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Measurement of Hemoglobin Adducts in Female Mice Inhaled with 1,3-butadiene by Using GC/MS

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Abstract : 1,3-butadiene(DB) is a well-established rodent carcinogen, and a probable carcinogen to humans. This study was investigated the formation of hemoglobin adduct in ICR female mice inhaled with BD for 3 weeks (5 hr/day, 5 days/week). Body weights of mice were significantly low from onward day 4 or 9 of exposure comparing the control. Hemoglobin adducts formed by DB exposure were (N-2-hydroxy-3-butenyl) valine (HB Val adduct) and (N-2,3,4-trihydroxy-butyl)valine(THB Val adduct). The levels of HB Val adducts were 1.8, 3.7 and 6.2 pmol/mg globin for the 500 ppm BD inhalation group, and 5.7, 7.4 and 16.0 pmol/mg globin for the 1000 ppm BD inhalation group, when observed on the 1st, 2nd, and 3rd week after inhalation exposure, respectively. The levels of THBVal adducts were 32.0, 42.0 and 55.0 pmol/mg globin for the 500 ppm DB inhalation group, and 67.8, 72.7 and 83.5 pmol/mg globin for the 1000 ppm BD inhalation group, when observed on the 1st, 2nd, and 3rd week after inhalation exposure, respectively. Ratios of THBVal and HBVal adducts were higher at earlier exposure period and lower concentration. Ratios on the 1st, 2nd, and 3rd week were 17.8, 11.4 and 8.87 for the 500 ppm BD inhalation group, and 11.9, 9.8 and 5.2 for the 1000 ppm BD inhalation group, respectively. In conclusion, THB Val and HB Val adducts were the important hemoglobin adducts in ICR female mice inhaled with BD, and the latter was more appropriate biomarker than the other for biological monitoring of BD exposure.

Keywords : 1,3-butaiene(BD), biomarker, (N-2-hydroxy-3-butenyl)valine(HB Val adduct), (N-2,3,4-trihydroxy-butyl) valine(THB Val adduct), GC/MS

Introduction

1,3-butadiene(BD) is a colorless, inflammable and easily liquid organic chemical. Its melting, boiling and ignitable point are −108°C, −4.4°C and −85°C, respectively. It is an important pollutant at the petrochemical products, particularly in the preparation of polymers, i.e., styrene/butadiene synthetic rubber (SBR), acrylonitrile/butadiene/styrene thermóplastic (ABS) and styrene/butadiene/styrene block copolymer (SBS). It also occurs as an environmental contaminant. It has been estimated that most BD emissions derive from mobile sources, although leaks and waste emissions from manufacturing facilities may be locally important. All burning of organic material produces emissions containing minor amounts of BD, and cigarette smoke contains small amount of BD. Low exposure to BD is thus a common characteristics of whole human population, although the exposure levels in industry might be several orders of magnitude higher.

BD is a well-established rodent carcinogen. Although both sexes of B6C3F1 mice and Sprague -Dawley rats developed tumors in 2 years inhalation bioassay (Huff *et al.*, 1985; Melnick *et al.*, 1990; Owen *et al.*, 1987; Osterman-Golkar *et al.*, 1998). The most striking aspect of butadiene-induced carcinogenicity in rodent is the high sensitivity of mice compared with rats. Rats developed tumors from exposure to butadiene concentrations (1,000 -8,000 ppm) that were as much as three orders of magnitude higher than that caused cancer in mice (6.25-1,250 ppm)(Osterman-Golkar *et al.*, 1998).

Based on sufficient evidence of carcinogenicity in laboratory and partial evidence of carcinogenicity in epidemiological studies in humans, the International Agency for Research on Cancer (IARC), in 1999, classified BD as a probable carcinogen to humans (group 2A)(IARC, 1999). The European Union also labels BD as a potential carcinogen (R45: "may cause

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cancer"). In 2000, the ninth report on carcinogens edited by the Department of Health and Human Services, US, described BD as "known to be a human carcinogen" (Department of Health and Human Services, 2000). In 2002, the US Environmental Protection Agency classified BD as a human carcinogen by inhalation (EPA, 2002).

