Arch Pharm Res Vol 29, No 2, 172-177, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Identification of Glutathione Conjugates of 2, 3-Dibromopropene in Male ICR Mice

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(Received June 27, 2005)

Hepatotoxic potential of 2, 3-dibromopropene (2, 3-DBPE) and its conjugation with glutathione (GSH) were investigated in male ICR mice. Treatment of mice with 20, 50, and 100 mg/kg of 2, 3-DBPE for 24 h caused elevation of serum alanine aminotransferase and aspartate aminotransferase activities. The hepatic content of GSH was not changed by 2, 3-DBPE. Meanwhile, the GSH content was slightly reduced when mice were treated with 2, 3-DBPE for 6 h and significantly increased 12 h after the treatment. Subsequently, a possible formation of GSH conjugate of 2, 3-DBPE was investigated *in vivo*. After the animals were treated orally with 20, 50, and 100 mg/kg of 2, 3-DBPE, the animals were subjected to necropsy 6, 12, and 24 h later. A conjugate of *S*-2-bromopropenyl GSH was identified in liver and serum treated with 100 mg/kg of 2, 3-DBPE by using liquid chromatography-electrospray ionization tandem mass spectrometry. The protonated molecular ions $[M+H]^+$ of *S*-2-bromopropenyl GSH were observed at *m/z* 425.9 and 428.1 in the positive ESI spectrum with a retention time of 6.35 and 6.39 min, respectively. In a time-course study in livers following an oral treatment of mice with 100 mg/kg of 2, 3-DBPE for 6, 12, and 24 h, the 2, 3-DBPE GSH conjugate was detected maximally 6 h after the treatment. The present results suggested that 2, 3-DBPE-induced hepatotoxicity might be related with the production of its GSH conjugate.

Key words: 2, 3-Dibromopropene, Hepatotoxicity, Glutathione conjugate, LC/ESI-MS

INTRODUCTION

Alkyl dihalides including 2, 3-dibromopropene (2, 3-DBPE) have been used in industry as chemical intermediates, extraction solvents, degreasing compounds, copolymer cross-linking agents and pesticides. 2, 3-DBPE was a soluble and stable end product formed by chemical degradation of 1, 2-dibromo-3-chloropropane (2, 3-DBCP) in buffered aqueous solution (Burlinson *et al.*,1982). 2, 3-DBPE was highly mutagenic to the bacteria both in the absence and presence of metabolic activation system, such that its mutagenicity was similar to that of 2, 3-DBCP (Låg *et al.*, 1994).

By conjugation with glutathione (GSH), 2, 3-DBCP could be metabolized to reactive episulfonium ions (Søderlund *et al.*, 1995). Likewise, other halide compounds, such as 1-bromopropane (1-BP), 2-bromopropane (2-BP), 1,2-dibromopropane (1,2-DBP), and 1,3-dibromopropane (1,3-DBP), have been investigated on their oxidative biotransformations and conjugations with GSH (Barnsley *et al.*, 1966; Zoetemelk *et al.*, 1986; Tornero-Velez *et al.*, 2004; Lee *et al.*, 2005). Meanwhile, a possible formation of GSH conjugate(s) of 2, 3-DBPE has not been investigated yet.

GSH conjugation is generally a detoxicating process. In some cases, however, the toxicity or genotoxicity of a compound could be increased by this metabolic process. For examples, the conjugation of 2, 3-DBCP and 1, 2-dibromoethane, with GSH could form DNA adducts (Kim and Guengerich, 1990; Kim *et al.*, 1990; Humphreys *et al.*, 1991).

In the present study, the formation of GSH conjugate was investigated following a treatment of mice with 2, 3-DBPE by using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS). To characterize hepatotoxic potential of 2, 3-DBPE, dose

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response and time course effects of 2, 3-DBPE on hepatotoxicity parameters were initially studied in male ICR mice.

MATERIALS AND METHODS

Animals

Specific pathogen-free male ICR mice (28 to 33 g) were obtained from Orient (Seoul, Korea). The animals received at 4 weeks of age were acclimated for at least 2 weeks. Upon arrival, animals were randomized and housed 5 per cage. The animal quarters were strictly maintained at 23 ± 3°C and 50 ± 10% relative humidity. A 12 h light and dark cycle was used with an intensity of 150 - 300 Lux. All animal procedures were followed based on a guideline recommended by the Society of Toxicology (U.S.A.) in 1989.

