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GLUTATHIONE CONJUGATION OF THE α-BROMOISOVALERIC ACID ENANTIOMERS IN THE RAT *IN VIVO* AND ITS STEREOSELECTIVITY

PHARMACOKINETICS OF BILIARY AND URINARY EXCRETION OF THE GLUTATHIONE CONJUGATE AND THE MERCAPTURATE

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Abstract—The glutathione (GSH) conjugation of (*R*)- and (*S*)-α-bromoisovaleric acid (BI) in the rat *in vivo*, and its stereoselectivity, have been characterized. After administration of racemic [1-^{14}C]BI two radioactive metabolites were found in bile: only one of the possible diastereomeric BI-GSH conjugates, (*R*)-I-S-G (35 ± 2% of the dose), and an unidentified metabolite "X" (6 ± 1%). In urine, only one of the possible BI-mercapturates, (*R*)-I-S-MA (14 ± 1%), minor unidentified polar metabolites (5 ± 1%) and unchanged BI (13 ± 2%) were excreted. When (*R*) or (*S*)-BI were administered separately, the same metabolites were found. However, a ten-fold difference in excretion half lives of the biliary metabolites was observed following (*S*)- and (*R*)-BI administration, (*S*)-BI being more rapidly excreted. The excretion of the mercapturate in urine shows the same difference in excretion rate: its half life after administration of (*R*)-BI was more than 10 times longer than after a dose of (*S*)-BI. More of the dose of (*S*)-BI was excreted after 5 hr in bile and urine: 58% and 23% respectively for (*S*)- and (*R*)-BI. Therefore, a pronounced stereoselectivity in GSH conjugation exists for the (*R*) and (*S*) enantiomers of BI in the rat *in vivo*, which is a major determinant of their pharmacokinetics. The results suggest that (slow) inversion of the chiral centre of BI occurred in the rat *in vivo*.

In the detoxication of many xenobiotics, glutathione conjugation plays an important role [1, 2]. Recent work from our group showed that α-bromoisovalerylurea (BIU)† is a suitable model substrate to study the pharmacokinetics and stereoselectivity of the glutathione conjugation *in vivo* [3, 4]. Beside conjugation with glutathione (GSH), hydrolysis to α-bromoisovaleric acid (BI) and urea [5] occurs. Such alternative metabolism is a disadvantage for a model substrate, which, ideally, should be converted only by one pathway.

The product of this hydrolysis, BI, also is a substrate for GSH conjugation [5–7]; it is, however, not a substrate for the amidase catalysed hydrolysis exhibited by BIU [5]. Therefore, we considered its use as an alternative model substrate for GSH conjugation *in vivo*. BI also contains a chiral center, so (*R*) and (*S*) enantiomers exist. Incubations of (*R*)- and (*S*)-BI with isolated hepatocytes and purified glutathione-*S*-transferases (GST) showed stereoselectivity in the conjugation with glutathione [5, 7].

BI may have several advantages compared to BIU as a model substrate for the GSH conjugation: it has a higher water solubility, is more stable and less competing metabolism is expected. The present work was done to characterize BI glutathione conjugation in the rat *in vivo*, including its stereoselectivity.

MATERIALS AND METHODS

Chemicals. α-Bromoisovaleric acid (2-bromo-3-methylbutanoic acid) was purchased from Aldrich Chemicals Co. (Milwaukee, WI). The (*R*) and (*S*) enantiomer of BI were separated as described elsewhere [7]. The sodium salt of [1-^{14}C]isovaleric acid (52 mCi/mmol, aqueous solution) was purchased from Medgenix BV IRE (Soesterberg, The Netherlands). 1-Decanesulfonic acid, sodium salt (98%), was from Janssen Chimica (Beerse, Belgium). Liquid scintillation fluid (Emulsifier-Safe) was from Packard Instrument Company, Inc. (Downers Grove, IL).

Animals. Male Wistar rats (SPF, 200–250 g body wt), of the Sylvius Laboratories, University of Leiden, were used. The rats were housed in Macrolon cages on standard hard wood bedding. The animals had free access to tap water and standard lab chow (SRM-A, Hope Farms, Woerden, The Netherlands). Lights were on from 6 a.m. to 6 p.m.

