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Comparison of Hydrolytic and Conjugative Biotransformation Pathways in Horse, Cattle, Pig, Broiler Chick, Rabbit and Rat Liver Subcellullar Fractions

F. Gusson[1], M. Carletti[1], A. Giuliano Albo[1], M. Dacasto[2] and C. Nebbia[1]*
[1] *University of Turin, Department of Animal Pathology, Division of Pharmacology and Toxicology, Via Leonardo da Vinci 44, I-10095 Grugliasco;* [2] *University of Padua, Department of Public Health, Comparative Pathology and Veterinary Hygiene, Legnaro, Italy*
**Correspondence: E-mail: carlo.nebbia@unito.it*

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ABSTRACT

To complete a study aimed at investigating the pattern of the basal activities of liver xenobiotic-metabolizing enzymes in major and minor species intended for meat production, microsomal carboxylesterases and some conjugating enzyme activities were determined and compared in liver preparations from horses, cattle, pigs, rabbits and broiler chicks, using the rat as a reference species. Horses and broiler chicks exhibited a lower microsomal carboxylesterase activity towards indophenyl or *p*-nitrophenyl acetate than that measured in cattle or pig subfractions. Among food-producing species, the rate of glucuronidation of either 1-naphthol or *p*-nitrophenol was in the order pigs ∼ rabbits > horses > > cattle > broiler chicks. The widest variations were observed in the acetylation capacity towards *p*-aminobenzoic acid or isoniazid, which in rabbits was 3-fold to 11-fold greater than that displayed by any other examined species; low but measurable activities were found in equine and bovine cytosols. The activity of cytosolic glutathione *S*-transferase (GST) accepting the general substrate 1-chloro-2,4-dinitrobenzene was significantly higher in rabbits, horses and pigs than in rat, broiler chicks and cattle. Finally, an uneven pattern of activity towards the other tested GST substrates – 3,4-dichloronitrobenzene, ethacrinic acid or 1,2-epoxybutane – was observed, possibly reflecting the species-related expression of different GST classes; in this respect, the conjugative capacity displayed by horses was higher than or comparable to that found in the other food-producing species.

Keywords: comparative studies, food-producing species, glutathione *S*-transferases, liver, microsomal carboxylesterases, *N*-acetyltransferases, rat, uridinediphosphoglucuronyltransferases, xenobiotic-metabolizing enzymes

Abbreviations: BOX, 1,2-epoxybutane; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloronitrobenzene; ETA, ethacrinic acid; GST, glutathione *S*-transferase; INH, isoniazid; IPA, indophenyl acetate; NAT, *N*-acetyltransferase; NPSH, non-protein sulphydryls; PABA, *p*-aminobenzoic acid; PNPA, *p*-nitrophenyl acetate; UGT, uridinediphosphoglucuronyltransferase; XMEs, xenobiotic- metabolizing enzymes

INTRODUCTION

For the elimination of a large array of chemicals, living organisms have developed, in virtually all tissues enzyme systems (xenobiotic-metabolizing enzymes, XMEs) that transform exogenous and also endogenous compounds into more hydrophilic derivatives through reactions collectively known as biotransformations. It is well recognized that species-related differences in XME expression may affect not only the persistence in the body of drugs and poisons but also susceptibility to them (Nebbia, 2001). However, food-producing species have been the subject only of a limited number of studies (Watkins and Klaassen, 1986; Short *et al.*, 1988; Nebbia *et al.*, 2003; Sivapathasundaram *et al.*, 2003), the majority of which have been concerned with cytochrome P450-dependent monooxygenases. In contrast, relatively less attention has been paid to hydrolytic and conjugative pathways.

In recent years, the horse and the rabbit have gained considerable importance as minor food-producing species in certain EU countries. There is a need for comparative data because the relative lack of drugs specifically registered for such species commonly results in the extra-label use of veterinary medicinal products already authorized for other major species. As far as the horse is concerned, the majority of the published investigations have focused on the fate of specific classes of drugs (for a review see Schmid and Schmid, 1994) rather than on study of the *in vitro* metabolism of model substrates (Lakritz *et al.*, 2000; Nebbia *et al.*, 2004); in addition, the comparative approach has been adopted only very rarely (Chauret *et al.*, 1997; Nebbia *et al.*, 2003). Furthermore, although the rabbit has been the subject of numerous studies aimed at investigating interspecies differences in XME activity, almost invariably the animals used belonged to the New Zealand White breed and have been included as laboratory species (Watkins and Klaassen, 1986; Short *et al.* 1988).

