

**QUANTIFICATION OF EPOXIDE METABOLITE SPECIFIC N-TERMINAL  
GLOBIN ADDUCTS: A BIOMARKER OF INTERNAL DOSIMETRY OF  
1,3-BUTADIENE EXPOSURE AND METABOLISM**

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## **ABSTRACT**

**NARISA BORDEERAT: Quantification of Epoxide Metabolite Specific N-terminal Globin Adducts: A biomarker of Internal Dosimetry of 1,3-Butadiene Exposure and Metabolism**

(Under the direction of Dr. James A Swenberg)

Butadiene (BD) carcinogenicity in rodents shows gender, species and concentration dependency, making the extrapolation of animal results to humans complex. BD is a multispecies multisite carcinogen, with mice being a much more sensitive species than rats. This is considered to be related to the metabolism of BD to its epoxy metabolites, 1,2-epoxy-3-butene (EB), 1,2;3,4-diepoxybutane (DEB) and 1,2-epoxy-butanediol (EB-diol). The mutagenic potency of individual epoxides varies up to 200-fold, with DEB being the most mutagenic metabolite. For accurate risk assessment it is important to elucidate species differences in the internal formation of the individual epoxides in order to assign the relative risks associated with their different mutagenic potencies. N-terminal globin adducts have been widely used for measurements of the formation of BD derived epoxides. In this study, the formation of each epoxide was evaluated in globin samples from both genders of mice and rats exposed to BD by inhalation for 10 days. The numbers of globin adducts were then converted into EB dose equivalents. These were calculated on the basis of the combined internal dose (globin adducts) and the relative genotoxic potency of the respective epoxides inferred from the efficiency of inducing mutations at the Hprt locus.

Then, the multiplicative cancer risk model was applied to quantitatively estimate tumor incidence by using the EB dose equivalent and long-term cancer bioassay data. Based on the EB equivalent, higher exposures formed lower amounts per ppm BD. This indicates that metabolism of BD to epoxides is most effective at low exposures. The EB equivalent for mice was about 40-fold higher than that of rats at similar exposures. No gender differences were noted in globin adducts of mice or rats at all exposures. As such, EB dose equivalents provide quantitative data on biomarkers of exposure that can be extended to a scientific basis for BD risk assessment.

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## LIST OF ABBREVIATIONS

BD	1,3-Butadiene
U.S. EPA	U.S. Environmental Protection Agency
IARC	International Agency for Research on Cancer
NIOSH	National Institute for Occupational Safety and Health
OSHA	United States Occupational Safety and Health Administration
PEL	Permissible exposure limit
TWA	Time-weighted average
STEL	Short-term exposure limit
BMD	Butadiene monomer
SBR	Styrene-butadiene rubber
WHO	World Health Organization
CML	Chronic myelocytic leukemia
CLL	Chronic lymphocytic leukemia
Hb	Hemoglobin
EB	1,2-epoxybutene
DEB	1,2:3,4-diepoxybutane
GSH	Glutathione
GST	Glutathione-S-transferase
EH	Epoxide hydrolase
EB-diol	3,4-epoxy-1,2-butanediol
ADH	Alcoholdehydrogenase
HMVK	Hydroxymethylvinylketone
M1	1,2-dihydroxy-4-(N-acetylcysteiny)-butane

M2	1-(N-acetylcysteinyl)-2-hydroxy-3-butene
M3	1,3,4-trihydroxy-2-(N-acetylcysteinyl)-butane
NTP	National Toxicology Program
IISRP	International Institute of Synthetic Rubber Producers
MF	Mutant frequencies
HB-Val	N-(2-hydroxy-3-butenyl)-valine
<i>pyr</i> -Val	N, N-(2, 3-dihydroxy-1, 4-butadiyl)-valine
THB-Val	N-(2,3,4-trihydroxybutyl) valine
PFPTH	Pentafluorophenylthiohydantoin
IA	Immunoaffinity
HPLC	High pressure liquid chromatography
LC-MS/MS	Liquid chromatography mass spectrometry
N1-THB-A	N1-(2,3,4-trihydroxybut-1-yl)adenine
THB-G	N7-(2',3',4'-trihydroxybut-1'-yl)guanine
HEB	2-hydroxy-3,4-epoxybut-1-yl
DPC	DNA-Protein crosslink
AUC	Area under curve

# CHAPTER I

## LITERATURE REVIEW

### 1.1 1,3-Butadiene: Overview

1,3-butadiene (BD), CAS No. 106-99-0, is a colorless gas with a mild aromatic gasoline-like odor. The structure and molecular formulae (**Figure 1.1**) is that of an aliphatic compound and the relative molecular mass is 54.09 (1). BD is flammable at atmospheric concentrations and spontaneously forms a dimer at room temperature. It can also form explosive peroxides on contact with air, thus an inhibitor of peroxidation (4-t-butyl catechol) is added to commercial butadiene (2).

#### 1.1.1 Source of 1,3-Butadiene

BD is mainly used in the production of plastics and synthetic rubber. It is manufactured primarily as a co-product of ethylene production. This process accounts for over 95% of global butadiene production, with a demand of 9.3 million metric tons worldwide in 2005 (3, 4). The major uses include styrene-butadiene rubber (30 – 35%), polybutadiene rubber (25%), adiponitrile (15%), and styrene-butadiene latex (10%) (1). BD is also an environmental pollutant, present in gasoline, motor vehicle exhaust, and cigarette smoke (4, 5, 6).



The U.S. EPA and IARC classified BD as a human carcinogen (class 1) based on animal studies in which laboratory rodents exposed to BD by inhalation and epidemiologic evidence suggesting that human BD industry workers have an increase incidence of leukemia (7–11). The most common exposure route for humans in the workplace is inhalation. According to the National Occupational Exposure survey (NIOSH, 1999), approximately 50,000 workers in the US were potentially exposed to BD (12). The United States Occupational Safety and Health Administration was adjusted permissible exposure limit (OSHA PEL) from 1,000 ppm to 1 ppm for 8 h time-weighted average (TWA) in 1996, while the short-term exposure limit (OSHA STEL) was 15 ppm as a 15-min exposure (13). BD has been identified in cigarette smoke and delivery levels in the mainstream smoke of cigarettes ranges from 1.3 to 100 µg/cigarette (14, 15). Additionally, small amounts of BD are found in gasoline vapor, automobile exhaust, and fossil fuel incineration products. The U.S. EPA estimated that the mean concentration of BD in the ambient air of 19 U.S. cities in 1990 was 1.4 µg/m<sup>3</sup> (16). Therefore, the general population, smokers and those nearby are exposed to this compound.

### *1.1.2 Epidemiologic studies*

#### *1.1.2.1 Mortality and Cancer studies*

Over the past 40 years, the relationship between exposure to butadiene and human health effects has been investigated in numerous studies. The most relevant investigations focused on working populations who were employed in butadiene monomer (BMD) and styrene-butadiene rubber (SBR) production.

Three cohorts of BMD production workers in the US have been studied: at two Union Carbide plants in West Virginia (17), at a Texaco plant in Texas (18), and at Shell plants in Texas (19, 20). The standard mortality ratio (SMR) for all cancers as a whole was 10% less than that for the US population rates. The first study focus on mortality among male workers who had been employed for at least six months at a 1,3-butadiene production facility between 1943 and 1979 (21). The incidence of death from lymphosarcoma was increased 2-fold, but leukemia mortality rate was not evaluated. The SMR for lymphoma was 1.5 (21). These data showed no increase in adverse hematological parameters in relation to BD exposure.

A follow up study in 1996 on the same group of workers, based on exposure concentration and duration showed that the leukemia SMR was 1.1 for workers employed more than 10 years and 1.8 for those employed less than 10 years. The SMR for lymphosarcoma in short term exposure (<5 years) was 3.3-fold higher than workers with 10 or more years of employment (22). Since there was no evidence on elevation of mortality rates with increased exposure duration, Divine and Hartman (1996) concluded that there was no casual relationship between leukemia occurrence and duration of employment in the BDM industry (22).

Two groups of SRB production workers have been studied. One was studied by the National Institute of Occupational Safety and Health (NIOSH) in a two-facility complex in Ohio (23–25), and the other comprised of workers from eight facilities in the USA and Canada who were studied by the Johns Hopkins University's researchers (26–28). Subsequently, researchers from the University of Alabama at Birmingham (UAB) (29) studied the same facility complex

investigated by NIOSH in addition to the seven facilities studied by the Johns Hopkins University. The most recent updated results published from these groups have been reviewed (30–34). The epidemiology data indicate that the worker mortality rates from all cancers as a whole were equal or less than the US average; however, mortality rates from leukemia were increased in the male SRB workers populations (31, 35). The UAB investigators also conducted analyses using the new World Health Organization (WHO) disease classification scheme for leukemias and lymphomas. Based on this WHO classification, chronic myelocytic leukemia (CML) was most strongly associated with peak exposures, while chronic lymphocytic leukemia (CLL) showed an opposite pattern, i.e., a stronger association with cumulative versus peak exposures to BD (31, 32). Additionally, the UAB findings represent the effect of BD exposure in the presence of styrene and other SRB chemicals (e.g. dimethyldithiocarbamate, DMDTC). Since there was a high correlation between BD and styrene exposure in the cohort, a possible role for styrene as a co-exposure factor could not be ruled out.

Mortality rate for female workers employed in the SRB industry from 1991 to 1994 are currently studied (36). However, results were unavailable due to delays in accessing mortality follow-up information. The UAB investigators anticipate approximately 30-35 lymphohematopoietic cancer deaths among 5,000 female SRB workers; this study will contribute significant new information once completed.

In conclusion, epidemiological studies for both SRM and BDM industries report an overall deficit in cancer mortality in BD-exposed workers. An elevated incidence of leukemia mortality was observed for long-term workers routinely

exposed to BD in SBR industries (29, 30, 37). On the other hand, no increase in leukemia deaths and no correlation between leukemia incidence rate and BD exposure were observed in BDM cohort (22). This finding is inconsistent between the results for workers employed in SRB and BDM industries. This may reflect generally lower exposures in the BDM or the presence of the co-exposure to BD and other chemicals (styrene and DMDC) in SRB industries. Since the UAB data set provides the largest and most comprehensive consistent data set available on occupationally BD exposed workers, the weight of evidence indicates that BD is weakly leukemogenic in humans with long term exposure (30-40 years) (38).

#### *1.1.2.2 Molecular studies*

While studies of the relationships between BD exposure and cancer in humans have given various results, a number of studies have been found an association with leukemia and lymphomas. All increases in leukemia deaths have been confined to the sub-group classified as CML. Molecular epidemiological studies in humans can provide information analogous to cancer data that can be derived from animal studies, using biomarkers to assess metabolism and measure internal doses of BD metabolites to examine potential pre-neoplastic effects and identify issues related to increased susceptibility to BD.

Molecular epidemiology studies on 41 SRB workers in China exposed to BD demonstrated that exposed workers had greater amounts of hemoglobin (Hb) adducts and white blood cell counts when exposed to 2 ppm of BD (6-h TWA). However, the levels of hemoglobin N-(2,3,4-trihydroxybutyl) valine (THB-Val) adducts were not associated with genotoxic effects evaluated as sister chromatic exchanges, glycophorin A or *Hprt* mutations. The study suggests that neither

short-term, nor long-term exposures to BD in these workers induced specific genotoxic effects at the chromosomal or gene levels (39–42).

A large-scale international molecular epidemiological study of BD exposed Czech workers was initiated in 1998 and reported in 2003 (43–45). Albertini et al. reported a clear no-observed-adverse-effect level (NOAEL) for biomarkers of effect; *Hprt* mutations and chromosome aberrations, at mean BD exposure concentration of 0.8 ppm. This study utilized a comprehensive series of biomarker responses, including urinary metabolites and Hb adducts, *Hprt* mutations, sister-chromatid exchange frequencies and chromosomal aberrations. Both urine metabolites and Hb adduct concentrations proved to be excellent biomarkers of exposure.

A second study of Czech workers was conducted at the same facility to compare biomarker responses in female and male employees (46, 47). Mean BD exposure concentrations were lower in this study than in the first study, being 0.18 ppm and 0.37 ppm for females and males, respectively. There were no BD-associated elevation above background for *Hprt* mutations or chromosome aberration frequencies in either sex. Similarly, there was no difference between genders in the pattern of BD detoxication, as indicated by urinary M2/(M1 + M2) ratios levels. Females appeared to absorb or metabolize less BD than males per unit of exposure. Workers also were genotyped for polymorphisms in several metabolic genes in both Czech studies. Although some genotype-associated differences in metabolic patterns were observed, there were no such differences for biomarkers of effect such as *Hprt* mutations or chromosome aberrations in either study.

### 1.1.3 Metabolism of 1,3-butadiene

BD is metabolically activated by cytochrome P450 monooxygenases (P450) forming reactive electrophiles. As shown in **Figure 1.2**, BD is initially oxidized to the 1,2-epoxybutene (EB), a reactive metabolite mediated primarily by cytochrome P450 isozyme CYP2E1, although other isozymes such as CYP2A6 have also been shown to be involved (48). Further P450 oxidation of EB produces 1,2:3,4-diepoxybutane (DEB). Detoxification of EB proceeds by conjugation with glutathione (GSH) (mediated by glutathione-S-transferase, GST) or by hydrolysis (mediated by epoxide hydrolase, EH), the latter producing the 1,2-dihydroxy-3-butene (butanediol, B-diol) metabolite. Both DEB and B-diol undergo further conversions *in vivo*, DEB by EH hydrolysis and B-diol by CYP2E1 oxidation to produce the 3,4-epoxy-1,2-butanediol (EB-diol). B-diol can also be metabolized by alcoholdehydrogenase (ADH) and CYP2E1 to form hydroxymethylvinylketone (HMVK) (38).

All three reactive epoxides of BD (EB, DEB, and EB-diol) can react with nucleophilic sites in biomolecules, including DNA and proteins. The DNA adducts can result in one or more genotoxic events relevant to the carcinogenic mode of action (MOA) for BD (49). DEB, while not the major metabolite formed, is a bifunctional alkylating agent. It is capable of binding to two cellular macromolecules [e.g., DNA-protein crosslinks; (50)] or to the same molecule twice [e.g., DNA-DNA crosslinks; (51, 52)]. DEB is considered the most potent carcinogenic metabolite of BD and is by far the most mutagenic metabolite of BD (53, 54). DEB is 50 times more mutagenic than EB and 100 times more mutagenic than EB-diol (53).

The major detoxication enzyme for epoxide metabolites of BD are EH and GST conjugation. Direct GST-mediated conjugation of EB with GSH leads to 1-hydroxy-2-(N-acetylcysteinyl)-3-butene as a racemic mixture with 1-(N-acetylcysteinyl)-2-hydroxy-3-butene known as M2 , a urinary metabolite and a biomarker for this pathway. The GST-mediated conjugation of HMVK with GSH leads to the production of 1,2-dihydroxy-4-(N-acetylcysteinyl)-butane, known as M1, a second urinary metabolite. The GSH conjugation product of DEB forms 1,3,4-trihydroxy-2-(N-acetylcysteinyl)-butane, known as the M3. M1 is a biomarker of the hydrolytic pathway because EH-mediated hydrolysis of EB is the initial step, followed by reaction with alcohol dehydrogenase and cytochrome P450 to form HMVK. The ratio  $M1/(M1 + M2)$  in urine provides an estimate of the relative importance of hydrolysis versus conjugation in the detoxication of EB (55, 56).

#### 1.1.4 Physiologically based pharmacokinetic (PBPK) model

PBPK models can be applied as a tool to use *in vitro* and *in vivo* rodent and *in vitro* human data to predict the dosimetry of BD in humans. The goal of PBPK models is to aid extrapolation of rodent results to humans to understand species differences in metabolism and pharmacokinetics. PBPK models of BD and its metabolites have been developed at the Chemical Industry Institute of Toxicology (CIIT) (57) and other laboratories (58–68).

The CIIT PBPK model has used rates of BD and EB metabolism determined *in vitro* (69). The *in vitro* metabolic rates were adapted to account for metabolism by the entire liver rather than just a subcellular fraction. This model demonstrated that *in vitro* metabolism parameters could predict *in vivo*

disposition in animals. It suggested that *in vitro* parameters derived from human tissue might also be predictive of the disposition of BD in humans (70). Furthermore, the Medinsky model included metabolism of BD in the lung. To gain insight into the potential importance of lung metabolism, several different models simultaneously were conducted and concluded that lung metabolism may be important in defining lung tissue (57).

Elfarra et al. (1996) described studies in which the isozyme specificity of the BD to EB and the EB to DEB reactions were determined with human cytochrome P450s. Human myeloperoxidase was observed to catalyze formation of EB from BD, but not DEB from EB (58).

Kohn and Melnick et al. (1996) discussed the effects of the structure of a PBPK model for BD and EB and how different descriptions of the compartments, flows, and kinetic constants can affect the predictions. They concluded that reliable physiological and kinetic data must be used to build the model before it can be useful for interspecies extrapolation and risk assessment (59). In 2001, Kohn and Melnick et al. revised their model to include a transient complex between cytochrome P450 and microsomal EH (60). The model was further enhanced by the addition of equations for the production and detoxication of EB-diol in the liver, lungs, and kidneys in mice and rats. The results demonstrated that the model predicted accumulation of EB-diol in animals exposed to BD, which is consistent with observations that most of the DNA adducts arise from EB-diol.

Bond et al. (1996) discussed data used to set parameters in a PBPK model for BD and EB, as well as data being developed on DEB that could be used in PBPK models (61). *In vivo* studies of epoxide disposition after BD



exposure were fit a PBPK model to show the importance of reliable *in vitro* data for accurate predictions, and the role of lung metabolism in mice.

Csanady et al. (1996) developed a PBPK model to describe disposition and metabolism of BD and EB in rats, mice and humans (62). In addition, DEB disposition and metabolism was also described in mice. The model described the formation of EB and DEB, intrahepatocellular first-pass hydrolysis of EB, conjugation of EB with GSH and GSH turnover in the liver. The authors reported that species differences in EB dosimetry were not sufficiently large to explain the observed species differences in carcinogenic potency for BD, suggesting that additional factors are involved.

Johnson and Filser et al. (1996, 2010) developed a PBPK model for BD and its metabolites in mice, rats, and humans (63–65). The model includes compartments for lung, liver, fat, and a vessel-rich group. It incorporated three important features: (1) reduce alveolar ventilation; (2) intrahepatic first-pass hydrolysis of EB; and (3) a two substrate Michaelis-Menten kinetic description of EB conjugation with GSH. The authors concluded that relatively small differences in EB between mice and rats as internal EB doses could only partly explain the marked species difference in cancer response between mice and rats exposed to BD.

Sweeney et al. (1996, 1997, 2001) demonstrated *in vivo* and *in vitro* BD metabolism data in laboratory animals that were integrated into a PBPK model in rats with flow- and diffusion- limited compartments (66, 67). The model described experiments from closed chamber inhalation and nose-only flow through inhalation exposures. Blood concentrations of BD were accurately predicted for mice and rats exposed by inhalation to constant concentrations of BD. A

preliminary human model was developed that accurately predicted published data on exhaled breath BD concentrations in a human volunteers exposed to BD inhalation (68). Human blood DEB was found to be sensitive to rates of epoxidation of EB to DEB and hydrolysis of EB and DEB.

In conclusion, the PBPK models are proving to be very useful for testing, and given their quantitative nature, could be a key tool in risk assessment of BD. PBPK models can be used to address questions of extrapolation of critical metabolites in tissues from high to low exposure concentrations. These models will also be useful in resolving conflicting observations about interspecies differences in metabolism.

