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Role of Glutathione in the Biliary Excretion of the Arsenical Drugs Trimelarsan and Melarsoprol

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ABSTRACT. After administration of the inorganic sodium arsenite or arsenate to rats, the biliary excretion of arsenic is rapid, is accompanied by the biliary output of large amounts of GSH, and is completely arrested by the GSH depletor diethyl maleate (DEM). We studied the biliary excretion of trimelarsan (TMA) and melarsoprol (MAP) in rats in order to determine whether biliary excretion is also significant in the disposition of these trivalent organic arsenicals that are used as therapeutic agents and whether GSH is also involved in their hepatobiliary transport. After injection of either drug (100 μmol/kg, i.v.), arsenic was rapidly excreted in bile (up to 1 μmol/kg · min, ~55% of dose/100 min). Concurrently, TMA and MAP increased the biliary output of GSH 3- and 6 fold, and lowered the hepatic GSH content by 24% and 27%, respectively. In TMA-injected rats, pretreatment with DEM or buthionine sulfoximine decreased the initial biliary excretion of arsenic by 75% and 40%, respectively, whereas in MAP-injected rats these GSH depletors diminished arsenic output by 45% and 20%. Both arsenicals reacted with GSH *in vitro*, giving rise to the same product, which was also shown by HPLC analysis to be a major biliary metabolite of both TMA and MAP. This metabolite was sensitive to γ-glutamyltranspeptidase *in vitro* and its biliary excretion was virtually prevented by the GSH depletors, confirming that it is a GSH conjugate (purportedly melarsen–diglutathione). Some TMA was excreted in the bile unchanged, whereas a significant amount of MAP also appeared there as two glucuronides. The biliary excretion of unchanged TMA and MAP glucuronides was increased by experimental depletion of GSH. These studies indicate that the biliary excretion of TMA and MAP (1) is very significant in their disposition, (2) is partially dependent on the hepatic availability of GSH, as these arsenicals are excreted in part as a GSH conjugate, and (3) is concomitant with the increased appearance of GSH in bile, probably originating from dissociation of the unstable GSH conjugate of these arsenicals. Thus, conjugation with GSH is important in the elimination of both TMA and MAP, although glucuronidation is also involved in the fate of MAP. BIOCHEM PHARMACOL **59**;11:1375–1385, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. arsenic; biliary excretion; glucuronidation; glutathione; melarsoprol; trimelarsan

Arsenicals comprise a heterogeneous group of chemicals, many of which are highly poisonous after acute exposure and carcinogenic following chronic exposure. Some are environmental contaminants, while others have gained biological application as pesticides or drugs [1]. Although arsenicals have long been used therapeutically, they have aroused increasing interest recently by the discovery that arsenic trioxide, an inorganic compound of trivalent arsenic, can cause complete remission of acute promyelocytic leukemia by inducing apoptosis in the leukemic cells [2, 3]. MAP,† a melaminophenylarsine-type trivalent organic arsenical and an important drug in the treatment of African trypanosomiasis [4], is also active against leukemia cell lines [5] and plasma cells from myeloma patients [6].

When administered to rats, arsenite and arsenate as well as related metalloids are rapidly excreted into bile [7–11]. Although the biliary forms of the inorganic arsenicals have not yet been identified, we suggested earlier that they are transported from liver into bile as unstable GSH conjugates of trivalent arsenic, because DEM, a chemical depletor of hepatic GSH, completely arrests their biliary excretion and because their excretion is concomitant with a large increase in the biliary output of GSH and related non-protein thiols [9, 10]. Information on the fate of arsenical drugs in the body is limited. Clinical studies indicate that elimination of MAP is relatively slow [12] and that its excretion into urine is minimal [13, 14]. Data on the biliary excretion and biotransformation of MAP and its water-soluble congener, TMA, are apparently lacking.

The present study was devised to determine the significance of biliary excretion in the fate of TMA and MAP and to analyse the role of GSH in their hepatobiliary transport in rats. Specifically, it was studied whether, similarly to the hepatobiliary transport of inorganic arsenicals, the biliary excretion of these organic arsenical drugs is (1) also rapid, (2) likewise accompanied by a large output of GSH into

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† *Abbreviations:* BSO, D,L-buthionine-[*S*,*R*]-sulfoximine; DEM, diethyl maleate; GGT, γ-glutamyltranspeptidase; MAP, melarsoprol; TMA, trimelarsan.

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bile and depletion of GSH from liver, and (3) is also absolutely dependent on hepatic GSH availability, and (4) whether these arsenicals are transported into bile as GSH conjugates. To this end, we first examined the biliary excretion of arsenic after administration of TMA and MAP to control rats and rats pretreated with the GSH depletors DEM or BSO [15, 16], then studied the effect of TMA and MAP on the biliary excretion and hepatic concentration of GSH, and finally analysed the bile for TMA and MAP metabolites by HPLC. To facilitate comparison of the present and previous findings, the administered doses of TMA and MAP were in the same range as those of the inorganic arsenicals [9, 10], despite the fact that these doses are significantly higher than those used in human therapy. Chemical formulas of TMA and MAP are shown in Fig. 7.

MATERIALS AND METHODS

Chemicals

TMA, melarsen, and melarsen oxide were provided by Rhone-Poulenc Rorer, whereas Arsobal™ in an injection containing 180 mg MAP in 5 mL propylene glycol was a gift from Specia. BSO, 5,5′-dithiobis(2-nitrobenzoic acid), GGT (type II from bovine liver), and glutathione reductase (type III) were purchased from Sigma Chemical Co. and reduced glutathione, NADPH, and phosphoric acid from Reanal. Acetonitrile for gradient HPLC was obtained from Spektrum-3D, DEM from Koch-Light Laboratories, D-glucaric acid-1,4-lactone monohydrate from Fluka, β-glucuronidase/aryl sulfatase (from *Helix pomatia*, B grade) from Calbiochem, and metaphosphoric acid from Alfa Products.