Materials

2, 3-DBPE (purity, >99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 5, 5-Dithio-bis(2-nitrobenzoic acid) and 5-sulfosalicylic acid were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). The kits for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays were purchased from Asan Pharm. Co. (Hwasung, Korea). Acetonitrile (ACN) was HPLC-grade from Merck Ltd. (Poole, UK). All other chemicals were of analytical grade and used as received.

Animal treatments

2, 3-DBPE in corn oil was treated once orally to animals at 20, 50, and 100 mg/kg. One day after the dose, the animals were euthanized. For time course studies, 2, 3-DBPE at 100 mg/kg was treated orally. Animals were subjected to necropsy at 0, 6, 12 or 24 h after the oral administration. Following the blood collection, the livers were removed and homogenized with 4 volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4. Aliquots of tissue homogenates and sera were stored at -80°C until use.

Hepatotoxicity parameters

For assaying the activities of ALT and AST, the serum was prepared by a centrifugation of the blood at 3,000×g at 4°C for 15 min. The activities of the enzyme were determined according to an instruction manual prepared by the manufacturer.

Content of reduced GSH

Liver GSH levels were determined by the Ellman's method (Ellman, 1959). The content of liver homogenate protein was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Sample analysis by LC/ESI-MS

The conjugates in liver homogenates and sera were extracted by an addition of 4 volumes of ACN. After a vortex mixing and a centrifugation at 15,000 × g for 20 min at 15°C to remove the proteins, the resulting supernatant was evaporated under a stream of nitrogen in a water bath maintained at 60°C. The resulting residue was reconstituted in a mixture of 50/50 of 0.1% aqueous formic acid/ACN by vortexing and a subsequent centrifugation at 15,000 × g for 10 min at 15°C. Then, a 100 μL aliquot was used for the LC/ESI-MS analysis.

The HPLC consisted of an surveyor system (Thermo Finnigan, San Jose, CA, U.S.A.) with the LCQ advantage trap mass spectrometer (Thermo Finnigan, San Jose, CA, U.S.A.) equipped with an electrospray ionization source. The column used for the separation was an Xterra C18 (2.1 × 150 mm, 5 μm). The HPLC mobile phases consisted of 0.1% aqueous formic acid, pH 4.0 (A), and 50% ACN in 0.1% formic acid (B). A gradient program was used for the HPLC separation with a flow rate of 210 μL/min. The initial composition was 10% B and programmed linearly to 100% B after 6 min. Nitrogen was used both as the sheath gas at 70 L/min and as the auxiliary gas at 6.6 L/min with a capillary temperature of 215°C and the spray voltage set to 4 kV. The mass spectrometer was operated in the positive ion mode in *m/z* range 100-400. Helium was used as the collision gas for the tandem mass spectrometric experiments, followed by the isolation of ions over a selected mass window of 1 Da.

Statistics

The mean value ± standard error (S.E.) was determined for each treatment group of a given experiment. Dunnett's t-test was used to compare the statistical significance of data obtained. The significant values at either $P<0.05$ (*) or $P<0.01$ (**) were represented as asterisks.

RESULTS

Hepatotoxicity induced by a single treatment with 2,3-DBPE

To characterize the acute effects of 2, 3-DBPE on general toxicity, changes in body and liver weights were determined following a single oral treatment of male ICR mice with 2, 3-DBPE at 20, 50, and 100 mg/kg for 24 h (Table I). The body and liver weights and hepatic content of GSH were not changed by the single treatment with 2,3-DBPE. When mice were treated orally with 100 mg/kg of 2, 3-DBPE once for 6, 12, and 24 h, the body and liver weights were not altered (Table II). Interestingly, however, the hepatic GSH level was slightly decreased 6 h after a single treatment with 2, 3-DBPE , and the contents were over recovered 12 h after the treatment (Table II).

Table I. Effects of 2, 3-DBPE on body and liver weights and contents of reduced GSH in male ICR mice : a dose-response study

Dose, mg/kg	Body weight (g)	Relative liver weight (%)	Reduced GSH (nmole/mg protien)
Vehicle	33.5 ± 1.4	5.46 ± 0.17	160 ± 10
20	30.6 ± 0.6*	4.87 ± 0.07	137 ± 8
50	30.9 ± 0.2	5.06 ± 0.15	138 ± 5
100	30.5 ± 0.3*	6.05 ± 0.26	192 ± 23

Male ICR mice were orally treated with 20, 50, and 100 mg/kg of 2,3-DBPE in corn oil once. All animals were subjected to necropsy 24 h after the treatment. Each value represents the mean ± S.E. of 5 animals. The asterisks indicate the values significantly different from vehicle control at P<0.05 (*).