## **1.2 BD toxicity studies**

### *1.2.1 Acute toxicity*

Acute toxicity effects have been observed at high BD exposure concentrations. The median lethal concentration (LC50) in rats for a 4-h exposure was 129,000 ppm or 12.9% and in mice for a 2-h exposure was 123,000 ppm or 12.3% (71). Early toxicology studies indicated that BD caused irritation to mucous membranes, skin, and eyes, or narcosis only at high BD concentrations (8,000 ppm). Human volunteers exposed to 2,000, 4,000, or 8,000 ppm BD for 6 to 8 h experienced minor irritation to eyes and difficulty in visual focusing (72). In addition acute inhalation of 2,000 to 8,000 ppm, BD can cause irritation of upper respiratory tract. Coughing and bronchospasm can occur, especially in susceptible individuals, such as persons with asthma. Bradycardia and hypotension secondary to CNS depression is possible with exposure to very high concentrations (150,000-250,000 ppm) of BD (73).

### *1.2.2 Chronic toxicity*

BD has been considered to have a low and noncumulative toxicity in animals and human. Melnick et al. (1990) demonstrated nonneoplastic lesions associated with chronic inhalation of BD (74). B6C3F<sub>1</sub> mice exposed to 6.25 – 625 ppm BD for 40–65 weeks exhibited epithelial hyperplasia of the forestomach, endothelial hyperplasia of the heart, alveolar epithelial hyperplasia, hepatocellular necrosis, testicular atrophy, ovarian atrophy, and toxic lesions in nasal tissues (chronic inflammation, fibrosis, cartilaginous metaplasia, and atrophy of the olfactory epithelium) (74). Testicular atrophy was induced at 625 ppm BD, and ovarian atrophy was observed at concentration as low as 6.25 ppm.

In a study of hematological parameters in mice exposed to BD at 62.5–625 ppm, decrease in RBC count, Hb concentration, and packed red cell volumes were found (75–77). These changes were not accompanied by increases in reticulocyte counts or in the frequency of polychromatic erythrocytes in peripheral blood, and may indicate a poor regenerative response in the bone marrow to reduced levels of circulating erythrocytes.

Thurmond and associates (1986) observed significant extramedullary hematopoiesis and erythroid hyperplasia in spleens of male B6C3F<sub>1</sub> mice exposed to 1,250 ppm BD for 24 weeks (78). One explanation of these results is that exposure to BD caused suppression of hematopoiesis in the bone marrow, and that young large red cells were released into the blood from extramedullary sites, such as the spleen. These findings establish the bone marrow as a target of BD toxicity in mice (78). No BD exposure-related gross or microscopic changes or effects on growth, survival, hematologic and blood biochemical

parameters, urinary measurements, or neuromuscular functions were observed in male and female Sprague-Dawley (SD) rats exposed to BD at concentrations ranging from 1,000 to 8,000 ppm (6 hours/day, 5 days/week for 13 weeks) (79).

### *1.2.3 Carcinogenicity studies in laboratory animals*

The carcinogenicity of inhaled BD was studied in B6C3F<sub>1</sub> mice by the National Toxicology Program (NTP) and in SD rats by the International Institute of Synthetic Rubber Producers (IISRP) (**Table 1.1**). The NTP studies demonstrated that BD is a potent multi-site carcinogen. In the first NTP study, male and female mice were exposed 6 h/day, 5 days/week, to air containing 0, 625, or 1,250 ppm BD (80, 81). This study was terminated after 60 to 61 weeks because of reduced survival due to malignant neoplasms at multiple sites. Malignant lymphomas, observed as early as week 20, were the major cause of early deaths in both sexes. In addition, there was early induction and increased incidences of hemangiosarcomas of the heart and neoplasms of the lung and forestomach in males and females, and of the mammary gland, ovary, and liver in females. The high incidence of hemangiosarcomas of the heart was a particularly unusual finding because this neoplasm is extremely uncommon in B6C3F<sub>1</sub> mice.

In an expanded NTP study, male and female mice were exposed to 0, 6.25, 20, 62.5, 200, or 625 ppm BD for up to 2 years (82). Consistently, lymphomas were the major cause of early deaths for both sexes exposed to 625 ppm. The incidence of lymphomas was also increased in females exposed to 200 ppm. Hemangiosarcomas of the heart were observed at concentrations as low as 20 ppm in males and 62.5 ppm in females. Incidences of lung neoplasms in

female mice were increased at all concentrations, including the 6.25 ppm exposure. The harderian gland was also identified as a site of BD-induced neoplasia.

Stop-exposure experiments after BD exposure at one year were also conducted in mice to assess the relationship between concentration and duration of exposures on BD-induced carcinogenicity (83). These studies demonstrated that tumors were induced at multiple organ sites even only 13 weeks of exposure to 625 ppm BD, and that at comparable total exposures, the incidence of lymphoma was greater with exposure to a higher concentration of BD for a shorter time (e.g. 625 ppm for 26 weeks) compared with exposure to a lower concentration for a longer time (e.g. 312 ppm for 52 weeks). Thus, for the development of lymphomas, the concentration of BD is a greater contributing factor than is the duration of exposure.

In the IISRP study, rats of each sex was exposed to 0, 1,000 or 8,000 ppm BD 6 h/day, 5 days/week, for 2 years (10). Results from the study were also reported later by the same author in 1993 (84). Minor clinical effects, including excessive eye and nose secretions plus slight ataxia, were observed between 2 and 5 months in rats exposed to 8,000 ppm. Alterations in organ weight were also observed in this high exposure group. Following exposures up to 2 years, BD was carcinogenic at multiple organ sites, as evidenced by increased incidences or dose-response trends for neoplasms of the pancreas and testis in males, and of the mammary gland, thyroid gland, uterus, and zymbal gland in females. In addition, the average number of mammary gland fibroadenomas was increased in both exposure groups compared to control. The increased number of uncommon glial cell tumors of the brain in exposed male rats, though not

statistically significant, may also have been related to BD exposure (85). These studies reveal that the carcinogenic effects of BD differ markedly between rats and mice with respect to sites of tumor induction.

#### *1.2.4 Species and gender differences in BD metabolism*

Carcinogenicity studies in animals have clearly shown differences in species susceptibility to BD induced tumor formation. For example, in chronic BD exposure studies, mice developed tumors at 6.25 ppm BD, while rats developed tumors at exposures of 1,000 ppm BD (9, 10). These notable interspecies differences in susceptibility to BD-induced cancer are believed to be due to differences in the extent of formation of DEB in rat and mouse tissues. The rate of P450 oxidation of BD to EB and EB to DEB is roughly 3.5-fold faster in mice compared to rats (85). Moreover, the rate of EH hydrolysis of EB to B-diol is 2.7 fold faster in rats compared to mice (85). Furthermore, microsome studies of BD metabolism in mouse, rat, and human microsomes have shown that mice indeed form greater levels of DEB than both rat and human microsomes (85). This is due to a greater rate of oxidation of BD to EB and EB to DEB in mouse tissues, as well as a slower rate of hydrolysis of EB and DEB in mice as compared to rat and human microsomes (85). In contrast, rat cytochrome P450 2E1 monooxygenase, the major isoform of mixed function monooxygenase responsible for BD metabolism to EB and DEB, can form covalent binding with Tyr and His residues within its active site (86). This could partially explain why BD-exposed rats form more EB but less DEB than mice and are less susceptible to BD induced cancer.

Multiple BD inhalation studies have revealed gender differences in metabolism and carcinogenicity. Female mice developed lung and liver tumors

after exposure to 6.25 ppm BD, whereas male mice did not form tumors until exposure to 62.5 ppm BD or higher (38). In another study, female rats exposed to a single 6-h exposure of 62.5 ppm BD had 4.75-fold higher blood concentrations of DEB than did male rats that received the same exposure (87). Similarly, female mice had greater DEB concentrations in blood, femur, lung and fat tissue as compared to male mice receiving the same BD exposure (87).

A possible cause of the observed gender differences in BD metabolism is differences in enzymes involved in the metabolism, P450 monooxygenases and EH. P450s are responsible for the production of DEB as it oxidized BD to EB, and EB to DEB. There is evidence that some isoforms of cytochrome P450 (CYP2C12) are expressed only in females, while others (CYP2C6 and CYP2A1) are expressed in both sexes, but not more abundant in females (88). In addition, an increased level of expression of BD-oxidizing enzymes in females as compared to males may be responsible for the observed differences in toxicity of BD between the sexes (89).

Another key enzyme involved in the metabolism of BD is EH, which is involved in detoxication of BD epoxides to the corresponding diols. In general, testosterone has been noted to stimulate cytosolic EH expression leading to overall activity of EH in male mice than in female (90, 91). In contrast, female mice had greater activity of cytochrome P450 monooxygenases, resulting in increased formation and decreased hydrolysis of reactive BD metabolites, specifically DEB. Similarly, results were presented for BD exposed rats, blood of female animals contained higher concentrations of DEB as compared to males (89).

However, a recent study of hemoglobin adducts showed no significant gender differences at the exposure concentrations studied (151). In addition, there were no gender differences in the amount of DNA monoadducts indicate that there is no difference in BD metabolism in rodents at multiple exposure concentrations and durations. This comprehensive data set strongly suggests that a deficiency in DNA cross-link repair in females is the most plausible hypothesis for the gender differences in mutagenic and carcinogenic susceptibility.

### **1.3 Genotoxicity and mutagenicity studies**

BD has been tested for genotoxicity in a number of *in vitro* and *in vivo* studies. Furthermore, molecular and genetic toxicity of BD and its metabolites have been extensively evaluated in wide variety of test systems including bacterial cells, yeasts, *Drosophila*, mammalian cell and rodents (92–97). In order to summarize the huge amount of genotoxicity information, this review is organized according to the mutational endpoints in *in vivo* and *in vitro* test systems.

#### *1.3.1 In vitro studies of mutagenicity of BD and its metabolites*

BD metabolites have been studied for mutagenicity in a variety of test systems. Positive results have been found in the reverse mutation assay in *Salmonella typhimurium*, the first test organisms employed to test parent BD and its metabolites (98–107). On the basis of these data, BD appears to require metabolic activation to produce genotoxicity. DEB induced gene mutations, mitotic recombination, and gene conversions in eukaryotic microorganism (108–



112). Mammalian cells in culture have been extensively employed in studies of the mutagenicity of BD's metabolites. Each of the major metabolites has been shown to induce gene mutations in endogenous genes (*Hprt* and *TK*) and intrans-genes (lac I and c II) using different cell systems, i.e., mouse lymphoma, fibroblasts, CHO cells, TK6 human fibroblasts, mouse and rat transgenic fibroblasts (113–116). It is noteworthy that EB primarily induces point mutations (A:T to T:A transversions) and small deletions, whereas DEB, a bifunctional alkylating agent, results in large deletions (113, 114). The relative mutagenic potencies of the three major electrophilic metabolites of BD have been determined in several studies of gene mutations employing both endogenous and transgenes as targets. The findings have always shown these potencies to be DEB >> EB > EB-diol (117). In mutagenic studies using endogenous gene targets in cultured human lymphoblast TK6 cells and the cII transgene in mouse fibroblasts, DEB was approximately 50-fold more potent than EB and 100-fold more potent than EB-diol (57, 117–119).

Chromosome-level mutations were studied both as chromosome aberrations and as micronuclei (MN) in several cell types; including rat embryo fibroblasts, rat hepatocytes, and human peripheral blood lymphocytes (PBLs) (120–125). Most studies concentrated on DEB, perhaps because it is the most efficient of the metabolites for inducing chromosome-level changes.

The studies exploring the effect of DNA repair were revealed that repair was usually effective in correcting EB lesions before they could produce chromosome aberrations (121, 123–124). DEB induced chromosome-level mutations (aberrations and MN) were shown to be influenced by the Fanconi anemia gene, where mutations confer sensitivity in both homozygotes and

heterozygotes (125–129). When BD metabolites were evaluated for relative potencies in inducing micronuclei in mouse and rat transgenic fibroblasts, the relative order was again DEB >> EB > EB-diol, with ratios being approximately the same as for introducing gene mutations (119). In other studies, DEB was also shown to induce heritable (stable) translocations in human lymphoblastoid cells (130–131).

In summary, BD metabolites have induced gene mutations in different prokaryotic assay systems. BD itself has been positive only in the presence of external metabolic activation. DEB has always been the most potent mutagen of the BD metabolites.

### 1.3.2 *In vivo* mutagenicity of BD and its metabolites

Studies of mutations arising *in vivo* at either the gene- or chromosome-level provide a mode of action for the carcinogenic potential of BD. *Hprt* mutations in splenic lymphocytes were studied in mice exposed to 625 ppm BD by inhalation for 10 days (6 h/day, 5 days/week for 2 weeks), or total doses of 180, 240, and 500 mg/kg of EB, or to total doses of 21, 28, and 42 mg/kg DEB by intraperitoneal injections (i.p.) (53, 117). Induced mutations, as indicated by increased *Hprt* mutant frequencies (MFs) above background, were observed at all doses of BD and its metabolites in young animals, who were 7 to 10 days of age at the initial of exposures.

Analysis of mutational spectra can detect only “point mutations” in exon 3 and not deletions encompassing larger portions of the gene. They showed an increase in +1 frameshift mutations for all epoxides metabolites and transversion and transitions involving both GC and AT base pair for EB and DEB, but was

limited to AT base pairs for BD. In contrast to EB and BD, the yield of mutations with DEB showed the induction of deletions.

In a later study, Tates et al. (1994, 1998) used older animals (11 – 12 weeks of age) that were exposed to BD by inhalation (200, 500, and 1,300 ppm for 6 hours/day for 5 days) or to EB (33 or 100 mg/kg for each of three i.p. injections), or to DEB (various doses by i.p. injection) did not give such positive results in *Hprt* MFs in splenic lymphocytes for BD and DEB at any dose tested (132). However, EB gave a significant increase in MF only at the highest dose (132, 133).

Later, a series of studies then were applied a modified assay system that included larger numbers of young (4–5 weeks) animals. They were measuring mutations at optimum expression times and defining BD's relative mutagenicity in mice and rats. These studies identified the roles of EB and DEB for BD's mutagenicity at different doses and better established the BD induced *Hprt* molecular mutation spectrum (134–143). When *Hprt* MFs were studied at multiple times postexposure to BD by inhalation at 1,250 ppm 6 h/day, 5 days/week for 1 or 2 weeks, the expression times for mutation manifestation in thymic lymphocytes were 2 or 3 weeks postexposure for mice and rats, respectively, and for mutation manifestation in splenic lymphocytes were 5 or 4 weeks postexposure, for mice and rats, respectively.

In summary, there is a clear evidence that BD induces mutagenic effects in *in vivo* test systems primarily through its reactive metabolites, EB and DEB. *In vivo* studies indicate that EB primarily induces point mutations (A:T to T:A transversion) and small deletions, whereas DEB, also results in large deletions.

### 1.3.3 Species and gender differences in BD mutagenicity

Several studies of mutations suggest that BD, a genotoxic agent, induces cancer by a mutagenic mode of action (MOA). Such events show not only that BD epoxides reach critical targets *in vivo* and that they produce the kinds of irreversible genetic effects that alter genetic information. Because of their importance in an overall mutagenic evaluation of BD that incorporates metabolism in the different species and genders, the reviews in this section are discussed in greater detail.

BD's relative mutagenic potency in mice and rats were explored by exposing both species by inhalation at 20, 62.5, or 625 ppm on the same daily schedule for 2 or 4 weeks (134, 136). In addition, they exposed additional group of mice to 3 ppm BD for 2 weeks. (135, 138, 142). Mice showed a significant increase in *Hprt* MFs in splenic lymphocytes at the lowest exposure tested, i.e., 3 ppm, whereas a significant increase in MFs in rat splenic lymphocytes was observed only at 62.5 ppm. It is noteworthy that mice exposed to 3 ppm for 2 weeks and rats exposed to 62.5 ppm for this same duration were subsequently shown to have approximately the same number of DEB globin adducts in blood, as determined by the DEB-specific, *pyr*-Val, hemoglobin adduct assay (86). This suggests strongly that the important BD metabolite for inducing gene mutations at low BD exposures is DEB. These comparative studies also showed that gender, as well as species, influences the mutagenic response to BD, with the order being female mice > male mice > female rats > male rats.

Analyses of dose response curves for *Hprt* mutations in the two species showed that the curve of mice was supralinear, whereas in rats curve was shallow but linear. This influences the determination of relative mutagenic

potencies of BD in the two species, which will differ at different exposure concentrations. At 625 ppm this potency was estimated to be 9-fold greater in mice than in rats, compared to a 5-fold difference at 1,250 ppm (135, 142).

The relative roles of racemic EB and DEB in determining BD's mutagenicity at different exposures in mice and rats were specifically investigated by administering EB or DEB by inhalation for 6 h/day, 5 days/week for 4 weeks (136). It was observed that the mutagenic potency of racemic EB administered by inhalation in mice was much lower than the mutagenic potency of BD at the exposure levels that would give the corresponding EB blood levels, indicating that EB is not the major mutagenic metabolite of BD in mice at either 62.5 or 625 ppm. In contrast, the mutagenic potency of DEB administered by inhalation at both 2 and 4 ppm exposure levels to mice was approximately equivalent to BD's mutagenic potency at an exposure level of 62.5 ppm. DEB was the major contributor to BD's mutagenicity in mice at exposure levels of 62.5 and below. Rats showed a different picture in the study. Exposure to racemic EB did not induced significant mutation, whereas DEB at both exposures showed greater mutagenicity than in mice. This illustrates the potent mutagenicity of DEB in rats but also that rats do not produce high levels of DEB from BD at 62.5 ppm, as that level of BD exposure was only minimally mutagenic in rats (135–136, 142).

A recent study provided important data on the mutagenic potency of BD at low exposure (3 ppm) and the metabolites B-diol in different genders and species of rodents (143). The study included a series of experiments in which male and female rats and mice were exposed BD or its metabolites DEB and B-diol via inhalation. These studies confirmed and extended earlier observations that female rodents are more susceptible than male rodents to BD exposure. In

addition, mice are more susceptible than rats to inhaled BD. The investigators demonstrate that in both species the contribution of the metabolites B-diol and EB-diol to mutagenicity induced by BD exposure is most prominent at high BD concentrations, whereas the metabolite DEB is probably more important at lower concentrations. Therefore, the difference in sensitivity to BD between species and genders in all mutagenicity tests points towards differences in production and detoxication of DEB and EB-diol.

#### **1.4 1,3-Butadiene biomarkers**

Over the past 10 years, several protein and DNA adducts of BD reactive metabolites have been measured as biomarkers of BD exposure and effect (73, 144). Quantification of biomarkers has been performed by various methods. The studies are briefly reviewed below.

##### *1.4.1 Hemoglobin adducts*

Hemoglobin (Hb) adducts have been extensively used as biomarkers for evaluating the “internal dose” of various chemicals (145–151). They can provide quantitative dose estimates in blood in experimental animals and in occupationally exposed humans. N-terminal valine adducts of Hb are recognized as good surrogate markers to study carcinogen metabolism across species, since they offer many advantages. Blood samples are easier to obtain than tissues, and globin adducts accumulate over the life-span of erythrocytes, which is about 45, 63, and 120 days for mice, rats, and humans, respectively. Since they represent cumulative exposure prior to sampling, the time of sample collection is less critical. In addition, the N-terminal valine is freely accessible to

alkylating agents in all species, allowing interspecies comparisons relative to metabolism and cancer risk. Therefore, N-terminal valine adducts are valuable biomarkers to study the internal formation of the reactive metabolites of BD-derived epoxides (86, 151–152).