Animal Experiments

Male Wistar rats (Charles River Hungary) weighing 230–270 g were used. The animals were kept at 22–25° room temperature, at 55–65% relative air humidity, and on a 12-hr light/dark cycle and provided with tap water and lab chow (Altromin, LATI) *ad lib*. The rats were anaesthetised by i.p. injection of a mixture of fentanyl, midazolam, and droperidol (0.045, 4.5, and 5.5 mg/kg, respectively), and their body temperature was maintained at 37± by means of heating lamps. To maintain patent airways, tracheotomy was performed on each animal. After median laparotomy, the common bile duct was cannulated with the shaft of a 23-gauge needle attached to a PE-50 tubing (Clay Adams). TMA dissolved in saline and MAP in propylene glycol were injected in a dose of 100 μmol/kg into the right saphenous vein. Control animals were given the vehicle of these drugs, i.e. saline (3 mL/kg, i.v.) or propylene glycol (1.1 mL/kg, i.v.). Subsequently, bile was collected in 20-min periods for 100 min into preweighed 1.5-mL microcentrifuge tubes. These tubes were embedded in ice and contained 400 μL 5% metaphosphoric acid when bile was collected for analysis of arsenic and glutathione in order to prevent oxidation and hydrolysis of glutathione. For HPLC analysis of the arsenicals and their metabolites, however, bile was collected into empty tubes kept at subzero temperature in a cooling box. After determination of the volume of bile gravimetrically (assuming unity for specific gravity), the bile samples were stored at −20° until analysis, except those intended for metabolite analysis, which were stored in liquid nitrogen. At 100 min after administration of TMA or MAP, the rats were exsanguinated and their livers removed for quantification of hepatic glutathione concentrations. When studying the effect of glutathione depletion on the biliary excretion of arsenicals, rats were pretreated with DEM (4 mmol/kg, i.p.) or BSO (5 mmol/kg i.p.) for 1 and 4 hr before injection of the drugs, respectively.

Analysis

For quantification of total arsenic in bile, 50-μL aliquots of bile were diluted with water to 5 mL. When bile collected into 5% metaphosphoric acid was analysed for arsenic, the samples were vigorously vortexed, 100 μL of 0.5 M sodium hydroxide solution was added to 100 μL of the thus homogenised sample in order to resolubilise the acid-precipitated proteins, and the mixture was diluted with water to 5 mL. Arsenic in the diluted bile samples was quantified by inductively coupled plasma atomic emission spectrometry using a Spectroflame ICP spectrometer (Spectro Analytical Instruments). Arsenic in bile collected from rats not injected with arsenicals was below the detection limit (0.05 μg/mL).

Concentrations of total glutathione (GSH + 2GSSG) in bile and liver were quantified according to the method of Tietze [17] using appropriate dilutions of the supernatants of the bile collected in 5% metaphosphoric acid and of the liver homogenates made by homogenising the liver in 9 volumes of 10% metaphosphoric acid. Known amounts of GSH added to the diluted bile samples were completely recovered by the Tietze assay. Biliary excretion rates of arsenic and glutathione were calculated as the products of bile flow and the biliary concentrations of arsenic and glutathione, respectively.

HPLC analysis of biliary metabolites of TMA and MAP was based on the description of Ericsson *et al*. [18]. The HPLC equipment (Waters) included two pumps (Model 501), a Rheodyne 7125 injector with a 20-μL sample loop, a reversed-phase guard column (Nova-Pak C18, 4 μm, 3.9 × 20 mm), an analytical column (Nova-Pak C18, 4 μm, 3.9 × 150 mm), and a tuneable absorbance detector (Model 486) operated under Millennium Chromatography Manager. The eluents (A = 75 mM phosphoric acid in water, B = 30% acetonitrile in 75 mM aqueous phosphoric acid) were pumped at a combined flow rate of 1 mL/min, and the absorbance of the effluent from the column was monitored at 283 nm. After injection of the analytes, the initial composition of the eluents (100% A, 0% B) was maintained for 0.5 min, then was changed linearly to 83% A and 17% B by 1 min, where it was maintained till 3 min. From 3 to 10.5 min, this composition was modified according to profile 7 to 0% A and 100% B, which was

maintained till 14 min, after which the initial condition was re-established. A 7-min equilibration period was kept between injections. This analysis was not devised to separate the two diastereoisomers of MAP present in the authentic preparation at a ratio of 3:1 [18, 19]. Therefore, MAP appeared in the chromatograms as a split peak. For HPLC analysis, the bile samples were diluted and deproteinised with 49 volumes of 75 mM phosphoric acid. These solutions were centrifuged for 2 min in a Beckman Microfuge E, and 20 μL of supernatant was injected into the HPLC.

In order to identify their GSH conjugates, TMA or MAP (50 μM) was reacted with GSH (1.5 mM) in 0.1 M phosphate buffer, pH 7.4. This mixture was kept at room temperature for 2 hr before HPLC analysis. A stock solution from the water-insoluble MAP (1 mM) was made with dimethylformamide. This could be diluted appropriately with 0.1 M phosphate buffer, pH 7.4. Presence of GSH conjugates of the arsenicals in the bile was confirmed by incubation of the bile samples from rats given TMA or MAP with GGT. The incubation mixture consisted of 10 μL bile and 60 μL 0.1 M sodium phosphate buffer (pH 7.4) containing 2 μmol glycylglycine and 0.25 units GGT. After incubation at 25° for 30 min, the mixture was diluted with 75 mM phosphoric acid to 500 μL, centrifuged, and the resultant supernatant analysed by HPLC as described above. Presence of MAP–glucuronide in the bile was confirmed by incubation of the bile sample from rats given MAP with β-glucuronidase/aryl sulfatase in the absence and presence of the β-glucuronidase inhibitor D-glucaric acid-1,4-lactone. The incubation mixture consisted of 10 μL bile and 60 μL 0.2 M sodium acetate buffer (pH 5.0) containing 0.02 IU β-glucuronidase and 0.06 IU aryl sulfatase with or without 1 mg D-glucaric acid-1,4-lactone. After incubation at 25° for 30 min, the mixture was diluted with 75 mM phosphoric acid to 500 μL, centrifuged, and the resultant supernatant analysed by HPLC as described above.

Statistics

Data were analysed by ANOVA followed by Duncan's test with $P < 0.05$ as the level of significance.

RESULTS

Biliary Excretion of Arsenic and Glutathione after Administration of TMA or MAP

As arsenic in control rat bile was not detected, arsenic in bile after administration of TMA and MAP originates entirely from the injected arsenicals. Therefore, biliary excretion of arsenic following administration of TMA or MAP reflects excretion of these arsenicals and/or their arsenic-containing metabolites into bile. As indicated in Fig. 1 (top), the biliary excretion of arsenic increased dramatically immediately after injection of TMA or MAP (100 μmol/kg, i.v.), reaching maximal rates of approximately 1100 nmol/kg · min within 20 min. Thereafter, the rate of biliary arsenic excretion gradually decreased below 200 nmol/kg · min by 100 min after administration of either arsenical. It is estimated that rats injected with TMA or MAP excreted as much as 53% and 56%, respectively, of the dose of these arsenicals within 100 min as parent compounds and/or arsenic-containing metabolites.

