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The metabolism of drugs by the gut flora

M. MIKOV

Department of Pharmacology, Toxicology and Clinical Pharmacology, Medical Faculty, Novi Sad, Yugoslavia

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SUMMARY

Gut flora and gut contents can be considered as a system with huge metabolic capacity, qualitatively and quantitatively different from the body cells and organs. That system changes along with life and nutrition, but despite broad investigation has not yet been defined satisfactorily. In many cases inter individual and intra individual differences in drug metabolism could be linked to variations in the gut flora metabolism. Gut flora metabolism of drugs and other xenobiotic metabolites excreted in bile is the key phase responsible for enterohepatic circulation.

In the last decade there has been more and more evidence for the crucial role of the gut flora cysteine conjugate β-lyase in the metabolism of cysteine conjugates. A new pathway for paracetamol cysteine conjugate metabolism has been directly linked with gut flora activity, as demonstrated in our studies.

Nowadays, it is quite clear that gut flora metabolism must be considered an integral part of drug metabolism and toxicity studies.

INTRODUCTION

There are considerable differences in gut flora composition with quantitative and qualitative metabolic changes influenced by species, age, diet, xenobiotics and disease (1,2).

Gut flora are mainly comprised of anaerobes, primarily absolute anaerobes, which makes the study of gut flora metabolism dependent on methods designed for anaerobic cultivation. Methods introduced by Drasar (3) and Aranki et al. (4) have greatly improved the probability of the correct assessment of gut flora metabolism.

The distribution and composition of gut flora along the gastrointestinal tract in different animal species and man has been reviewed (5–15), with an awareness of the limitations in the number of microbial species which can be studied and followed. Studies of distribution and composition of gut flora have been restricted to *Bacteroides, Eubacteria, Peptococcaceae, Bifidobacteria, Lactobacilli, Clostridia, Fusobacteria, Enterobacteriaceae, Streptococci,* but from current work it is likely that bacterial strains within the same bacterial species behave differently in different animal species.

The complexity of gut flora distribution is complicated by differences not only along the gastrointestinal tract, but also within the cross section of the intestinal lumen (13).

Several reviews have been published on gut flora metabolism of drugs and other xenobiotics (1,16–23) with the common conclusion that metabolic changes by gut flora are far more extensive than any part of the body. Scheline (1) stressed that 'gut flora have the ability to act as an organ with a metabolic potential equal to, or sometimes greater than, that of liver'.

Initially, the toxicological significance of gut flora metabolism was stressed. However, a broad spectrum of metabolic reactions performed by gut flora has been identified (1,16,23–25), such as hydrolysis, dehydroxylation, decarboxylation, dealkylation, dehalogenation,

Please send reprint request to : Prof. Momir Mikov, Department of Pharmacology, Toxicology and Clinical Pharmacology, Medical Faculty, PO Box 380, 21000 Novi Sad Yugoslavia.

deamination, heterocyclic ring fission, reduction, aromatization, nitrosamine formation, acetylation, esterification, isomerisation, and oxidation. Gut flora metabolism of bile excreted metabolites in many cases is the crucial phase of enterohepatic xenobiotic circulation.

Most of the drug metabolic changes by gut flora are considered in the light of enzymatic changes, but attention should also be paid to chemical reactions and the production of H_2S and methanethiol in the gut contents (26,27).

Drugs and other xenobiotics interfere with the ecology of the gut contents and the problem can be considered along similar lines to other investigations on the contamination of different ecosystem.

There are several approaches to the study of gut flora (1,28) and their role in the in vitro and in vivo metabolism of drugs and their metabolites in the light of their pharmacological and toxicological activation and deactivation, including:

1. The direct action of gut flora (contents) incubations
2. The action of pure cultures, mixed cultures, continuous cultures and cell-free bacterial products
3. The difference in metabolism after oral and parenteral drug application
4. The difference between control animals and animals treated with antibiotics
5. The difference between control and gnotobiotic animals (germ-free and animals with known gut flora strains).

The method of expression of enzyme activity by gut flora can greatly influence the impression of their impact on metabolism. The best method for animal studies is the expression of enzyme activity per total gut contents, which shows the metabolic capacity of the gut flora. But in humans the most appropriate way of gut flora activity assessment is the expression of the activity per gram body weight (29).

The result of gut flora activity could be drug activation to pharmacologically active drug, or toxic product, or deactivation. One of the early examples of pharmacological activation by microorganisms was given for prontosil (30) and the significance of gut flora for the conversion of prontosil and neoprontosil to sulfanilamide was subsequently shown (31).