We have recently published a report on the hepatic expression and activity of P450-dependent hepatic monooxygenases in the horse and in some agricultural and laboratory species (Nebbia *et al.*, 2003); here we report the results of further investigations on hydrolytic and conjugative reactions performed in liver subfractions from the same animals, in order to complete a reference metabolic pattern and to compare the basal activities of some of the main XMEs in major and minor species intended for meat production.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Animals

Liver samples, free from pathological lesions, were obtained from horses, cattle, pigs, broiler chicks and rabbits (n = 6) not subjected to any pharmacological treatment during the 90 days prior to slaughtering, which in all cases took place according to the Italian law in EU-certified abattoirs in the Turin area. All animals were sexually mature males, crossbred for meat production, except for pigs that were castrated. Male Wistar rats (100–120 g) were purchased from Morini (San Polo d'Enza, RE, Italy) and were sacrificed after light CO_2 anaesthesia.

Preparation of subcellular fractions

Immediately after slaughter, liver samples (20–30 g for larger species, the whole liver for the smaller ones) were collected, washed in isotonic KCl, blotted dry, placed on ice and brought to the laboratory within 2 h. Cytosolic and microsomal fractions were isolated by differential ultracentrifugation as detailed by Nebbia and colleagues (2003), frozen in liquid nitrogen and stored at –80°C. The protein content of tissue preparations was analysed using Lowry's method (Lowry *et al.*, 1951).

Carboxylesterase assays

Carboxylesterase activity was determined on microsomal fraction with two substrates, namely indophenyl acetate (IPA) (0.33 mmol/L) and *p*-nitrophenyl acetate (PNPA) (0.33 mmol/L) according to Zemaitis and Greene (1979) and Nousiainen and colleagues (1984), respectively. The protein concentration ranged between 0.002 and 0.0125 mg/ml (IPA esterase) and 0.001 and 0.002 mg/ml (PNPA esterase). Product formation was monitored spectrophotometrically at 522 nm and 400 nm, respectively.

Phase II enzyme activities and non-protein sulphydryl content

Uridinediphosphoglucuronyltransferase (UGT) activity was assayed on 0.25% Triton X-100 activated microsomes (0.13 mg/ml protein) with 1-naphthol 0.3 mmol/L or *p*-nitrophenol 0.3 mmol/L as substrates according to Antoine and colleagues (1988). The remaining phase II activities were assayed in the cytosolic fractions. The *N*-acetyltransferase (NAT) activity was measured using isoniazid (INH) or *p*-aminobenzoic acid (PABA) as substrates as detailed by Kaddouri and colleagues (1990). The *N*-acetylation rate of INH 1 mmol/L was determined by measuring the formation of acetylisoniazid at 302 nm with a protein concentration of 0.1 mg/ml; when PABA 0.4 mmol/L was used as the substrate, enzyme activity was determined with 0.6–2 mg/ml of protein by measuring the amount of unconjugated substrate. Glutathione *S*-transferase (GST) assays were performed using different substrates, namely 1-chloro-2,4-dinitrobenzene (CDNB) 1 mmol/L, measuring the so-called

'total' GST, or ethacrinic acid (ETA) 0.2 mmol/L at pH 6.5 and 3,4-dichloronitrobenzene (DCNB) 1 mmol/L at pH 7.5 as described by Habig and colleagues (1974). The amount of protein used for each assay was as follows: 0.004–0.026 mg/ml for CDNB-GST, 0.1–1.3 mg/ml for DCNB-GST, and 0.005–0.19 mg/ml for ETA-GST; the formation of the GSH adduct was followed spectrophotometrically at 340 nm (CDNB), 345 nm (DCNB) and 270 nm (ETA), respectively. GST accepting 1,2-epoxybutane (BOX) as the substrate was determined using a protein concentration of 0.5 mg/ml and a substrate concentration of 0.66 mmol/L according to Baars and colleagues (1978). The enzyme activity was expressed as the amount of the conjugated glutathione after subtraction of appropriate blanks to account for spontaneous conjugation. Non-protein sulphydryl (NPSH) content was determined with dithiobis (nitrobenzoic) acid on deproteinized cytosolic fractions as described elsewhere (Ugazio *et al.*, 1993).