Synthesis of [1-^{14}C]*α-bromoisovaleric acid.* The sodium salt of [1-^{14}C]isovaleric acid was provided in an aqueous solution; the water had to be removed because it would interfere with the synthesis. The tracer amount of 100 μCi of the sodium salt of [1-^{14}C]isovaleric acid (200 μl) was mixed with a solution of the sodium salt of unlabelled isovaleric acid in ethanol (300 μl; 0.25 mmol; the ethanol was dried on a molecular sieve of 3 A). This mixture was dried

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† Abbreviations: BI, α-bromoisovaleric acid; BIU, α-bromoisovalerylurea; I-S-G, α-[glutathion-*S*-yl]-isovaleric acid; I-S-MA, α-[*N*-acetyl-L-cystein-*S*-yl]-isovaleric acid; GSH, glutathione; GST, glutathione-*S*-transferases; γ-GT, γ-glutamyl transpeptidase; ECD, electrochemical detection.

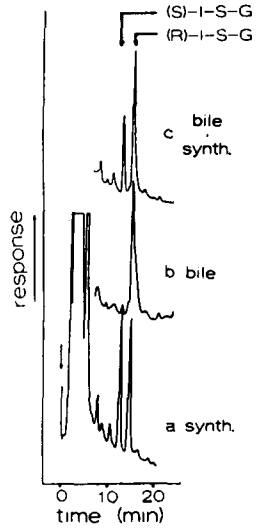

Fig. 1. HPLC/ECD chromatograms of (a) the two chemically synthesized GSH conjugates; (b) a bile sample [60–75 min after administration of a dose of (*RS*)-BI]; and (c) cochromatography of this bile sample with the two chemically synthesized GSH conjugates.

under vacuum. The residue was dissolved in unlabelled isovaleric acid (65 μl; 0.60 mmol). PBr_3 (10 μl) was added and the mixture was stirred for 30 min. To start the synthesis, Br_2 (60 μl; 1.10 mmol) was added and the mixture was stirred for another 18–20 hr at 70–80°, under reflux [8]. After the reaction, 5 ml water was added and the BI was purified by preparative HPLC. The compound was eluted from a Nucleosil 7C18 column (25 × 1) using water (pH 3 with HCl):acetonitrile, 70:30 as eluent at a flow rate of 2.0 ml/min. Detection was at 210 nm. The fractions containing BI were combined and the eluent was removed under vacuum while the pH was kept at 7 in order to prevent evaporation of BI. The specific radioactivity, radiochemical yield and purity (68 μCi/mmol, 35% and 96% respectively) were determined by the above mentioned HPLC system.

Synthesis of the α-[glutathion-S-yl]-isovaleric acid diastereomers. These metabolites of BI were chemically synthesized as described elsewhere for BIU [3, 6]. The mixture contained the two diastereomers (Fig. 1), which could be separated in the HPLC system for the GSH conjugates as described below. In previous work it was established that only (*S*)-BI was conjugated with GSH by purified hepatic GST isoenzymes [7]. The resulting conjugate eluted from the column with the same retention time as the second diastereomer from the synthetic mixture. Because GSH conjugation involves complete inversion of the configuration, (*S*)-BI gives rise to (*R*)-I-S-G. The retention times of the diastereomers (*S*)- and (*R*)-I-S-G were 12.5 and 14.5 respectively (see below).

Synthesis of the α-[N-acetyl-L-cystein-S-yl]-isovaleric acid diastereomers. These metabolites were synthesized by mixing solutions of the separate (*R*) or (*S*) enantiomers of BI (0.2 M) with *N*-acetyl-L-cysteine in water (0.2 M); final pH was adjusted with NaOH to 10. The mixture was left under argon for 4 days at room temperature under constant shaking in an Eppendorf Mixer 5423 from Merck. At the end of the reaction each mixture contained only one diastereomer, which could be separated and visualised with a HPLC/ECD system as described below for the mercapturates.