The bioavailability and pharmacological effect of numerous drugs, like opiates, digoxin, hormones, and antibiotics, can be changed by the activity of gut flora and the contents of the gut as well as the gut wall (23).

Geographic and ethnic differences in drug metabolism could be the result of differences in gut flora composition and activity as was shown for digoxin (32,33).

The role of gut flora is significant in entero-entero recirculation (34) and entero-hepatic recirculation (1,35). This is of clear benefit for gut flora and the host for certain endogenous substances, like bile acids and steroids (36–38). But for drugs and other xenobiotics the benefit for the host and the gut flora is not always clear, sometimes it is harmful. One very important metabolic activity of gut flora is the deconjugation of glucuronide, sulphate and cysteine conjugates which decreases polarity and makes products more convenient for reabsorbtion, enterohepatic circulation and prolonging retention in the body (35,39–46).

In the last decade there has been growing evidence for the key role of the gut flora cysteine conjugate β-lyase in the further metabolism of drug and other xenobiotic cysteine conjugates originating from glutathione conjugates excreted via bile into gut lumen (47–54). Glutathione conjugates, as a result of conjugation of electrophilic metabolites, are extensively excreted in bile. They are metabolised via γ-glutamyl transferase and dipeptidase to the corresponding cysteine conjugates in tissues as well as in the gut lumen. Cysteine conjugates can be reabsorbed from intestinal contents or cleaved by β-lyases of bacterial origin (48,49,57,58,60–62).

β-Lyases transform xenobiotic cysteine conjugates to toxic metabolites which has been shown for many conjugates (63–67) These toxic metabolites are thiols or other metabolites derived from thiols, in most cases with unknown structure. Every cysteine conjugate is a potential toxicant because of this activation mechanism and it is worth considering its metabolism and toxicity.

Our work has been dedicated to answering the question of the role of the gut flora in the further metabolism of paracetamol 3-glutathione as a main thioconjugate excreted in bile. In our preliminary work it was shown that, both in germ-free and conventional mice, paracetamol 3-glutathione conjugate incubated in caecum contents was degraded to paracetamol 3-cysteine conjugate.

Attention has been given to the specificity of further cysteine conjugate metabolism, especially the role of gut flora in the further processing of cysteine conjugate metabolites with the presentation of our most recent investigation on the metabolism of paracetamol

3-cysteine conjugate in germ-free and conventional mice.

MATERIALS AND METHODS

Materials

Paracetamol, silver oxide, L-cysteine (all analytical grade) and [ring-^{14}C]-paracetamol (specific activity 7.3 mCi/mmol) were purchased from Sigma Chemical Co. [^{35}S]-L-cysteine (specific activity 600 Ci/mmol) was purchased from Amersham Int. Paracetamol and their metabolite standards were gifts from St. Winthrop. All solvents were HPLC grade.

Synthesis

Paracetamol cysteine was prepared by the method of Hoffmann and Baillie (69) using the reaction of L-cysteine with N-acetyl-*p*-benzoquinone imine prepared by the method of Dahlin and Nelson (70). [ring-^{14}C]-paracetamol-3-cysteine and [^{35}S]-paracetamol-3-cysteine were prepared by semi-preparative purification of hydrolysed urine collected after dosing mice with [^{14}C]-paracetamol or [^{35}S]-cysteine and paracetamol. HPLC purity of unlabelled products and radiochromatographic purity of [ring-^{14}C]-paracetamol-3-cysteine was 99%, but radiochromatographic purity of [^{35}S]-paracetamol-3-cysteine was 70% due to unidentified peak in the solvent front, but without any other detectable peak apart from paracetamol-3-cysteine.

Analytical HPLC

Analytical HPLC was performed by the radiochromatographic method for hydrolysed urine developed previously (71).

Semi-preparative HPLC

The HPLC system used was a Waters M-45 pump, UV detector and chart recorder. The HPLC column used was Technicol RP-18, 10 µm 30 cm x 10 mm, eluted with water:methanol:acetic acid (85:14:1, v/v/v). Fractions with retention times as for the paracetamol cysteine conjugate standard were collected, freeze dried and, after redissolving, purified once more on the same system and column but with water:methanol (90:10) as a mobile phase. Fractions were collected, freeze dried and kept at –20°C. The purified products had identical HPLC retention times and the characteristic UV and NMR spectra of authentic standards.