Statistics

Statistical calculations were performed with GraphPad Instat version 3.00 (GraphPad Instat software, San Diego, CA, USA). Data were expressed as means $\pm$ SD and subjected to Kolgomorov–Smirnov test for normal distribution. Differences between species were assessed by means of one-way ANOVA followed by Tukey's post-test, and p-values less than 0.05 were considered statistically significant.

RESULTS

Carboxylesterase activities

As shown in Table I, the rate of the *in vitro* metabolism of IPA was of the same order of magnitude in horse, cattle, broiler chick and rat microsomes, while pigs and rabbits exhibited 7-fold to 10-fold higher values. A similar trend was seen with PNPA as substrate, in that activities measured in horses, chicks and rats were again much lower than those recorded in rabbits and pigs; in contrast, cattle preparations were comparatively more active in metabolizing PNPA with respect to IPA.

Phase II enzyme activities and non-protein sulphydryl content

Table II illustrates the comparative activity of glucuronidating and acetylating enzymes in the examined species. As regards glucuronidation, broiler chicks apparently lacked any measurable activitiy of the UGT accepting *p*-nitrophenol as substrate and bovine microsomes showed values ranging from about 1/4th to 1/5th of those displayed by pig, rabbit and rat preparations. In the horse, the glucuronidation capacity towards the same substrate was roughly twice as high as that of cattle and half that of pigs. The equine species showed a similar trend also using 1-naphthol as substrate, towards which pig and rat subfractions displayed the highest glucuronidation rate. A

TABLE I
Carboxylesterase activity towards two different substrates in liver microsomes from horses, cattle, pigs, broiler chicks, rabbits and rats

Parameter	Horses	Cattle	Pigs	Broiler chicks	Rabbits	Rats
Indophenyl acetate esterase*	330 ± 84^{a}	355 ± 29^{a}	2550 ± 845^{b}	259 ± 25^{a}	3399 ± 601^{c}	280 ± 34^{a}
p-Nitrophenyl acetate esterase**	2.81 ± 0.67^{a}	12.96 ± 2.33^{b}	15.23 ± 3.05^{b}	2.42 ± 0.27^{a}	14.40 ± 2.70^{b}	6.49 ± 3.17^{a}

Data are expressed as means $\pm$ SD, $n = 6$ for each group of animals

[a,b,c]Values with different superscripts in a row are significantly different ($p < 0.05$ or less)

*nmol/min per mg protein; **μmol/min per mg protein

TABLE II
Conjugative enzyme activities in hepatic subfractions from horses, cattle, pigs, broiler chicks, rabbits and rats

Parameter*	Horses	Cattle	Pigs	Broiler chicks	Rabbits	Rats
Isoniazid *N*-acetyl transferase	7.03 ± 1.15^{a}	4.04 ± 1.28^{a}	8.78 ± 3.27^{a}	2.28 ± 0.57^{a}	25.0 ± 7.5^{b}	6.83 ± 0.75^{a}
p-Aminobenzoic acid *N*-acetyltransferase	0.60 ± 0.30^{a}	0.57 ± 0.45^{a}	11.8 ± 3.6^{b}	ND	22.5 ± 5.6^{c}	0.88 ± 0.65^{a}
1-Naphthol UGT	21.4 ± 7.0^{a}	7.47 ± 2.21^{b}	34.7 ± 4.4^{c}	13.0 ± 2.7^{bd}	17.8 ± 4.0^{d}	38.5 ± 3.3^{c}
p-Nitrophenol UGT	9.66 ± 4.72^{a}	4.01 ± 0.77^{b}	19.2 ± 3.0^{c}	ND	16.7 ± 2.4^{c}	20.4 ± 2.3^{c}

Data are expressed as means $\pm$ SD, $n = 6$ for each group of animals

[a,b,c]Values with different superscripts in a row are significantly different ($p < 0.05$ or less)

