Biochemical Pharmacology, Vol. 37, No. 18, pp. 3461–3465, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

IMPAIRMENT OF SULFOBROMOPHTHALEIN BILIARY EXCRETION AND INHIBITION OF GLUTATHIONE *S*-TRANSFERASE ACTIVITY INDUCED BY PERHEXILINE MALEATE IN RATS

MARIA A. MARISCAL, MARIA E. MUÑOZ, PILAR S. COLLADO, ALEJANDRO ESTELLER and JAVIER GONZALEZ*

Department of Physiology and Pharmacology, University of Salamanca and *Department of Physiology, Pharmacology and Toxicology, University of León, Spain

Abstract—The effect of the antianginal agent perhexiline maleate (160 mg/kg i.g., daily for 4 days) on the biliary excretion of sulfobromophthalein (BSP) and BSP-glutathione and the hepatic activity of glutathione *S*-transferases was investigated in Wistar rats. Perhexiline maleate caused a significant reduction in the maximal biliary excretion of BSP (−28%). The decrease corresponded to a lowered excretion of the conjugated dye whereas the excretion of the parent compound did not change significantly. Administration of the drug caused no effect on the maximal biliary excretion of infused BSP-glutathione. Liver glutathione concentrations were similar in control and treated rats. Perhexiline maleate significantly reduced liver glutathione *S*-transferase activities toward BSP (−25%), 3,4-dichloronitrobenzene (DCNB) (−21%) and 1-chloro-3,4-dinitrobenzene (DNCB) (−27%). Kinetic studies of the enzyme in liver cytosol showed that perhexiline maleate induced an uncompetitive inhibition for the BSP substrate with a reduced V_{max} and K_m. The results indicate that the reduction in glutathione *S*-transferase activity plays an important role as a factor determining the impairment in the hepatobiliary transport of BSP caused by perhexiline maleate.

Perhexiline maleate, 2-(2,2-dicyclohexylethyl)-piperidine maleate is a useful antianginal agent [1] that is excreted primarily in faeces after biotransformation by hydroxylation of the cyclohexyl rings [2, 3]. Following treatment with the drug the occasional development of hepatic lesions has been described, accompanied by storage in the liver of phospholipids, gangliosides, triglycerides and fatty acids [4]. Because of this hepatic toxicity the drug has been retired from clinical use in many countries. The alterations in the hepatic metabolism have also been found in the rat [5] and in this species it has been reported a depressed liver transport of BSP with increased plasma retention of the dye [6].

The elimination of BSP from plasma depends on a series of processes including the transfer from plasma to liver, conjugation with reduced glutathione by means of a glutathione *S*-transferase and canalicular secretion of the conjugated dye [7]. Alteration of any of these processes can change the rate of BSP excretion. Conjugation plays an extremely important role and it has been demonstrated that the depletion of hepatic glutathione [8, 9] or inhibition of glutathione *S*-transferase activity [10, 11] can significantly reduce BSP excretion into bile.

The purpose of this study was to investigate further the effect of perhexiline maleate on the hepatic transport of BSP in the rat and to elucidate whether a reduction in the conjugating capacity of the liver might be responsible for the impaired excretion of the organic anion induced by the drug.

MATERIALS AND METHODS

Chemicals. Sulfobromophthalein, 1-chloro-3,4-dinitrobenzene, 5,5′-dithiobis-(2-nitrobenzoic acid), glutathione, glutathione reductase and perhexiline maleate were purchased from Sigma Chemical Co. (St. Louis, MO). 3,4-Dichloronitrobenzene was obtained from Aldrich Chemie (Steinheim, F.R.G.). BSP-glutathione conjugate was synthesized from BSP by the method of Whelan *et al.* [12] and separated from the BSP and BSP-diglutathione in a Sephadex G-10 column [8]. All other reagents were of the highest quality available. Distilled deionized water was used throughout.

Animals and experimental procedures. Male Wistar rats (Sepal, Madrid, Spain) weighing 210–250 g were housed in stainless steel cages in a temperature controlled room (20–22°) under a 12 hr dark/light cycle. The animals were provided with commercial pelleted food (standard diet from Panlab, Barcelona, Spain) and water *ad libitum* until used. Perhexiline maleate (160 mg/kg) suspended in 4 ml/kg of undiluted propylene glycol were given daily for 4 days by gastric intubation. Controls received propylene glycol.