Excretion of α-bromoisovaleric acid metabolites in bile and urine of anaesthetized rats. After anaesthetizing the rats with sodium pentobarbital (60 mg/kg, i.p.), the bile duct, urine bladder and the external jugular vein were catheterized as described elsewhere [9]. During the experiment, the animals were kept on a heating pad to keep the body temperature at 38°. In order to get sufficient urine production, a D-mannitol infusion was given to the animals [9]. When the urine flow had stabilised, urine and bile were collected during 15 min to determine the basal production rate. Hereafter a BI-solution (BI dissolved in 250 mM sodium phosphate buffer, pH 7.4; 2.5 ml/kg) was injected in the jugular vein over approx. 1 min. For the separate (*R*) and (*S*)-BI enantiomers the dose was 50 μmol/kg and for the racemic (*RS*)-BI 100 μmol/kg. In the case of [1-^{14}C](*RS*)-BI, 3.2 μCi/kg (100 μmol/kg) was administered. During 5–6 hr bile and urine samples were collected and stored at −20° until analysis of the metabolites could take place.

Excretion of metabolites of [1-^{14}C]α-bromoisovaleric acid in urine and faeces of unanaesthetized rats. Rats were administered a (*RS*)-[1-^{14}C]α-BI solution (100 μmol/kg and 1.5 μCi/kg, dissolved in 250 mM sodium phosphate buffer, pH 7.4; 2.5 ml/kg) in the lateral tail vein under mild diethyl ether anaesthesia. The animals were placed in metabolism cages for 24 hr and the urine and faeces were collected separately and stored at −20° until analysed.

Analysis of BI and its metabolites. BI in urine could only be measured using [1-^{14}C]BI, because no alternative detection method was available that was sensitive enough to measure the low BI concentrations. It was determined by the HPLC system used for the BIU mercapturates [10], with one modification: 20% (v/v) methanol was used instead of 15%. A stainless steel column (25 × 0.3 cm) packed with 5 μM ODS 2 from Phase Sep (Queensferry, Clwyd, U.K.), was used. The mobile phase consisted of an aqueous buffer (0.1 M $NaNO_3$/0.01 M KBr/0.01 M citric acid, pH 2.5):methanol, 80:20 at a flow rate of 0.45 ml/min. [1-^{14}C]BI eluted from the column with a retention time of 27 min. Quantification was done by liquid scintillation counting of the BI containing fractions: 450 μl of the eluent was mixed with 3 ml of scintillation fluid (Emulsifier SAFE, Packard Indust.) and counted with a Packard Tri-Carb 4640 scintillation counter. Quench correction was by the spectral index external standard method.

Routinely, the mercapturate (*R*)-I-S-MA in urine samples was estimated with the same HPLC system as described above for BI, but with 15% methanol in the eluent. The retention time of the mercapturate was 23 min; it was detected with electrochemical detection [10]. In a number of experiments to determine which diastereomer of I-S-MA was present in urine, the same HPLC system but with 5% methanol instead of 15% was used. This system resolved the

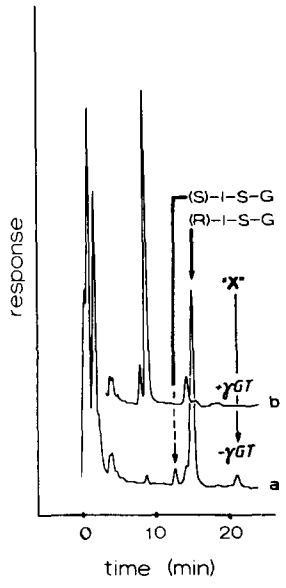

Fig. 2. HPLC/ECD chromatograms of a bile sample [60–75 min after administration of a dose of (*RS*)-BI] incubated (a) without or (b) with γ-GT.

two diastereomers: (*R*)-I-S-MA [originating from (*S*)-BI] had a retention time of 71 min and (*S*)-I-S-MA [originating from (*R*)-BI] of 75 min.