*Activities are expressed as nmol/min per mg protein

ND = no detectable activity

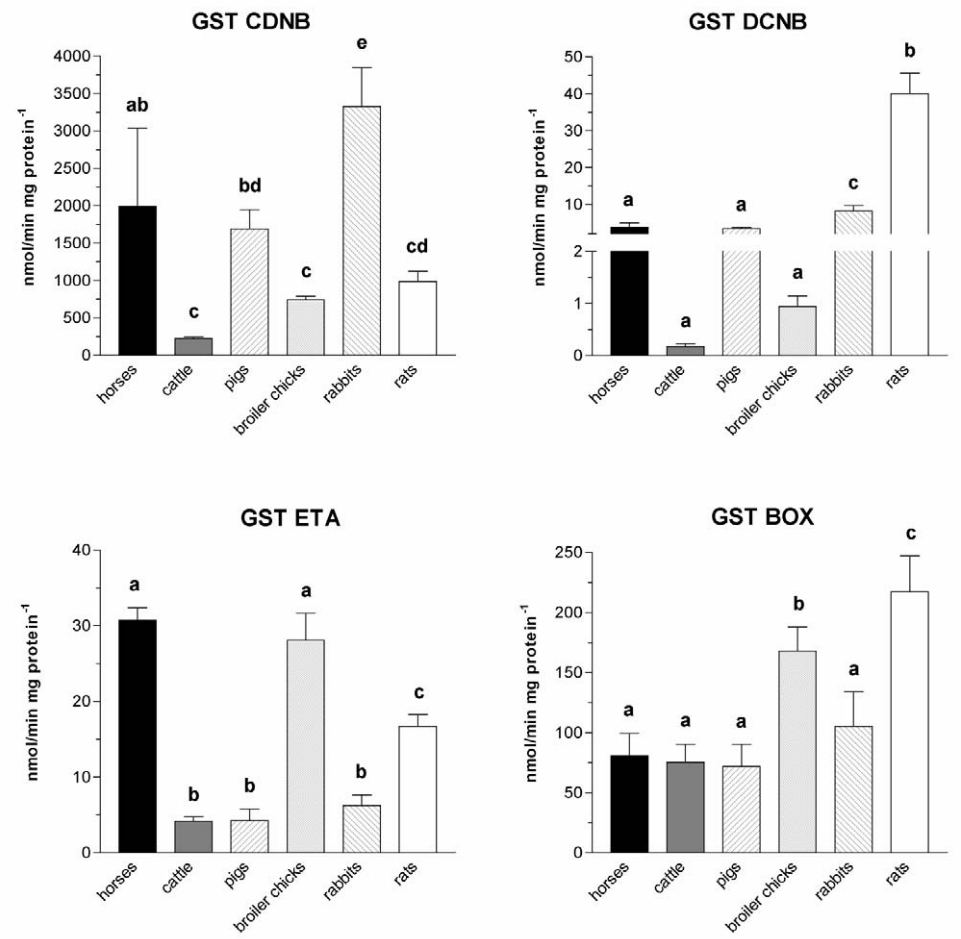

Figure 1. Comparative glutathione *S*-transferase activities measured with different substrates in liver cytosolic subfractions from horses, cattle, pigs, broiler chicks, rabbits and rats; values are mean $\pm$ SD, $n = 6$ for each species; [a,b,c,d]Bars with different superscripts vary significantly ($p < 0.05$ or less)

comparatively low but detectable activity was found in broiler chick microsomes.

Rabbit cytosols acetylated INH to a 3-fold to 11-fold greater extent than those from any other species. This difference became even wider with PABA: rabbit NAT activity towards this substrate exceeded that recorded in the other species by a factor of at least 25, aside from pigs, which showed statistically significant lower ($p < 0.05$) but comparable values. Unlike broiler chicks, horses displayed low but measurable activity values.

Figure 1 depicts the comparative activity of GSTs accepting four different substrates. For total GST (CDNB), rabbits, horses and pigs had the highest values. Most notably,

cattle preparations were characterized by very low activities ranging from about 1/3rd to 1/15th of those recorded in the other food-producing species. A similar pattern occurred also for GST-DCNB. Much lower differences between species were noted in the conjugation of ETA. In this respect also, equine cytosols exhibited a remarkable capacity, together with broiler chick and rat subfractions. Rabbits, cattle and pigs displayed statistically significantly ($p < 0.05$) lower values with a 4-fold to 5-fold variation with respect to the horse. The extent of BOX conjugation was similar in all cytosolic subfractions, except for rat and chicken, which showed activities about twice as high as the other species.