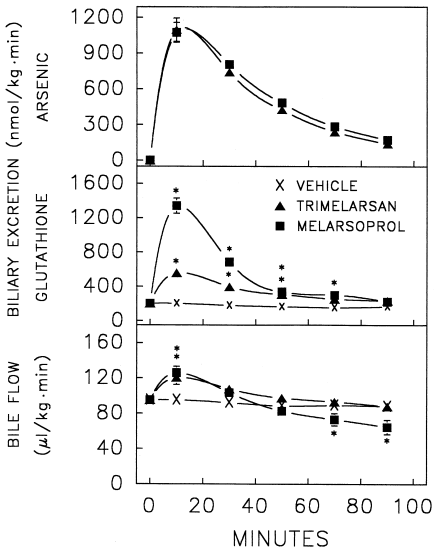

FIG. 1. Biliary excretion of arsenic and glutathione and the biliary flow after administration of TMA or MAP. Anaesthetised bile duct-cannulated rats were injected intravenously with TMA or MAP (100 μmol/kg) or their respective vehicle (3 mL/kg saline or 1.1 mL/kg propylene glycol) at time 0, and bile was collected subsequently in 20-min periods for 100 min. As the rates of glutathione (GSH + 2GSSG) excretion and bile flow did not differ significantly in the two groups of vehicle-injected rats, the values of all vehicle-injected animals were combined. To illustrate the rise in glutathione excretion and bile flow after injection of the arsenicals, the respective 0–20-min values of vehicle-injected rats were also plotted at 0 min for the arsenical-injected animals. Symbols represent means ± SEM of 5–8 rats. Asterisks indicate values significantly different ($P < 0.05$) from the respective values of the vehicle-injected rats.

Simultaneously with excretion of arsenic, the biliary output of endogenous glutathione (GSH + 2GSSG) was also increased immediately after administration of the drugs (Fig. 1, middle panel), reaching maximal rates within 20 min. MAP, however, was more effective in this respect than TMA, as the maximal MAP-induced enhancement in biliary excretion of glutathione was more than 6-fold, whereas that for TMA was barely 3-fold. Subsequently, the rate of the biliary glutathione output gradually receded in rats receiving either drug, and by 100 min was only slightly higher than the rate in the control rats. We observed similar increases in the biliary excretion rate of non-protein thiols (data not shown), as determined by a non-enzymatic assay using Ellman's reagent [20].

Both arsenicals induced acute choleresis (Fig. 1, bottom), increasing bile flow by 26–33% within 20 min after

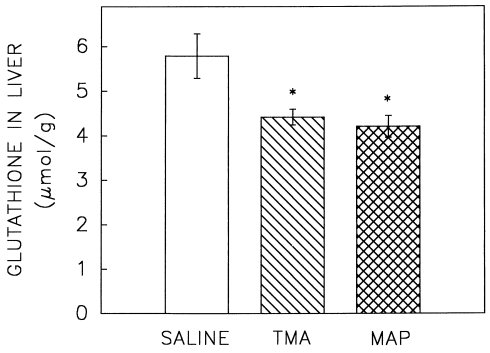

FIG. 2. Effect of TMA and MAP on hepatic glutathione concentration. Experimental conditions are described under Fig. 1. Bars represent hepatic concentrations of glutathione (GSH + 2GSSG) (mean ± SEM; N = 8) 100 min after intravenous injection of the vehicles, TMA, or MAP (100 μmol/kg). As the hepatic glutathione concentrations did not differ significantly in the two groups of vehicle-injected rats, the values of all vehicle-injected animals were combined. Asterisks indicate values significantly different ($P < 0.05$) from the values of the vehicle-injected rats.

administration. Thereafter, the bile formation gradually decreased, returning to the basal rate by 100 min in TMA-injected rats and declining significantly below the basal bile flow in MAP-injected animals.

Effect of TMA and MAP on Hepatic Glutathione

The glutathione (GSH + 2GSSG) concentration in the liver 100 min after injection of TMA, MAP, or the vehicle is demonstrated in Fig. 2. Both arsenicals brought about partial depletion of hepatic glutathione, as TMA and MAP lowered glutathione levels in the liver by 24% and 27%, respectively.

Effect of Glutathione Depletors on the Biliary Excretion of TMA and MAP

The effects of pretreatments with BSO or DEM on the biliary excretion of TMA- and MAP-derived arsenic are depicted in Fig. 3. The GSH depletors transiently diminished the biliary excretion of arsenic after administration of either TMA or MAP, with maximal effects seen immediately after arsenical injection. The degree of diminution in the biliary excretion of arsenic varied with respect to both the GSH depletor used and the arsenical injected, with DEM being more effective than BSO and with the excretion of TMA and/or its metabolites being more responsive than the excretion of MAP and/or its metabolites. For example, in TMA-injected rats, pretreatment with DEM or BSO decreased the initial biliary excretion of arsenic by 75% and 40%, respectively, whereas in MAP-injected rats these GSH depletors diminished the initial arsenic output by 45% and 20%.

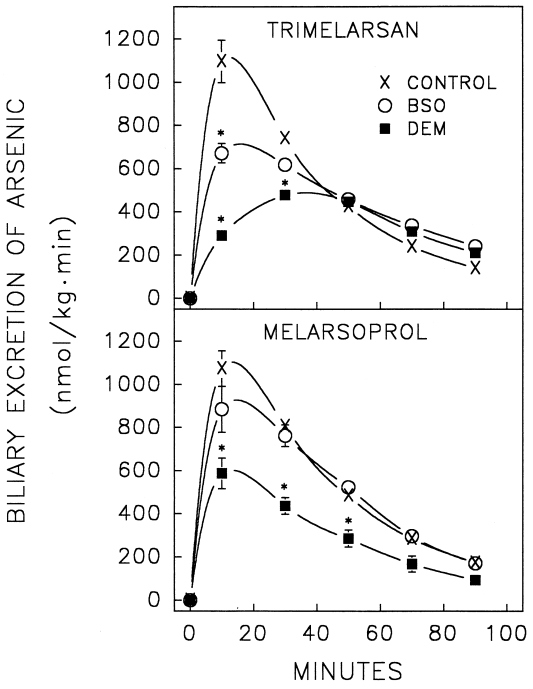

FIG. 3. Biliary excretion of total TMA and MAP expressed as arsenic in control rats and in rats pretreated with GSH depletors. TMA or MAP (100 μmol/kg, i.v.) was injected into the rats at time 0 and bile was collected in 20-min periods thereafter. The rats were pretreated intraperitoneally with saline (2 mL/kg; control), BSO (5 mmol/kg), or DEM (4 mmol/kg) at 1, 4, and 1 hr before administration of the arsenicals, respectively. Symbols represent means ± SEM of 5–8 rats. Asterisks indicate values significantly different ($P < 0.05$) from the respective values in control rats.