On the 5th day, the rats were anaesthetized i.p. with sodium pentobarbitone (Nembutal, Abbott Laboratories, Madrid, Spain; 50 mg/kg). The left carotid artery and left jugular vein were catheterized and the common bile duct was cannulated with PE-50 tube. Rectal temperature was monitored via a

thermistor probe and maintained at 37° by means of a thermostatically controlled heating table.

After collecting two 10 min baseline samples of bile, rats were infused intravenously for 60 min with 215 nmol/min/100 g of BSP after a priming dose of 2.15 μmol/100 g or with 500 nmol/min/100 g of BSP-glutathione conjugate after a priming dose of 5 μmol/100 g. Blood and bile samples were collected at 10 min intervals. The animals were killed by exsanguination at the end of the experiments and the livers excised, washed with 0.154 M NaCl and weighed.

To study plasma BSP disappearance, blood samples from the carotid artery were collected in heparinized micro-tubes at 1, 3, 5, 10, 20 and 30 min following injection of a single intravenous dose of the dye (120 μmol/kg) in both the untreated and perhexiline maleate-treated rats.

Analytical procedures. Bile flow was measured gravimetrically assuming a specific gravity of 1.0 g/ml. Blood and bile BSP concentration were determined spectrophotometrically at 580 nm after appropriate dilution with 0.1 M NaOH. Dye content in the liver was determined by the method of Whelan and Combes [13]. The relative amounts of conjugated and free BSP in bile were estimated by ascending chromatography on Whatman No. 1 paper using butan-1-ol/acetic acid/ethanol/water (120:1:20:40) as solvent system. The spots were cut, eluted with water and read at 580 nm after alkalinization. Bile acid concentration in bile was determined enzimatically by the 3-α-hydroxysteroid dehydrogenase method of Paumgartner *et al.* [14] with sodium taurocholate as standard. Determination of total glutathione content of the liver homogenates prepared in cold 5% (w/v) trichloroacetic acid in 0.01 N HCl was carried out as described by Tietze [15] with the modification of Griffith [16]. Glutathione *S*-transferase activities were determined using BSP, DCNB or DNCB [17] as substrates. All the enzyme assays were run under conditions of maximal initial velocity and were linear with time. Protein concentration was measured by the method of Lowry *et al.* [18].

Pharmacokinetic parameters of plasma BSP disappearance were calculated with a two compartment open model [19].

Means and SEM were calculated for all data. Significant differences were determined by the non parametric Mann–Whitney U test. P values of less than 0.05 were considered to be significant.

RESULTS

Perhexiline maleate treatment slightly but non-significantly modified the liver/body weight ratio (3.3 ± 0.3 g/100 g vs 3.5 ± 0.2 g/100 g in untreated rats). Administration of perhexiline maleate caused a significant decrease both in basal bile flow (5.7 ± 0.4 μl/min/100 g vs 6.8 ± 0.6 μl/min/100 g in the controls) and the basal secretion of bile acids (148 ± 8 nmol/min/100 g vs 189 ± 10 nmol/min/100 g).

The maximal biliary excretion of total BSP following its intravenous infusion for 60 min was significantly lowered by perhexiline maleate compared with the untreated rats (−28%), while plasma levels were significantly higher (Fig. 1). Bile flow was also significantly reduced to a greater extent than that of the basal period (−28%) (Fig. 1). The decrease in BSP excretion was exclusively due to a lowered excretion of conjugated BSP whereas the excretion of the parent compound did not change significantly (Table 1). These modifications were accompanied by a significant rise in hepatic BSP (Table 1).

When pharmacokinetic parameters of plasma BSP disappearance were calculated following a single 120 μmol/kg injection, no significant difference was found for the value of K_α between untreated and perhexiline maleate-treated rats ($K_\alpha = 0.284 \pm 0.015\ min^{-1}$ vs $K_\alpha = 0.277 \pm 0.045\ min^{-1}$; N = 4).

Figure 1 also demonstrates the effect of perhexiline maleate treatment on the biliary excretion of BSP-glutathione. Maximal biliary excretion and plasma concentration of the dye were not affected by the drug. Bile flow was significantly reduced although the change detected was similar to that of the basal period (−1 μl/min/100 g). BSP-glutathione liver concentrations at the end of the experiments were similar in the untreated and perhexiline maleate treated rats (0.20 ± 0.02 and 0.23 ± 0.03 μmol/g liver).

