Toxicology and Applied Pharmacology 232 (2008) 190–202

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Characterization of biliary conjugates of 4,4′-methylenedianiline in male versus female rats

Kan Chen [b], Richard B. Cole [b], Vicente Santa Cruz [c], Ernest W. Blakeney [d], Mary F. Kanz [c], Tammy R. Dugas [a,*]

[a] Department of Pharmacology, Toxicology and Neuroscience, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130, USA
[b] Department of Chemistry, University of New Orleans, New Orleans, LA, USA
[c] Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA
[d] Department of Chemistry, Centenary College, Shreveport, LA, USA

ARTICLE INFO

Article history:
Received 23 May 2008
Accepted 20 June 2008
Available online 1 July 2008

Keywords:
Methylene dianiline
Bile duct cells
Xenobiotic metabolism
Sex differences
Conjugation
Glutathione
Biliary excretion

ABSTRACT

4,4′-Methylenedianiline (4,4′-diaminodiphenylmethane; DAPM) is an aromatic diamine used in the production of numerous polyurethane foams and epoxy resins. Previous studies in rats revealed that DAPM initially injures biliary epithelial cells of the liver, that the toxicity is greater in female than in male rats, and that the toxic metabolites of DAPM are excreted into bile. Since male and female rats exhibit differences in the expression of both phase I and phase II enzymes, our hypothesis was that female rats either metabolize DAPM to more toxic metabolites or have a decreased capacity to conjugate metabolites to less toxic intermediates. Our objective was thus to isolate, characterize, and quantify DAPM metabolites excreted into bile in both male and female bile duct-cannulated Sprague Dawley rats. The rats were gavaged with [^{14}C]-DAPM, and the collected bile was subjected to reversed-phase HPLC with radioisotope detection. Peaks eluting from HPLC were collected and analyzed using electrospray MS and NMR spectroscopy. HPLC analysis indicated numerous metabolites in both sexes, but male rats excreted greater amounts of glutathione and glucuronide conjugates than females. Electrospray MS and NMR spectra of HPLC fractions revealed that the most prominent metabolite found in bile of both sexes was a glutathione conjugate of an imine metabolite of a 4′-nitroso-DAPM. Seven other metabolites were identified, including acetylated, cysteinyl–glycine, glutamyl–cysteine, glycine, and glucuronide conjugates. While our prior studies demonstrated increased covalent binding of DAPM in the liver and bile of female compared to male rats, in these studies, SDS-PAGE with autoradiography revealed 4–5 radiolabeled protein bands in the bile of rats treated with [^{14}C]-DAPM. In addition, these bands were much more prominent in female than in male rats. These studies thus suggest that a plausible mechanism for the increased sensitivity of female rats to DAPM toxicity may be decreased conjugation of reactive DAPM metabolites, leading to greater levels of protein adduct formation.

Introduction

4,4′-Diaminodiphenylmethane (DAPM, 4,4′-methylenedianiline) is an aromatic diamine used as a precursor to 4,4′-methylenediphenyl-diisocyanate (MDI) in the production of a number of polyurethane products. The toxicity of DAPM was first documented following the Epping jaundice incident of 1964, in which 84 people accidentally consumed DAPM and subsequently suffered symptoms of toxic hepatitis (Kopelman et al., 1966). This and other accidental and occupational exposures to DAPM have resulted in fever, jaundice, toxic hepatitis, cholangitis with cholestasis, skin rash, and in two acute cases, cardiomyopathy and retinopathy (Brooks et al., 1979; McGill and Motto, 1974; Bastian, 1984). In rats, chronic treatment with DAPM increased the incidence of thyroid and hepatic carcinomas (Weisburger et al., 1984; Lamb et al., 1986). Although studies of acute DAPM exposure indicated that biliary epithelial cells of the liver and common bile duct are the early site of injury (Kanz et al., 1992, 1995), little is known about its mechanism of toxicity. One prerequisite for elucidating the mechanism of DAPM toxicity is an understanding of its metabolism.