To prevent health risks in humans arising from exposure to BD, occupational exposure limits were established or recommended in different countries. A concentration of 2 ppm (4.4 mg/m^3) as a threshold limit value time-weighted average (TLV-TWA) during an 8 h shift is recommended by the American Conference of Governmental Industrial Hygienists (ACGIH, 2002). Concentration of 15 ppm (34 mg/m^3) and 5 ppm (11 mg/m^3) are established as technical exposure limit (TRK) by the German Federal Ministry for Employment and Social Affairs (AGS Committee for hazardous substances) for processing after polymerization or loading and other applications, respectively (Deutsche Forschungsgemeinschaft, 2002). At the present time, BD is under evaluation by the Scientific Committee for Occupational Exposure Limits (SCOEL) of the European Union (Fustinoni *et al.*, 2004). A concentration of 10 ppm (22 mg/m^3) as a permission limit value time-weighted average during 8 h shift is established by the ministry of labor in Korea.

Once inhaled and absorbed through the respiratory tract, BD is distributed through the blood to various organs. Its elimination may occur either via exhaled air, for the un-metabolized compound, or via urinary excretion, after transformation in hydrophilic metabolites. The metabolism of BD has been studied in rats and mice by several different investigators and reviewed by Himmelstein *et al.* (1997), Albertini *et al.* (2001) and Swenberg *et al.* (2001).

It is generally accepted that BD is first metabolized to 1,2-eopxy-3-butene(EB), a process that is primary associated with CYP2E1 and CYP2A6 (Albertini *et al.*, 2001). This electrophilic metabolite can be detoxified by congjugation with glutathione and subsequent excretion in the urine as 1-hydroxy-2-(N-acetylcysteinyl)-3-butene(M-2) and 1-(N-acetylcysteinyl)-2-hydroxy-3-butene. It can also undergo hydrolysis by epoxide hydrolase(EH) to form 1,2-dihydroxy-3-butene(BD-diol). BD-diol is a major metabolite of BD in liver. It can be conjugated with glutathione and subsequently excreted in the urine as 1,2-dihyroxy-4-(N-acetylcysteinyl)-butane(M-1). It can be further oxidized by CYP to the 1,2-dihydroxy-3,4-epoxybutane(epoxybutane diol, EBD). Alternative pathway for the metabolism of EB is its further oxidation to 1,2,3,4-diepoxybutane (DEB), which can be further hydrolyzed to EBD or conjugated by glutathione and excreted in the urine (Henderson *et al.*, 1993). This series of epoxidation and detoxification steps generates three electrophilic metabolites, such as EB, DEB and EBD.

The main goal of biological monitoring of exposure is to ensure that the current or past exposure of workers and citizens is safe, i.e., does not entail an unacceptable health risk. Biological monitoring of exposure is clearly distinguished from health surveillance. Whereas it attempts to detect unhealthy exposure condition (health risk), health surveillance evaluates the health status and aims at identifying individuals with early signs of adverse health effects, i.e., effects which are likely to be reversible or which do not progress to significant functional impairments when the exposure conditions are improved.

Biological monitoring of exposure can be classified in three categories, but of different importance (Bernard and Lauwerys, 1986; Lauwerys and Hoet, 1993): 1) determination of the chemical or its metabolites in biological fluid; 2) quantification of non-adverse biological effects related to the internal dose; and 3) direct measurement of the amount of active chemical interacting with the target molecules. Biological responses include biomarkers of exposure (urinary metabolite concentrations, hemoglobin adducts and DNA adducts), biomarkers of effects (in vivo somatic gene mutations and chromosome changes) and biomarkers of susceptibility (metabolic genotypes)(Albertini *et al.*, 2001; Committee of biological Markers of the National Research Council, 1987).

The purposes of this study were to measure hemoglobin adducts of BD by using GC/MS and to investigate the appropriate biomarker in female mice inhaled with 1,3-butadiene. Metabolites of DB formed hemoglobin adduct are (N-2-hydroxy-3-butenyl)valine(HB Val) and (N-2,3,4-trihydroxy-butyl)valine(THB Val) which are produced by the EB and EBD, respectively.