Table II. Effects of 2, 3-DBPE on body and liver weights and contents of reducted GSH in male ICR mice: a time-course study

Time, h	Body weight (g)	Relative liver weight (%)	Reduced GSH (nmole/mg protien)
0	33.5 ± 1.4	5.46 ± 0.17	160 ± 10
6	31.1 ± 0.9	6.30 ± 0.06*	113 ± 11
12	31.3 ± 0.6	5.57 ± 0.12	392 ± 30**
24	30.5 ± 0.3	6.05 ± 0.26	192 ± 23

Male ICR mice were orally treated with 100 mg/kg of 2, 3-DBPE in corn oil once. All animals were subjected to necropsy 0, 6, 12, and 24 h after the treatment. Each value represents the mean ± S.E. of 5 animals. The asterisks indicate the values significantly different from 0-h control at either P<0.05(*) or P<0.01 (**).

In Fig. 1, the hepatotoxic effects of 2, 3-DBPE were determined. Following a single oral treatment with 100 mg/kg of 2, 3-DBPE for 24 h, the activities of serum ALT and AST were increased by 86- and 29-folds when compared with the vehicle-treated control, respectively. The other groups did not affect the activities of ALT and AST. In a time course study, the activities of serum ALT and AST were elevated gradually by 100 mg/kg of 2, 3-DBPE from 6 to 12 h after the treatment and rapidly increased at 24 h.

Identification of 2, 3-DBPE-GSH conjugate *in vivo*

Based on the LC/ESI-MS analyses, the fragmentation patterns of individual conjugates could be proposed. The protonated molecular ions $[M+H]^+$ of *S*-2-bromopropenyl GSH were observed at *m/z* 425.9 and 428.1 in the positive ESI spectrum with a retention time of 6.35 and 6.39 min, respectively (Fig. 2). The product ions at *m/z* 350.6 and 352.7 were believed to be due to the elimination of glycine moiety from the corresponding GSH conjugates (Fig. 3, Loughlin *et al.*, 2001). The fragment ions at *m/z* 296.8 and 298.7 were believed to be formed by the loss of the glutamate moiety (Prabhu *et al.*, 2002) and the ions at *m/z* 279.7 and 281.7 corresponded to the loss of amino-glutamyl moiety from GSH conjugates (Lee *et al.*, 2005).