The effect of perhexiline maleate on glutathione *S*-transferase activity and glutathione concentration on liver is shown in Table 2. In the group receiving the drug, glutathione S-transferase activities dropped significantly with BSP (−25%), DNCB (−27%) and DCNB (−21%) as substrates. The levels of liver glutathione and cytosol protein were not significantly modified (Table 2). Figure 2 illustrates the apparent kinetic behaviour of BSP glutathione *S*-transferase activity in liver cytosol isolated from untreated and perhexiline maleate-treated rats. Both the apparent K_m (0.059 ± 0.004 mM) and the apparent V_{max} (32 ± 2.2 nmol/min/mg protein) from treated rats were significantly lower than the apparent K_m (0.096 ± 0.007 mM; $P < 0.05$) and the apparent V_{max} (54.82 ± 4.2 nmol/min/mg protein; $P < 0.05$) from untreated animals. To confirm the inhibitory effect of the drug on transferase activity, additional assays with liver cytosol from untreated rats were carried out in the presence of different concentrations of perhexiline maleate (20–40 μM). The results obtained further support the uncompetitive type of inhibition of the enzyme activity (data not shown) with an apparent K_i of $2.8\ 10^{-5}$ M.

DISCUSSION

The antianginal agent perhexiline maleate occasionally produces liver disturbances in some patients [4]. In the rat, short term administration of high doses of the drug has been reported to depress maximum liver transport of BSP [6], but the exact mechanism responsible for the impairment of BSP excretion has not been elucidated.

As shown by our results, perhexiline maleate treatment in rats significantly reduces the maximal biliary excretion of BSP and this is accompanied by a decrease in conjugated BSP in bile and an accumulation of dye in the liver. Nevertheless, when BSP-glutathione (which does not require bio-

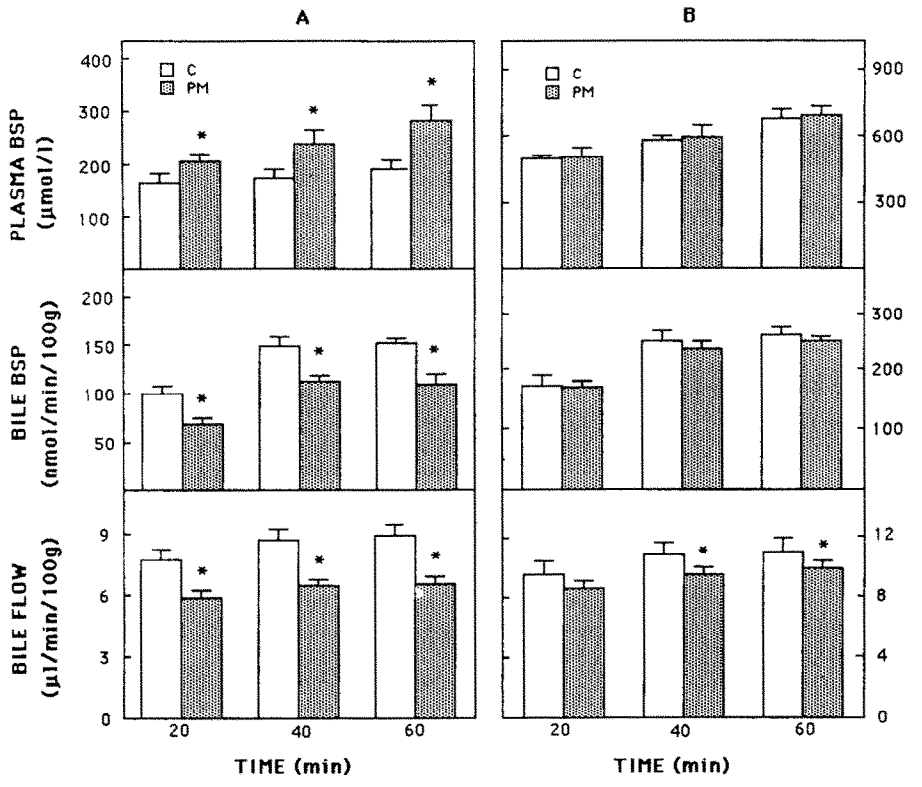

Fig. 1. (A) Effect of perhexiline maleate on plasma concentration and biliary excretion of BSP. After a priming dose of 2.15 μmol/100 g BSP was infused for 60 min at 215 nmol/min/100 g in untreated (C) and perhexiline maleate-treated (PM) (160 mg/kg i.g., daily for 4 days) rats. Means ± SEM for six to eight animals. *P < 0.05. (B) Effect of perhexiline maleate on plasma concentration and biliary excretion of BSP-glutathione. After a priming dose of 5 μmol/100 g BSP-glutathione was infused for 60 min at 500 nmol/min/100 g in untreated (C) and perhexiline maleate-treated (PM) (160 mg/kg i.g., daily for 4 days) rats. Means ± SEM for six to eight animals. *P < 0.05.

transformation prior to its excretion into bile) is infused, no differences in excretion are found between the control and perhexiline maleate-treated rats.