Current knowledge about DAPM metabolism has been ascertained from the characterization of metabolites isolated from rabbit, rat or human urine following DAPM exposure (Cocker et al., 1986, 1988a; Morgott, 1984). The major urinary metabolite found in humans and in rabbits exposed to DAPM is *N*-acetylated DAPM (Cocker et al., 1986, 1988a). This metabolite was also found to be the major metabolite conjugated to hemoglobin in humans (Bailey et al., 1990). *N*-acetylation has therefore been proposed as one major pathway in the metabolism of DAPM in humans. In addition to the acetylated metabolites, Kautiainen et al. (1998), demonstrated a hemoglobin adduct to a DAPM imine, formed following treatment of rats with

* Corresponding author.
E-mail address: tdugas@lsuhsc.edu (T.R. Dugas).

0041-008X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2008.06.008

DAPM. Because they were able to generate a similar product by incubating DAPM with peroxidase enzymes, they hypothesized that the imine was formed by extrahepatic peroxidases.

While these studies identified metabolites in urine and blood, the possibility exists that different, perhaps more reactive, metabolites of DAPM are excreted into bile, the proposed route of bile duct epithelial cell exposure to the proximate toxicant of DAPM (Kanz et al., 1995). Biliary excretion is an important mechanism for the elimination of xenobiotics. Conjugates formed following phase II reactions are the most common types of metabolites excreted in bile (Klaassen and Watkins, 1984). Thus, it is highly likely that reactive metabolites of DAPM formed following phase I oxidation reactions would be excreted into bile once they become conjugated, e.g., to glutathione, glucuronic acid, or even protein (Hobara et al., 1988).

Recent studies conducted in our laboratory demonstrated an increased sensitivity of female compared to male rats to DAPM-induced hepatobiliary injury (Dugas et al., 2001). Sex differences have been documented in metabolism involving isoforms of cytochrome P450 (Mugford and Kedderis, 1998; Sundseth and Waxman, 1992; Skett, 1988) sulfotransferases (Mugford and Kedderis, 1998), esterases (Los et al., 1996), as well as glucuronyl and glutathione-*S*-transferases (Zhu et al., 1996; Rao et al., 1977) and glutathione (Srivastava and Waxman, 1993). Thus, we hypothesize that the greater susceptibility to injury observed in female rats is due to differences in DAPM metabolism between the sexes. The aim of this study was to isolate, identify, and compare metabolites of DAPM excreted into the bile of male and female rats.

Experimental procedures

Materials

DAPM (4,4′-diaminodiphenylmethane) was purchased from Aldrich (Milwaukee, WI), and aromatic ring-labeled [^{14}C]-DAPM (specific activity 6.8 mCi/mmol) was synthesized by American Radiolabeled Chemicals (St. Louis, MO). Unless otherwise noted, all other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or higher. Stock solutions of DAPM containing 2–4% [^{14}C]-DAPM were dissolved with gentle warming in absolute ethanol and then diluted with deionized water to achieve a final concentration of 12.5 or 25 mg/mL DAPM in 35% ethanol. All doses of DAPM were administered at 2 mL/kg volumes.

Methods

Animals. Male and female Sprague Dawley (Harlan, Indianapolis, IN) rats of similar ages (12–15 weeks) were housed under controlled temperature and humidity (18–21 °C, and 55±5%, respectively). All animals were maintained in wire-bottomed cages over absorbent paper in 12 h light/dark cycles and were acclimated to the animal room for at least one week prior to DAPM treatment. [^{14}C]-DAPM or vehicle was administered to a total of 16 male (330–380 g) and 18 female (290–310 g) rats for the assessment of biliary metabolite excretion.

Animal surgery and bile collection procedure. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and biliary, duodenal, and peritoneal cannulas were implanted by standard procedures (Dugas et al., 2001; Kanz et al., 1995). Following surgery, taurocholate was infused into the duodenum to maintain continuous bile flow for 6–8 h, and when needed, pentobarbital diluted with saline was infused slowly into the peritoneum. Animals were equilibrated to stabilize physiological parameters such as respiration and body temperature, and basal bile was collected for 1 h at 15 min intervals. Rats were next gavaged with either 25 or 50 mg/kg [^{14}C]-DAPM, or vehicle (35% ethanol), and bile was collected for an additional 6 h in tared tubes stored on dry ice (Dugas et al., 2001). Bile samples thus collected were stored at −80 °C until analysis.