In vivo formation of 2, 3-DBPE-GSH conjugate in liver and serum

To characterize the formation of GSH conjugate *in vivo,*

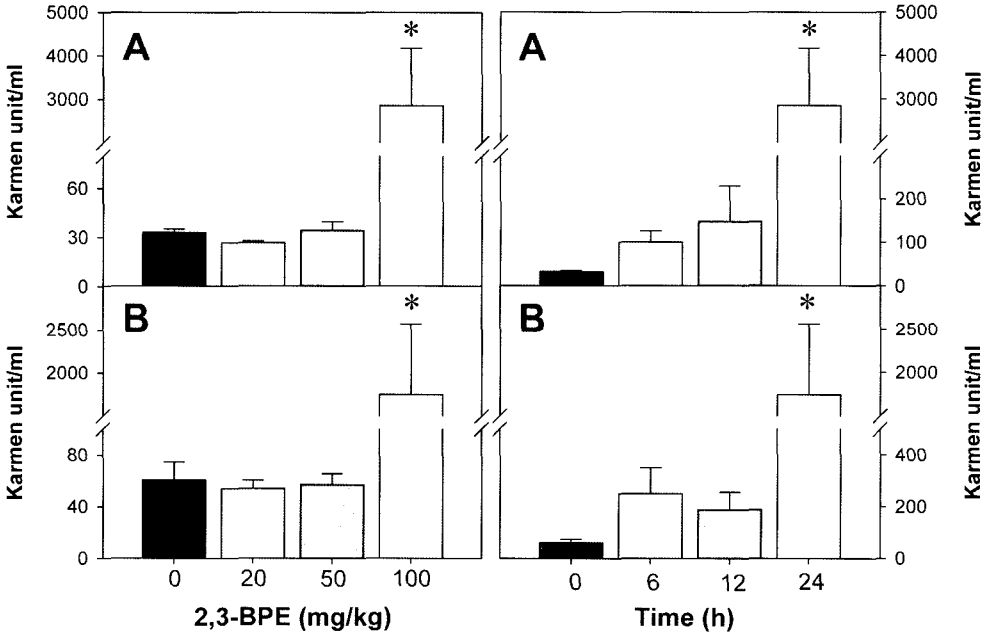

Fig. 1. Effects of 2, 3-DBPE on serum activities of ALT (A) and AST (B). For the dose-response study, male ICR mice were treated orally with 20, 50 and 100 mg/kg of 2, 3-DBPE in corn oil once. All animals were subjected to necropsy 24 h after the treatment. For the time-course study, animals that treated with 100 mg/kg of 2, 3-DBPE in corn oil were subjected to necropsy 6, 12, and 24 h after the treatment. Each value represents the mean ± S.E. of 5 animals. The asterisks indicate the values significantly different from the control at P < 0.05 (*).

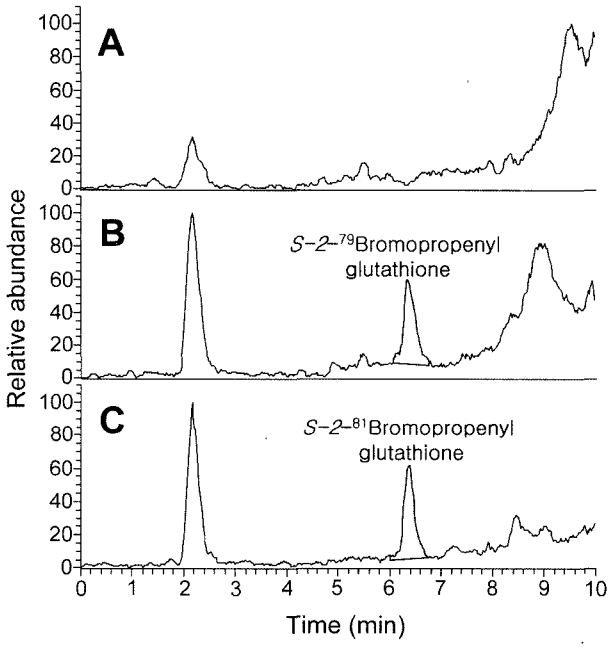

Fig 2. Total ion chromatogram of vehicle group (A) and total ion chromatogram of *S*-2-bromopropenyl GSH, at *m/z* 426 MS^2 (B) and 428 MS^2 (C). The conjugates in liver homogenates were extracted by 4 volumes of acetonitrile (ACN). After a vortex mixing and a centrifugation at 15,000 × g for 20 min at 15°C, the resulting supernatant was evaporated under a stream of nitrogen as described in the Materials and Methods. The resulting residue was reconstituted in a mixture of 0.1% aqueous formic acid/ACN and a subsequent centrifugation. Then, a 100 mL aliquot was used for the LC/ESI-MS analysis.

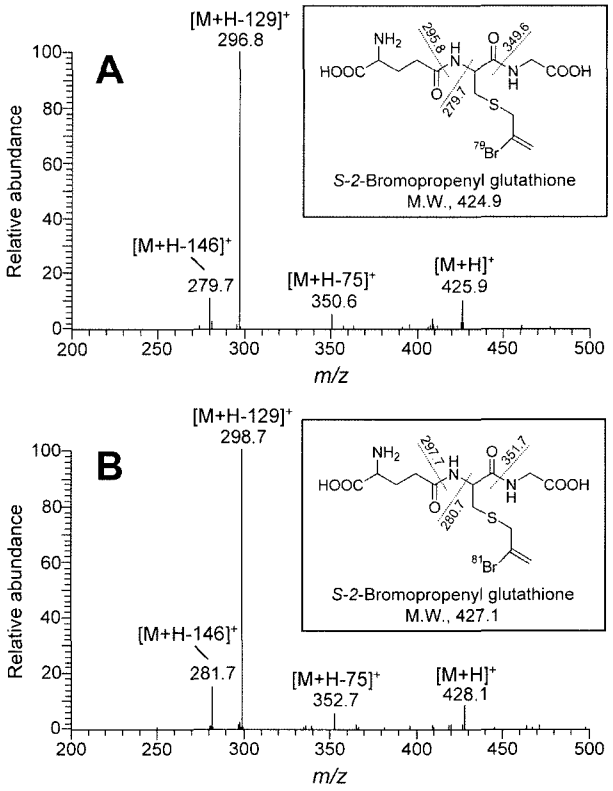

Fig. 3. CID spectra of protonated *S*-2-^{79}bromopropenyl GSH (A) and *S*-2-^{81}bromopropenyl GSH (B). The sample preparation for the mass analysis was the same as in Fig. 2. All the detailed procedures for the analyses were described in the Materials and Methods.