Urine hydrolysis

2 ml of each urine collection and cage washing was hydrolysed with 15 units of sulphatase and 300 units of β-glucuronidase (Sigma sulphatase type H1 from *Helix pomatia*) added in an equal volume of an acetate buffer, pH 5.0. Hydrolysed urine was freeze dried, stored at –20°C and, before HPLC analysis, the residuum was redissolved in 200 µl of water and 20 µl analysed by analytical radio-HPLC.

Animals and treatment

Standard male CD1 mice (body weight 22–25 g, Charles River Laboratories), germ-free male BALB/c mice (body weight 18–22 g, MRC Laboratory Animal Unit, Carshalton, Surrey, UK) and ex-germ free mice (produced by housing germ-free mice in the same cage with conventional mice for 1 week) were treated orally with sterile saline solution with [ring-^{14}C]-paracetamol-3-cysteine or [^{35}S]-paracetamol-3-cysteine (360 mg/kg, 10 µCi/kg). After dosing, each mouse was kept for 24 h in a glass metabolism cage (Mini Metabowls, Jecons Ltd) for urine collection. Urine was collected in dark, ice cold tubes. At the end of the collection period, cages were washed, the washings added to collected urine and kept at –20°C until analysed. The animals had free access to food and water. In the experiment with germ-free mice, food, water and handling devices were sterile.

[^{14}C] and [^{35}S] activity determination

Urinary excretion of [^{14}C] and [^{35}S] were determined by liquid scintillation spectrometry with correction for loss of counts due to isotopic decay of [^{35}S] according to the nomogram provided by the supplier (Amersham International).

Statistical analysis

Results were expressed as mean ± SD and the significance of difference between groups was established by unpaired 2-tailed Student's t-test.

RESULTS

From Table I it can be seen that in conventional mice there is a lower urinary excretion of [ring-^{14}C]-paracetamol-3-cysteine and [ring-^{14}C]-paracetamol-3-mercapturate but higher excretion of [ring-^{14}C]-paracetamol-3-methylsulphoxide and [ring-^{14}C]-3-thiomethyl paracetamol. Free paracetamol was detected in conventional mice urine but not in germ-free mice urine. In ex-germ free mice the pattern of excretion was close to conventional mice but in somewhat different proportions.

From Table II it can be seen that in conventional mice, excretion of [^{35}S]-paracetamol-3-cysteine and [^{35}S]-paracetamol-3-mercapturate was lower and [^{35}S]-paracetamol-3-methylsulphoxide and [^{35}S]-3-thiomethyl paracetamol was higher. Free paracetamol was detected only as a UV trace in conventional mice but not in germ-free mice. In the ex-germ-free mice, the urinary excretion was similar to conventional mice, with somewhat different proportions, but distinct from germ-free mice.

The specific radioactivity (Table III) of urinary excreted [^{35}S]-paracetamol-3-cysteine and [^{35}S]-3-thiomethyl paracetamol in conventional and ex-germ free mice was lower than the initial specific activity, but not in germ-free mice urine. This finding, together with our previous finding of free paracetamol and decreased [^{35}S]-cysteine and [^{35}S]-thioparacetamol specific activity in conventional mice incubates (72), suggests a mechanism linked to the activity of intestinal microflora.

Table I: Urinary metabolites of [ring-^{14}C]-paracetamol-3-cysteine in the conventional and germ-free and ex-germ-free mice after a dose of 360 mg/kg [ring-^{14}C]-paracetamol-3-cysteine.

Paracetamol	*% of 24 h urinary [^{14}C]*			
	Conventional $n = 4$	*Germ-free* $n = 5$	*Ex-germ-free* $n = 3$	
Paracetamol-3-cysteine	40.0 ± 9.94	74.2 ± 8.07*	15.6 ± 0.57*	g*
Paracetamol-3-mercapturate	3.98 ± 0.79	13.9 ± 8.49	1.59 ± 0.88*	g§
Free	1.65 ± 1.18	ND	1.27 ± 1.01	
Paracetamol-3-methylthiosulphoxide	9.01 ± 8.35	1.91 ± 1.11	12.87 ± 9.02	g§
Paracetamol-3-methylthio	25.3 ± 5.66	1.52 ± 0.78*	31.92 ± 2.81	g*
Unidentified peaks	13.78 ± 5.59	7.07 ± 5.02	33.4 ± 11.99	
Urinary [^{14}C] recovery	53.8 ± 4.24	46.7 ± 9.64	50.3 ± 2.83	

*$P < 0.01$, §$P < 0.05$ g towards germ free. Figures quoted are means ± SD.