The GSH conjugate of BI in bile and the unknown metabolite "X" were determined with the same HPLC/ECD system as was used for BI, except that the buffer present in the mobile phase contained, in addition to the above mentioned components, 0.1 mM 1-decanesulfonic acid. The retention time of metabolite "X" was 22 min.

The quantification of the metabolites (*R*)-I-S-G, "X" and (*R*)-I-S-MA was done by ECD. A calibration curve was made of ECD peak height against radioactivity in the peak of the radioactive metabolites, derived from [^{14}C]BI of known specific radioactivity. As external standards IU-S-G and IU-S-MA were used.

In the experiments with the anaesthetized animals the urine was diluted six times and most bile samples twenty times with the eluent buffer. The urine from the metabolism cage experiment with [1-^{14}C](*RS*)-BI needed a clean-up procedure before injection. A Sep-pack column was acidified with 100 μl 8 N HCl. Then 5 ml of urine was applied, followed by 4 × 5 ml 0.1 N HCl and 3 × 5 ml methanol. The first methanol fraction contained almost the total radioactivity; the methanol was evaporated. The residue was taken up into 100 μl buffer of the urine eluent, injected into the HPLC system; fractions of 450 μl were collected and counted after mixing with 3 ml liquid scintillation fluid.

RESULTS

Excretion of [1-^{14}C](RS)-*BI and its metabolites in urine, bile and faeces*

After intravenous administration of racemic [1-^{14}C]BI to rats in metabolism cages, urine and faeces were collected for 24 hr. In the faeces 1.3 ± 0.3% of the dose of ^{14}C was found. The urine contained 75 ± 1%, of which 34 ± 2% was identified as (*R*)-I-S-MA and 37 ± 3% as unchanged BI.

Table 1. Cumulative excretion of metabolites of (*R*)-BI and (*S*)-BI in anaesthetized rats after 5 hr

Metabolite	(*R*)-BI (% of dose)	(*S*)-BI (% of dose)
Bile		
(*R*)-I-S-G	8 ± 1	34 ± 2
"X"	3 ± 1	4 ± 0
Urine		
(*R*)-I-S-MA	12 ± 1	20 ± 4
Total	23	58

BI was injected intravenously. Data are presented as mean ± SE (N = 4).

Racemic [1-^{14}C]BI was also administered to anaesthetized animals from which bile and urine were collected. Three radioactive metabolites were found in bile; these compounds could also be detected with electrochemical detection (Fig. 2) [10]. Two major radioactive compounds were found in urine, but only one could be detected by ECD [10].

Identification of the metabolites in bile

To identify these metabolites, bile samples were incubated with γ-glutamyl transpeptidase (γ-GT), to find out whether they were GSH conjugates. After this incubation, the three peaks had disappeared from the HPLC chromatogram and three new peaks at different retention times had appeared (Fig. 2), indicating that all three metabolites most likely contained a γ-glutamyl group. Therefore, their chromatographic behaviour was compared with that of the synthetic GSH conjugates of BI. This mixture contained the two diastereomers, (*S*)- and (*R*)-I-S-G, which could be separated in the HPLC system used (Fig. 1) [10]. Two metabolites in bile had the same retention time as the synthetic diastereomers (Fig. 1); cochromatography of the synthetic GSH conjugates with bile samples showed that the two synthetic GSH conjugates and the two metabolites were identical. The major metabolite in bile cochromatographed exactly with (*R*)-I-S-G (Fig. 1). The other GSH conjugate, (*S*)-I-S-G, was also present in the bile samples, but only as a very minor compound (<1%). The structure of the third metabolite (metabolite "X") is as yet unidentified. Unchanged BI was virtually absent in bile (≪1%).

Identification of metabolites in urine

After administration of racemic (1-^{14}C]BI two major radioactive peaks were found in the HPLC chromatogram of a urine sample. The chemically synthesized mercapturate of (*S*)-BI, (*R*)-I-S-MA, cochromatographed with one of the metabolites in urine. (*S*)-I-S-MA was not found in urine of rats that had received the racemate, nor when only (*R*)-BI had been administered. The other major radioactive compound in urine, that did not give a positive response in the ECD detection, was unchanged BI.

