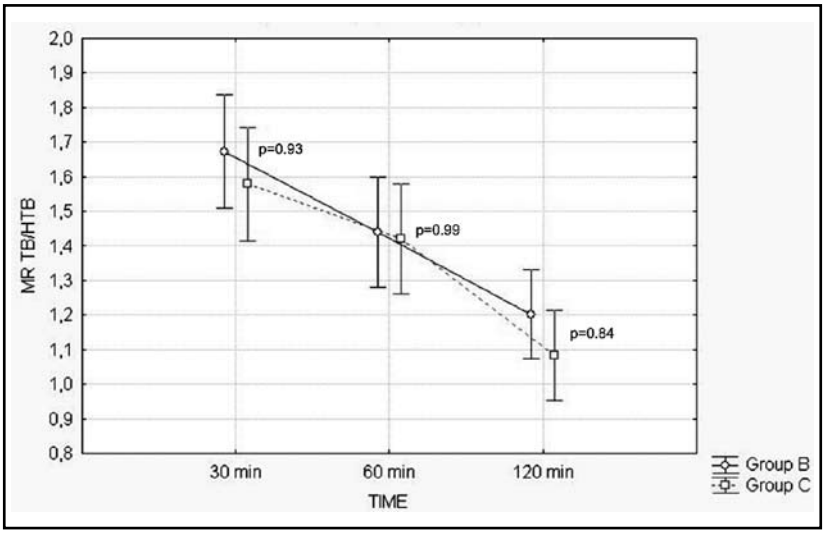

Fig. 1. Log $MR_{TB/HTB}$ data for groups B (marker: TB) and C (marker: TB + PHE). Graph displays statistically insignificant differences in log $MR_{TB/HTB}$ values in the 30th, 60th and 120th min of perfusion, which means that PHE had no effect on metabolic turnover of TB in the group C.

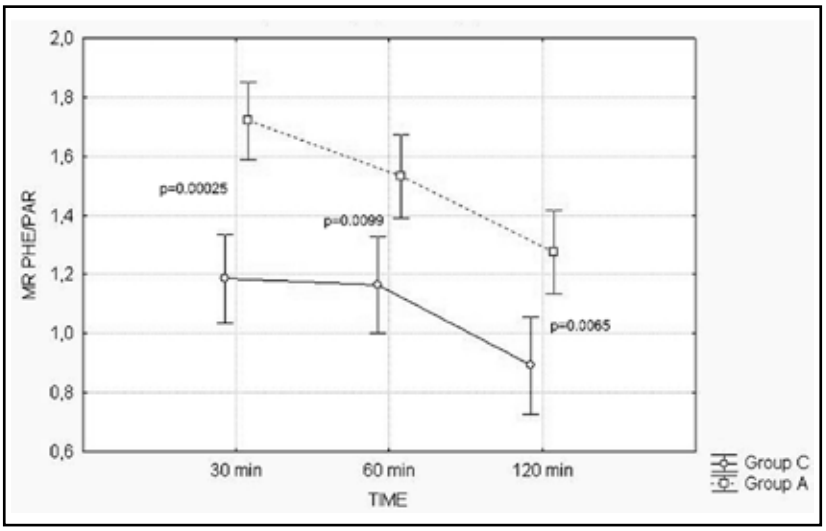

Fig. 2. Log $MR_{PHE/PAR}$ data for groups A (marker: PHE) and C (marker: TB + PHE). Graph displays the statistically significant (p <0.01) differences in log $MR_{PHE/PAR}$ values in the 30th, 60th and 120th min of perfusion, which means higher metabolic turnover of PHE in the group C.

Table 1. Concentrations of TB and HTB in perfusion medium and appropriate MR values in groups B and C

Group	Minute of perfusion	TB concentration * (mg/L)	HTB concentration * (mg/L)	$MR_{TB/HTB}$ *
B	30	8.17 ± 0.63	0.17 ± 0.08	48.77 ± 20.39
B	60	8.14 ± 0.66	0.26 ± 0.12	31.91 ± 13.37
B	120	7.73 ± 0.73	0.49 ± 0.22	15.73 ± 5.45
C	30	7.74 ± 0.57	0.25 ± 0.09	30.62 ± 11.29
C	60	7.35 ± 0.81	0.35 ± 0.21	21.16 ± 13.94
C	120	6.64 ± 0.71	0.66 ± 0.27	10.13 ± 5.06

* Values are expressed as arithmetic mean ± SD

Table 2. Concentrations of PHE and PAR in perfusion medium and appropriate MR values in groups A and C