Some explanations can be put forward to account for this effect. Cholephilic anions have been classified as bile acid-dependent or -independent according to whether its excretion is affected or not by modifications in bile acid secretion. BSP is included in the first group because its biliary excretion is either increased following injection of bile acids [20] or depressed by cholestyramine treatment [21]. On the contrary, BSP-glutathione excretion is not affected by bile acids [20, 21]. It could be speculated that the impairment found for bile acid secretion in perhexiline maleate-treated rats might be responsible for the lowered excretion of BSP. However, although this would explain the slight reduction in the excretion of the unconjugated dye it could not account for the decrease in that of conjugated BSP. Neither could the reduction in bile flow be responsible for the lowered excretion of BSP-glutathione, because changes in bile acid-dependent [21, 22] or -independent bile flow [23] do not apparently modify the maximal biliary excretion of the conjugated, administered as such, in different species.

Table 1. Effect of perhexiline maleate on biliary excretion of conjugated and unconjugated BSP and on BSP liver storage

Treatment	Conjugated BSP (nmol/min/100 g)	Unconjugated BSP (nmol/min/100 g)	Liver BSP (μmol/g liver)
Untreated	139 ± 7	18 ± 3	0.47 ± 0.03
Perhexiline maleate	103 ± 6*	16 ± 2	0.82 ± 0.05*

Perhexiline maleate was given at 160 mg/kg i.g., daily for 4 days. BSP was infused 60 min at 215 nmol/min/100 g after a priming dose of 2.15 μmol/100 g.

Values are means ± SEM for five to eight animals and correspond to 20–40 min after beginning of infusion. * P < 0.05.

Table 2. Effect of perhexiline maleate on glutathione S-transferase activities and glutathione concentration in liver

Treatment	Cytosolic protein (mg/g liver)	Glutathione S-transferase activity (nmol/min/mg protein) DNCB	DCNB	BSP	Glutathione (μmol/g liver)
Untreated	89.4 ± 3.0	678 ± 22	309 ± 14	32.0 ± 1.5	4.04 ± 0.29
Perhexiline maleate	93.7 ± 3.1	492 ± 20*	244 ± 9*	24.0 ± 0.8*	3.75 ± 0.25

Perhexiline maleate was given at 160 mg/kg i.g., daily for 4 days. Values are means ± SEM for five to eight animals. * $P < 0.05$.

Another mechanism that might significantly affect biliary BSP excretion would be that of a modification in the conjugation of the organic anion. The conjugation process, although it may be the result of non-enzymatic binding to glutathione, mainly involves reaction with reduced glutathione by a cytosolic enzyme, glutathione S-transferase [24]. The importance of conjugation for biliary excretion is clearly shown by the fact that BSP excretion is decreased following depletion of glutathione hepatic content by diethyl maleate [8, 9], iodomethane [25] and administration of a protein-free diet [26] or when glutathione S-transferase activity is inhibited by benzodiarone [27], organic analogs of metals [28] and hypolipidemic drugs [10, 11].

In the present study no significant changes in the hepatic concentration of glutathione were found following treatment with perhexiline maleate, which allows to rule out a depletion of the tripeptide as the cause for the lowered transport of BSP. However, the activities of different isoenzymes of glutathione S-transferase were clearly inhibited. This would reduce the quantity of conjugated BSP available for excretion and it is known that the conjugate shows a higher excretion rate than the parent compound [13, 29]. Additionally the inhibition of BSP-glutathione excretion induced by BSP [30] would be favoured. When studying the kinetics of the inhibitory action of perhexiline maleate on glutathione S-transferase activity, it was found that the inhibition was uncompetitive for BSP, the decrease in the conjugating activity mainly being due to a decrease both in the apparent V_{max} and the K_m. In this sense our results differ from the noncompetitive inhibition found for indomethacin [31], 1,2-dibromoethane [32] or clofibrate [10] with respect to DCNB and BSP and from those found for propylthiouracyl [33] or sulfasalazine [34] which show competitive kinetics with respect to glutathione.