Materials and Methods

Chemicals

1,3-butadiene(BD) was obtained from Sigma (St. Louis, MO, USA). Analytical hydroxide, potassium bishydrogen phosphate, sodium sulfate, acetyl chloride and acetic ethanol, acetone and ethyl acetate (E. Merck, Darmstadt, Germany) were used as solvents. All other chemicals were of the highest purity available from Sigma and Merck.

Synthesis and Identification of HB Val. and THB Val.

N-(2-hydroxy-3-butenyl)valine(HB Val) was synthesized by incubation of butadiene monoxide with L-valine in 3 m*l* of water, pH 10 for overnight at 37°C. N-(2,3,4-trihydroxybutyl)valine(THB Val) was synthesized by incubation of butadiene dioxide with L-valine in 3 m*l* of water, pH 10 for overnight at 37°C. They were dry with N_2 stream, dissolved in acetone and identified with GC/MS. Peaks and spectra of HB Val are Fig. 1 and Fig. 2, and peaks and spectra of THB Val are Fig. 3 and Fig. 4, respectively.

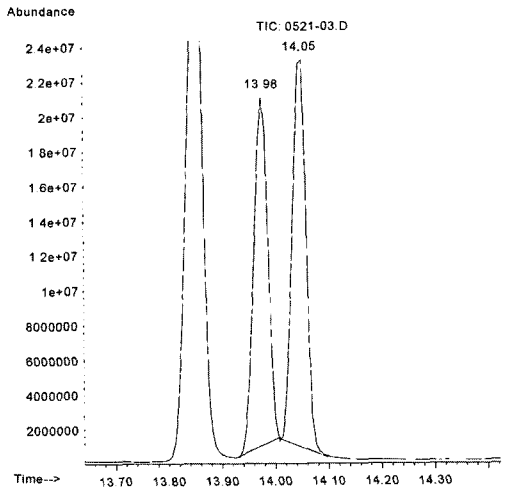

Fig. 1. Chromatogram of N-(2-hydroxy-3-butyl)Valine (HB Val.) by using GC/MS.

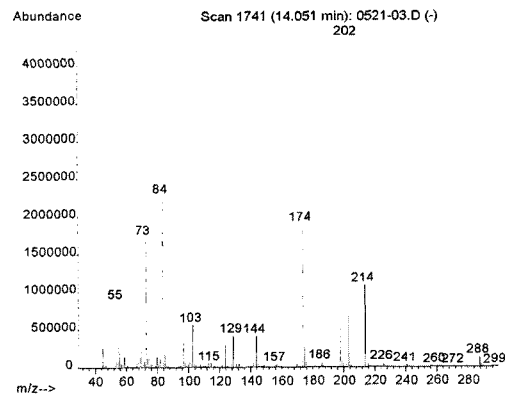

Fig. 2. Mass spectra of N-(2-hydroxy-3-butyl)Valine(HB Val.).

Animals and Treatment

Forty five female ICR mice with a body weight

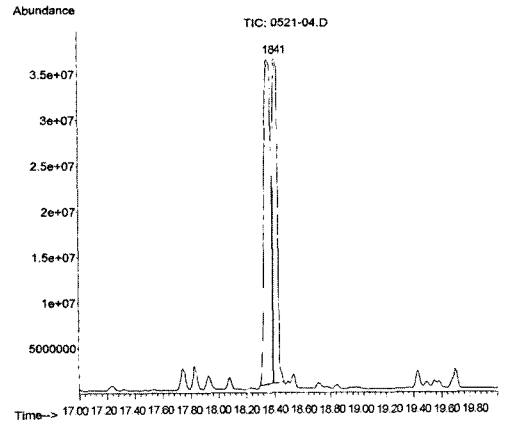

Fig. 3. Chromatogram of N-(2,3,4-trihydroxy-3-butyl) Valine (THB Val.) by using GC/MS.