Amounts of NPSH averaging 6–7 μmol/g liver were measured in horse, rabbit and pig cytosols; statistically significantly ($p < 0.05$ or less) lower contents (2–5 μmol/g liver) were measured in rat, chicken and cattle preparations (data not shown).

DISCUSSION

The main aim of this study was to complete a previous investigation about the comparative oxidative biotransformation pattern in some of the most important food-producing species, with rat as the reference species (Nebbia *et al.*, 2003).

Relatively few comparative studies concerning carboxylesterases are cited in the literature (Satoh and Hosokawa, 1995). The rate of hydrolysis of IPA or PNPA showed a considerable interspecies variation according to the nature of the substrate. While rabbit and pig preparations on the one hand, and horse and broiler chick preparations on the other were respectively the most and the least active with either substrate, on a comparative basis bovine microsomes metabolized PNPA to a far greater extent than IPA. These results point to the involvement of different isoenzymes in the metabolism of these esters. Indeed, several carboxylesterase isoforms have been purified from liver microsomes of various mammalian species. In particular, rat liver microsomes were found to contain three different isoenzymes, named RL1, RL2 and RH1, which differ considerably from each other in inducibility, substrate specificity and immunological properties (Hosokawa *et al.*, 1987), and a cross-reaction with anti-RH1 rat antibodies was detected in the same preparations from cows, pigs and rabbits. For these species, activities towards PNPA were reported to be very similar to those recorded in the present study (Satoh and Hosokawa, 1995). It is worth noting that the horse proved to be among the least active in the metabolism of either substrate and that there appear to be remarkable sex-related differences in enzyme activity (Carletti, 1999). The (carboxyl)esterase-mediated hydrolytic reactions represent a very important step in the activation/deactivation of many drugs as well as in the detoxification of several pesticides including pyrethroids, carbamates and organophosphorus compounds (Jokanovic, 2001). Interestingly, Atterberry and colleagues (1997) reported in male rats of different ages a strong correlation between the activity of microsomal tissue esterase accepting *p*-nitrophenylvalerate as substrate and the sensitivity to parathion or chlorpyrifos.

Glucuronidation represents one of the major phase II reactions in the metabolism of drugs and toxicants as well as of endobiotics. Mammalian microsomal UGTs have

been grouped into two distinct families, UGT1 and UGT2, which in humans are reported to metabolize mainly bilirubin and phenols (UGT1) or steroids (UGT2). They are characterized by overlapping substrate specificities so that, at least in humans, both are involved in steroid glucuronidation (Bock, 2002). The probes used in our study are both reported to be specific for the UGT1A subfamily in humans (Radominska-Pandya *et al.*, 1999). On the whole, among the food-producing species examined in this study, rabbits, pigs and horses showed the maximal glucuronidating capacity towards the phenolic substrates employed. In this respect, it should be noted that proteins cross-reacting with anti-human UGT1A have recently been detected in liver microsomes from all the above species (Court, 2001). As regards the pig, the glucuronidative pathway may serve to compensate for the relative poor ability in the sulphation of several aromatic derivatives displayed by the swine species (Glatt, 2002). Conversely, in line with observations by Coulet and colleagues (1996) and Watkins and Klaassen (1986), respectively, microsomes from broiler chicks and cattle displayed a relatively low conjugation rate.