Table II: Urinary metabolites of [^{35}S]-paracetamol-3-cysteine in the conventional and germ-free and ex-germ-free mice after a dose of 360 mg/kg [^{35}S]-paracetamol-3-cysteine.

Paracetamol	*% of 24 h urinary [^{35}S]-*			
	Conventional $n = 4$	*Germ-free* $n = 2$	*Ex-germ-free* $n = 3$	
Paracetamol-3-cysteine	18.0 ± 7.20	58.9 ± 9.55*	16.8 ± 1.70	g*
Paracetamol-3-mercapturate	1.62 ± 0.30	4.28 ± 1.60†	0.50 ± 0.33*	g§
Free	ND	ND	ND	
Paracetamol-3-methylthiosulphoxide	18.6 ± 13.10	1.91 ± 2.70	11.3 ± 1.15	g§
Paracetamol-3-methylthio	18.9 ± 11.84	ND	30.9 ± 9.96	
Unknown peaks	13.9 ± 3.70	3.28 ± 4.64	12.89 ± 7.8	
Urinary [^{35}S] recovery	59.9 ± 5.02	49.7 ± 14.28	49.3 ± 4.64	§

*$P < 0.01$, †$P < 0.2$, §$P < 0.05$ g towards germ free. Figures quoted are means ± SD.

Table III : [^{35}S]-paracetamol-3-cysteine and [^{35}S]-paracetamol-3-methylthio metabolite specific activity in the conventional and germ-free mice in 24 h urine as % applied [^{35}S]-paracetamol-3-cysteine specific activity.

Mice caecal incubate		*% of specific activity in paracetamol* *3-cysteine*	*3-thiomethyl*
Conventional	(n = 4)	72.3 ± 27.50	59.6 ± 26.7
Germ-free	(n = 2)	100	–
Ex-germ-free	(n = 3)	42.4 ± 5.81	61.6 ± 5.90

– peak not detected. Figures quoted are means ± SD.

Pathohistology examination of livers did not find changes, but renal medullary blood vessel congestion was prominent in germ-free mice but not in conventional mice. Also, during 24h urine collection, two germ-free mice died, but none of the conventional mice. This indicated possibly higher toxicity of paracetamol-3-cysteine for germ-free than for the conventional mice.

DISCUSSION

The question arising from our findings is why, in germ-free mice, in whose tissues there are all the necessary enzymes for the further metabolism of cysteine conjugates (β-lyase, methyltransferase and N-acetyl-transferase), the only considerable metabolite was paracetamol-3-mercapturate but not 3-thiomethyl paracetamol.

The mechanisms which have been shown for 2-mercaptobenzothiazole (73) – exchange of mercapto group with glutathione – and for 2-methylthiobenzotiazole (74) – methylthio group displacement with glutathione – could exist for paracetamol and could explain the decreased specific radioactivity of urinary excreted [^{35}S]-3-thiomethyl paracetamol in conventional mice urine. One reason for the extremely small quantities of paracetamol-3-thiosulphoxide and 3-thiomethyl paracetamol could be thiomethyl group turnover. The extent of this pathway is less important for the conventional mice, where 3-thiomethyl paracetamol is generated within the intestinal tract, by the action of intestinal contents and intestinal mucosa, reabsorbed and conjugated with sulphate and glucuronide and, as hydrophilic metabolites, excreted in urine. In germ-free mice, *in situ* formed thiol and thiomethyl products are susceptible to further oxidation, for example in the kidneys, and as methylsulphinyl and methylsulphonyl could be good leaving groups for the reconjugation with glutathione, and thus serve again as a source of thiols.

To judge the benefits or disadvantages of metabolism by one complex and metabolically very active organ – conventional mice gut contents with microflora as the most important part – is not easy. First findings show that it seems that by activating to reactive metabolites and their deactivation within the intestinal tract protects other sensitive organs, especially kidneys, from the toxic metabolites which would result from the metabolic activity within the organ.

Gut flora and gut contents in the organ, which changes with life and nutrition, have not been defined satisfactorily, despite intensive investigation during the last decades. The extrapolation of findings on the role of gut flora in animals to their role in humans is also uncertain.

Clear data should be available about the influence of gut flora on drug metabolism and kinetics especially in view of the influence of antibiotics.

The main aim of the gut flora metabolic studies should be, in the first place, to reveal what is its influence on the host, and secondly, what part of the gut flora and contents are essential for this influence. That makes the appropriate interaction of microbiology, pharmacology and toxicology – research tools, laboratories and specialized personnel – essential for studies on gut flora metabolism.

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