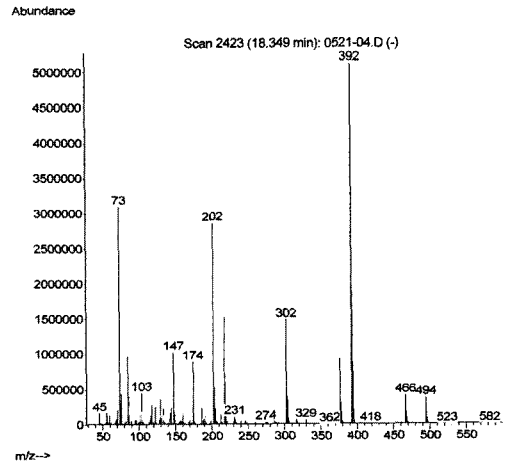

Fig. 4. Mass spectra of N-(2,3,4-trihydroxy-3-butyl)Valine (THB Val.).

of about 20 g, were obtained from Daehan biolink (Chongju, South Korea). They were acclimatized for one week in Maecrlone cages (temperature, 20°C ; humidity, 30-70%; illumination time, 06:00 h to 18:00 h), and had free access to tap water and food. Fifteen mice in each group were randomized with respect to body weight before onset of exposure and then weighted three times weekly. The animals were exposed for 1, 2 or 3 weeks, 5 h/day, 5 day/week, to nominal butadiene concentration of 0, 500 or 1,000 ppm. The animals were exposed in inhalation chamber with 1,3-butadiene dilution systems. Butadiene was generated directly from a liquid-gas storage tank and passed through a calibrated flow control device prior to mixing with clean air (99.999%). Air flow through the inhalation chambers were maintained at 5 ± 2 *l*/min to ensure 5 to 7 air changes per hour. The animals had free access to water and food during non-exposure periods, but were deprived of water and food during the 5 h exposures. The animals were exsanguinated by cardiac puncture. Blood samples were collected in heparinized syringes from groups of five mice at the end of each week during the 3 week inhalation exposure period. The erythrocytes were isolated by centrifugation, washed three times with 0.9% NaCl solution, and stored at −20°C before isolation of globin.

Quantitative Determination of Hemoglobin Adducts

Erythrocytes were separated from the plasma by centrifugation at 1,000 × g for 10 min, and were then washed twice with saline. The cells were lysed by adding one volume of distilled water for each volume of packed saline.

Samples of 50 mg of globin were dissolved in 1 m*l* of formamide. 0.1 M NaOH and 10 u*l* MSTFA (N-Methyl-N-(trimethylsilyl)-(trifluoroacetamide, Aldrich, USA) were added and the samples were left on a rocking mixer at room temperature over night and then 45°C for 1.5 h. 2 m*l* of water was added to the formamid phase and MSTFA derivatives were extracted with ethyl ether (3 times with 3 m*l*). The ether extract was evaporated to dryness, dissolved in 2 m*l* toluene and washed with 0.1 m*l* of water. The toluene extracts were pooled in a new test tube, and analyzed with GC/MS (Osterman-Golkar *et al.*, 1996).

Conditions of GC/MS

All mass spectra were obtained with 6890/5973 GC/MS (Agilent Techologies; Palo Alto, CA, USA). The ion source was operated in the electron ionization mode (EI: 70 eV, 230°C). Full-scan mass spectra (m/z 30-800) were recorded for identification of analysts. Column was HP-5MS (30 m × 0.25 mm × 0.25 um F.T.). Samples were injected in the pulsed split ratio (1/10). The flow rate of helium was 1.0 m*l*/min. The GC operating temperatures were: injector temperature, 280°C; transfer line temperature, 280°C; oven temperature, programmed from 50°C at 10°C/min to 300°C (hold for 5 min)(Lee *et al.*, 2002).

Results

Body Weight Changes

Table 1 is the changes of body weights in mice inhaled with BD for 3 weeks (5 hr/day, 5 days/week). Body weights were significantly lower from 9 exposure post-day in 500 ppm inhalation group, and from 4 exposure post-day in 1000 ppm inhalation group than in control

Hemoglobin Adducts

Table 2 is the levels of N-(2-hydroxy-3-butenyl) valine(HB Val) and N-(2,3,4-trihydroxybutyl)valine (THB Val), which separated from hemoglobin adducts of female mice inhaled with BD for 3 weeks (5 hr/day, 5 days/week).