For internal dose measurements of epoxides, their adducts to Hb can be used as dosimeters (152). All of the epoxide metabolites of BD react with N-terminal valine in Hb, mainly by nucleophilic attack on the least hindered carbon (152). They form three different adducts: N-(2-hydroxy-3-butenyl)-valine (HB-Val); N, N-(2, 3-dihydroxy-1, 4-butadiyl)-valine (*pyr*-Val), and N-(2, 3, 4-trihydroxybutyl)-valine (THB-Val), originating from EB, DEB, and EB-diol, respectively (**Figure 1.3**).

#### *1.4.1.1 Quantitation of BD derived N-terminal valine Hb adducts*

In the last several years, Hb adducts from EB, HB-Val, were initially analyzed by the N-alkyl Edman method (148) in rats treated with different concentrations of BD. The HB-Val adducts were shown to be stable and they increased linearly with exposure concentration. In following studies both regioisomers of HB-Val adduct were quantified in rats and mice exposed to different concentrations of BD (153–155).

THB-Val mainly forms from the reaction of EB-diol with the N-terminal valine of globin. This adduct has been analyzed in both laboratory animals and humans exposed to BD in an occupational setting. THB-Val was quantified by using the N-alkyl Edman method with modification in the extraction of formed pentafluorophenylthiohydantoin (PFPTH) according to Perez et al. (1997) (156). In rats exposed to BD at different concentrations, the THB-Val was formed in 3 –

32 times greater amount than the HB-Val adducts formed from EB by reaction at carbon position 1 (C<sub>1</sub>) (153, 156). The given ratio was higher at the lowest BD exposure concentrations. THB-Val has also been monitored in BD workers in China and Czech Republic (162, 163). The mean THB-Val concentration was significantly higher in BD exposed group (2 -21 ppm) than unexposed group (40).

Recently, a DEB-specific valine adduct, N, N-(2, 3-dihydroxy-1, 4-butadiyl)-valine (*pyr*-Val), has been developed as a biomarker (165). Unlike THB-Val and HB-Val, *pyr*-Val adduct is quantified as a peptide by immunoaffinity (IA)-LC-ESI-MS/MS (86, 157). For these methods hemoglobin is digested with trypsin to release N-terminal peptides (1-7), which has been spiked with a synthetic deuterated *pyr*-Val peptide (1-11) as an internal standard. The *pyr*-Val peptides are purified and enriched from the trypsin digest with immunoaffinity column prior to LC-ESI-MS/MS analysis. *Pyr*-Val has been monitored in laboratory animals exposed to BD (151, 157). A 10 to 60 fold greater concentration of *pyr*-Val was observed in mice as compared to rats ranging from low to high BD exposure ( 0.1 – 625 ppm BD) (151).

In addition, the same approach was developed for analysis of the EB-specific; HB-Val, adduct in rats exposed to 1,000 ppm BD for 90 days (158). The analysis of HB-Val by using (IA)-LC-ESI-MS/MS has advantages over N-alkyl Edman method. The new method demonstrated excellent reproducibility with a CV < 25% and sensitivity with LOQ 100 fmol/injection. However, this current method described above is not available for analysis of THB-Val. Consequently, the application of the method for *pyr*-Val and HB-Val are labor intensive requiring extensive sample processing time.



Therefore, this research describes the development of sensitive and specific combined method based on IA coupled with LC-ESI- MS/MS which makes it possible to measure all three BD globin adducts in rodents samples exposed to BD. This information may be used to estimate rodent cancer incidence.

#### 1.4.2 DNA adducts

DNA adduct formation by the three major BD metabolites is complicated by the fact that the three electrophilic intermediates exist in more than one stereoisomeric form (85, 159–160). EB has one such asymmetric center that allows for two stereoisomeric forms, referred to as (S) and (R) isomers. DEB has two asymmetric centers that allow for three stereoisomeric forms: the (S,S), and (R,R) forms, and a third *meso* form (**Figure 1.4**), in which one center has the S and the other center has the R configuration to produce overall symmetry in the molecule. An isomeric mixture of DEB contains both the (S,S) and (R,R) forms. Hydration of these stereoisomeric forms of DEB can result in four stereoisomers of EB-diol: the (2S,3R), (2R,3S), (2R,3R), and (2S,3S) forms. Therefore, the metabolism of BD can yield nine different stereoisomeric forms of three electrophilic intermediates which can potentially form DNA adducts (161).

##### 1.4.2.1 EB-produced DNA adducts

*In vitro* studies that have reacted various nucleobase/DNA targets with EB have repeatedly identified regioisomeric adducts at the N7 position of guanine (N7G) as the most abundant adducts (162–170). These adducts are hydrolytically unstable, with half-lives varying from 50 to 90 hours. EB also forms

two regioisomeric N3 adenine (N3A) adducts *in vitro* in calf thymus DNA that are lost by spontaneous depurination even more rapidly than the N7G adducts (167). Regioisomeric N1A and N6A adducts, being a Dimroth rearrangements of the N1 adducts also occur(171). Tretyakova et al. (1997c) identified adenine adducts in EB-treated DNA formed at approximately 1/10 the abundance of the guanine adducts (169).

Several N7-guanine DNA monoadducts of BD, N7-(2-hydroxy-3-buten-1-yl) guanine (HB-GI) have been quantified in laboratory animals exposed to <sup>14</sup>C-BD and <sup>14</sup>C-EB (163, 172). Although somewhat greater numbers of adducts were observed in mice compared to rats exposed to the same concentrations of BD, these differences cannot explain the ~100 fold greater sensitivity of mice to BD-induced cancer. Furthermore, because in these studies adduct concentrations were determined by measuring radioactivity of DNA and DNA hydrolysates, no structural information on the adduct was obtained. Later, Koivisto et al. (1995) employed <sup>32</sup>P-postlabeling to quantify N7G and, N1A adducts in laboratory animals exposed to 1, 3-butadiene (171, 173–176). N7G adduct levels were ~100 fold higher than the concentrations of N1A adducts. In 1999, Koc et al. measured N-7 guanine adduct formation, N-7-(2-hydroxy-3-buten-1-yl)guanine (EB-Gua I), and N-7-(1-hydroxy-3-buten-2-yl)guanine (EB-Gua II), using neutral thermal hydrolysis, desalted on solid-phase extraction cartridges, and quantitated by LC/ESI(+)/MS/MS (190). The studied was performed in liver, lung, and kidney tissue of B6C3F1 mice and F344 rats exposed to 0, 20, 62.5, or 625 ppm BD. EB-Gua adducts had a linear exposure-response relationship in both species. When the numbers of EB-Gua adducts in rats and mice were compared, the numbers in mice were about twice those in

liver and lung DNA at 625 ppm, while the difference in kidney at this exposure level was not significant.

#### 1.4.2.2 DEB-produced DNA adducts

DEB produces both monoadducts and bifunctional adducts. Reaction of the two epoxide sites of DEB with sites on two nearby nucleobases joins the two nucleobases, forming an inter- or intrastrand cross-links in double-stranded DNA. DEB treatment of deoxyguanosine has produced seven major and a variety of additional monofunctional adducts at the N7, N1, or an N position, with two being ring-structured compounds (177–179). Alkylation at the N7 position was the most rapid, producing abundant N7-trihydroxybutylguanine (N7THBG). DEB also produces an adenine monoadduct involving the N6 position after treating deoxyadenosine, calf thymus DNA, or Chinese hamster ovary (CHO) cells in culture (180 -181). Subsequent *in vitro* studies have verified that DEB produces monofunctional adenine adducts at this position, as well as at the N1, N3, N7, and N9 positions (170, 182–183). Extensive investigations have demonstrated that DEB produces bifunctional DNA adducts linking guanine with guanine and guanine with adenine, producing both inter- and intrastrand DNA cross-linking (184–186). Park and Tretyakova (2004) showed that low concentrations of DEB reacted with calf thymus DNA yielding equivalent amounts of the bis-N7G-BD bifunctional adducts and the corresponding N7THBG monoadducts, but that the former were more stable (81.5-h half-life) than the latter (48.5-h half-life).

Stereochemistry is important in the formation of bis-N7G-BD inter- and interstrand DNA cross-links (187). (S,S)-DEB produced 8- and 10-fold more of the interstrand lesions than did the (R,R)- and meso-DEB, respectively. The

intrastrand cross-links formed by (R,R)- and meso-DEB accounted for 19% and 51% of the total bis-N7G-BD adducts produced by these two isomeric forms of DEB, respectively. Racemic DEB was reacted with calf thymus DNA to produce four asymmetrical guanine-adenine bifunctional adducts (188). Two were hydrolytically stable, indicating that these lesions have the potential to accumulate in DNA *in vivo*.

Chronic BD inhalation studies in B6C3F1 mice demonstrated male and female mice had tumors of similar tissues, but for the most part females developed tumors at lower exposure concentrations. For example, lung and liver tumors in female mice were observed at exposure concentrations of 6.25 and 20 ppm BD, respectively. By comparison with male mice, the respective exposure concentrations required a 10-fold increase in concentration. There are also gender differences in the formation or repair of *bis*-N7G-BD adduct in male and female exposed to 625 and 200 ppm BD for two weeks. *Bis*-N7G-BD adduct amounts in female mice are 2- to 2.5- fold higher than in male mice (189). Some studies have explained this gender difference in neoplasia, MFs, and amount of adduct formed due to differences in BD metabolism, especially of DEB.

#### 1.4.2.3 EB diol-produced DNA adducts

Zhao et al. (1998) were still able to detect N1-(2,3,4-trihydroxybut-1-yl)adenine (N1-THB-A) adducts in human lymphocytes obtained from 14 butadiene industry workers. N1-THB-A adducts ( $4.5 \pm 7.7$  adducts/ $10^9$  nucleotides) were observed in BD-exposed workers, while only  $0.8 \pm 1.2$  adducts/ $10^9$  nucleotides were measured in non-BD exposed individuals (183).

The problem with using radiolabeled carcinogens and <sup>32</sup>P-postlabeling analysis of DNA adducts is that these methodologies do not yield any structural information and they do not have internal standards to assure accurate quantitation.

N7-(2',3',4'-trihydroxybut-1'-yl)guanine (THB-G) adducts of BD have been quantified in rodents exposed to BD using isotope dilution mass spectrometry (170, 190, 191). These adducts can result either from guanine reactions with DEB, followed by hydrolysis of the second epoxide, or directly by guanine alkylation by EB-diol. EB-diol is much more abundant than DEB, and 98% of THB-G observed *in vivo* is produced from the reaction of EB-diol with DNA (190). Furthermore, only a 2 fold difference in THB-G levels was observed between mice and rats (190), which is inconsistent with the 100-fold difference in mutagenicity among the two species and suggests that DNA adducts other than THB-G monoadducts are responsible for the genotoxic effects of BD.

#### 1.4.3 DNA-Protein crosslink (DPC)

The adverse biological effects of DEB have been attributed to its ability to cross-link cellular biomolecules. Initial alkylation of adenine and guanine bases in DNA by DEB produces 2-hydroxy-3,4-epoxybut-1-yl (HEB) lesions, which contain an inherently reactive oxirane group that can alkylate neighboring nucleobases within the DNA duplex to form DNA–DNA cross-links (184). Alternatively, the 3,4-epoxy ring can be subject to nucleophilic attack by amino acid side chains of neighboring proteins, giving rise to DPCs (**Figure 1.5**) (50, 192). Therefore, measuring DPC can indicate the dose of DEB at a critical target site. DPC by DEB was first observed by Jelitto et al. (1989) who employed alkaline elution methodology to detect DPC formation in liver and lung tissue of B6C3F<sub>1</sub> mice

and SD rats following exposure to BD (193). This observation was subsequently confirmed by other groups that utilized biophysical methods for detecting DEB-induced DPCs in mice, but not to the same extent in the rat (194). Vangala et al. (1993) detected an increase in DPC activity in lung and liver, with increasing exposure concentrations of BD (100 ppm -2000 ppm). DPCs are more pronounced in mouse lung than in mouse liver. No such signs of genotoxicity could be observed for the lungs of rats (194).

Michaelson-Richie et al. (2010) have observed DPCs in human cervical carcinoma cells (HeLa nuclear protein extracts) using an HPLC-ESI-MS/MS method (195). This observation positively identified DPC between cysteine thiols within proteins and the N-7 guanine positions within DNA. Similar studies have not been reported in rodent or human tissues, but it is likely that they will form proportionate to DEB formation. The ability of specific proteins to form DPCs is likely to be influenced by a number of factors, including protein abundance, structure, and cellular and nuclear localization. For example, DNA-binding proteins are in close proximity to DNA and readily available to form DPCs in the presence of cross-linking agents such as DEB. Several of the proteins found to form DEB-mediated DPCs, such as those involved in DNA replication and repair, are known to associate with DNA in the nucleus. For example, the DNA repair protein poly [ADP-ribose] polymerase I (PARP) is involved in the DNA damage signaling response, and has been previously shown to form cross-links to DNA as a result of exposure to nitrogen mustards (196), ionizing radiation (197), and formaldehyde (198). No DPC lesions involving histone proteins were detected, which could be a result of their low concentrations in nuclear protein extracts, their inefficient cross-linking to DNA, or their disrupted association with DNA

(199). Although the biological relevance of DEB-mediated DPC is poorly understood and very limited, certain types of DPCs persist through several cycles of DNA replication (200–201), potentially leading to cytotoxic and mutagenic outcomes such as sister chromatid exchanges and large deletions. The formation of these bulky, helix-distorting lesions would have the potential to interfere with the critical cellular process such as replication, transcription, and triggering programmed cell death or inducing genotoxicity.

## **1.5 Multiplicative risk model for 1,3-butadiene cancer risk estimation**

### *1.5.1 Internal formation dose (D)*

A dose concept for genotoxic electrophiles agents has been developed (202) where the dose is defined as the time-integrated concentrations of ultimate genotoxic agents. It is expressed in  $\text{mmol} \times \text{Kg}^{-1} \times \text{h}$  (mMh) that are proportional to frequencies of potentially genotoxic events.

The key event in the carcinogenic action of genotoxic agents (reactive chemicals) is mutation (203, 204) induced via chemical changes in DNA.

The internal dose at target tissue of chemical is defined by:

$$\int_{t_i} D = \int C(t) dt \quad (\text{Eq. 1})$$

Where D is the integral over time (t) of the concentration, C(t) in target cells of a reactive chemical or metabolite (205). A chemical dose thus defined will be proportional to the frequency of potentially mutagenic DNA changes. At the present state of knowledge, the dose accordingly will be proportional to the area under the curve of the concentration and time curve (AUC).

In this research, the internal dose of the reactive epoxide metabolites (EB, DEB, and EB-diol) will be calculated from levels of hemoglobin adducts in rodents exposed to BD in short-term experiments (10 days). Proportionalities between rates of formation of Hb adducts in different organs provide a good surrogate monitor for target doses (206). Internal formation dose in blood could be inferred from measurement of Hb adduct levels in short-term experiments with animals, if the rate constant for the reaction is known (in this case, the formation of an adduct to NH<sub>2</sub>-terminal valine in Hb; therefore, the simple expression for internal formation dose (D) when the adduct level is measured a short time after acute exposure.

$$D = A / K_{val} \quad (Eq.2)$$

Where A is the Hb adduct level for the individual reactive epoxide in [ mol × g<sup>-1</sup>], K<sub>val</sub> is the rate constant of adduct formation to NH<sub>2</sub>-terminal valine in [ L × g<sup>-1</sup> × h<sup>-1</sup> ] specific for the individual reactive epoxide in each species.

### 1.5.2 1,3-Butadiene cancer risk estimation

Cancer risk estimation on the basis of internal dose of a genotoxic chemical is important for the detection and the assessment of cancer risk factors. An improving approach for risk estimation, the multiplicative risk model, is being developed, which is based on experience from ionizing radiation (207, 208). Evaluations of animal cancer tests using this approach indicate that a multiplicative cancer risk model could be applied to genotoxic chemical (202). According to that result, the cancer risk increment is proportional to the internal dose of causative genotoxic agent and its genotoxic potency specific for the studied species, strain and gender. In addition, it is indicated that the relative risk



increment according to this model is common for different responding sites. Therefore, the model has been shown to provide a prediction of a tumor incidence in long-term animal cancer tests with genotoxic agents, and this model can be used for extrapolation of human cancer risk (208). Moreover, the applicability of the multiplicative risk model to cancer test data for ethylene oxide (207), acrylamide (209), and 1,3-butadiene (210) has been evaluated. It has been shown that internal dose of genotoxic agent and the observed tumor incidence in animal cancer test are well correlated with the predicted incidences from the multiplicative risk model. Furthermore, the model can be applied to genotoxic agents and the interspecies extrapolation can be performed.

In the assessment of cancer risk estimation from exposure to genotoxic agent, the multiplicative risk model is applied by:

$$P_i(D) = 1 - e^{-P_i^0(1+\beta D)} \quad (\text{Eq.3})$$

Where **D** denotes the internal dose in target tissue, and **P<sub>i</sub><sup>0</sup>** represent the cumulative hazard for tumor formation in the target tissue, and **i** is tumor incidence among unexposed animals (the background risk), and **β** is the common relative excess risk per unit of dose. The parameters of this nonlinear function will be estimated by a binomial regression model with 95% confidence intervals. The fit of model is presented as observed versus predicted tumor incidence.

## 1.6 Research goal and specific aims

In cancer epidemiology and cancer studies of BD, three epoxides are associated with the carcinogenic potency in rodents and humans. This is considered to be related to the metabolism of BD to its individual epoxide

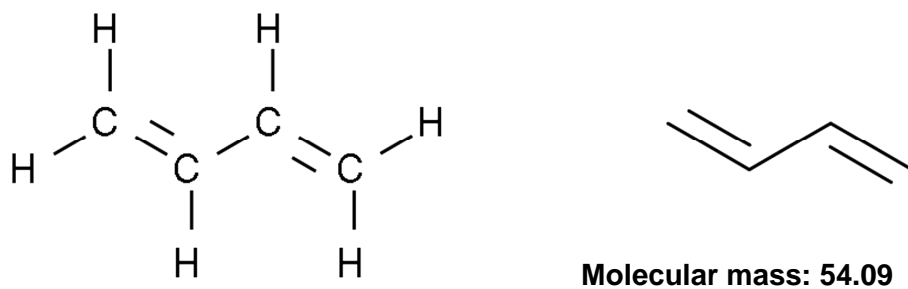
metabolites and their different mutagenic potency. Therefore, it is important to measure the internal dose of individual epoxides in order to understand species and gender differences in metabolism and mutagenesis. N-terminal globin adducts of the three BD epoxides is an effective biomarker used to monitor the exposure and internal dose. One of the main aims of this research is to develop specific analytical methods that allow for quantification of all three BD epoxides in a single processing. Furthermore, the prediction of the response in cancer tests to the internal dose (globin adducts levels) and the relative mutagenic potency (from *in vitro* data) of the BD epoxide, will be evaluated with the application of a multiplicative cancer risk model.