N-Acetyltransferases are widely distributed among animal species and are active in the detoxification of arylamine and arylhydrazine drugs, including sulfonamides and isoniazid. In humans and in other mammalian species, two families of the enzyme have been recognized and called NAT1 and NAT2 (Levy and Weber, 2002). Isoniazid and PABA, the substrates selected for this study, are believed to be specific for NAT2 and NAT1, respectively, in mice and humans (Levy and Weber, 2002). As expected (Watkins and Klaassen, 1986), interspecies variations in NAT activities were wider than for any other tested enzyme system. Our results confirmed the extremely high acetylating capacity of rabbits (Levy and Weber, 2002), as well as the poor acetylation tendency of broiler chicks (Coulet *et al.*, 1996), which apparently lack any activity towards the NAT1 substrate PABA. Pigs also appeared to be good acetylators, and lower but measurable activities towards either substrate were observed in equine preparations. Interestingly, the acetylating capacity of horses has been questioned (Schmid and Schmid, 1994), although the N^4-acetylation of several sulfonamides has been reported in this species (Van Duijkeren *et al.*, 1994). Acetylation defects have been documented in other species: the domestic dog and other canids lack cytosolic NAT owing to the absence of NAT genes (Trepanier *et al.*, 1997), whereas in domestic and wild felids only NAT1 appears to be expressed. In addition, at least in humans and rabbits, the most common cause of altered NAT activity is genetic polymorphisms of the NAT enzymes, resulting in 'low' or 'fast' acetylator phenotypes (Meyer and Zanger, 1997). Although a low or absent NAT activity may enhance the toxicity of certain drugs like sulfonamides, isoniazid or procainamide, other metabolic routes involving microsomal *N*,*O*-acyltransferases may adequately overcome these apparent deficiencies (Levy and Weber, 2002).

The GSTs are a multigene family of enzymes whose isoforms exhibit different but often overlapping specificity for both endogenous and exogenous compounds and are expressed to very high levels in liver. They catalyse the nucleophilic attack of GSH on a wide array of electrophilic substrates and have been implicated in the detoxification of plant phenols, mycotoxins (including aflatoxin B_1), many chemical carcinogens and pesticides as well as certain reactive oxygen species (Eaton and Bammler, 1999). In

TABLE III
Substrate specificity of rat, mouse, human, cattle and rabbit GST classes

GST class	Substrate	Species
μ	DCNB	Rat, mouse, man, rabbit
π	ETA	Rat, mouse, man, cattle
θ	BOX	Rat, mouse, man

addition, they are involved in steroid metabolism and in prostaglandin synthesis (Sheehan *et al.*, 2001). Based on physical, catalytical and sequence similarities, four main classes of cytosolic GSTs (α, μ, π and θ) have been identified in humans, rats and mice, each of them containing different isoenzymes (Sherratt and Hayes, 2002). Relatively little information is available for species such as cattle (Hayes *et al.*, 1989), rabbits (Primiano and Novak, 1993), pigs (Rouimi *et al.*, 1996), chicks (Hsieh *et al.*, 1999) and horses (D'Silva, 1990). Taking into account the already mentioned overlapping substrate specificities, CDNB is classified as the more general substrate for all GST classes, while DCNB, ETA, and BOX are generally considered markers for μ, π and θ classes, in a number of species (Table III) (Ketterer and Mulder, 1990; Geisler and Olshan, 2001).

In line with the results of previous investigations (Juskevich, 1987; Sivapathasundaram *et al.*, 2003), in our study the GST activity towards CDNB in livestock species was comparable to or higher than that found in the rat, with the notable exception of cattle, which exhibited a consistently reduced efficiency in the conjugation of this and of the other tested substrates, especially when compared with rabbits, horses and pigs. This may have practical implications; for example, it was reported that the GST-CDNB activity in rat hepatocytes was about 6-fold higher than in bovines, and this feature has been inversely related to the different hepatic cytotoxic potency of aflatoxin B_1 in primary cultures from either species (Kuilman *et al.*, 2000). More to the point, as detected by measuring the rate of DCNB or ETA conjugation, cattle would also express the μ and the π GST classes to a very low extent. Conversely, according to our results, horses showed a comparatively good ability in conjugating all the tested GSH-dependent substrates, and particularly ETA, a specific marker for the π class. It remains to be established whether these apparent wide variations could have practical implications other than those envisaged above for aflatoxin B1, especially because the role played by GSTs in endobiotic metabolism is not yet well elucidated for livestock species.

In conclusion, remarkable differences in the activity and possibly the expression of several hydrolytic and conjugative XMEs appear to occur not only between the tested food-producing species and the rat, but also between minor and major species. Taking due consideration of the limitations of the *in vitro* approach adopted in this study, and being aware of the fact that a number of factors including age, sex, diet, and exposure

to drugs and environmental pollutants, as well as the occurrence of genetic polymorphisms, may modulate XME activity, the results generated from this investigation may provide a rationale for the understanding of the species-related susceptibilities to a number of poisons; and they stress once again the need for caution in extrapolating to minor species data on kinetic, efficacy and safety of drugs derived from studies performed in the major species.

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