The levels of HB Val adducts on 1st, 2nd, and 3rd week were 1.8, 3.7 and 6.2 pmol/mg globin for 500 ppm BD, and 5.7, 7.4 and 16.0 pmol/mg globin for 1000 ppm BD inhalation exposure, respectively. The levels of THB Val adducts on 1st, 2nd, and 3rd week were 32.0, 42.0 and 55.0 pmol/mg globin for 500 ppm BD, and 67.8, 72.7 and 83.5 pmol/mg globin for 1000 ppm BD inhalation exposure, respectively.

Ratio of THB Val and HB Val Adducts

Table 3 is the ratios of THB Val and HB Val separated from hemoglobin for BD inhalation exposure (5 hr/day × 5 days/week). Their ratios were higher at earlier exposure period and lower concentra-

Table 1. Changes of body weight in ICR female mice inhalation exposure with 1,3-butadiene (5 hr/day, 5 days/week)
Mean ± S.D., g

Exposure post-days	Control	Inhalation exposure groups		F-value	p-value
		500 ppm BD	1,000 ppm BD		
0	26.35 ± 1.4661	26.12 ± 1.7135	25.55 ± 2.1371	2.588	0.120
2	26.82 ± 1.3058	25.82 ± 1.6246	24.37 ± 1.6193	2.253	0.108
4	27.03 ± 2.1240	25.69 ± 1.8317	24.54 ± 2.1781*	3.317	0.058
7	27.61 ± 1.7860	25.47 ± 1.1870	24.81 ± 2.2521*	4.720	0.019
9	27.83 ± 1.7247	25.34 ± 1.4811*	24.83 ± 2.3838*	4.849	0.017
11	28.06 ± 2.2483	25.29 ± 1.2974*	24.26 ± 2.3552*	7.112	0.009
14	28.15 ± 3.2005	25.16 ± 1.8215*	23.92 ± 1.2988*	7.292	0.005
16	28.30 ± 2.2913	25.24 ± 1.6502*	24.36 ± 1.4258*	7.136	0.003
18	28.66 ± 2.6312	26.30 ± 1.5379*	24.66 ± 1.6906*	7.807	0.001

*; $p<0.05$, Significantly different from control.

Table 2. The levels of N-(2-hydroxy-3-butenyl)valine(HBVal adduct) and N-(2,3,4-trihydroxybutyl)valine(THBVal adduct) extracted from hemoglobin of ICR female mice inhalation exposure with 1,3-butadiene (5 hr/day, 5 days/week)
Unit : pmol/mg globin

	Inhalation exposure	1st week	2nd week	3rd week
HBVal adduct	500 ppm	1.8 ± 0.6	3.7 ± 0.3	6.2 ± 0.4
	1,000 ppm	5.7 ± 0.9	7.4 ± 1.5	16.0 ± 1.5
THBVal adduct	500 ppm	32.0 ± 2.3	42.0 ± 4.1	55.0 ± 2.1
	1,000 ppm	67.8 ± 3.4	72.7 ± 5.3	83.5 ± 3.9

Table 3. The ratios of N-(2-hydroxy-3-butenyl)valine(HBVal adduct) and N-(2,3,4-trihydroxybutyl)valine(THBVal adduct) separated from hemoglobin of ICR female mice inhalation exposure with 1,3-butadiene (5 hr/day, 5 days/week)

	Inhalation Exposure	1st week	2nd week	3rd week
Ratio of THB Val and HB Val adduct	500 ppm	17.78	11.35	8.87
	1,000 ppm	11.89	9.82	5.22

tion. They were 17.8, 11.4 and 8.87 in 1st, 2nd, and 3rd week for 500 ppm BD, and 11.9, 9.8 and 5.2 in 1st, 2nd, and 3rd week for 1000 ppm BD inhalation exposure, respectively.

Discussion

Hemoglobin adducts were offered as an effective biomarker for biological monitoring of exposure to reactive intermediates of chemicals. They have several advantages for molecular epidemiology studies including that they were accumulated over the life of the red cell, which are ~43, 63 and 120 days in mice, rats and humans, respectively(Van Putten, 1958).

1,2-epoxy-3-butene(EB), the primary metabolite of BD, may react with the N-terminal valine of hemoglobin, such as N-(2-hydroxy-3-butenyl)valine (HB Val adduct) and N-(2,3,4-trihydroxybutyl) valine(THB Val adduct)(Swenberg *et al.*, 2001).