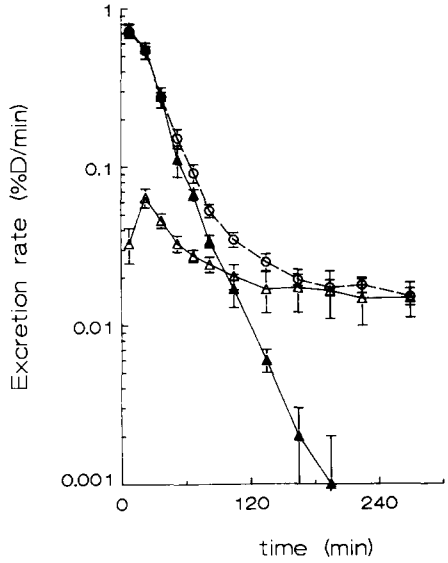

Fig. 3. Biliary excretion rates of (*R*)-I-S-G after administration of (*R*)-BI (△), (*S*)-BI (▲) or racemic BI (○) to anesthetized rats. Mean ± SE; N = 4; %D; percentage of the dose.

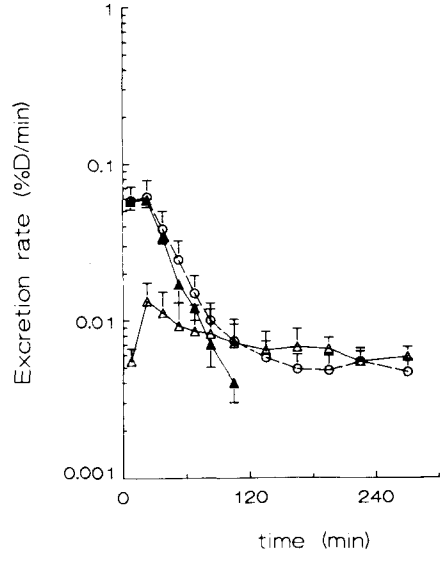

Fig. 4. Biliary excretion rates of metabolite "X" after administration of (*R*)-BI (△), (*S*)-BI (▲ or racemic BI (○) to anesthetized rats. Mean ± SE; N = 4.

Biliary and urinary excretion kinetics of BI metabolites in anaesthetized rats

After administration of (*S*)-BI, the above mentioned metabolites were found after 5 hr: (*R*)-I-S-G (34% of the dose) and "X" (4%) in bile, and (*R*)-I-S-MA (20%) in urine (Table 1). (*S*)-I-S-G was almost absent: less than 1% of the dose. The biliary excretion of (*R*)-I-S-G was rapid: the half life of the excretion was 16 min (Table 2, Fig. 3). The excretion of "X" in bile followed almost exactly that of (*R*)-I-S-G (Fig. 4) and the half life of its excretion (20 min) was also very similar (Table 2). (*R*)-I-S-G was further metabolized to the mercapturate, (*R*)-I-S-MA, that had an excretion half life of 43 min in urine (Table 2, Fig. 5).

The same metabolites were found after administration of (*R*)-BI. Surprisingly, in bile very little (*S*)-I-S-G was found, but mainly (*R*)-I-S-G was present. The major difference between the two BI enantiomers was the pronounced difference in the excretion half lives of their metabolites. The metabolites of (*R*)-BI were excreted very slowly, compared to those of (*S*)-BI (Figs 3–5). The excretion half lives were 180, 220 and more than 650 for (*R*)-I-S-G, "X" and (*R*)-I-S-MA respectively (Table 2). As a result, a major difference in the percentage excreted as GSH derived metabolites (in bile and urine), was found: 58% and 23% for (*S*) and (*R*)-BI respectively (Table 1).