Identification of a Biliary Glutathione Conjugate Formed from TMA and MAP

In order to explore the mechanism that may underlie the responsiveness of arsenic excretion in TMA- and MAP-injected rats to depletors of GSH, it was examined whether these arsenicals form GSH conjugate(s) *in vitro*, and if they do, whether the conjugate(s) are detectable in the bile of TMA- or MAP-injected rats. As demonstrated by the HPLC analyses shown in the top panels of Fig. 4, both MAP and TMA formed a product (X) when these arsenicals were incubated with excess GSH. Product X eluted considerably earlier than MAP (M) or TMA (T) and at identical elution times (8.1 min) irrespective of whether the incubate of TMA or MAP was analysed. These findings indicate that incubation of TMA or MAP with GSH results in formation of the same product that is considerably more polar than either parent arsenical.

The middle panels in Fig. 4 demonstrate HPLC analyses of bile collected from rats injected with TMA (left) or MAP (right). Analyses of both the TMA- and MAP-injected rat bile samples revealed a major metabolite (X) eluted at 8.1 min, i.e. at the same time as that for the common product of the reaction of TMA or MAP with GSH. Besides this metabolite, the bile from TMA-injected

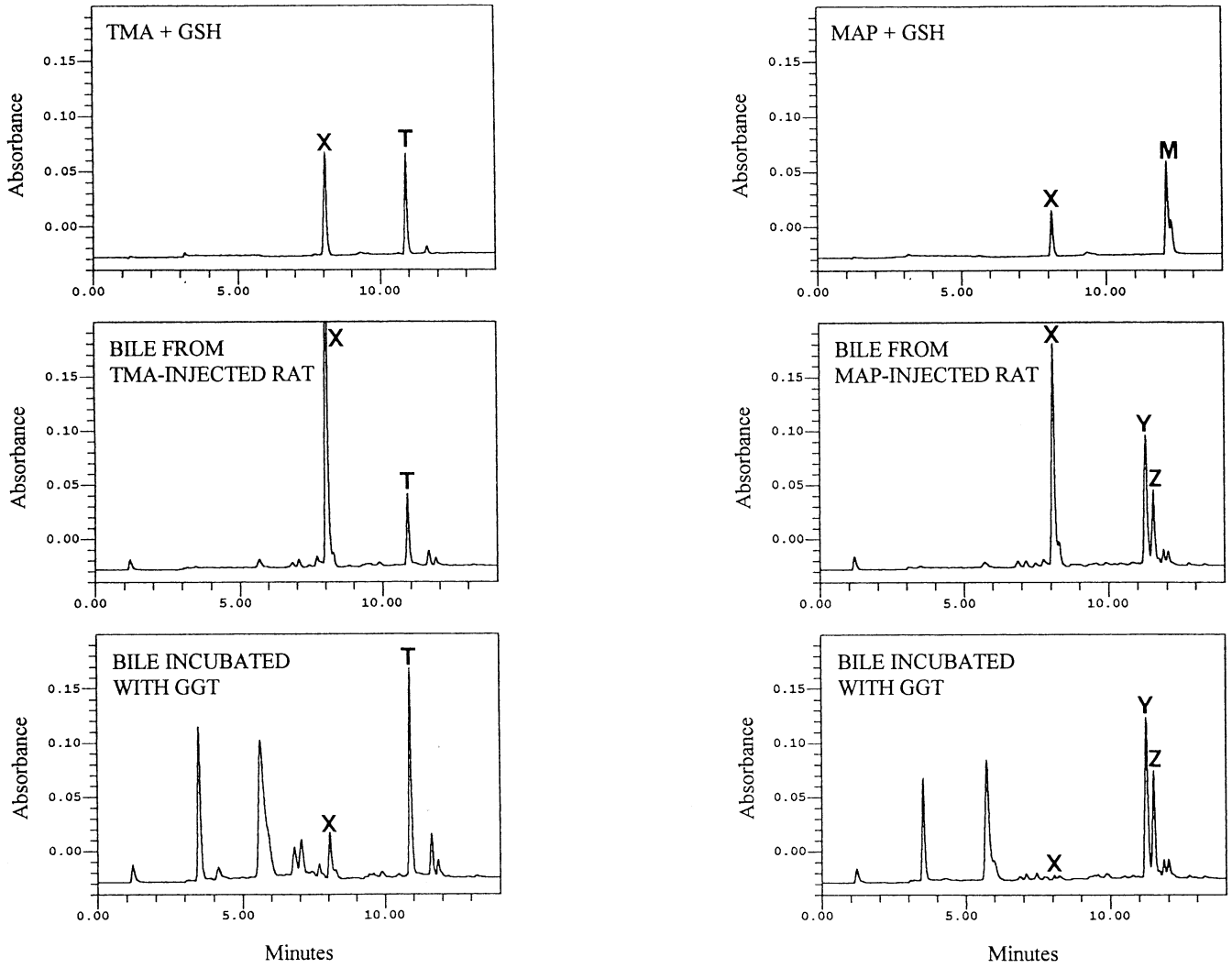

FIG. 4. Identification by HPLC analysis of a GSH conjugate in the bile of rats injected with TMA (left) and MAP (right). *Top panels*: Analysis of the incubate of 50 μM TMA (left) or MAP (right) with 1.5 mM GSH at pH 7.4. *Middle panels*: Analysis of a representative bile sample collected from a rat 0–20 min after i.v. injection of 100 μmol/kg TMA (left) or MAP (right). *Bottom panels*: Analysis of a representative bile sample collected from a rat 0–20 min after i.v. injection of 100 μmol/kg of TMA (left) or MAP (right) that was incubated with GGT. *Peak labels*: T = trimelarsan, M = melarsoprol diastereoisomers, X = the main biliary metabolite of both TMA and MAP identified here as a GSH conjugate, and Y and Z = main biliary metabolites of MAP demonstrated in Fig. 6 to be MAP-glucuronides.

rats contained TMA (T) (Fig. 4, middle left), whereas the bile from the MAP-injected rat also contained two prominent metabolites (Y and Z) that eluted earlier than MAP, which itself did not appear in bile unchanged in significant quantities (Fig. 4, middle right). Authentic melarsen and melarsen oxide, potential metabolites of both TMA and MAP, eluted at 3.62 and 6.04 min, respectively (not shown). Thus, these compounds are not biliary metabolites of TMA or MAP, because the HPLC analysis of bile from TMA- or MAP-injected rats did not reveal any peaks appearing at these time points (Fig. 4, middle panels).