For HB Val adduct, Boogaad *et al.* (2001) reported it was a sensitive method for monitoring cumulative exposure to BD at or above 0.35 ppm (0.771.1 mg /m^3), its concentration was 0.2 pmol/g globin in workers exposed with 1.1 mg/m^3 BD. Albrecht *et al.* (1993) reported the adduct was 17 nmol/g globin in mice exposed at 500 ppm BD, 6 h/day, 5 days/week, and it was five times higher in rats (3.5 nmol/g globin). This was similar to our results, of which HB Val adduct on 1st, 2nd, and 3rd week were 1.8-6.2 pmol/mg globin for 500 ppm BD, and 5.7-16.0 pmol/mg globin for 1000 ppm BD

inhalation exposure (5 hr/day, 5 day/week). HB Val adduct concentration, determined in Amsterdam, were 0.47, 2,23, 0.22 pmol/g globin for the monomer production, polymerization and administration workers, respectively (Albertini *et al.*, 2001).

THB Val adduct were potentially produced by either DEB or EB-diol, although current evidence indicates that they derive almost entirely from the latter (Perez *et al.*, 1997). THB Val adduct concentrations, determined in Chapel Hill, were 178.73, 716.7, 94.77 pmol/g globin for the monomer production, polymerization and administration workers, respectively (Albertini *et al.*, 2001). This was similar to our results, of which THB Val adducts on 1st, 2nd, and 3rd week were 32.0-55.0 pmol/mg globin for 500 ppm BD, and 67.8-83.5 pmol/mg globin for 1000 ppm BD inhalation exposure (5 hr/day, 5 day/week).

After exposure to 1000 ppm (2210.0 mg/m^3) of BD by inhalation, the average concentrations of THB Val adducts on both sex were 4.3 fold greater in mice than in rats (Swenberg *et al.*, 2000). The ratios of THB Val to total HB Val concentration were 6.3 for male mice and 3.4 for female mice. At high exposure of BD (1000-1250 ppm, for 90 or 10 days, respectively), the ratios of THB Val : HB Val adducts were 2-6 in mice and rats. The ratio of THB Val : HB Val adducts was 39:1 at 62.5 ppm BD exposure, while at 1250 ppm BD, it was 5.7:1 (Swenberg *et al.*, 2001). This was similar to our results, of which ratios were 17.8:1 at 1st week for 500 ppm BD, and 5.2:1 at 3rd week for 1000 ppm BD inhalation exposure, when mice was exposed by inhalation

Conclusion

1,3-butadiene(DB) is a well-established rodent carcinogen, and a probable carcinogen to humans. The purpose of this study was to investigate hemoglobin adducts after 500 ppm and 1000 ppm DB inhalation exposure to ICR female mice for 3 weeks (5 hr/day × 5 days/week). They were (N-2-hydroxy - 3-butenyl)valine(HB Val adduct) and (N-2,3,4-trihydroxy-butyl)valine(THB Val adduct).

Body weights of mice were significantly lower from 4 or 9 exposure post-day in 500 or 1000 ppm inhalation group than in control. The levels of HB Val adducts were 1.8, 3.7 and 6.2 pmol/mg globin for the 500 ppm BD inhalation group, and 5.7, 7.4 and 16.0 pmol/mg globin for the 1000 ppm BD inhalation group, when observed on the 1st, 2nd, and 3rd week after inhalation exposure, respectively. And the levels of THBVal adducts were also 32.0, 42.0 and 55.0 pmol/mg globin for the 500 ppm BD inhalation group, and 67.8, 72.7 and 83.5 pmol/mg globin for 1000 ppm BD inhalation group, on the 1st, 2nd, and 3rd week after inhalation exposure, respectively. Their ratios were higher at earlier exposure period and lower concentration. Ratios on the 1st, 2nd, and 3rd week were 17.8, 11.4 and 8.87 for the 500 ppm BD inhalation group, and 11.9, 9.8 and 5.2 for the 1000 ppm BD inhalation group, respectively.

In conclusion, THB Val and HB Val adducts were the important hemoglobin adduct after BD inhalation exposure, and the latter was more appropriate biomarker than the other for biological monitoring of DB exposure.

Acknowledgement

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