The higher BSP plasma levels observed in rats receiving perhexiline maleate are apparently not a consequence of the depressed primary uptake, because no alteration was found in K_a values, that have been assumed to represent transfer from plasma to liver [35]. The effect induced by perhexiline maleate would result solely from the diminished transport of BSP from liver to bile and the increased plasma concentrations of the organic anion could represent a sinusoidal release and/or regurgitation of the dye via permeabilized paracellular pathways.

In summary, this study demonstrates that perhexiline maleate reduces liver glutathione S-transferase activities and that this effect would be responsible for the impairment in the biliary excretion of compounds that are biotransformed by hepatic conjugation with glutathione.

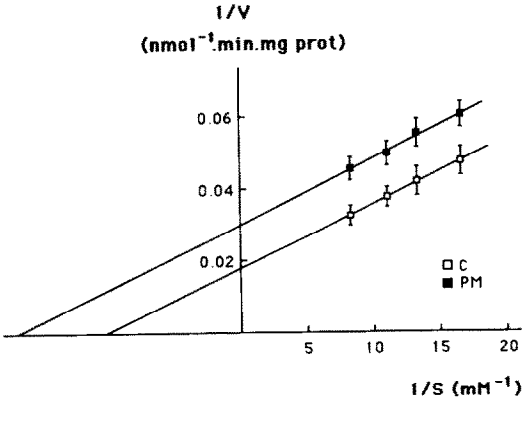

Fig. 2. Lineweaver–Burk plots showing the BSP-glutathione S-transferase activity of liver cytosol from untreated (C) or perhexiline maleate-treated (PM) (160 mg/kg i.g., daily for 4 days) rats in the presence of various concentrations of BSP. Means ± SEM for six to eight animals.

REFERENCES

1. Lyon LF, Nevins MA, Fisch S and Henry S, Perhexiline maleate in treatment of angina pectoris. *Lancet* **1**: 1272–1274, 1971.
2. Leeson GA, Lang JF, Zeiger AV, Hudek WJ and Wright G, Excretion, blood levels and tissue retention of perhexiline-^{14}C maleate in dogs. *Pharmacologist* **11**: 28, 1969.
3. Wright GJ, Leeson GA, Zeiger AV and Lang JF, The absorption, excretion and metabolism of perhexiline maleate by the human. *Postgrad Med J* **49**: 8–15, 1973.
4. Poupon M, Rosensztajn L, Prudomme de Sant Maur P, Lagenon H, Gombean P. and Darnin F, Perhexiline maleate associated hepatic injury. Prevalence and characteristics. *Digestion* **2**: 145–150, 1980.
5. Hoenig W and Werner Fr, Effect of perhexiline maleate on lipid metabolism in the rat. *Arzneim Forsch* **29**: 1395–139, 1979.
6. Hoenig W and Werner Fr, Effect of perhexiline maleate on bile formation and liver disposition of sulfobromophthalein in the rat. *Pharmacol Res Commun* **12**: 931–93, 1980.
7. Klaassen CD and Plaa GL, Species variation in metabolism, storage and excretion of sulfobromophthalein. *Toxicol Appl Pharmacol* **12**: 132–13, 1967.