In order to ascertain that biliary metabolite X is a GSH conjugate, bile from rats injected with TMA or MAP was incubated with GGT, which is known to hydrolyse GSH conjugates to cysteinylglycine conjugates. The analyses of these incubates (Fig. 4, bottom) revealed that GGT treatment of the bile samples resulted in complete and almost complete disappearance of metabolite X from the bile samples of rats injected with TMA (left) or MAP (right), respectively. Simultaneously, incubation with GGT brought about the appearance of new early-eluting unidentified compounds. These changes were caused largely by GGT, because incubation of the same bile samples under similar conditions, but with omission of GGT, brought about only a small diminution in the size of peak X and yielded a comparatively small amount of unidentified breakdown products (not shown).

In order to obtain further evidence for the identity of metabolite X as a GSH conjugate formed from both arsenical drugs, bile samples collected from rats pretreated

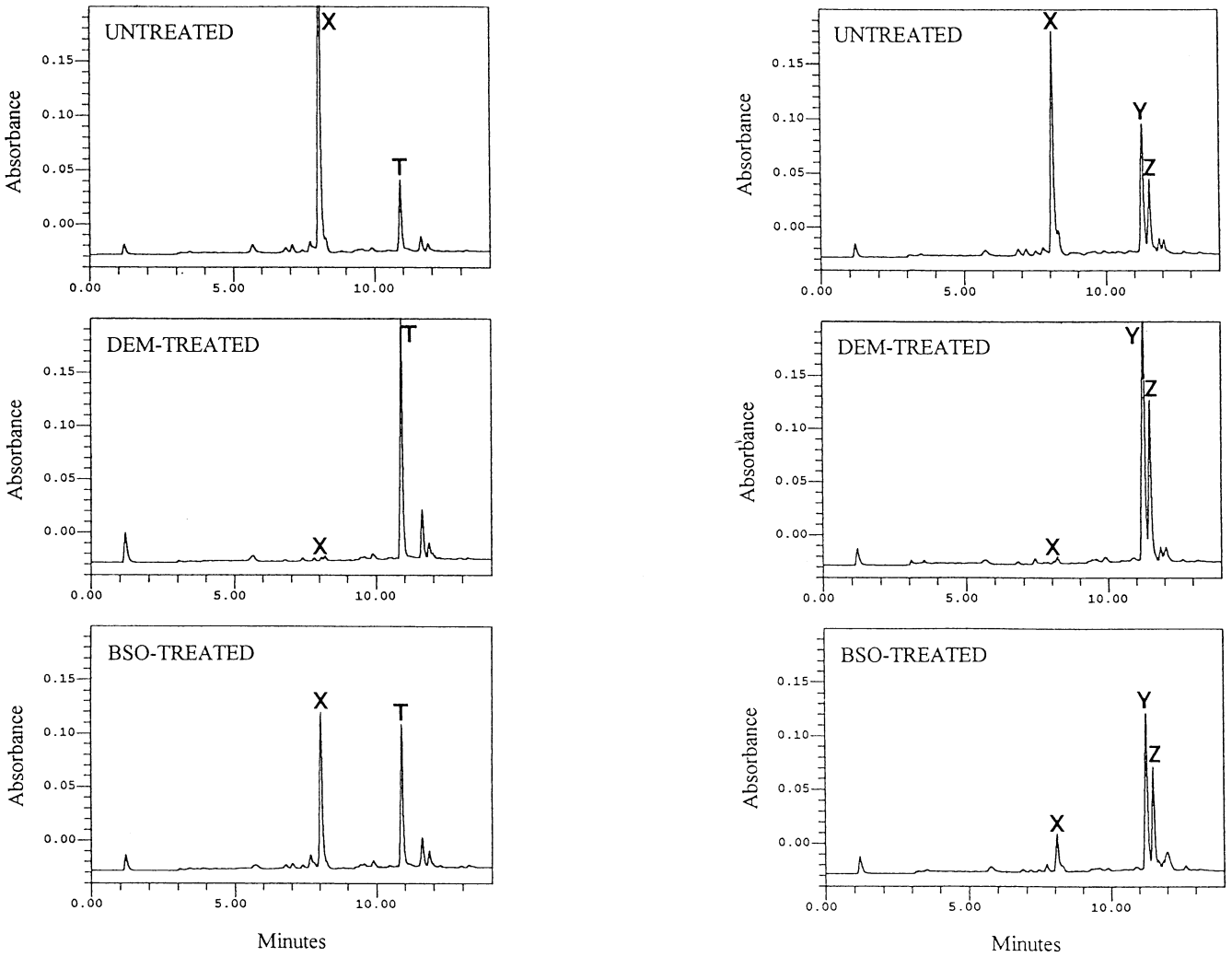

FIG. 5. HPLC analysis of the biliary metabolites of TMA (left) and MAP (right) in rats pretreated with GSH depletors. The figure demonstrates analyses of representative bile samples collected from untreated rats (top panels) and rats pretreated with DEM (middle panels) or BSO (bottom panels) 0–20 min after i.v. injection of 100 μmol/kg TMA (left panels) or MAP (right panels). Pretreatments are described in Materials and Methods. Peak labels are given under Fig. 4.

with GSH depletors and injected with TMA or MAP were also analysed (Fig. 5). These analyses revealed that rats pretreated with DEM and injected with TMA (Fig. 5, middle left) or MAP (Fig. 5, middle right) excreted practically no metabolite X, whereas in rats pretreated with BSO and injected with TMA or MAP, the biliary concentration of metabolite X (as judged from the size of peak X) decreased significantly (Fig. 5, bottom panels). In contrast, rats pretreated with the GSH depletors and injected with TMA tended to excrete more TMA (as judged from the size of peak T) (Fig. 5, left middle and bottom panels) than the non-pretreated rats given TMA (Fig. 5, top left). Similarly, the GSH depletor-treated and MAP-injected rats appeared to excrete more metabolite Y and Z (as judged from the sizes of peaks Y and Z) (Fig. 5, right middle and bottom panels) than the non-pretreated rat given MAP (Fig. 5, top right).