The goal of this research is to gain the information of the cancer risk estimation of animal cancer tests on the basis of internal dose, using globin adducts as a biomarker, to help us to better understand BD metabolism and carcinogenicity. This information will provide critical data to improve the cancer risk assessment for BD. The specific aims for this research are as follows:

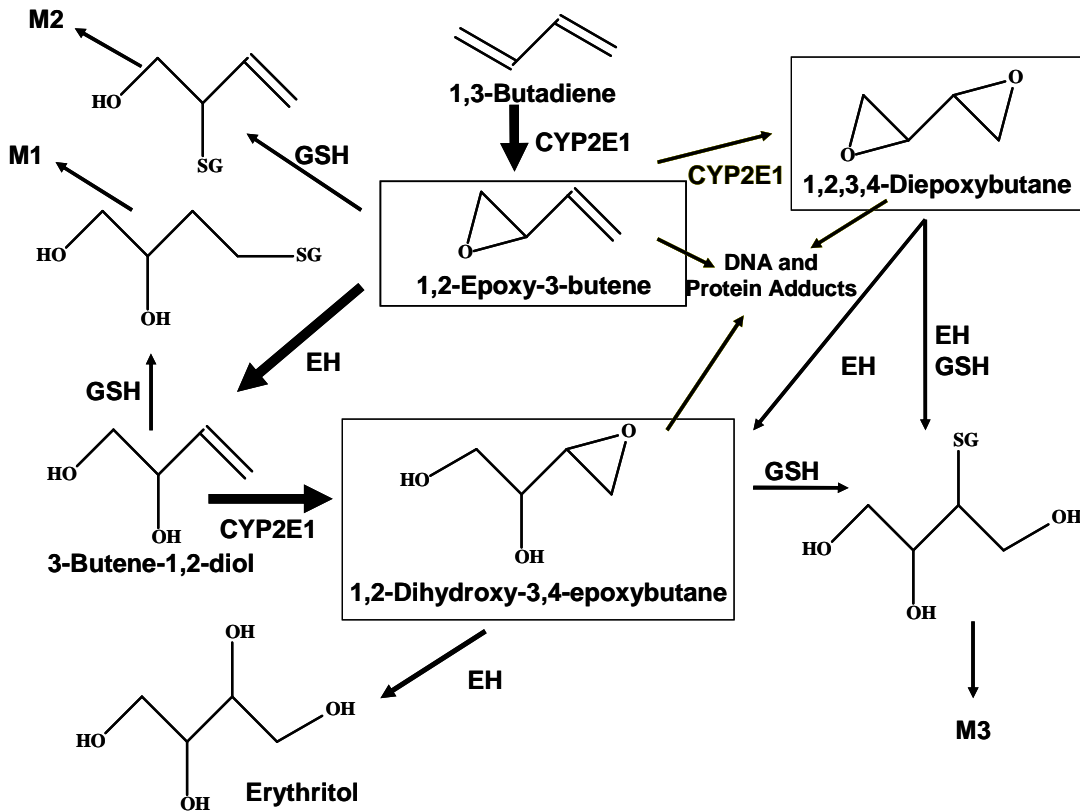
1. Develop a highly specific method for synthesis and characterization of butadiene epoxide alkylated peptide utilizing adducted peptides as analyte standards and internal standards for quantification of globin adduct in animal samples.
2. Establish a sensitive method for peptide quantitation based on the analysis of their amino acid components using ultra performance UPLC-MS/MS.
3. Develop an improved method for simultaneous analysis of all three adducts formed by butadiene epoxides at the N-terminal valine of globin alpha chain in animals exposed to butadiene via inhalation.

4. Evaluate differences in cancer outcome of animals exposed to BD using an internal adduct dose of epoxides metabolites; EB, DEB, and EB-diol, and their relative genotoxic potency as the estimators.

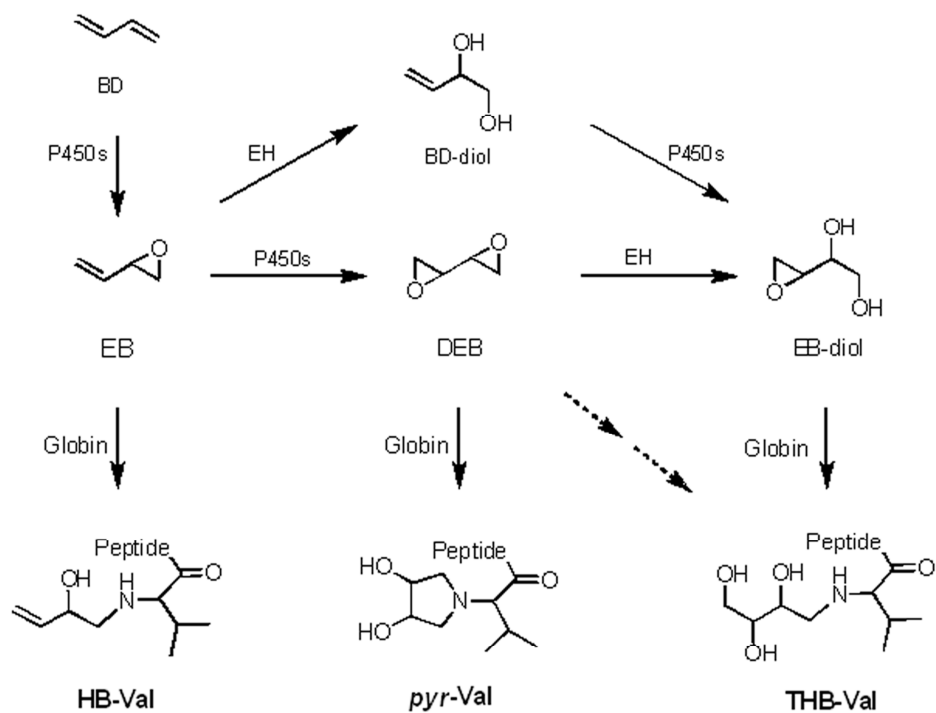
## FIGURES



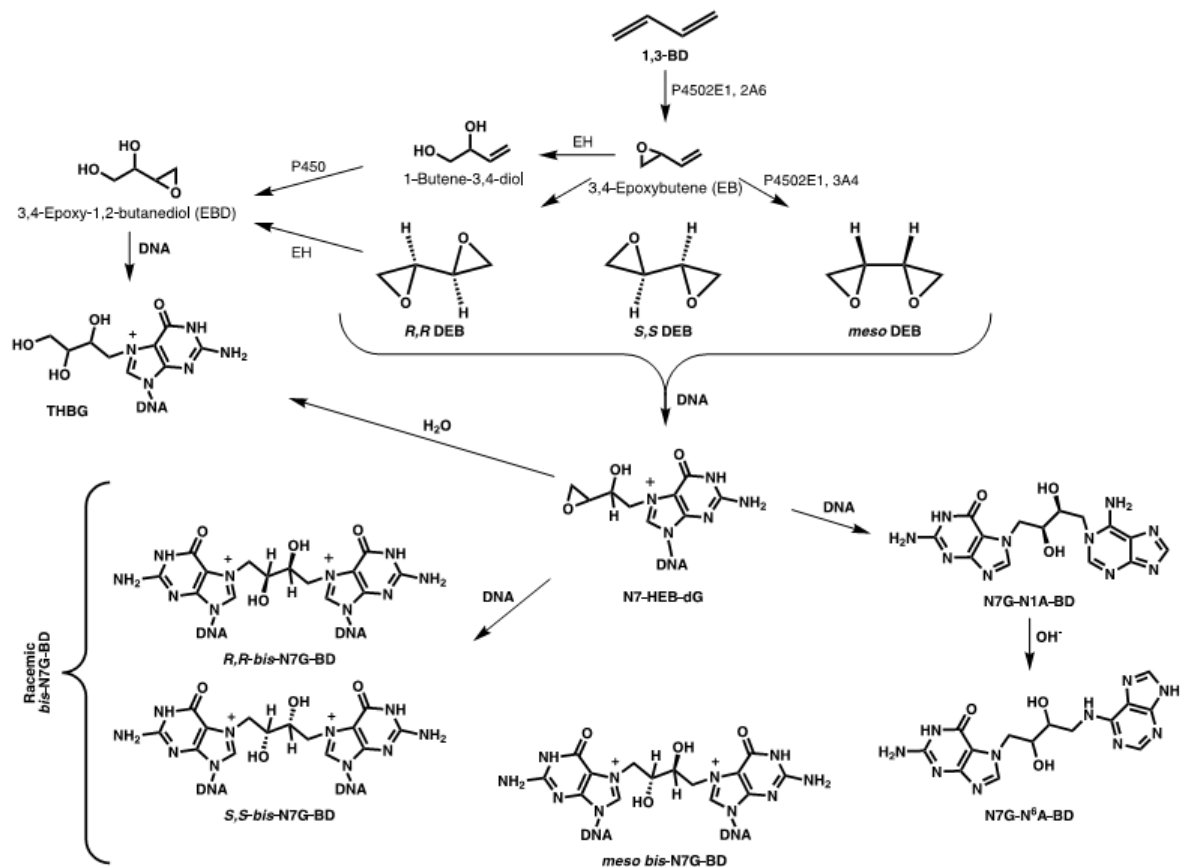
**Figure 1.1** Molecular structure and relative molecular mass of BD



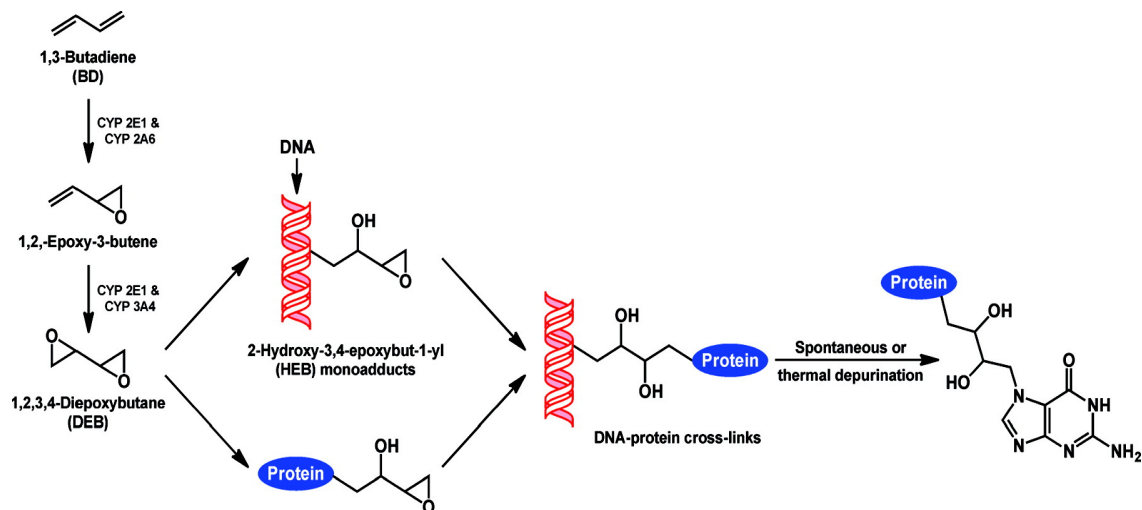
**Figure 1.2** Metabolism of BD. Showing enzymes involved in BD metabolism and the major detoxification metabolites excreted in urine



**Figure 1.3** BD metabolism and formation of *N*-terminal valine adducts



**Figure 1.4** Metabolic activation of BD to reactive electrophiles and the formation of DNA adduct



**Figure 1.5** Metabolic activation of BD to reactive electrophiles and the formation of DNA-Protein crosslink (DPC)



**Table 1:** Survival-adjusted percentage for B6C3F1 mice and F344 rats in the long-term inhalation studies of BD [NTP 1993]

Sex	Mice		Exposure concentration (ppm)					
	Neoplastic lesion	Target organ	0	6.25	20	62.5	200	625
Males								
Initial number <sup>a</sup>			70	70	70	70	70	70
Number of survivors			35	39	24	22	3	0
-	Malignant lymphomas	All	4 (9%) <sup>b</sup>	3 (4%)	8 (10%)	11 (15%) <sup>c</sup>	9 (27%) <sup>c</sup>	69 (97%) <sup>c</sup>
-	Haemangiosarcoma	Heart	0 (0%)	0 (0%)	1 (3%)	5 (14%) <sup>c</sup>	20 (64%) <sup>c</sup>	6 (53%) <sup>c</sup>
-	Alveolar/bronchiolar carcinoma	Lung	22 (48%)	23 (49%)	20 (45%)	33 (75%) <sup>c</sup>	42 (88%) <sup>c</sup>	12 (45%) <sup>c</sup>
-	Papilloma and carcinoma	Forestomach	1 (2%)	0 (0%)	0 (0%)	5 (3%)	12 (29%) <sup>c</sup>	13 (54%) <sup>c</sup>
-	Adenoma and adenocarcinoma	Harderian gland	6 (14%)	7 (15%)	11 (22%)	24 (51%) <sup>c</sup>	33 (81%) <sup>c</sup>	7 (64%) <sup>c</sup>
-	Hepatocellular carcinoma	Liver	31 (45%)	27 (48%)	35 (65%)	32 (62%)	40 (86%)	12 (61%)
Females								
Initial number <sup>a</sup>			70	70	70	70	70	70
Number of survivors			37	33	24	11	0	0
-	Malignant lymphomas	All	10 (13%)	14 (27%)	18 (27%)	10 (20%)	19 (40%) <sup>c</sup>	43 (86%) <sup>c</sup>
-	Haemangiosarcoma	Heart	0 (0%)	0 (0%)	0 (0%)	1 (3%)	20 (72%) <sup>c</sup>	26 (83%) <sup>c</sup>

Mice			Exposure concentration (ppm)					
Sex	Neoplastic lesion	Target organ	0	6.25	20	62.5	200	625
-	Alveolar/bronchiolar carcinoma	Lung	4 (9%)	15 (33%)	19 (47%)	27 (61%) <sup>c</sup>	32 (81%) <sup>c</sup>	25 (83%) <sup>c</sup>
-	Papilloma and carcinoma	Forestomach	0 (0%)	0 (0%)	3 (8%)	4 (6%)	7 (23%) <sup>c</sup>	28 (83%) <sup>c</sup>
-	Adenoma and adenocarcinoma	Harderian gland	9 (18%)	10 (23%)	7 (17%)	16 (41%)	22 (71%) <sup>c</sup>	7 (58%) <sup>c</sup>
-	Hepatocellular carcinoma	Liver	17 (33%)	20 (30%)	23 (36%)	24 (51%) <sup>c</sup>	20 (65%) <sup>c</sup>	3 (22%)
-	Adenocarcinoma	Mammary gland	0 (0%)	2 (5%)	2 (10%)	6 (33%) <sup>c</sup>	13 (57%) <sup>c</sup>	13 (67%) <sup>c</sup>
-	Benign and malignant granulose cell	Ovarian	1 (2%)	0 (0%)	2 (3%)	9 (26%) <sup>c</sup>	11 (41%) <sup>c</sup>	6 (47%)

Rats			Exposure concentration (ppm)		
Sex	Neoplastic lesion	Target organ	0	1,000	8,000
Males					
Initial number <sup>a</sup>			110	110	110
Number of survivors			55	50	69
-	Exocrine adenoma	Pancreas	3 (3%) <sup>b</sup>	1(1%)	10 (10%)
-	Glial cell	Brain	1 (1%)	4 (4%)	5 (5%)
-	Leydig cell tumor	Testis	0 (0%)	3 (3%)	8 (8%) <sup>c</sup>

Sex	Rats		Exposure concentration (ppm)		
	Neoplastic lesion	Target organ	0	1,000	8,000
Females					
Initial number <sup>a</sup>			110	110	110
Number of survivors			60	68	76
• Sarcoma		Uterus	1 (1%)	4 (4%)	5 (5%)
• Follicular cell adenoma		Thyroid gland	0 (0%)	2 (2%)	10 (10%) <sup>c</sup>
• Benign or malignant		Mammary gland	50 (50%)	79 (79%)	81 (81%) <sup>c</sup>
• Carcinoma		Zymbal gland	0 (0%)	0 (0%)	4 (4%)

From Melnick et al (1990); National Toxicology Program (1993); Owen (1987); Owen (1993)

<sup>a</sup>Initial numbers include animals removed from study for interim sacrifices.

<sup>b</sup>Mortality-adjusted tumor rates are given in parentheses.

<sup>c</sup>p < 0.05, based on regression analysis with adjustment for intercurrent mortality.

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## CHAPTER II

### SYNTHESIS AND CHARACTERIZATION OF STANDARD PEPTIDES SPECIFIC FOR 1,3-BUTADIENE N-TERMINAL VALINE ADDUCTS

#### 2.1 Abstract

Butadiene (BD) is an industrial chemical used in the production of synthetic rubber and found in gasoline and combustion products. It is a multi-site and multi-species carcinogen in rodents with mice being much more sensitive than rats. BD is metabolized mainly by P450 2E1 to 1,2-epoxy-3-butene (EB), 1,2;3,4-diepoxybutane (DEB) and 1,2-epoxy-3,4-butanediol (EB-diol). For risk assessment, the internal formation of each epoxide can inform their relative risks because they show significantly different mutagenicity. The measurement of N-terminal valine adduct in globin chains can be used as a biomarker to determine the internal formation dose of each epoxide after exposure to BD. In this chapter, we needed to prepare pure and rigorously characterized epoxide alkylated N-terminal glycopeptides of rat and mouse for using in an immunoaffinity purification step and to standardize the assay. In addition, we needed a pure isotope-labeled peptide to serve as an internal standard for quantitation by LC-MS. We therefore synthesized the alkylated peptides using *in vitro* direct alkylation of the peptide reaction with EB and EB-diol.

The peptides were purified by HPLC and characterized by their fragmentation patterns obtained by tandem mass spectrometry (MS/MS). Use of isotope labeled [ $^{13}\text{C}^{15}\text{N}_5$ ] on N-terminal valine in the oligopeptide synthesis provided the required isotopic labeled peptide for use as an internal standard. This strategy enabled us to obtain pure and well characterized alkylated analyte standard peptides for quantification of globin adducts in rodent samples exposed to BD.

## 2.2 Introduction

1,3-Butadiene (BD) is an important industrial chemical widely used for synthetic rubber and resin production (1). It has been identified as an environmental contaminant in gasoline, motor vehicle exhaust, and cigarette smoke (2, 3). BD was recently reclassified as a human carcinogen following inhalation exposure by the US EPA (4) and IARC (5). This classification was based on epidemiologic studies of exposed workers in the butadiene rubber industry and laboratory animal data. Human epidemiologic studies of occupationally exposed workers have presented evidence for an increased incidence of leukemia, lymphatic and hematopoietic cancer (6–8). In chronic inhalation experiments with B6C3F<sub>1</sub> mice and Sprague-Dawley rats, BD was carcinogenic in both species (9, 10). However, while mice developed tumors after chronic exposure to as little as 6.25 ppm BD (9), much higher concentrations (1,000 ppm) were reported to induce tumors in rats (10). These interspecies differences in sensitivity have been attributed to differences in BD metabolism in mice and rats, in particular, the increased formation of reactive epoxide metabolites in the mouse (9).

BD requires metabolic activation by the cytochrome P450 forming reactive epoxides: 1,2-epoxybutene (EB), 1,2:3,4-diepoxybutane (DEB) and 3,4-epoxy-1,2-butanediol (EB-diol) (1) that are known to covalently bind to DNA and hemoglobin. Hemoglobin adducts, especially adducts at the N-terminus, have been successfully used as biomarkers of exposure to reactive chemicals such as acrylamide, ethylene oxide, and butadiene (11–14). The BD derived *N*-terminal globin adducts 2-hydroxy-3-butenyl-valine (HB-Val), *N,N*-(2,3-dihydroxy-1,4-butadiyl)-valine (*pyr*-Val), and 2,3,4-trihydroxybutyl-valine (THB-Val) are used as biomarkers for EB, DEB, and EB-diol, respectively. A simplified pathway for metabolism and globin adduct formation of BD is shown in **Figure 2.1**.