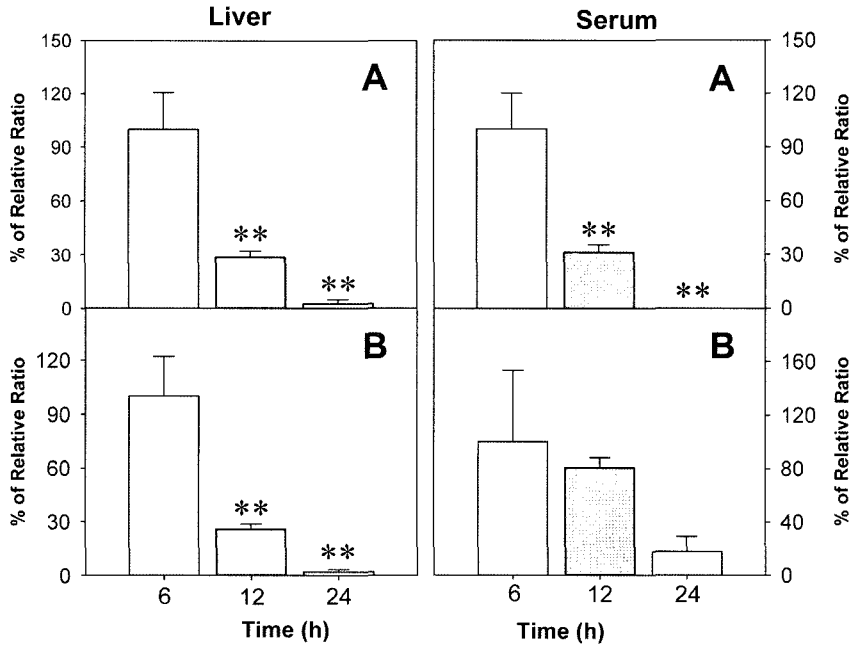

Fig. 4. Time course effects of 2, 3-DBPE on the formation of *S*-2-^{79}bromopropenyl GSH (A) and *S*-2-^{81}bromopropenyl GSH (B) in liver and sera. 2, 3-DBPE in corn oil was treated once orally to animals at 100 mg/kg for 0, 6, 12, or 24 h. Following the blood collection, the livers were removed and homogenized with 4 volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The sample preparation for the mass analysis was the same as in Fig. 2. All the detailed procedures for the analyses were described in the Materials and Methods. The asterisks indicate the values significantly different from the 6-h data at $P < 0.01$ (**).

dose response and time course studies were performed in the same strain of mice. Following an oral treatment of mice with 20, 50, and 100 mg/kg of 2, 3-DBPE for 24 h, the sera and liver homogenates were analyzed. In livers and sera isolated from 2, 3-DBPE-treated mice, no GSH conjugates were detected except the group of 100 mg/kg-treated (data not shown). Meanwhile, when the production of GSH conjugates was investigated in livers following an oral treatment with 100 mg/kg of 2, 3-DBPE for 6, 12, and 24 h, all metabolites were detected maximally 6 h after the treatment (Fig. 4).

DISCUSSION

Sometimes the conjugation of xenobiotics with GSH would enhance the their toxicities, as mentioned in cases of alkyl dihalides, polyhaloalkene, isocyanates and certain quinines. In a particular case of 2, 3-DBPE, the GSH conjugates could from a reactive metabolites which can covalently bind to macromolecules in livers (Søderlund *et al.*, 1995). In addition, 1, 2-dibromoethane could form the adenine adduct following conjugation with GSH (Kim *et al.*, 1990). In the present studies, possible hepatotoxic potential of 2, 3-DBPE and its conjugation with GSH were investigated to understand the toxic mechanism of 2, 3 DBPE *in vivo*.