Identification of Biliary Glucuronic Acid Conjugates of MAP

As MAP was thought to be a likely candidate for glucuronidation (see Discussion), evidence was sought for biliary MAP metabolites Y and Z as glucuronides. For this purpose, bile samples collected from rats injected with MAP were incubated with β-glucuronidase/aryl sulfatase in the absence or presence of the β-glucuronidase inhibitor D-glucaric acid-1,4-lactone. Figure 6 demonstrates that peaks Y and Z present in the chromatogram of the bile of MAP-injected rat (top) almost completely disappeared upon incubation of this bile sample with β-glucuronidase/aryl sulfatase (middle). Concomitantly, two new and incompletely separated peaks (M) appeared at elution times identical with the elution times of authentic MAP diastereoisomers (Fig. 4, top right). Both of these changes brought about by β-glucuronidase/aryl sulfatase were pre-

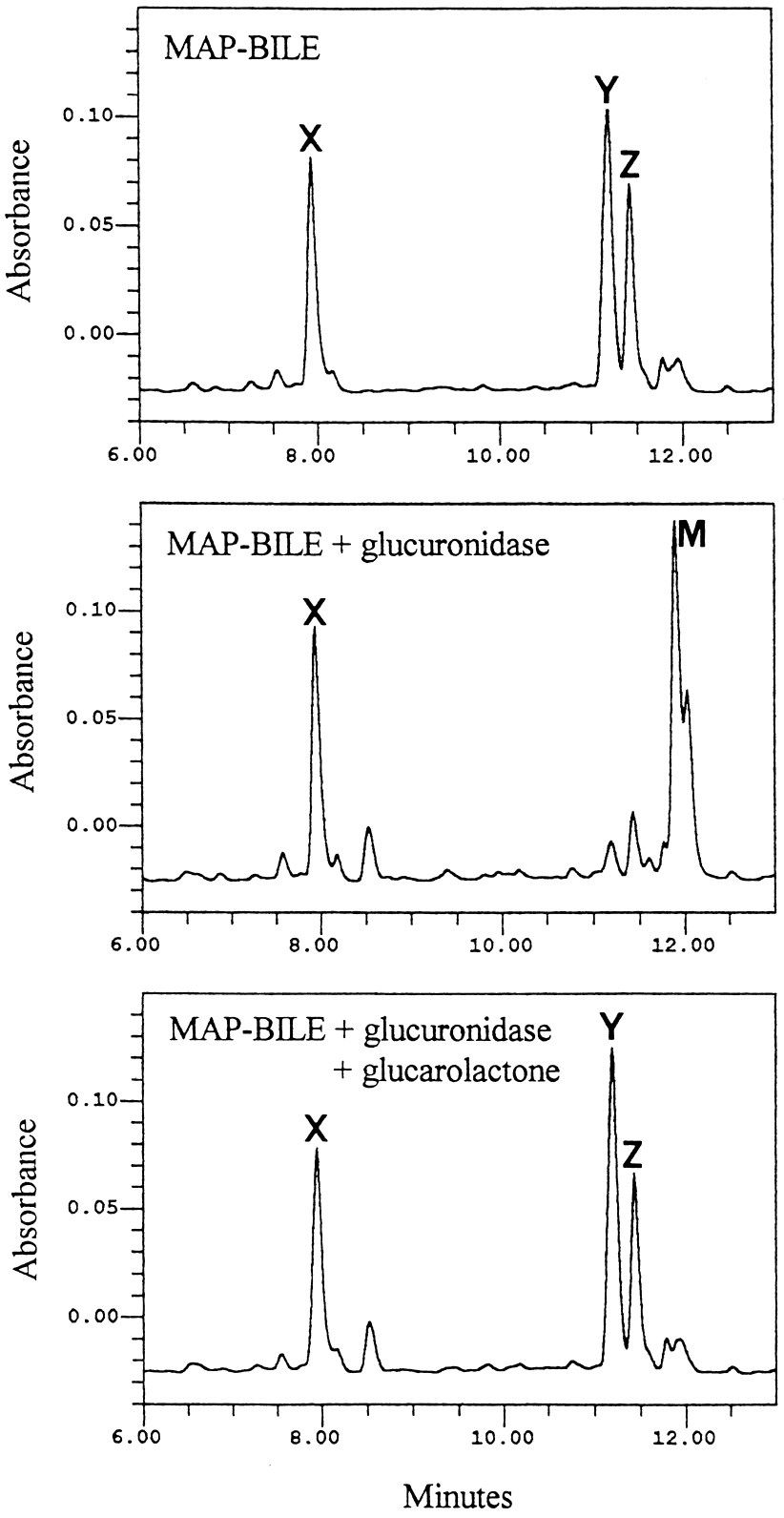

FIG. 6. Identification by HPLC analysis of glucuronides as major biliary metabolites of MAP. The figure demonstrates analyses of a representative bile sample collected from a rat 0–20 min after injection of MAP (100 μmol/kg, i.v.). This bile sample was analysed directly (top), after incubation with β-glucuronidase/aryl sulfatase (middle), and after incubation with β-glucuronidase/aryl sulfatase in the presence of D-glucaric acid-1,4-lactone (glucarolactone; bottom). Peak labels are given under Fig. 4.

vented by inclusion of D-saccharic acid-1,4-lactone into the incubation mixture (Fig. 6, bottom).