Currently, a sensitive immunoaffinity (IA)-LC-MS/MS technique for quantitation of the cyclic adduct, *pyr*-Val, has been refined to the point of practical application to the routine analysis (15). In addition, Georgeiva et al. (2010) provided the basis for a more sensitive ultra performance liquid chromatographic – mass spectrometry (UPLC-MS/MS) assay for quantitative measurement in globin of rodents exposed to BD (16). Both HB-Val and THB-Val adducts in rodents and humans have been quantitated using the “modified Edman degradation method” (17, 18), with modifications in the extraction of formed pentafluorophenylthiohydantoin (PFPTH) (19) and analysis by GC-MS/MS. This method had lacked sensitivity for HB-Val analysis and had a poor recovery of analyte and internal standard for THB-Val analysis. Therefore, the objective of this research was to develop a specific and sensitive assay for detecting all three reactive epoxides adducts using IA-LC-MS/MS.

IA-LC-MS/MS has been developed based on enzymatic hydrolysis of globin for measurement of adducts to N-terminal valine formed from BD reactive epoxides (13). The most commonly used protease enzyme is trypsin, which hydrolyses the peptide bonds at the carbonyl groups of lysine (K) and arginine (R). The N-terminal peptides of the alpha-chain formed after tryptic hydrolysis of globin are heptapeptides, specific to mouse and rat (**Table 2.1**).

Key to adapting the trypsin digestion procedure for application to rodent samples is the availability of a pure and well characterized, isotope-modified N-terminal sequence of rodent globin alpha chain to serve as an internal standard peptide. There are several approaches available to synthesize the analyte standard and internal standard peptide. However, these approaches are not suitable for synthesis of all three epoxides standard peptides due to the different structure and position of the carboxylic group on an alkylated peptide chain. Recently, the corresponding alkylated *pyr*-Val (1-11) and *pyr*-Val [<sup>2</sup>H<sub>3</sub>] (1-11) peptides were synthesized by our group and used as analyte and internal standards, respectively (20). Valine at the N-terminal was alkylated with DEB to give *pyr*-Val after protection of the carboxylic group with di-O-tert-butyl derivatives (t-boc). However, the method was not efficient enough to synthesize the HB-Val and THB-Val standards. In the present work, the analyte and internal standard peptides were synthesized using direct alkylation reaction between (1-11) globin peptides and 1,2-epoxybutene (EB) or 3,4-epoxy-1,2-butanediol (EB-diol).

### 2.3 Materials and methods

**Materials.** (±)-1,3-butadiene monoxide (EB, 98%) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). 1,3-butadiene diepoxide (EB-diol)

was generously provided by Dr. Avram Gold and Dr. Louise M. Ball from Department of Environmental Science and Engineering, The University of North Carolina at Chapel Hill. The N-terminal valine alpha chain peptides of mouse and rat globin (1-11) (**Table 2.1**) were synthesized by the peptide synthesis facility core at the University of North Carolina at Chapel Hill. The corresponding [ $^{13}\text{C}^{15}\text{N}_5$ ] 1-11 peptides of mouse and rat were also obtained from the peptide synthesis facility core. The purity of the peptides was greater than 98% on the basis of high performance liquid chromatography (HPLC) analysis. Trypsin (biotin-agarose) from bovine pancreas and all other chemicals, mass spectrometry grade, were purchased from Sigma-Aldrich Chemical.

A Surveyor HPLC system (ThermoFinnigan, San Jose, CA) connected to an LCQ Deca ion trap mass spectrometer (ThermoFinnigan) was used in all analyses.

## ***Methods***

### *Alkylation and purification of standard peptides*

#### *Direct alkylation method*

N-terminal alpha chain (1-11) peptides and the stable isotope [ $^{13}\text{C}^{15}\text{N}_5$ ] labeled peptides were incubated with EB or EB-diol at 1:20, 1:50, 1:100, or 1:200 peptide to epoxide molar ratios for 3 days at 37°C. Total volume was adjusted to 1.5 mL with 0.1 M ammonium bicarbonate buffer with different pH levels; 6.5, 7.5, and 8.5, to determine the optimum peptide ratio and pH for EB and EB-diol peptide alkylation. After incubation, the reactions were stopped with 20% formic acid and the pH was adjusted to strong acid conditions (pH 2).

### *Purification of standard peptides*

Alkylated peptides were purified by HPLC equipped with a quaternary pump coupled with LCQ-Deca electrospray ionization (ESI)/ion trap mass-spectrometer. The fraction collection was automatically performed by LCQ-Deca system. Separation was obtained at room temperature with a Vydac C18 (150 × 2.1 mm) HPLC column (Grace, Deerfield, IL). The LC solvents were 15 mM ammonium formate (A) and methanol (B). Separation was achieved at 400  $\mu$ L/min with a gradient of 5% - 50% B over 20 minutes. After separation, the column was washed at 80% B for 5 minutes and equilibrated at 5% B for 5 min. The monoalkylated peptide fraction was collected every 30 min (0.4 mL per fraction). All fractions were pooled and assessed for the presence and purity of the monoalkylated peptide of interest by HPLC-MS as described below. Fractions containing alkylated peptides were stored at -20 °C until use.

### *Assessment of purified monoalkylated peptides*

The LC-MS was performed in a LCQ-Deca mass spectrometer equipped with a ESI ion source interface. Column and chromatographic conditions were the same as those in the previously described in purification step. The mass spectrometer was set up to obtain full scan MS spectra (Scan range: 200 – 2000) with a positive ion injection mode.

### *Characterization of standard peptides*

Following the HPLC-MS purification and assessment, purified monoalkylated peptides were characterized by full scan MS/MS to confirm ion fragments ( $\gamma$ -fragments and b-fragments). Full scan MS/MS spectra in the linear

ion trap were obtained by collision-induced dissociation (CID) at a normalized collision energy of 35% as the activation mode. The activation time of 30 ms was used for selecting the precursor ion.

## 2.4 Results and discussion

The aim of this study was to synthesize, purify, and rigorously characterize standard peptides in quantities for tuning analytical equipment and quantification of BD derived globin adducts. Standard peptides were successfully synthesized using the direct alkylation method.

### *Optimization of the reactions in direct alkylation method*

The purpose of this experiment was to optimize the conditions for direct alkylation of peptides with BD monoepoxide (EB, EB-diol) over concentrations (molar ratios) and pH. Concentration of the peptides and pH were adjusted in order to increase the reactivity of valine (V) at the N-terminal site and to minimize the alkylation of lysine (K) and also cross-linking between peptides (21). The highest yields of monoalkylated peptides was found at 1:100 and 1:20 molar ratios for EB and EB-diol standard peptides, respectively. These reactions were performed at 37 °C for 3 days, which was adjusted from Fred et al. (2004) (13). This experiment showed that the reaction of EB with peptides was less efficient in alkylation than the reaction of EB-diol. In order to obtain high yields for the EB alkylated product, higher amounts of EB was needed for the reaction.

In our experiment, the alkylated peptide reactions with EB and EB-diol was achieved at pH 6.5 (**Figure 2.2**). At this pH, the reaction mixture revealed three products, the major being monoalkylated peptide and the two minor products

were unalkylated and dialkylated peptides. It was important to adjust pH in 0.1 M ammonium bicarbonate buffer at about 6.5 - 7 to decrease the reactivity of epoxide with lysine (K) (pKa = 8.95) at both positions 7 and 11 in the peptide sequence.

#### *Formation and purification of standard peptides*

The alkylated peptides were synthesized by direct reacted the EB or EB-diol with 1-11 globin peptide and the yield of alkylation was analyzed by HPLC-MS. The (1-11) rat peptide (VLSADDKTNIK) and mouse peptide (VLSGEDKSNIK) incubated with EB, forming HB-Val analyte standard peptides, provided signals for singly-charged ion,  $(M + H)^+$ , at  $m/z$  1273.5 and 1259.5, respectively. These alkylation peptides were 70 Da higher than those of unalkylation peptides. Similarly, the (1-11) rat peptide and mouse peptide incubated with EB-diol gave signals for THB-Val analyte standard peptides singly charge ion,  $(M + H)^+$ , at  $m/z$  1306.5 and  $m/z$  1293.5, respectively, that were 104 Da higher than unalkylation peptide. An increase of 70 Da and 104 Da for both peptides agreed with the incorporation of one EB or EB-diol equivalent, respectively.

Alkylated stable isotope [ $^{13}\text{C}^{15}\text{N}_5$ ] peptides was then used as an internal standard for correction the possible variation in yields during globin sample digestion. EB-alkylated and EB-diol -alkylated [ $^{13}\text{C}^{15}\text{N}_5$ ] peptides were reacted using the same approach as analyte standard peptides. They were 6 Da higher than analyte standards due to the mass unit of [ $^{13}\text{C}^{15}\text{N}_5$ ]. The representative ESI<sup>+</sup>-MS ion spectrum of EB-alkylated internal standard peptide in rat is shown in **Figure 2.3**.



More than 70% of the peptide was converted into the monoalkylation product and about 25% was converted to dialkylation. Under the conditions of this experiment, the alkylation reaction always yielded monoalkylated and dialkylated peptides, which were well separated by HPLC at about 13 min from the total run time 30 min. The representative ESI<sup>+</sup>-MS ion spectrum of purified EB-alkylated internal standard peptide for rat is shown in **Figure 2.4**. The ion spectrum demonstrated purified monoalkylated rat internal standard peptide at m/z 1279.74. This purification protocol provided a high yield of pure monoalkylated standard peptide.

#### *Characterization of standard peptides*

All alkylated peptides were characterized by LCQ-DECA MS/MS. collision-induced dissociation (CID) of the singly-charged ions of EB-alkylated peptide and EB-diol-alkylated peptide using ESI<sup>+</sup>-MS/MS in the positive ion mode, providing further information on the location of the epoxide-derived group within the peptides. For both epoxides, the CID spectra predominantly yielded modified b-type fragment ions (b\*) and y-type fragment ions (y\*). Abundant modified ion fragments can be used to identify the alkylation position on peptides. Furthermore, CID of alkylated peptide also provides confirmation of amino acid position on the peptide chain, as several alkylated ions are observed. The representative ion fragments of EB alkylated peptide (HB-Val) and EB-diol alkylated peptide (THB-Val) in rat are shown in **Figure 2.5a** and **Figure 2.5b**, respectively. The scan range of MS/MS spectra was 350 -1500. For HB-Val standard peptide in rat (**Figure 2.5a**), the fragment ions at m/z 677, 805, and 906 correspond to mass of aspartic acid (D), lysine (K), and threonine (T) on alkylated

peptide, respectively. The fragment ion at  $m/z$  603 corresponds to only valine alkylation (\*V). The lack of lysine alkylation (K\*) in the peptide chain at  $m/z$  671 indicates that EB alkylation is located at N-terminal valine.

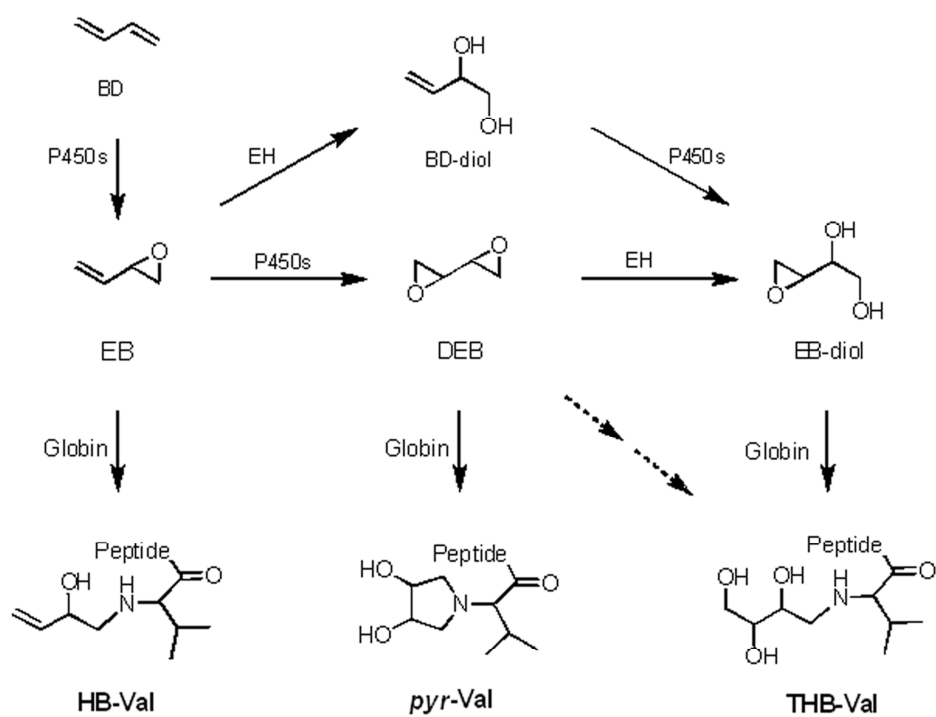
For the THB-Val standard peptide in rat (**Figure 2.5b**), consistent fragment ion chromatograms were obtained. The alkylation at N-terminal valine yielded an abundant  $y_5$  ion at  $m/z$  637. The  $b_6$  and  $b_7$  ions at  $m/z$  711 and  $m/z$  839 corresponded to the alkylated fragments THB-VLSADD and THB-VLSADDK, respectively. These findings indicated that EB-diol alkylation was located at N-terminal valine of peptide chain.

MS/MS chromatograms did not show any fragment ions correspond to the alkylation on both valine and lysine position at pH 6.5. This condition is well below the  $pK_a$  of lysine at 8.95 – 10.53. Therefore, the amino group is mainly protonated, which may explain the inhibition of alkylation on this amino acid. These findings agree with previous reports from Fred et al. (13).

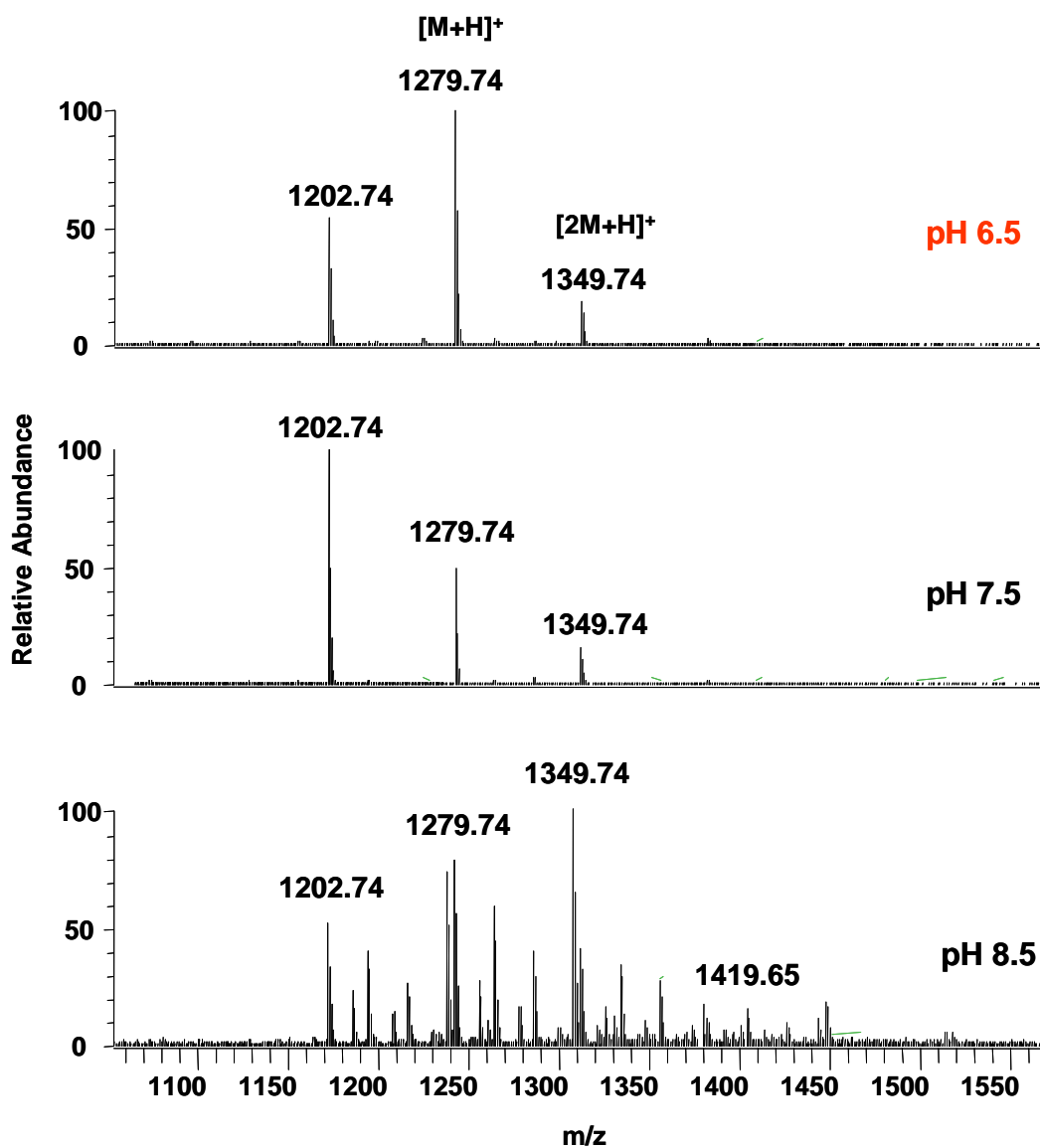
## 2.5 Conclusion

The availability of suitable analyte and internal standard peptides is critical for the development of our analytical approach to quantify hemoglobin adduct biomarkers in biological samples. In this study, we report a direct alkylation method for the synthesis of (1-11) peptide standards for rat and mouse. This method successfully provided proper syntheses, purification, and characterization of both the analyte and internal standards. Our future goal is to use these standard peptides in the immunoaffinity purification step for quantification of globin adducts in rodent samples exposed to BD.