When racemic [1-^{14}C]BI was injected, the same three metabolites were found: (*R*)-I-S-G (35 ± 2%), "X" (6 ± 1%) in bile and (*R*)-I-S-MA (14 ± 1%) in urine. The excretion rates of the three were biphasic (Fig. 6). Unchanged BI (13 ± 2%) and several unidentified minor polar compounds (5 ± 1%) could also be found in urine. Analysis of the curves shows a rapid and a slow phase in each curve. The excretion half lives are presented in Table 2. Total excretion after 6 hr was 73% of the dose of racemic BI. The excretion half lives of the metabolites were very similar to those in the experiment with the separate BI enantiomers (Table 2).

Table 2. Excretion half lives of the biliary and urinary metabolites of (*R*)-BI, (*S*)-BI and [^{14}C](*RS*)-BI in anaesthetized rats

	Compound administered					
		(*RS*)-BI			[^{14}C](*RS*)-BI*	
Metabolite	(*R*)-BI (min)	(*R*)-BI (min)	(*S*)-BI (min)	(*S*)-BI (min)	(*R*)-BI (min)	(*S*)-BI (min)
Bile						
(*R*)-I-S-G	180 ± 50	170 ± 10	13 ± 1	16 ± 1	290 ± 50	13 ± 1
"X"	220 ± 60	>300	14 ± 1	20 ± 1	—	18 ± 1
Urine						
(*R*)-I-S-MA	>650	—	—	43 ± 2	340 ± 14	34 ± 3

* Only in the experiment with [^{14}C](*RS*)-BI, metabolites were measured during 6 hr; in all other experiments for 5 hr. BI was administered intravenously. Data are presented as mean ± SE (N = 4).

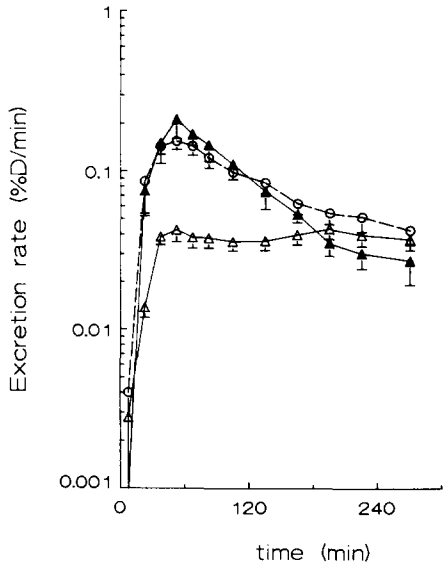

Fig. 5. Urinary excretion rates of (*R*)-I-S-MA after administration of (*R*)-BI (△), (*S*)-BI (▲) or racemic BI (○) to anesthetized rats. Mean ± SE; N = 4.

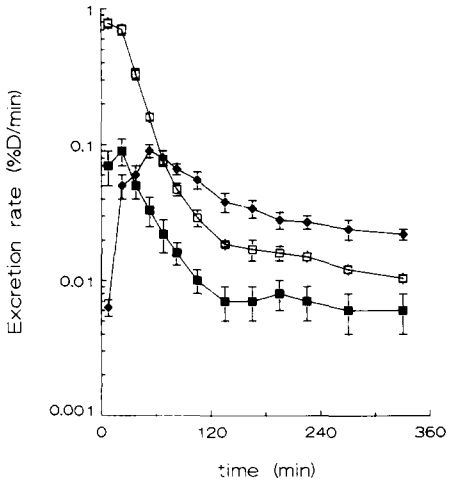

Fig. 6. Biliary and urinary excretion rates of metabolites of racemic [1-^{14}C]BI in anesthetized rats. Mean ± SE; N = 4. Biliary excretion of (*R*)-I-S-G (□) and "X" (■), and urinary excretion of (*R*)-I-S-MA (◆).

Racemization of BI in vitro

The fact that the same GSH conjugate was found in bile after administration of (*R*)- and (*S*)-BI, may be due to inversion of the configuration. To determine the rate of chemical racemization, a 55 mM solution of either (*R*)- or (*S*)-BI in Hanks buffer, pH 7.4 [containing 0.1% (w/v) sodium azide] was kept in a shaking waterbath at 37° during 6 days. The $[\alpha]^{20}$ was followed during this time (before measurement the samples were cooled down to 20°). However, no substantial chemical racemization was found: it amounted to only 5% per 24 hr for each enantiomer.