Group	Minute of perfusion	PHE concentration * (mg/L)	PAR concentration * (mg/L)	$MR_{PHE/PAR}$ *
A	30	4.06 ± 1.51	0.27 ± 0.12	15.75 ± 4.71
A	60	2.97 ± 0.82	0.24 ± 0.11	13.05 ± 5.79
A	120	2.72 ± 0.87	0.38 ± 0.23	7.16 ± 4.27
C	30	4.88 ± 1.57	0.10 ± 0.05	49.44 ± 19.64
C	60	4.60 ± 1.39	0.14 ± 0.06	32.57 ± 19.23
C	120	3.04 ± 0.75	0.17 ± 0.07	18.27 ± 8.78

* Values are expressed as arithmetic mean ± SD

was assessed as a concentration ratio (metabolic ratio, MR): model substrate/metabolite, in the 30th, 60th and 120th minute of liver perfusion. The concentrations of TB, PHE and their CYP-specific metabolites were assessed after extraction with diethyl ether. Ether was then evaporated under gentle stream of nitrogen, residues were dissolved in 150 µl of mobile phase (65% acetonitrile + 35% 10 mM potassium phosphate monobasic) and then injected on Luna C18 (2) reversed-phase chromatographic column (150 × 4.6 mm I.D.; 5 µm). The flow rate was set at 1.5 mL/min. Analytes were detected at 229 and 245 nm using diode array detector. All experimental procedures were approved by the Czech Central Commission for Animal Welfare according to the Czech Act No. 246/1992. After the log transformation, Repeated Measure ANOVA with Tukey post-hoc test for multiple comparisons was used for the data analysis using software Statistica 8 for Windows. Data are expressed as means ± S.E.M. Values of $p < 0.05$ were considered to be significant.

RESULTS

Table 1 and ***Fig. 1*** show the concentrations of TB and its metabolite hydroxytolbutamide (HTB) in group B (marker: TB) and C (substrates: PHE+TB), and the metabolic ratios ($MR_{TB/HTB}$). The differences in concentrations of TB and HTB or differences in $MR_{TB/HTB}$ between groups B and C were not significant. On the other side, the statistical analysis displayed significant difference between the concentrations of PHE and its metabolite paracetamol (PAR) and appropriate MR ($MR_{PHE/PAR}$) in groups A and C – ***Table 2*** and ***Fig. 2***. Our results suggest that PHE addition to the perfusion medium containing TB had no significant effect on CYP2C6/11-mediated TB hydroxylation. On the other hand, TB addition to the perfusion medium significantly increased the rate of CYP1A2-mediated *O*-deethylation of PHE. This effect was observed in the 30th, 60th and 120th minute of perfusion ($p \leq 0.01$).

DISCUSSION

We observed that TB addition to the perfusion medium together with PHE increased CYP1A2-mediated *O*-deethylation of PHE. Detailed mechanism of this phenomenon is unclear so far, it could be hypothesized that acute increase in the metabolic activity could not be explained by an enzyme induction (in its right sense - i.e. enhancement of the expression of an enzyme), but more likely by some allosteric interaction. Possible explanation could be heterotrophic positive cooperativity (the third substance influences interaction of substrate and enzyme) of these substrates (or their metabolites or both), since similar effects have been already observed in cytochrome P450 enzymes, namely CYP1A2 (Isin *et al.* 2008; Sohl *et al.* 2008), CYP3A4 (Emoto *et al.* 2001; Roberts & Atkins, 2007), CYP2B4 (Sulc *et al.* 2008) and CYP2C9 (Liu *et al.* 2005). The other explanation like enzyme induction can be omitted due to the acute

administration of substances, as well as the influence of tissue or perfusate binding as the concentrations of marker/metabolite correspond in both experimental groups. The decrease in phenacetin concentration correlates with the increase in paracetamol concentration. In case of the tissue binding of single phenacetin (or paracetamol), the decrease of marker concentration would not be followed by the increase of metabolite concentration and vice versa. We can see no other possibility, how could other CYP enzymes (isoforms) increase metabolic turnover of PHE to PAR (reaction catalyzed selectively by CYP1A2).

Combination of the markers of metabolic activity in evaluating the P450 enzymes activity is quite often in practice. From the presented results may be suggested that due to shift in CYP enzyme metabolic activity in the case of combining of model substrates (PHE+TB) the procedure can show to some extent differential results comparing to the single-marker use.

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