DISCUSSION

This study has revealed both similarities and differences in the biliary excretion of arsenite, an inorganic trivalent arsenical studied earlier [9, 10], and the two trivalent organic arsenical drugs, TMA and MAP. In rats, biliary excretion is significant for both arsenite and the melarsophenylarsine-type drugs. Nevertheless, both the extent and the maximal rate of biliary excretion of arsenic in rats injected with TMA or MAP exceed those in rats exposed to arsenite. Following injection of arsenite at the highest dose not causing overt toxic signs (50 μmol/kg, i.v.), the rate of biliary arsenic excretion peaked within 20 min at approximately 300 nmol/kg · min, then declined precipitously, with 19% of the dose appearing in bile within two hours [9, 10]. In contrast, after administration of TMA or MAP, which was well tolerated at a dose of 100 μmol/kg i.v., the rate of the initial biliary arsenic output exceeded 1000 nmol/kg · min, then declined gradually, with more than half of the dose delivered into bile by 100 min (Fig. 1). Thus, TMA and MAP are highly cholephilic compounds whose hepatobiliary transport is more efficient than that of arsenite. It is likely that the melarsophenylarsine compounds are predominantly transported from the liver into bile, whereas arsenite also undergoes extensive hepatovascular transport following hepatic uptake [21], which counteracts and soon terminates its rapid clearance into bile.

Cholephilic compounds, unless they adversely affect bile formation, commonly induce choleresis of osmotic origin. Therefore, the choleretic effect of TMA and MAP seen at the peak of arsenic excretion (Fig. 1) is probably caused by the osmotic activity of these drugs and/or their metabolites transported into bile. The diminished bile flow observed following administration of MAP (Fig. 1) indicates that this arsenical impairs bile formation after a latent period. Such a biphasic effect on bile flow, i.e. an immediate choleresis followed by cholestasis, also occurs in rats injected with arsenite [9].

The biliary excretion of inorganic arsenic as well as the arsenic originating from TMA and MAP depends on the availability of hepatic GSH. However, while dependence on the GSH supply of the hepatobiliary transport of arsenic in arsenite- and arsenate-exposed rats is absolute—DEM pretreatment completely abolished the biliary excretion of arsenic in such animals [10]—the hepatobiliary transport of arsenic in rats injected with the melarsophenylarsine drugs is only partially GSH-dependent, because experimental GSH depletion only diminished, but did not abolish, the biliary output of arsenic in the latter animals (Fig. 3). Circumstantial evidence suggests that the hepatobiliary transport of inorganic arsenic depends on hepatic GSH availability, because arsenic in trivalent form reacts with GSH [22–24] and the GSH complex(es) formed are readily transported into bile [9, 10]. The existence of arsenic–GSH complexes in bile has not been directly demonstrated, probably owing to their instability at the alkaline pH of bile. Nevertheless, evidence was sought whether TMA and MAP also form GSH conjugates and, if they do, whether such conjugates appear in bile following administration of these drugs.

HPLC analysis revealed the presence of a major metabolite, labelled X, in the bile of both TMA- and MAP-injected rats (Fig. 4, middle). The following observations indicate that this metabolite is a GSH conjugate produced from either melaminophenyl arsenical. First, when incubated with GSH in excess, both TMA and MAP formed a compound with chromatographic properties identical to those of metabolite X (Fig. 4, top). Second, incubation of the bile samples from TMA- or MAP-dosed rats with GGT, which hydrolyses GSH and GSH conjugates, resulted in an almost complete disappearance of metabolite X (Fig. 4, bottom). Third, when TMA or MAP was administered to rats pretreated with GSH depletors, the biliary excretion of metabolite X was practically prevented by DEM, a very effective GSH depletor, and was markedly decreased by BSO, a less effective GSH depletor [25, 26] (Fig. 5). Thus, these findings represent strong circumstantial evidence that both TMA and MAP form the same GSH conjugate both *in vitro* and *in vivo* and that this conjugate is their predominant biliary metabolite.

As to the structure of the common GSH conjugate of TMA and MAP, we can conclude that the conjugate cannot contain the dithiol moiety bound to the arsenic atom in these drug molecules. The moieties in these arsenicals are different, i.e. dimercaptosuccinate in TMA and dimercaptopropanol in MAP (Fig. 7). Therefore, TMA and MAP could not have formed the same GSH conjugate if their respective dithiol moieties had been retained in the conjugate. Consequently, the dithiols must have been exchanged by and substituted for GSH. Thus, the common GSH conjugate of TMA and MAP is most likely melarsen–glutathione (Fig. 7). Such a thiol-exchange reaction is not without example. It has been demonstrated that the dithiol dihydrolipoic acid can readily exchange with dimercaptopropanol in MAP, forming a lipoate–arsenical adduct [19]. Because monothiols form much less stable adducts with trivalent arsenicals than with dithiols, the monothiol GSH can replace the dithiol moiety in TMA or MAP only if present in large excess. GSH was indeed in large excess when TMA and MPA were converted in part to the GSH conjugate *in vitro* (Fig. 4, top). *In vivo*, GSH is also likely to be in excess of TMA and MAP in the liver, because the hepatic concentration of GSH is 5–7 mM in rats [27] (Fig. 2). Monothiols can in fact bind to the arsenic atom in melaminophenyl arsenicals as demonstrated by the structure of melarsamine hydrochloride (Cymelarsan), a representative of such drugs, in which two cysteamine moieties are linked to the arsenic atom [28].

Apparently, neither the GSH conjugate nor any other biliary metabolites of melaminophenyl arsenical drugs have been reported. Fairlamb and co-workers [29] demonstrated