DISCUSSION

We have shown previously that BI, the hydrolysis product of α-bromoisovalerylurea (BIU), can be conjugated with GSH [5–7]. This conjugation was highly stereoselective in isolated hepatocytes and with purified GST. The present work characterizes the GSH conjugation of BI and its stereoselectivity *in vivo*. Contrary to BIU, which is very extensively metabolised, BI was eliminated unchanged in the urine. This may be due to the fact that the conjugation of BI is slower, and its lipid solubility less than that of BIU, so that the substrate can "escape" in urine.

The major difference between the BI enantiomers was the pronounced difference in the excretion rate of the metabolites: the (*S*) enantiomer was excreted much faster than the (*R*) enantiomer. *In vitro* incubations with isolated hepatocytes and purified hepatic GST, showed that (*R*)-BI was a very poor substrate for GSH conjugation [6, 7], while (*S*)-BI was conjugated more readily. This, therefore, is in good agreement with the present *in vivo* data, showing that (*S*)-BI is conjugated more rapidly than (*R*)-BI.

Administration of (*R*)- and (*S*)-BI resulted in the same GSH conjugate in bile, (*R*)-I-S-G, but at different rates. This was surprising, since normally two different enantiomers would give rise to different diastereomeric GSH conjugates. Little chemical racemization of either (*R*)- or (*S*)-BI was observed *in vitro*: the racemization was only 5% per 24 hr. *In vivo*, however, in 5 hr already 8% of the dose of (*R*)-BI was found as (*R*)-I-S-G and 12% as (*R*)-I-S-MA, indicating at least 20% inversion in 5 hr. Also in the experiments with racemic BI, a slower elimination phase follows after the rapid elimination of (*R*)-I-S-G [representing conjugation of (*S*)-BI]. This slow phase presumably results from the metabolism of (*R*)-BI; it appears that (*R*)-BI, which is hardly conjugated directly, can be inverted into (*S*)-BI, giving rise to (*R*)-I-S-G at a slow rate. Under certain conditions, compounds with a labile proton at the asymmetric carbon atom (as the α-C proton in BI) may lose this proton and, thereby, inversion of the chiral centre can take place [11]. This could explain the limited chemical racemization that was found, but not the racemization in the *in vivo* situation. An alternative explanation is enzymatic inversion of the chiral centre *in vivo*, comparable to, for instance, the inversion of the (*R*) enantiomers of the 2-arylpropionic acids into the (*S*) enantiomer through formation of a coenzyme A derivative [12].

The structure of metabolite "X" is as yet unknown. After incubation with γ-GT, the peak moved in the HPLC chromatogram. Thus, this compound, most likely, contains a γ-glutamyl moiety. Furthermore, it can be detected by ECD and the excretion follows exactly the excretion of the GSH conjugate, (*R*)-I-S-G (Fig. 6). Thus, "X" could be derived from the GSH conjugate by a loss of its glycine moiety.

GSH conjugates can be further metabolized to mercapturates. (*R*)-I-S-G will give rise to (*R*)-I-S-MA, which can be excreted in urine. Its excretion appears to follow the pattern of excretion of (*R*)-I-S-G, in that, after administration of (*S*)-BI, it is more rapidly excreted than after (*R*)-BI.

One of the aims of the present paper was to assess whether BI is a more suitable model substrate than BIU to study the GSH conjugation (including its stereoselectivity). Advantages are that BI has a higher water solubility, is more stable and is not subject to amidase catalysed hydrolysis. However, the major disadvantage of BI is the fact that only one enantiomer, (*S*)-BI, is conjugated with GSH, while in the case of BIU, both enantiomers are conjugated, each by a different set of glutathione-*S*-transferase isoenzymes [7]. Therefore, racemic BIU as model substrate for *in vivo* studies is superior to BI because it provides more complete information on the various glutathione-*S*-transferases contributing to overall conjugation *in vivo*, since the separate diastereometric mercaptures and GSH conjugates reflect the contribution of different GST families.

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