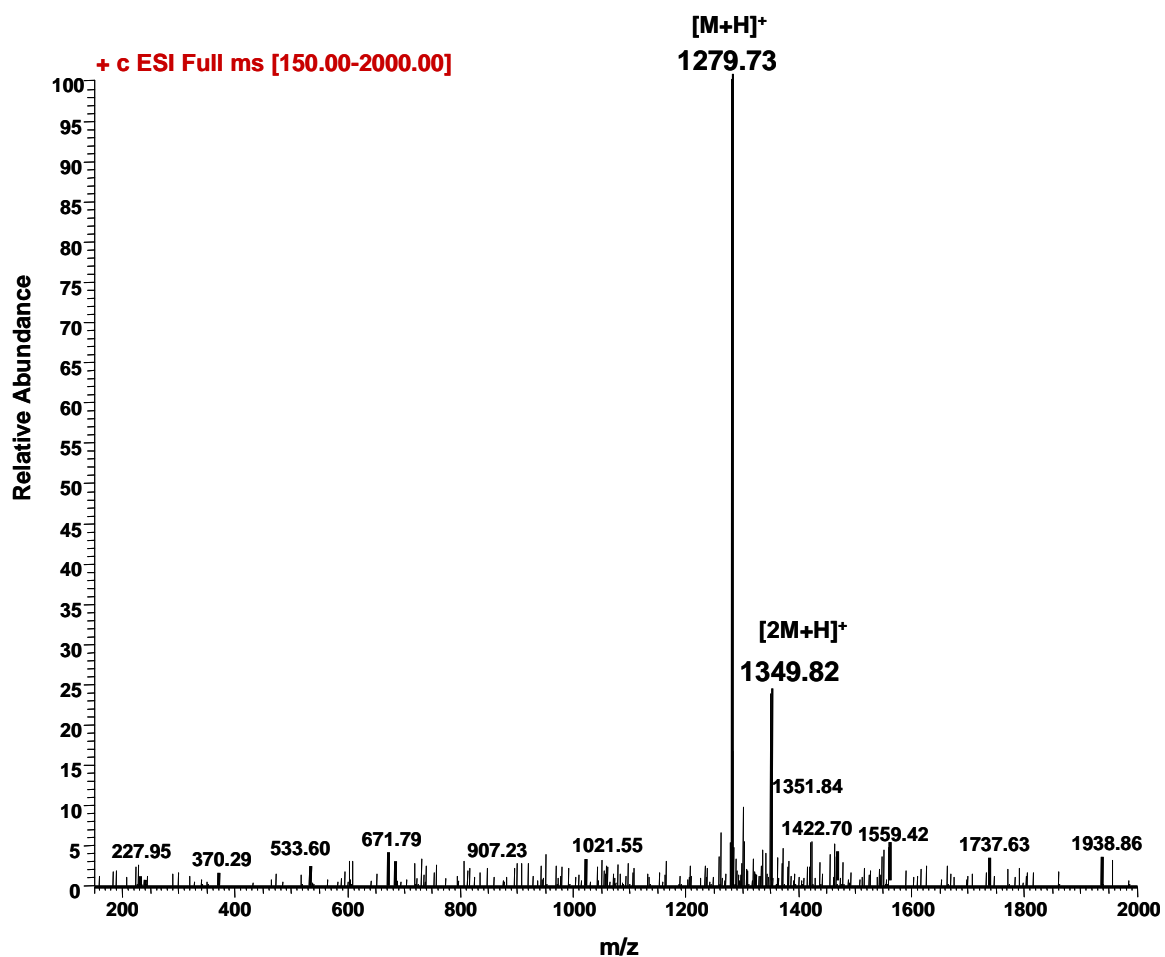
## FIGURES



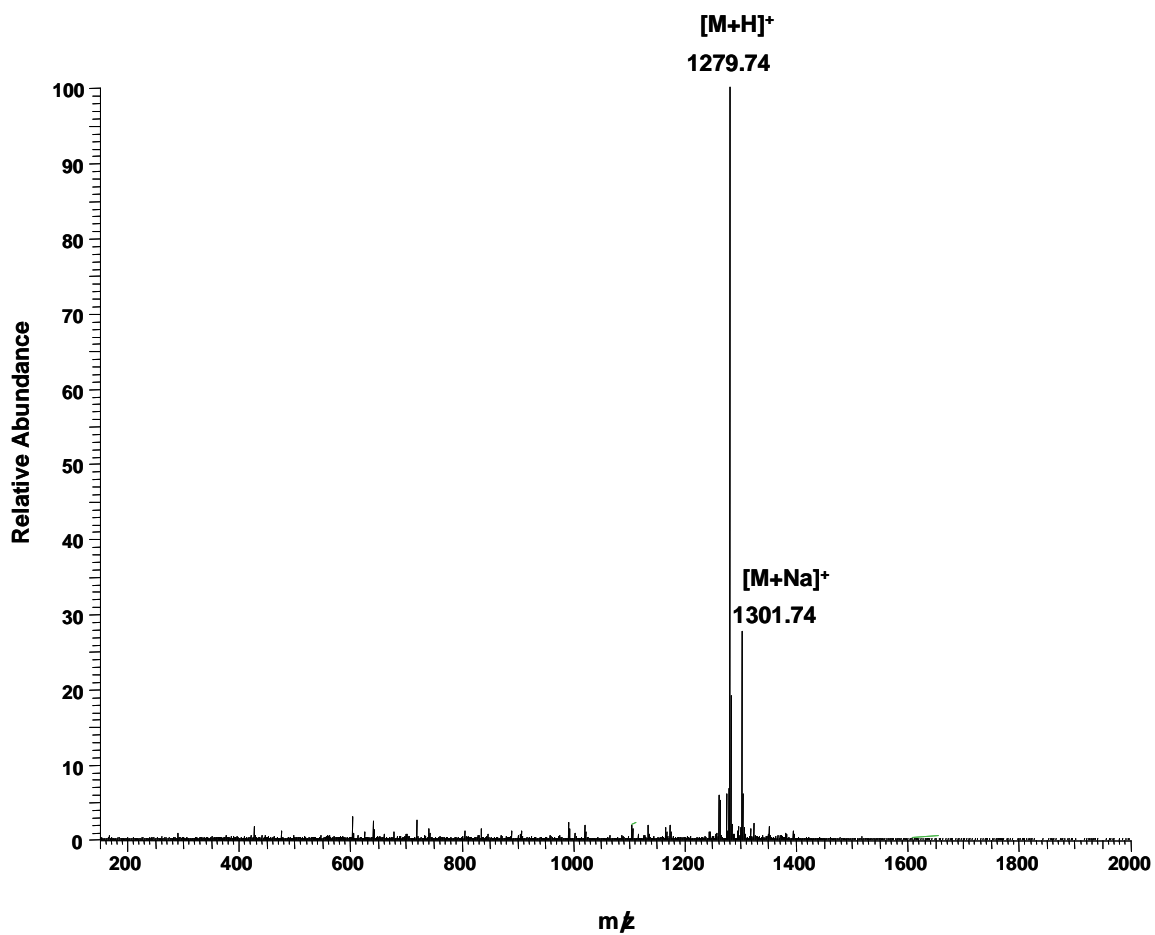
**Figure 2.1** BD metabolism and formation of *N*-terminal valine adducts



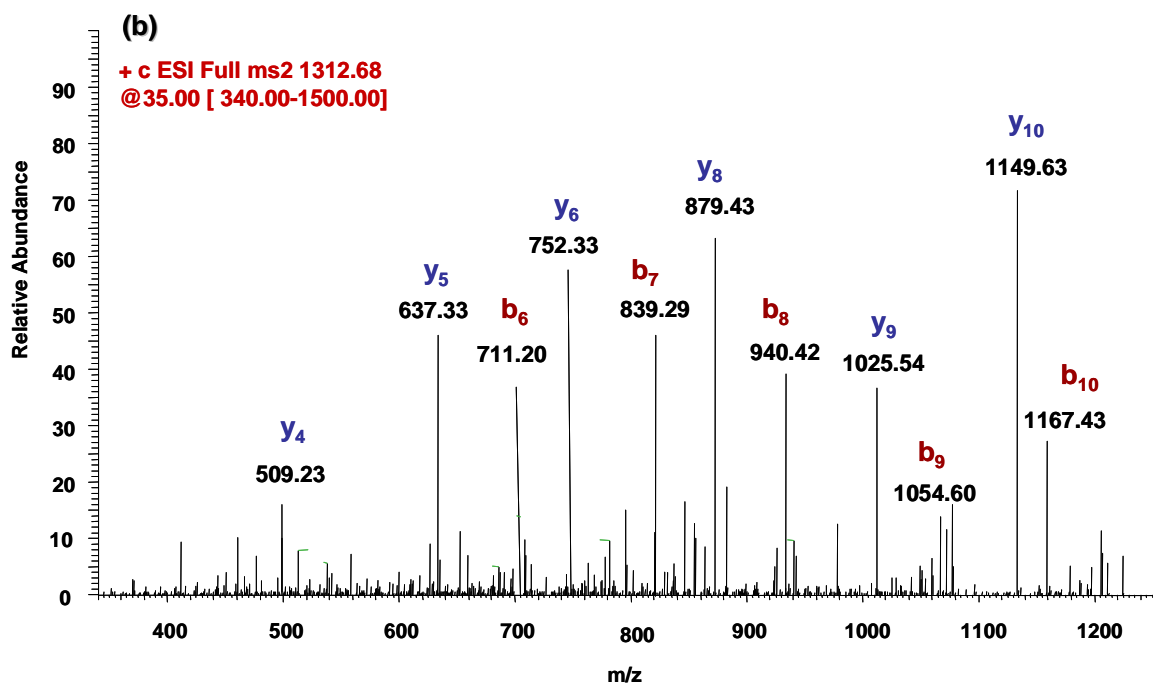
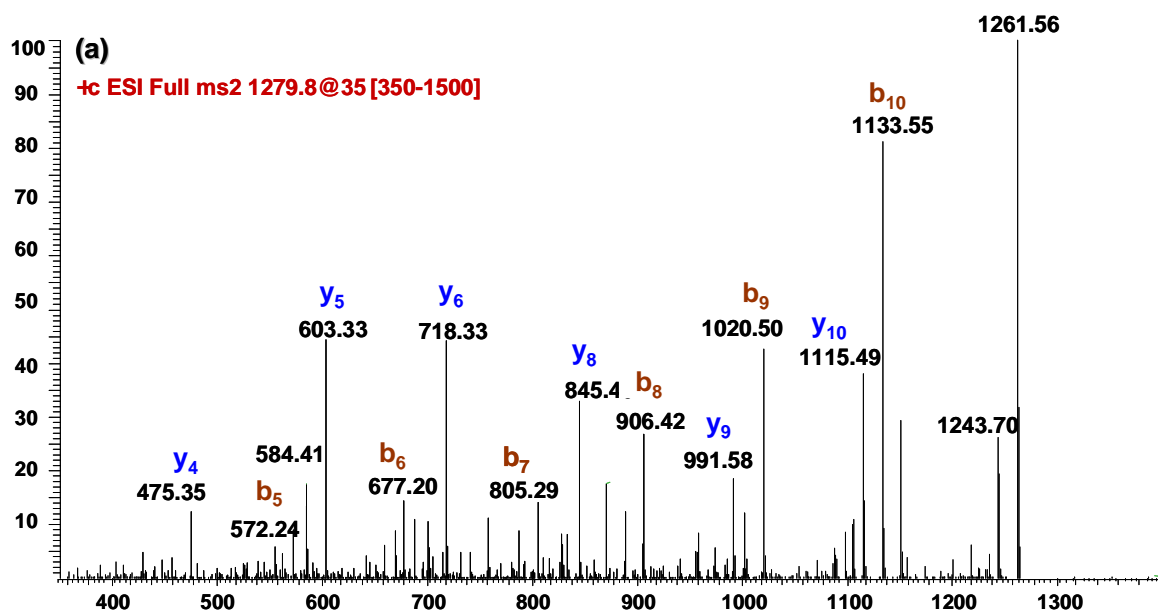
**Figure 2.2** Effect of pH on peptide alkylation. LC/ESI<sup>+</sup>-MS ion spectrum of EB-internal standard peptide show the spectrums corresponding to the unalkylated, monoalkylated  $[M+H]^+$ , and dialkylated  $[2M+H]^+$  peptide at 1202.74, 1279.74, and 1349.74, respectively



**Figure 2.3** Representative LC/ESI<sup>+</sup>-MS ion spectrum of EB-alkylated internal standard peptide in rat. The reaction was performed at molar ratio 1:100, pH 6.5 at 37°C for 3 days



**Figure 2.4** Representative LC/ESI<sup>+</sup>-MS ion spectrum of purified EB-alkylated internal standard peptide in rat



**Figure 2.5** MS/MS spectra of EB-alkylated peptide (a) and EB-diol-alkylated peptide (b)

## TABLE

**Table 2.1** Butadiene epoxide containing at N-terminal peptides (1-11).

**Mouse:** *EB or EB-diol*-\*Val-Leu-Ser-Gly-Glu-Asp-Lys/-Ser-Asn-Ile-Lys

**V - L - S - G - E - D - K - S - N - I - K**

**Rat:** *EB or EB-diol*-\*Val-Leu-Ser-Ala-Asp-Asp-Lys/-Thr-Asn-Ile-Lys

**V - L - S - A - D - D - K - T - N - I - K**

\* [<sup>13</sup>C<sup>15</sup>N<sub>5</sub>] in Internal standards



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## CHAPTER III

### ACCURATE QUANTITATION OF STANDARD PEPTIDES USED FOR QUANTITATIVE PROTEOMICS

*This paper has been published and therefore is reproduced with permission from [ Narisa K. Bordeerat, Nadia I. Georgieva, David G. Klapper, Leonard B. Collins, Tyra J. Cross, Christoph H. Borchers, James A. Swenberg, and Gunnar Boysen. Accurate quantitation of standard peptides used for quantitative proteomics. Proteomics, 2009, August; 9(15): 3939–3944.] Copyright [2009] Wiley-vch Verlag GmbH & Co. KGaA, Weinheim.*

#### **3.1 Abstract**

Mass spectrometry-based proteomics has become an indispensable tool in system biology generating a need for accurate and precise quantitation of peptide standards. The presented method utilizes ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to accurately quantify peptide standards at concentrations of 0.1-10  $\mu\text{M}$ . The ability for accurate quantitation of micro-molar concentrations has the advantages that quantitation can be performed routinely with high precision and the high sensitivity of the method minimizes the amounts required.

### 3.2 Introduction

Mass spectrometry-based proteomics has evolved into an indispensable tool for molecular and cellular biology that will significantly contribute to the emerging field of systems biology. Early work focused on qualitative identification of proteins and peptides. More recently, there has been increasing interest in quantitative proteomics (1). Progress in the area of mass-spectrometry-based proteomics has been made using stable isotopes. Stable isotopes can be introduced chemically by reacting peptide digests with an isotope tag (ICAT) (2), or the iTRAQ method (3) which currently allows the quantitative comparison of up to 8 samples in a single analysis. Whole proteomes can be labeled metabolically during cell culture (SILAC) by providing stable isotope precursors using either stable-isotope-labeled amino acids (1, 4–7) or stable-isotope-labeled salts (8). Although these isotopic labeling techniques are very powerful for biomarker discovery studies, their expense precludes their use in studies involving large numbers of samples, such as biomarker verification or validation. Moreover, SILAC can only be used in cell culture, and not for clinical studies.

Recently, there has been interest in label-free techniques, including GE Healthcare's DeCyder MS where two chromatograms are compared, the Empai scoring technique (9), ion accounting (10) and spectral counting (11, 12), where the relative abundances of peptides are used to give the relative abundances of proteins. Without stable-isotope-labeled standards, however, the effect of other components in the samples can cause suppression effects, as has been noted in our FIA-based metabolomics studies using high resolution FTICR (13), and even in other studies utilizing LC-MS/MS separation (14). This will probably mean that label-free methods will be limited to comparison of samples where the majority of

the components do not change – i.e., a set of samples where only the concentrations of a few components change, and the matrix is effectively the same for all of the samples in the set.

A successful absolute quantitation of proteins can be achieved using double exact matching isotope dilution mass spectrometry (IDMS) as shown in the excellent paper of Burkitt et.al (15). Alternatively, this can be applied using custom-synthesized stable-isotope labeled standard peptides (16–19). These labeled standards can include peptides which contain post-translational modifications (for example, phosphorylation and chemical alkylation) (20). We have studied globin alkylation by chemical carcinogens for several years, and have established assays for specific quantitation of alkylated peptides which can be used as biomarkers for exposure assessment (21, 22). These assays are based on proteolysis with trypsin, immunoaffinity enrichment, and LC-MS/MS. Recently, we used a similar approach to develop an immunoaffinity MALDI-MS/MS (iMALDI) assay for targeted protein quantitation (23).

During these studies, however, it became obvious that accurate quantitation of the standard peptides was challenging. The reason for this is that the amounts of standard peptides available are usually so small that accurate weights cannot be obtained. Peptides are hygroscopic and easily absorb moisture from the air, and the presence of water and salts leads to overestimation of peptide amounts. We and other researchers have been forced to use a combination of weight, HPLC-UV, and LC-MS/MS for quantitation of peptide standards (24). However, this method is labor-intensive and is therefore not suitable for routine quantitation of peptides for multiplex proteomic applications, which may require thousands of standard peptides.

To overcome these limitations, we have developed a new approach for the peptide quantitation based on the analysis of their component amino acids after acid hydrolysis, using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The high specificity of MS/MS combined with fast chromatography makes this a suitable approach for high-throughput analyses.

### **3.3 Materials and Methods**

Single standard amino acids were purchased from Sigma-Aldrich (St. Louis, MO), concentrated hydrochloric acid (12 N) from Fisher Scientific (Pittsburgh, PA), and all other reagents and solvents were ACS grade or higher. Amino acid standards for instrument calibration and the internal standard amino acid, norleucine, were prepared from single amino acid standards which are stable in 100 mM HCl solution. Stock solutions at 10 mM were made in water with 0.1 mM HCl. Norleucine was added to each calibration standard and samples at a final concentration of 2.5 mM.

The peptides were synthesized at a 5  $\mu$ mol scale using Fmoc chemistry on a Prelude peptide synthesizer (Protein Technology, Tucson, AZ). The C-terminal amino acids were conjugated to TentaGel R resin (Rapp Polymere, Tübingen, Germany) and subsequent residues, at a concentration of 100 mM, were double coupled using 20% piperidine as the deprotector and 1H-benzotriazolium 1-[bis(dimethylamino)methylene]-5chloro-,hexafluorophosphate (1),3-oxide (HCTU) as the activator. Cleavage and deprotection was performed with 95:2.5:2.5 TFA:water:triisopropylsilane. The peptides were resolubilized in 0.1% TFA and purified by reverse-phase HPLC Ultimate 3000 (Dionex, Sunnyvale, CA) monitoring peptide elution at 230 nm using a Vydac C<sub>18</sub> column (10 × 250 mm, 10

µm resin) with a linear gradient of 0.1% TFA in water (v/v) and 0.85% TFA in 50% acetonitrile (v/v) at a flow rate of 4 mL/minute over 60 min. The fractions of interest were spotted onto stainless steel MALDI plates and measured by MALDI-TOF (Applied Biosystems/MDS SCIEX, Foster City, CA) mass spectrometry. Fractions containing greater than 80% of the target peptide were pooled and lyophilized.

For HCl hydrolysis, peptide solutions were mixed with 10 µL of 20 µM norleucine, as internal standard, in a small culture vial and lyophilized in a vacuum centrifuge. The vials were placed inside a 10 mL glass container, 500 µL of 6N HCl were added to the bottom of the container, and the container was closed with an air tight PTFE lined cap. The vials were placed in an oven at 150°C for 4 h, then cooled to room temperature for 30 min in a water bath to condense the acid vapor on the inside of the sample vials. Prior to analysis the hydrolysates were dried in a vacuum centrifuge for 15 min to remove traces of acid.

The quantitative analysis of hydrolyzed peptide was performed with an UPLC (Waters, Milford, MA) coupled to a TSQ-Quantum ultra triple quadrupole mass analyzer (ThermoFinnigan, San Jose, CA). A 2.1 mm × 150 mm UPLC™ BEH SHIELD RP18 1.7 µm column was operated with a linear gradient from 20% methanol-0.1% formic acid to 80% methanol-0.1% formic acid for 7 min at a flow rate of 200 µL/min. The individual amino acids were monitored in selected reaction monitoring mode (SRM) (**Figure 3.1 and Supplemental Table S1**). Amounts of amino acids were calculated from the area ratio of the corresponding ion transitions of amino acids to ion transition of the internal standard, norleucine. Calibration curves of the area of amino acid over area of norleucine were

constructed daily, to guarantee linearity. Area ratios of analyte over internal standard were multiplied by the amount internal standard added and adjusted by a corresponding response factor to correct to difference in MS response. Calibration solution repeatedly showed linear response from 0.1 pmol up to 200 pmol/injection (high amount injected) representing 4 orders of magnitude.

### 3.4 Results and discussion

We have shown that all 20 common amino acids can be detected by UPLC-MS/MS analysis without any derivatization step (**Figure 3.1**). It is also shown that while accurate quantitation of all amino acids is desirable, using 1 to 3, 5 or 6 amino acids for quantitation produced CVs of <17% and, the mean did not significantly change by increasing the number of amino acids used for quantitation. Thus we concluded that three amino acids were sufficient for accurate quantitation of peptide solutions.