8. Varga F, Fischer E, and Szily TS, Biliary excretion of bromosulphthalein in rats pretreated with diethyl maleate. *Biochem Pharmacol* **2**: 2617–2623, 1974.
9. Aza MJ, González J and Esteller A, Effect of diethyl maleate pretreatment on biliary excretion and choleretic action of sulfobromophthalein in rats. *Arch Int Pharmacodyn Ther* **28**: 321–333, 1986.
10. Foliot A, Touchard D and Celier C, Impairment of hepatic glutathione *S*-transferase activity as a cause of reduced biliary sulfobromophthalein excretion in clofibrate-treated rats. *Biochem Pharmacol* **33**: 2829–2834, 1984.
11. Foliot A, Touchard D and Mallet L, Inhibition of liver glutathione *S*-transferase activity in rats by hypolipidemic drugs related or unrelated to clofibrate. *Biochem Pharmacol* **35**: 1685–169, 1986.
12. Whelan G, Hoch and Combes B, A direct assessment of the importance of conjugation for biliary transport of sulfobromophthalein sodium. *J. Lab Clin Med* **75**: 542–54, 1970.
13. Whelan G and Combes B, Competition by unconjugated and conjugated sulfobromophthalein sodium (BSP) for transport into bile. Evidence for a single excretory system. *J. Lab Clin Med* **78**: 230–24, 1971.
14. Paumgartner G, Horak W, Probst P and Grabner G, Effect of phenobarbital on bile flow and bile salt excretion in the rat. *Naunyn-Schmiedeberg's Arch Pharmacol* **270**: 98–10, 1971.
15. Tietze F, Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Applications to mammalian blood and other tissues. *Analyt Biochem* **27**: 502–522, 1969.
16. Griffith OW, Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* **106**: 207–212, 1980.
17. Habig WH, Pabst MJ and Jakoby WB, Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
18. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin Phenol reagent. *J Biol Chem* **193**: 265–272, 1951.
19. D'Argenio D and Schumitzky A, A program package for simulation and parameter estimation in pharmacokinetic system. *Comp Prog Biomed* **9**: 115–134, 1979.
20. Gregus Z and Fischer E, Effect of sodium taurocholate on hepatic uptake and biliary excretion of organic anions in rats. *Arch Int Pharmacodyn Ther* **24**: 180–192, 1979.
21. Gregus Z, Fischer E and Varga F, Effect of cholestyramine-induced bile acid depletion on the hepatobiliary transport of cholephilic organic anions in rats. *Arch Int Pharmacodyn Ther* **24**: 311–321, 1980.
22. Fischer E and Varga F, Effect of taurocholate pretreatment on the excretion of exogenous organic anions in rats. *Arch Int Pharmacodyn Ther* **267**: 1287–198, 1984.
23. Barnhart JL and Combes B, Biliary excretion of dye in dogs infused with BSP or its glutathione conjugate. *Am J Physiol* **231**: 399–407, 1976.
24. Combes B and Stakelum GS, Conjugation of sulfobromophthalein sodium with glutathione in thioether linkage by the rat. *J Clin Invest* **39**: 1214–1222, 1960.
25. Priestly BG and Plaa GL, Sulfobromophthalein metabolism in rats with iodomethane-induced depletion of hepatic glutathione. *J Pharmacol Exp Ther* **174**: 221–231, 1970.
26. Combes B, The importance of conjugation with glutathione for sulfoboromophthalein sodium (BSP) transfer from blood to bile. *J Clin Invest* **44**: 1214–1224, 1965.
27. Priestly BG and Plaa G, Effects of benzodiarone on the metabolism and biliary excretion of sulfobromophthalein and related dyes. *Proc Soc Exp Biol Med* **132**: 881–885, 1969.
28. Byington HK and Hansbrough E, Inhibition of the enzymatic activity of ligandin by organogermanium, organolead or organotin compounds and the biliary excretion of sulfobromophthalein by the rat. *J Pharmacol Exp Ther* **208**: 248–253, 1979.
29. Fischer E, Gregus Z and Gogl A, Hepatic transport of sulfobromophthalein and sulfobromophthalein glutathione conjugate in control and phenobarbital-pretreated rats. *Acta Physiol Acad Sci Hung* **51**: 61–66, 1978.
30. Gregus Z, Fischer E and Varga F, Inhibition by sulfobromophthalein of the biliary excretion of its glutathione conjugate. *Biochem Pharmacol* **261**: 1951–1952, 1977.
31. Wu C and Mathews KP, Indomethacin inhibition of glutathione *S*-transferases. *Biochem Biophys Res Commun* **112**: 980–984, 1983.
32. Botti B, Moslen MT, Trieff NM and Reynolds ES, Transient decrease of liver cytosolic glutathione *S*-transferase activities in rats given 1,2-dibromoethane or CCL_4 *Chem-Biol Interact* **42**: 259–270, 1982.
33. Kariya K, Sawahata T, Okuro S and Lee E, Inhibition of hepatic glutathione transferases by propylthiouracyl and its metabolites. *Biochem Pharmacol* **35**: 1475–1479, 1986.
34. Bach MK, Brashler JR and Johnson MA, Inhibition by sulfasalazine of LTC synthetase and of rat liver glutathione *S*-transferases. *Biochem Pharmacol* **34**: 2695–2704, 1985.
35. Hacki W, Burcher J and Preisig R, A new look at the plasma disappearance of sulfobromophthalein (BSP): Correlation with the BSP transport maximum and the plasma flow in man. *J Lab Clin Med* **88**, 1019–1031, 1976.