Metabolite profiling in rat bile was conducted either by fractionation after HPLC separation, followed by MS^n analysis, or by employing LC/ESI-MS^n. Separation and detection of the radioactive metabolites were performed by HPLC coupled to both UV and radioisotope detection. Once the retention time of each [^{14}C]-DAPM metabolite was identified, non-radiolabeled metabolites were collected, and these fractions were characterized by mass spectrometry.

HPLC fractionation of [^{14}C]-DAPM metabolites. Bile samples collected from the first 15 min of each half hour after DAPM treatment were evaluated for the presence of radiolabel using liquid scintillation counting. Bile samples collected from alternate 15 min intervals and determined to contain radiolabeled DAPM were thawed, deproteinized using Microcon 10,000 MW centrifugal filters (Fisher Scientific, Houston, TX), and then 20–50 μL samples were immediately injected onto HPLC. Alternatively, for some samples, 20 μL was injected and was directly analyzed using LC/ESI-MS^n. The collected fractions were concentrated by freeze-drying and reconstituted in a small volume of acetonitrile: water (50:50) for MS^n analysis. During the course of these studies, we noted that any additional incubation even at 4 °C resulted in increased numbers of peaks on HPLC, suggesting rapid decomposition of metabolites. The chromatography was accomplished using a Waters (Milford, MA) 626 pump and a 2487 dual-wavelength UV detector interfaced to an INUS Systems (Tampa, FL) β-RAM radioisotope detector. The separation was achieved using a 250×4.6 mm Ultrasphere (Beckman-Coulter, Fullerton, CA) reversed phase C18 column, a flow rate of 0.5 mL/min, and the following elution program: 1) gradient elution from 80% A (50 mM ammonium acetate, pH 3.5)/20% B (100% methanol) to 70% A/

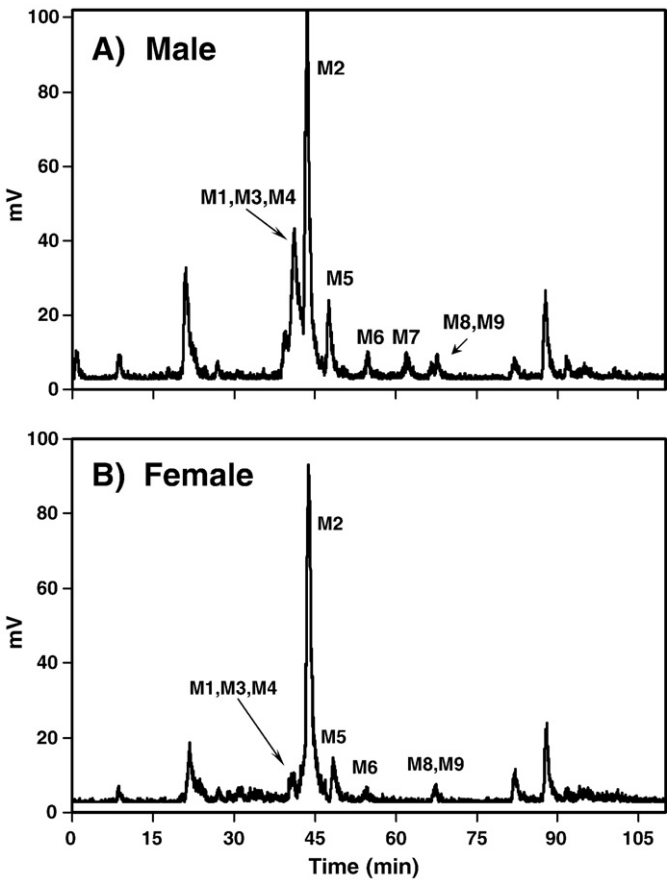

Fig. 1. Profile of radiolabeled metabolites detected using HPLC with radioisotope detection of bile samples from (A) a male and (B) a female rat, 1 h after treatment with 25 mg/kg [^{14}C]-DAPM.