Method validation was assessed by analyzing the NIST standard peptide reference material 8327 three times on the same day and on three different days to obtain intraday and interday results (**Supplemental Table S2**). Therefore, hydrolyzed peptide solutions were initially quantified by the amounts of alanine, serine, proline, threonine, valine, leucine or isoleucine, if present. The mean relative standard deviations (RSD) for intraday and interday assay reproducibility (n=3) were 8% and 5%, respectively. Accuracies of the validation samples ranged from 95% to 110%.

To illustrate that the complete hydrolysis of the standard peptide had been achieved, the release of the free amino acids from the peptide was monitored over time. The ratio of amino acid that was released from the peptide to the



internal standard amino acid (norleucine) that was added prior to hydrolysis was measured at several time points (1 h, 2 h, 4 h, 8 h, 16 h, and 24 h) after the start of the acid hydrolysis. Duplicate samples were analyzed for each time point and the release of six amino acids was monitored. Results for alanine, serine, proline, valine, threonine, and leucine are shown in supplement Figure S1. The result of this time course experiment indicated that hydrolysis need to be performed at 4 h since this time gave high yield of the six amino acids studied. Longer hydrolysis time (8-24 h) showed no difference in the yield.

UPLC-MS/MS was chosen for the analysis of peptide hydrolysates because of its high speed and resolution and the advantage of automation of sample preparation and processing. Initially, multiple stable isotope labeled amino acids were used as internal standards, but the addition of 20 extra ion transitions significantly reduces the number of points over the peaks. Comparison of results between multiple stable isotope label amino acid and single amino acid (Norleucine) as the internal standard was performed. The result of peptide quantitation using 4 amino acids, their isotope-labeled counterparts and norleucine are shown in **supplement Figures S2 & S3 and Table S3**. No difference was found in the value measured for the concentration of peptide. Therefore, we decided to use a single internal standard for all amino acids and to correct for different MS response using a response factor obtained from calibration solution made from single amino acid standards since this has been shown to be sufficient using the NIST standard peptide reference material (**Table S3**).

The study was extended to the analysis of standard peptides used for quantitative proteomic applications (**Figure 3.2, and Supplemental Table S4**).

These peptides were synthesized for projects that required absolute quantitation of proteins in biological samples. Usually, the peptides are quantified by micro-weight and a minimum amount of 100  $\mu\text{g}$  is required for these measurements. Quantitation by UPLC-MS/MS produces similar results to those previously determined by weight, but required less than 10 ng for accurate analysis. This represents a 10,000-fold increase in sensitivity without sacrifice of accuracy and specificity. Furthermore, ~30% of the peptide concentrations were not accurate based on weight quantitation. Thus, there is an unacceptable and unavoidable uncertainty using weight for standardization.

The present method is about 5-fold more sensitive than a previously reported LC-MS/MS method for underivatized amino acids (13). Alternatively, Amino acid analysis can be performed accurately at the femtomole level by a method employing fluorescence detection after derivatization with o-phthalaldehyde (OPA). However, inter-laboratories comparisons have shown high variability in the results due to inconsistency in derivatization conditions (14).

These discrepancies between the methods and laboratories are probably caused by impurities (such as TFA salts) of the peptides and absorption of moisture during peptide handling. Precautions can be implemented to better assure purity of the standard peptides; however, as the number of peptide standards increases, it will be difficult to maintain high purity and accuracy due to the chemical nature of peptides. Therefore, the ability of accurate quantitation of peptide solutions at micromolar concentrations, instead of dry material has the advantage of avoiding inaccuracy due to weighing, chemical derivatization and errors during preparation of dilutions. In addition, the increased sensitivity reduces the amounts needed for analysis and reduces costs, especially if chemically unique peptides are needed.

One of the problems with the MS/MS methodology is the fact that isobaric amino acids, such as leucine, isoleucine, and norleucine often fragment very similarly. This limitation was overcome by utilization of UPLC which achieves baseline separation for these amino acids (**Figure 3.3**). In addition asparagine and aspartic acid were separated to avoid cross peaks from natural  $^{13}\text{C}$  isotopes (**Figure 3.1**). Unfortunately, the reported fast separation did not separate glutamine and glutamic acid and, therefore, glutamic acid cannot be used for quantitation if the peptides also contain glutamine. Further, the method was optimized for speed and analysis of known peptides. Therefore, the current approach is unsuitable for analysis of amino acid content in unknown proteins or biological specimens. For such traditional amino acid analyses a modified UPLC gradient can be applied to separate all amino acids by time (Data not shown).

Peptide concentrations of 0.1-10  $\mu\text{M}$  could be analyzed with this method using as little as 10  $\mu\text{l}$  of sample. This new method is more sensitive than other LC-MS/MS (13) and common methods like ion chromatography (IC) using ninhydrin post column derivatization (25), and gas chromatography/mass spectrometry (GC/MS) (26). Moreover, the specificity of single reaction monitoring mode allowed specific analysis of twenty amino acids in less than 10 min per sample, making it suitable for high throughput analysis.

### **3.5 Conclusion**

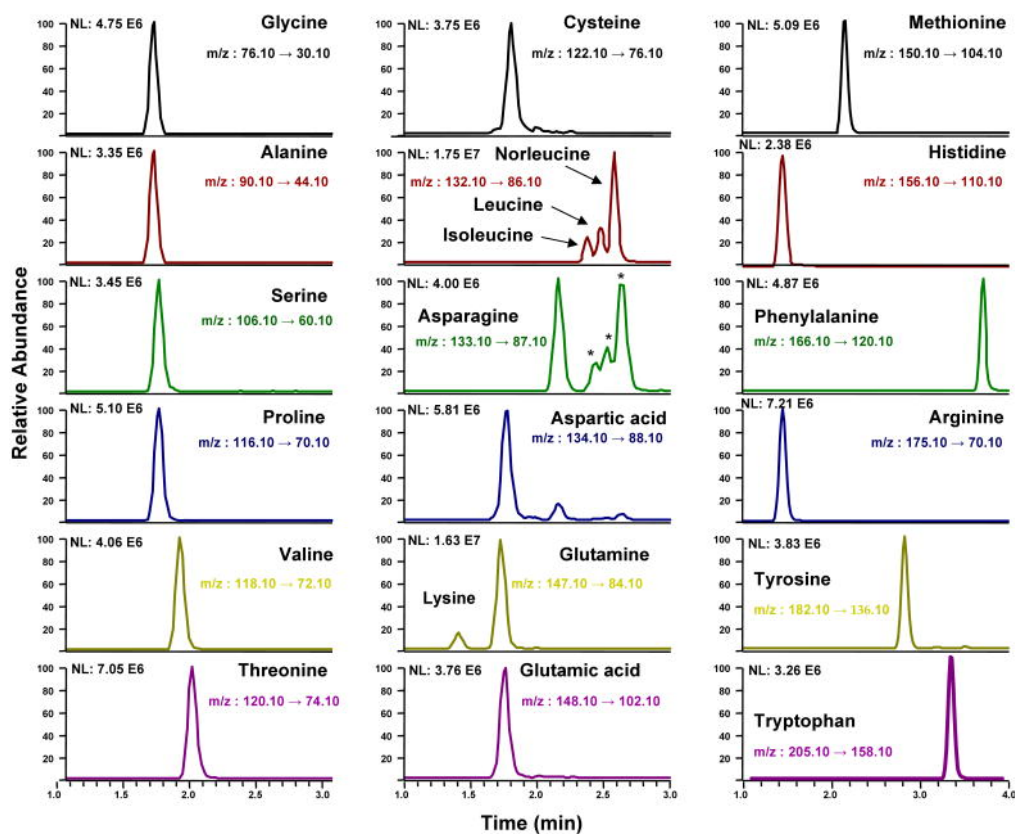
In conclusion, amino acid quantitation of peptide solutions by UPLC-MS/MS is a viable alternative to traditional amino acid analysis methods or quantitation by weight. The UPLC-MS/MS method utilizes a few simple steps that can be completed within 6 h, which represents a great advantage over previous

GC/MS and IC/ninhydrin methods requiring derivatization and numerous steps for sample preparation. The ability to accurately quantitate  $\mu\text{M}$  solutions of peptides significantly reduces the amounts needed and allows for high throughput quantitative analysis of peptide standards. This method is expected to become a standard repertoire in proteomic laboratories.

### **3.6 Acknowledgments**

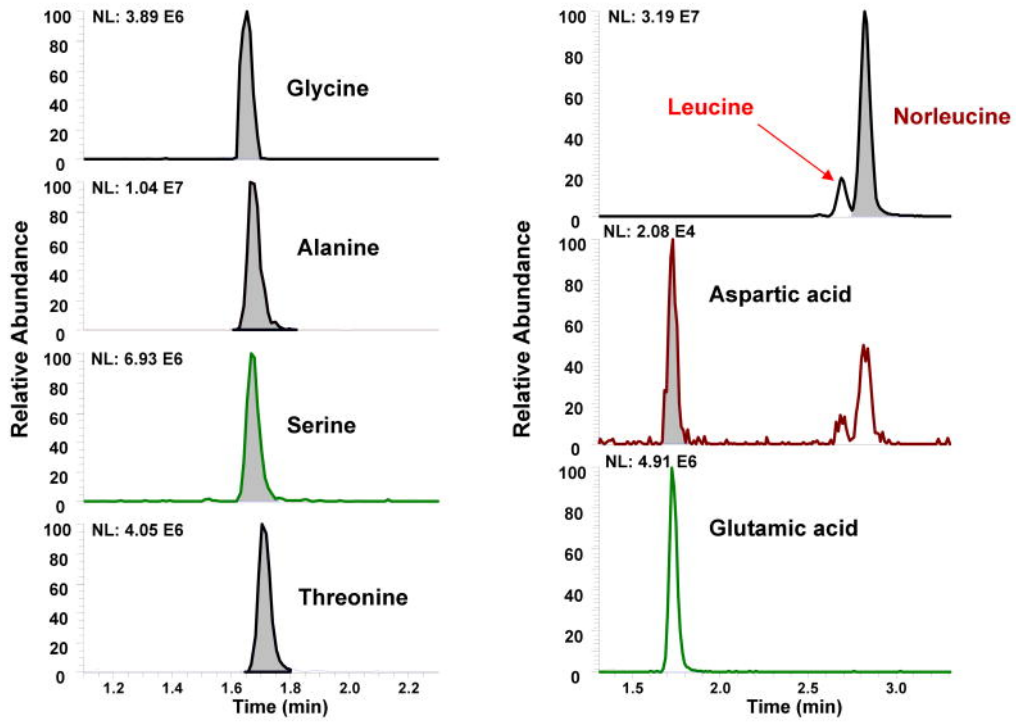
This work was supported in part by NIH grant P30 ES10126, P42-ES05948, and Genome Canada.

## FIGURES

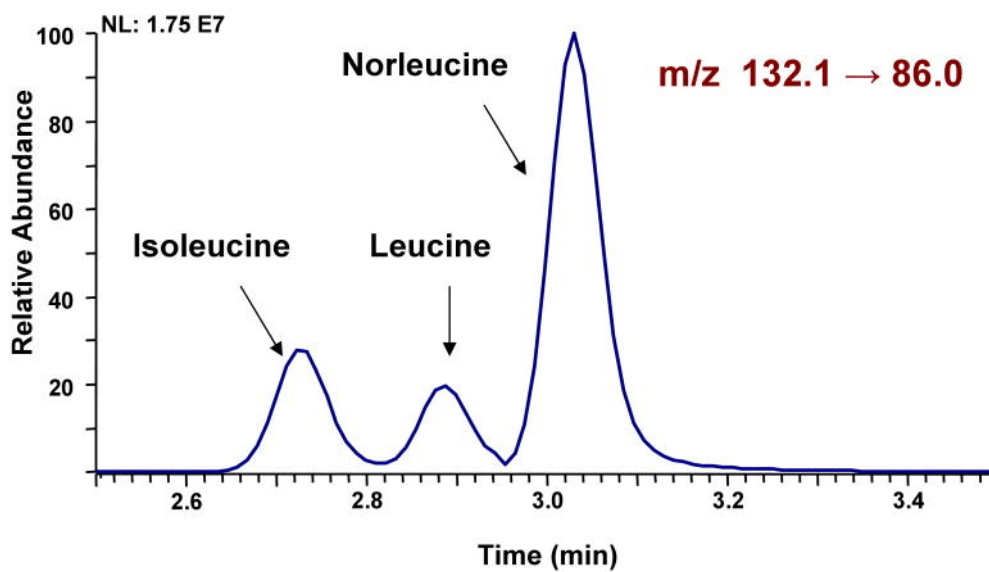


**Figure 3.1** Extracted ion chromatograms for common amino acid standards and norleucine used as internal standard. \* Indicates  $^{13}\text{C}$ -isotopes of isoleucine, leucine and norleucine

### ETAASLLQAGYK



**Figure 3.2** Representative extracted ion chromatograms of peptide hydrolysate determined by UPLC-MS/MS



**Figure 3.3** Separation of isobaric amino acids leucine, isoleucine, and norleucine by UPLC-MS/MS

## SUPPLEMENTAL TABLES AND FIGURES

**Table S1:** List of ion transitions monitored for amino acid quantitation

<u>Amino Acid</u>	<u>CID energy</u> <u>[eV]</u>	<u>MS/MS</u> <u>transitions</u>	<u>Retention time</u> <u>[min]</u>
<u>Glycine</u>	<u>10</u>	<u>76 → 30</u>	<u>1.67</u>
<u>Alanine</u>	<u>10</u>	<u>90 → 44</u>	<u>1.67</u>
<u>Serine</u>	<u>10</u>	<u>106 → 60</u>	<u>1.70</u>
<u>Proline</u>	<u>10</u>	<u>116 → 70</u>	<u>1.70</u>
<u>Valine</u>	<u>10</u>	<u>118 → 72</u>	<u>1.86</u>
<u>Threonine</u>	<u>10</u>	<u>120 → 74</u>	<u>1.95</u>
<u>Cysteine</u>	<u>10</u>	<u>122 → 76</u>	<u>1.81</u>
<u>Isoleucine</u>	<u>10</u>	<u>132 → 86</u>	<u>2.44</u>
<u>Leucine</u>	<u>10</u>	<u>132 → 86</u>	<u>2.53</u>
<u>Norleucine</u>	<u>10</u>	<u>132 → 86</u>	<u>2.65</u>
<u>Asparagine</u>	<u>10</u>	<u>133 → 87</u>	<u>1.97</u>
<u>Aspartic acid</u>	<u>10</u>	<u>134 → 88</u>	<u>1.78</u>
<u>Glutamine</u>	<u>15</u>	<u>147 → 84</u>	<u>1.73</u>
<u>Lysine</u>	<u>15</u>	<u>147 → 84</u>	<u>1.42</u>
<u>Glutamic acid</u>	<u>10</u>	<u>148 → 102</u>	<u>1.77</u>
<u>Methionine</u>	<u>10</u>	<u>150 → 104</u>	<u>2.17</u>
<u>Histidine</u>	<u>10</u>	<u>156 → 110</u>	<u>1.46</u>
<u>Phenylalanine</u>	<u>10</u>	<u>166 → 120</u>	<u>3.71</u>
<u>Arginine</u>	<u>20</u>	<u>175 → 70</u>	<u>1.46</u>
<u>Tyrosine</u>	<u>10</u>	<u>182 → 136</u>	<u>2.83</u>
<u>Tryptophan</u>	<u>10</u>	<u>204 → 158</u>	<u>3.14</u>



**Table S2:** Validation results for amino acid quantitation

		Sample [ $\mu\text{M}$ ]
Day 1	n	3
	mean ( $\mu\text{M}$ )	9.60
	RSD (%)	5
	accuracy (%)	96.0
Day 2	n	3
	mean ( $\mu\text{M}$ )	11.06
	RSD (%)	11
	accuracy (%)	110.6
Day 3	n	3
	mean ( $\mu\text{M}$ )	9.51
	RSD (%)	8
	accuracy (%)	95.1
<b>interday</b>	n	<b>3</b>
	mean ( $\mu\text{M}$ )	<b>10.06</b>
	RSD (%)	<b>8</b>
	accuracy (%)	<b>100.6</b>
<b>intraday</b>	n	<b>3</b>
	mean ( $\mu\text{M}$ )	<b>9.60</b>
	RSD (%)	<b>5</b>
	accuracy (%)	<b>96.0</b>

**Table S3:** Quantitative analysis of NIST peptide solutions (10  $\mu\text{M}$ ) by amino acid quantitation compared between isotopically labeled amino acid and single amino acid; Norleucine as the internal standard

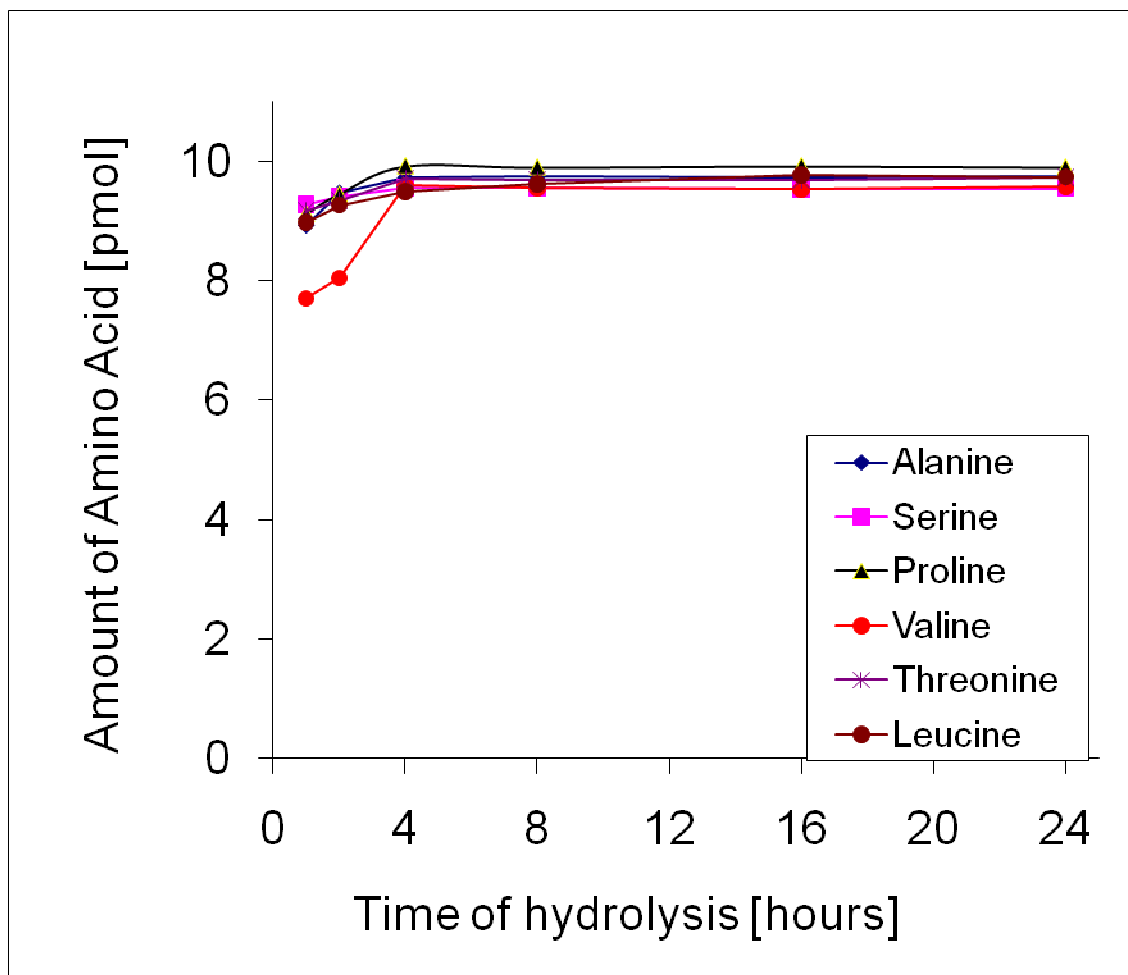
Peptide	Measured Amount ( $\mu\text{M}$ )	
	Amino acid	Norleucine as IST
<b>Peptide A</b>		
Alanine	9.2 $\pm$ 1.1	9.6 $\pm$ 1.0
Leucine	10.0 $\pm$ 0.7	9.5 $\pm$ 0.9
Arginine	9.3 $\pm$ 0.9	10.1 $\pm$ 0.8
<b>Mean <math>\pm</math> SD</b>	<b>9.5 <math>\pm</math> 0.9</b>	<b>9.7 <math>\pm</math> 1.0</b>
<b>Peptide B</b>		
Alanine	10.6 $\pm$ 1.5	9.9 $\pm$ 1.1
Valine	9.8 $\pm$ 0.7	10.0 $\pm$ 0.8
Leucine	10.5 $\pm$ 1.4	10.1 $\pm$ 0.9
Arginine	9.9 $\pm$ 0.8	9.6 $\pm$ 1.0
<b>Mean <math>\pm</math> SD</b>	<b>10.2 <math>\pm</math> 1.1</b>	<b>9.9 <math>\pm</math> 1.0</b>
<b>Peptide C</b>		
Alanine	9.5 $\pm$ 0.9	10.3 $\pm$ 1.5
Valine	10.2 $\pm$ 1.3	9.6 $\pm$ 0.6
Leucine	10.6 $\pm$ 0.6	9.8 $\pm$ 0.8
Arginine	8.9 $\pm$ 1.1	9.8 $\pm$ 1.4
<b>Mean <math>\pm</math> SD</b>	<b>9.8 <math>\pm</math> 1.0</b>	<b>9.9 <math>\pm</math> 1.1</b>

<sup>a</sup> The NIST peptide sequences were as flowed: Peptide A sequence: D-**A**-E-P-D-I-**L**-E-**L**-**A**-T-G-Y-**R** Peptide B sequence: K-**A**-Q-Y-**A**-**R**-S-**V**-**L**-L-E-K-D-**A**-E-P-D-I-**L**-E-**L**-**A**-T-G-Y-**R** and Peptide C sequence: **R**-Q-**A**-K-**V**-**L**-L-Y-S-G-**R**. Amino acids used for quantitation are indicated in bold.