When mice were orally treated with 100 mg/kg of 2, 3-DBPE, the activities of serum ALT and AST were elevated significantly 24 h after the treatment. However, the hepatic content of GSH was not significantly reduced by 2, 3-DBPE (Table I and Fig. 1). Meanwhile, the time-course study indicated the possible reduction of hepatic GSH level 6 h after the treatment and a rebound of the GSH content 12 h after the 2, 3-DBPE treatment, indicating the possibility of GSH conjugation (Table II). Subsequently, the formation of GSH conjugate(s) by 2, 3-DBPE and the structure of conjugate(s) were investigated.

Following an oral treatment of mice with 100 mg/kg of 2, 3-DBPE, the metabolites in liver was analyzed by using LC/ESI-MS. Fig. 2 and 3 showed the protonated molecular ion chromatograms of two conjugates of 2, 3-DBPE as *S*-2-bromopropenyl GSH. Each conjugates were observed at *m/z* 425.9 and 428.1 in the positive ESI spectrum with a retention time of 6.35 and 6.39 min, respectively. Although two different molecular weights were detected in the extracted ion chromatograms, we assigned these as a same structure because these ions would typically be within a cluster of Br^{79}/Br^{81} isotopic ions (Engen *et al.*, 1999; Lam and Ramanathan, 2002; Tornero-Velez *et al.*, 2004). Bromine-containing compounds as *S*-2-bromopropenyl GSH are simultaneously show two of the minor peaks due to the Br^+ ion in their mass spectra. Both conjugates could be detected in livers and sera prepared from 2, 3-DBPE-treated mice following an oral treatment with 100 mg/kg of 2, 3-DBPE for 6, 12, and 24 h. In addition, all metabolites were detected maximally 6 h after the treatment (Fig. 4).

One can possibly speculate that *S*-3-bromopropenyl GSH could be produced in this study. Generally, the GSH conjugation can be classified into two types: the displacement reaction, in which GSH displaces an electron-withdrawing group, and the addition reaction, in which GSH is added to an activated double or strained ring system (Klaassen, 2001). It was concluded that the conjugation of 2, 3-DBPE should be the former case due to the relatively strong electrophilicity of ^3C carbon of 2, 3-DBPE. The ^3C would be more electrophilic than ^2C because the electron would be provided from the C=C double bond. Thereby, the structure of conjugate was assigned as *S*-2-bromopropenyl GSH.

Although we found that the GSH conjugates of 2, 3-DBPE was produced in livers dose- and time-dependently, the hepatic GSH level was marginally changed. This discrepancy might be resulted from the experimental method to quantitate hepatic GSH contents. The method employed in this study for detecting hepatic GSH might not be so specific to the GSH. The reagent used could nonspecifically react with hepatic –SH group, so that the changes in GSH content would be masked significantly. To elucidate this possibility, more specific method for identifying the GSH is under application.

According to a previous study, 2, 3-DBPE was highly mutagenic to the bacteria both in the absence and presence of metabolic activation system, such that its mutagenicity was similar to that of 2, 3-DBCP (Låg *et al.*, 1994). It was suggested that 2, 3-DBPE might be metabolized by cytochrome P450 to the corresponding 1, 2-epoxide which can be spontaneously rearranged to form 1, 3-dibromo acetone, 2-bromoacrolein and 1, 3-dibromo acetone that are all potent direct mutagens (Låg *et al.*, 1994). In addition, it was also suggested that *S*-2-bromopropenyl GSH could easily form the reactive episulfonium ion capable of causing DNA damages, and that 2, 3-DBPE would be reactive toward nucleophiles, such as GSH and DNA, due to its α, β-double bond adjacent to the two vicinal halogens (Låg *et al.*, 1994). Therefore, the possible role of the metabolism mentioned above remained to be investigated. In the present study, the formation and structure of GSH conjugate with 2, 3-DBPE were identified. Although further studies should be done to understand 2, 3-DBPE-induced hepatotoxicity, the present results suggested that GSH conjugation may contribute to 2, 3-DBPE-induced hepatotoxicity. As in cases of 2, 3-DBPE, the possible formation of DNA adducts with 2, 3-DBPE-GSH conjugate should be studied in the future. In addition, a possibility of direct adduct formation

of 2, 3-DBPE with DNA should be investigated further.

ACKNOWLEDGEMENT

This work was supported by a grant from National Institute of Toxicological Research, Korea Food and Drug Administration.

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