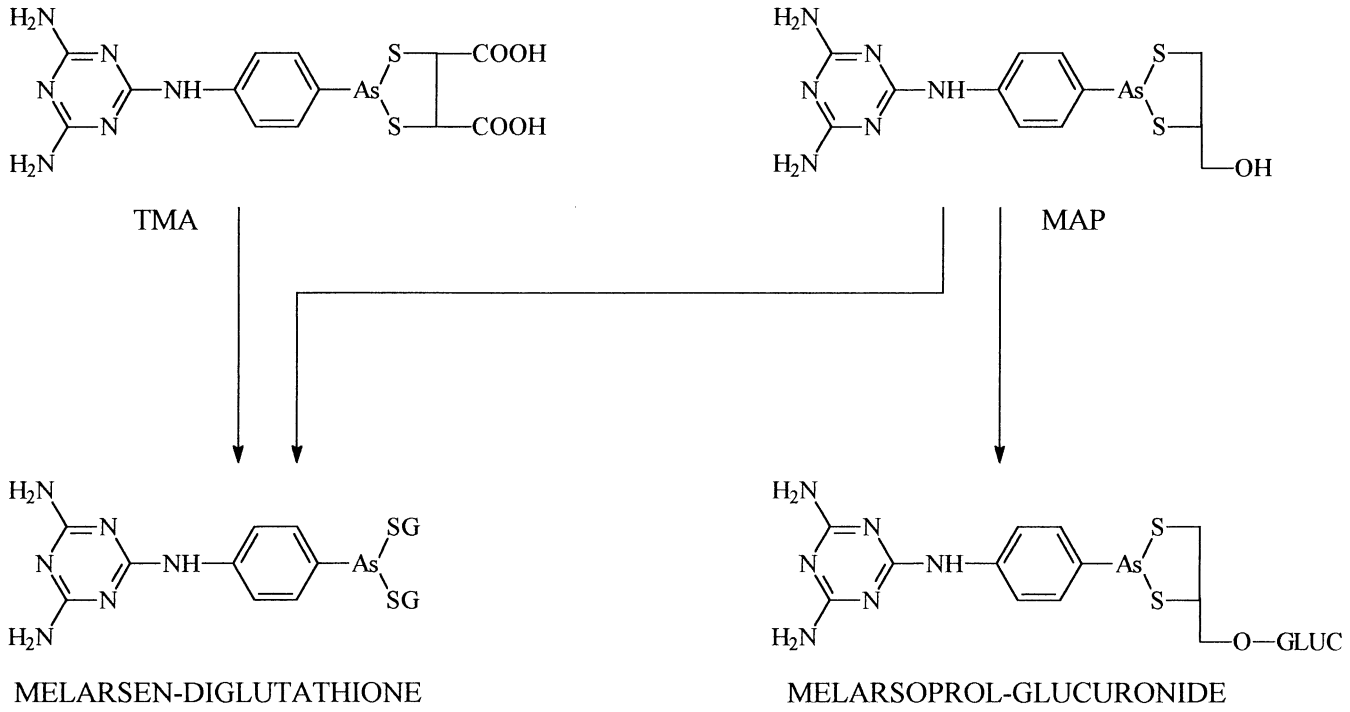

FIG. 7. The putative metabolites of trimelarsan (TMA) and melarsoprol (MAP) in rat bile.

the formation of a melaminophenyl arsenite–GSH complex spectrophotometrically when they incubated GSH with melarsen oxide, a chemical in which the dithiol moiety bound to the arsenic atom in TMA or MAP is substituted with an oxygen atom [29], although the product was not stable enough for separation by HPLC [19]. The latter authors were probably unable to demonstrate the GSH conjugate by HPLC analysis, seeing that they used an eluent with a high content of organic solvent in which, according to our experience, the GSH conjugate of the melaminophenyl arsenicals is indeed very unstable.

This study also demonstrates that the GSH conjugate is not the only form in which the arsenical drugs appear in bile. The finding that TMA, like its GSH conjugate, is also excreted unchanged (Fig. 4) is not unexpected, because the two carboxylate groups and the relatively large molecular weight (460) render this organic acid cholephilic. In contrast, MAP is not an organic acid and is not excreted into bile in significant quantities (Fig. 4). Nevertheless, HPLC analysis of the bile from MAP-injected rats revealed the presence of two prominent metabolites labelled with Y and Z (Fig. 4). Given that MAP contains a hydroxyl group in its dimercaptopropanol moiety (Fig. 7) and that hydroxyl group-containing compounds are often conjugated with glucuronic acid, we examined whether metabolites Y and Z are glucuronides of MAP, which would also be considered cholephilic organic acids. The following observations prove that metabolites Y and Z are indeed MAP glucuronides. First, they disappeared from the bile sample of MAP-injected rats upon incubation with β-glucuronidase/aryl sulfatase with concomitant appearance of the two diastereoisomers of MAP (Fig. 6). Second, the β-glucuronidase/aryl sulfatase-induced disappearance of metabolites Y and Z and appearance of MAP diastereoisomers was prevented by the β-glucuronidase inhibitor D-glucaric acid-1,4-lactone. These observations provide strong circumstantial evidence that metabolites Y and Z are glucuronides of the two diastereoisomers of MAP.

The finding that a GSH conjugate is the main biliary metabolite of both TMA and MAP readily accounts for the observation that biliary excretion of arsenic in rats given these drugs depends on the availability of GSH in the liver. On the other hand, the observations that the GSH conjugate is not the only form in which the melaminophenyl arsenicals are transported into bile account for the fact that the biliary excretion of TMA- and MAP-derived arsenic is only partially dependent on the hepatic GSH supply (Fig. 3). Moreover, when the lack of GSH compromised GSH conjugation, the biliary excretion of unchanged TMA and MAP–glucuronides appeared not only preserved but even increased (Fig. 5), suggesting that the hepatobiliary transport of unchanged TMA and the glucuronidation of MAP are in part complementary processes in the elimination of TMA and MAP, respectively, along with their GSH conjugation.

Immediately after injection in rats, arsenite (50 μmol/kg, i.v.) induced a more than 10-fold increase in the biliary excretion of non-protein thiols and glutathione (GSH + 2GSSG) [9, 30]. As this increase was related both in magnitude and time to the biliary excretion of arsenic, it was suggested that it was caused by dissociation of arsenic–GSH complexes (e.g. As-$(GSH)_3$) in bile. Like arsenite,

TMA and MAP also increased the biliary excretion of glutathione in a fashion temporally related to the biliary excretion of arsenic, but only 3- and 6-fold, respectively (Fig. 1). The mechanism of this increase in glutathione excretion and especially the reason for the difference between the two arsenicals in this respect is unclear. Under the conditions we performed the HPLC analysis of the biliary metabolites of TMA and MAP, the GSH conjugate of these arsenicals was stable. However, it is possible that during the assay of glutathione or non-protein thiols these conjugates partially decomposed, releasing GSH.

In summary, this study demonstrates that the melaminophenyl arsenicals TMA and MAP are highly cholephilic drugs. They are both excreted in bile partly as the same glutathione conjugate, which accounts for the partial dependence of their biliary excretion on hepatic GSH availability as well as for the partial depletion of hepatic GSH these drugs induce. In addition, TMA is also transported into bile unchanged and MAP as glucuronic acid conjugates. It is likely, therefore, that the biliary metabolites of TMA and MAP undergo reabsorption from the intestinal tract and enterophepatic circulation. These observations are of potential clinical relevance. Until confirmed in humans, they warn the clinician that elimination and detoxification of these drugs may also require GSH in patients and that therapy with such arsenicals may deplete GSH from tissues and compromise GSH-dependent defenses against oxidants and electrophiles, as well as against the arsenicals themselves. In addition, pharmacokinetic interactions with potential adverse outcomes may be expected between MAP and other drugs (e.g. acetaminophen) that are also glucuronidated.

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