**Table S4:** Quantitative analysis of peptide solutions (10  $\mu$ M) by amino acid

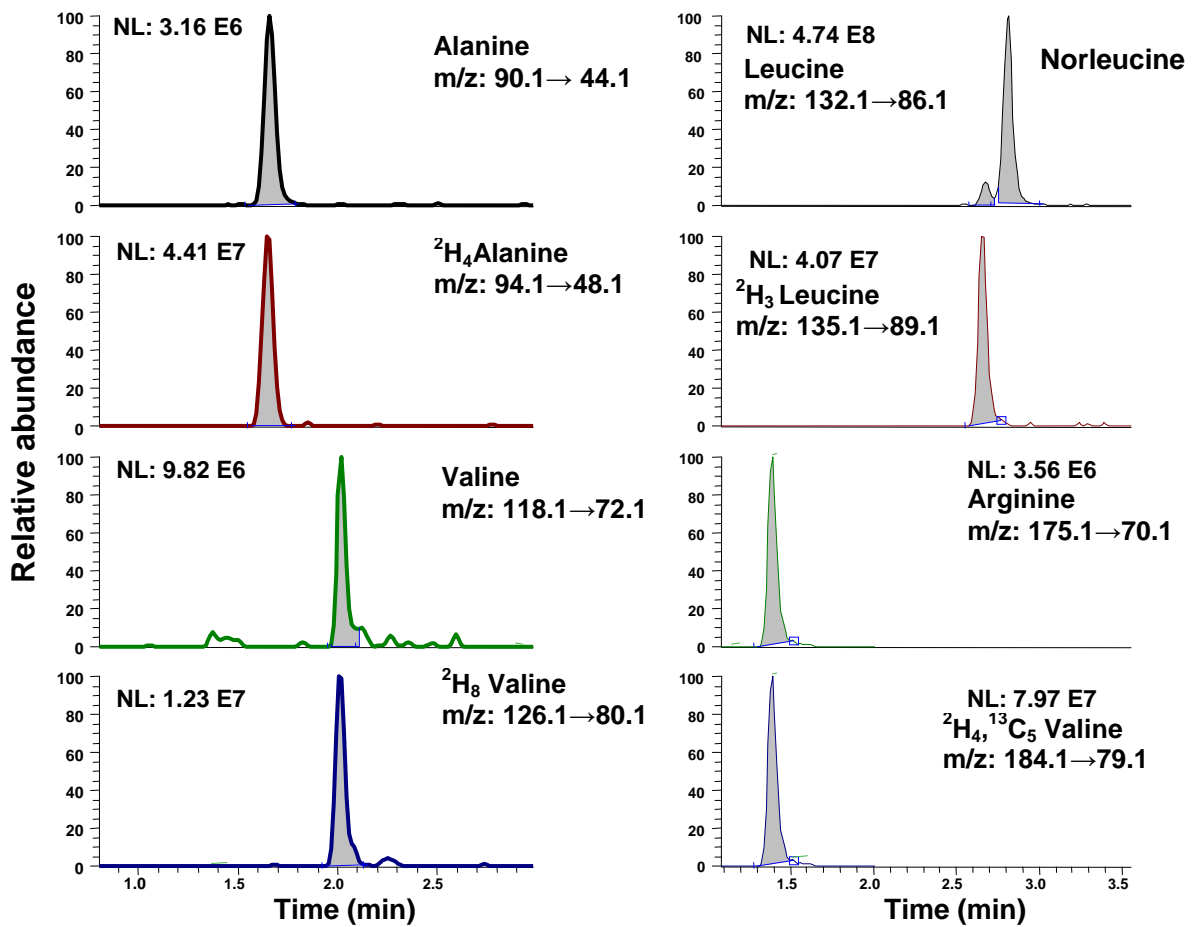
quantitation

Sample	AA used for quantitation	Determined Concentrations ( $\mu$ M)	Sequence
Sample 1	A,V,L	11.9 $\pm$ 1.7	AEEFLNTVVK
Sample 2	A,V,T,P,D	15.2 $\pm$ 1.8	AADEFPQTVVVYYK
Sample 3	A,T,L,S	9.7 $\pm$ 1.4	AEEHLLSSTTK
Sample 4	A,L,P,D	12.9 $\pm$ 1.7	ADEGKLNPSSTVK
Sample 5	A,L,S,P	12.0 $\pm$ 1.6	AAEELPQSK
Sample 6	V,S	7.7 $\pm$ 1.0	GGGSVVWYR
Sample 7	A,S,P,D	10.4 $\pm$ 1.4	AAADDEFPGSTWK
Sample 8	A,V,S,P,D	9.3 $\pm$ 1.2	AADFFNPPSVR
Sample 9	V,T,D,I	8.8 $\pm$ 0.9	DDGHIIQTTVK
Sample 10	I,S,T	14.7 $\pm$ 2.4	EFGGINSSTTK
Sample 11	A,V,S,L,I	10.4 $\pm$ 1.4	AADDGGIILLLPSTVVYK
Sample 12	A,V,S,P,L	8.2 $\pm$ 0.9	ADDLPSSTVK
Sample 13	P,L,I	10.0 $\pm$ 1.4	EEGILLPYK
Sample 14	V,L	14.5 $\pm$ 2.2	EEGKLQTVVK
Sample 15	V,P,D	9.5 $\pm$ 0.7	DDDEFGLPPVYR
Sample 16	A,V,S,L,I	4.0 $\pm$ 1.4	AAAEGGILNSVR
Sample 17	V,S,P,L	10.2 $\pm$ 1.1	EFHLLPSTVK
Sample 18	T,D,L	6.5 $\pm$ 0.8	DEFFLLPQTK
Sample 19	A,V,S,L	16.5 $\pm$ 1.8	ADDDFHLSVYK
Sample 20	L,S	8.9 $\pm$ 1.0	SVNQSLLELHK
Sample 21	D,L,S	6.4 $\pm$ 1.1	DSAFGLLR
Sample 22	G,A,V,T	11.2 $\pm$ 1.5	QGFGNVATNTDGK
Sample 23	A,S,P,T,L,D	10.4 $\pm$ 1.7	SLAPYAQDTQEK
Sample 24	S,P,V,I,D	9.6 $\pm$ 1.5	SPDVINGSPISQK
Sample 25	A,P,I,L,D	9.6 $\pm$ 1.4	EELLPAQDIK
Sample 26	G,A,T,L	11.0 $\pm$ 1.6	TGAQELLR
Sample 27	A,S,T,D	10.8 $\pm$ 1.3	EYTDASFTNR
Sample 28	A,P,V,T,I,D	9.0 $\pm$ 1.4	EIPAWVPFDPAAQITK
Sample 29	G,A,S,T,L	10.4 $\pm$ 1.3	ETAASLLQAGYK
Sample 30	G,A,L	9.8 $\pm$ 0.7	YWGVASFLQK
Sample 31	V,T,L,D	9.3 $\pm$ 1.5	VGDTLNLNLR
Sample 32	V,T,I,L	9.3 $\pm$ 1.1	ITQVLHFTK
Sample 33	P,V,T,L,D	9.4 $\pm$ 1.4	FPEVDVLTK
Sample 34	G,A,P,T,L	10.2 $\pm$ 1.4	LGNQEPGGQTALK
Sample 35	G,S,T	9.1 $\pm$ 0.7	TVGSDTFYSFK
Sample 36	A,V,L,D	9.2 $\pm$ 0.8	ALQDQLVLVAAK
Sample 37	A,I,L	9.5 $\pm$ 1.4	AEIEYLEK
Sample 38	A,V,T,L	8.8 $\pm$ 1.0	LVNEVTEFAK
Sample 39	A,S,T,L	9.8 $\pm$ 1.5	ATEHLSTLSEK
Sample 40	A,S,P,D	10.4 $\pm$ 1.4	AADDTWEPFASGK
Sample 41	A,S,V	10.6 $\pm$ 1.4	NFPSPVDAAFR
<b>Mean <math>\pm</math> SD</b>		<b>10.1 <math>\pm</math> 1.3</b>	

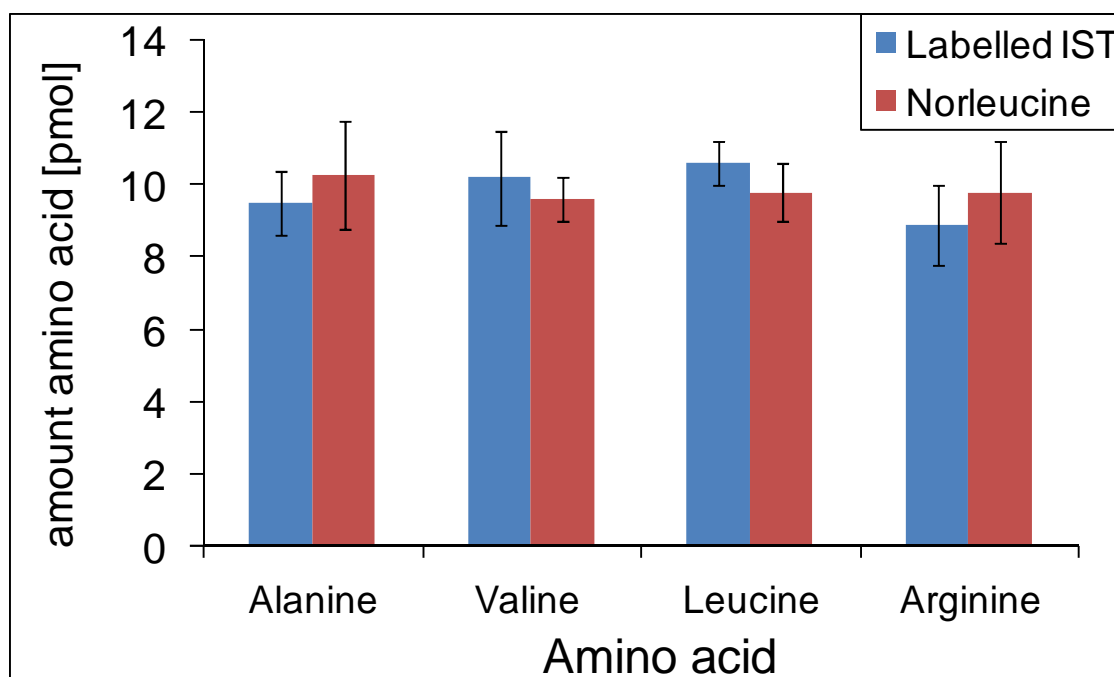


**Figure S1:** concentration of free amino acids as a function of time of hydrolysis of NIST standard peptide B. Hydrolysis was performed and measured in duplicate

# R-Q-A-K-V-L-L-Y-S-G-R



**Figure S2:** Representative extracted ion chromatograms for standard peptide and internal standard (isotopically-labeled amino acid and single amino acid; Norleucine)



**Figure S3:** Comparison of quantitation using stable isotope-labeled amino acids or single amino acid (Norleucine) as the internal standard. NIST peptide B solutions (10  $\mu$ M) were quantified as described using stable isotope-labeled amino acids (blue) or norleucine (red) as the internal standard

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## CHAPTER IV

### AN IMPROVED METHOD FOR QUANTITATIVE ANALYSIS OF N-TERMINAL VALINE ADDUCTS IN ANIMALS EXPOSED TO 1,3-BUTADIENE

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*[Manuscript]*

#### 4.1 Abstract

1,3-Butadiene (BD) is a well-characterized carcinogen that is both an occupational and environmental hazard. It is an important industrial chemical widely used in the production of rubber and plastic and is also present in automobile exhaust and cigarette smoke. BD is metabolized mainly by P450 2E1 to three epoxides, 1,2-epoxybutene (EB), 1,2:3,4-diepoxybutane (DEB) and 3,4-epoxy-1,2-butanediol (EB-diol). They have the ability to react with hemoglobin in blood. The globin adducts formed by EB, DEB, and EB-diol are *N*-(2-hydroxy-3-buten-1-yl)-valine (HB-Val), *N,N*-(2,3-dihydroxy-1,4-butadiyl)-valine (*pyr*-Val) and 1,2,3-trihydroxybutyl-valine (THB-Val), respectively.

The goal of this research was to develop an improved immunoaffinity UPLC-positive mode electrospray ionization-tandem mass spectrometry (UPLC-ESI<sup>+</sup> - MS/MS) method for the quantitative analysis of BD N-terminal valine adducts in globin extracted from BD-exposed rats. In our approach, N-terminal valine adducts are cleaved from globin proteins by trypsin hydrolysis. Following immunoaffinity enrichment, samples are subjected to nano-UPLC-MS/MS analysis with <sup>13</sup>C<sup>15</sup>N<sub>5</sub>-labeled internal standard peptides. The detection limit of our improved method is 5-10 fmol in globin (50 mg). This method allowed analysis of globin adducts in rats exposed to BD as low as 0.5 ppm via inhalation and demonstrated significant differences in the amounts and exposure response in BD epoxide adduct formation. The formation of BD epoxide adducts increased with exposure and was most efficient with regard to formation per ppm BD at low exposures (0.5-1.5 ppm). The amounts of *pyr*-Val and THB-Val formation saturated at exposures greater than 200 ppm for 10 days. In contrast, HB-Val concentrations linearly increased with exposure. Quantitative methods presented here may be used for studies of biological samples in animals as well as applied to analysis of samples collected from humans exposed to BD.

## 4.2 Introduction

1,3-Butadiene (BD) is an important industrial chemical used primarily in the production of synthetic rubbers and plastics. The general population is exposed to BD from cigarette smoke, burning of wood and automotive exhaust (1–3). Epidemiologic studies have linked occupational BD exposure to increased mortality

from lymphatic and hematopoietic cancers (4). In long-term (6 h/day, 5 days/week, 2 years) carcinogenicity studies, inhaled BD was weakly tumorigenic in rats exposed to 1,000 or 8,000 ppm (5) but was highly effective in mice exposed to BD concentrations ranging from 6.25 to 625 ppm (6). Therefore, the International Agency for Research on Cancer (IARC) has classified BD as group 1, a human carcinogen via inhalation (7). This categorization was based on epidemiologic studies and laboratory animal data. BD is a potent carcinogen in mice and considerably weaker in rats. In addition, species and gender differences related to BD metabolism and DNA repair can be examined by measuring suitable biomarkers in order to elucidate the formation of individual epoxides and assign their importance in mutagenesis and carcinogenesis.

Differences are generally related to species-specific metabolic activation to reactive epoxy metabolites, mainly 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxibutane (DEB) and 1,2-epoxy-3,4-butanediol (EB-diol). The primary pathways in BD metabolism are shown in **Figure 4.1**. Oxidation of BD by cytochrome P450 results in the formation of EB, which can be further oxidized by P450 to DEB. EB and DEB can be conjugated with glutathione (GSH) in presence of GSH transferase (GST) and can also be hydrolyzed by epoxide hydrolase (EH) to the detoxication pathways forming 3,4-dihydroxy-1-butene (B-diol) from EB and EB-diol from DEB. B-diol can be oxidized by P450 to EB-diol and by alcohol dehydrogenase to 1-hydroxy-2-butanone, 1-hydroxy-3-butene-2-one and 2-hydroxy-3-butenal.

Globin adducts have been widely used as surrogate biomarkers for the formation of each epoxide after exposure to chemicals. The globin adduct formed by

EB, DEB, and EB-diol are *N*-(2-hydroxy-3-buten-1-yl)-valine (HB-Val), *N,N*-(2,3-dihydroxy-1,4-butadiyl)-valine (*pyr*-Val) and 1,2,3-trihydroxybutyl-valine (THB-Val), respectively (**Figure 4.1**). Our laboratory has previously developed quantitative immunoaffinity (IA) LC-ESI<sup>+</sup>-MS/MS method for two adducts: the EB-specific adduct, HB-Val, and the DEB-specific adduct, *pyr*-Val, (8, 9). Both types of adducts were observed in globin extracted from laboratory animals that were exposed to BD by inhalation (9, 10). We have recently used an immunoaffinity ultra pressure liquid chromatography-tandem mass spectrometry (UPLC-ESI<sup>+</sup>-MS/MS) method for analysis of *pyr*-Val (10). This method allowed the measurement of *pyr*-Val in a wide range of BD exposed samples from mice and rats, ranging from concentrations as low as 0.1 ppm BD to 1250 ppm BD. However, this current method is not available for analysis of THB-Val specific adduct of EB-diol. Furthermore, the application of the method is labor intensive requiring extensive sample processing time since the analysis of all three adducts can not be measured in a single run. Here, we describe the development of a similar method for simultaneous analysis of all three BD derived N-terminal valine adducts in the globin of female F344 rats exposed to BD by inhalation.

### 4.3 Materials and Methods

**Materials.** *Chemical for standard peptides synthesis, Immunoaffinity (IA) enrichment, and UPLC-MS/MS analysis*

Butadiene monoxide (98%) and 1,3-Butadiene diepoxide (97%) were purchased from Sigma-Aldrich (St.Louis, MO). 3,4-epoxy-1,2-butanediol was

generously provided by Dr. Avram Gold and Dr. Louise M. Ball from the Department of Environmental Science and Engineering, University of North Carolina at Chapel Hill. The N-terminal alpha chain peptide from rat (VLSADDKTNIK; analyte) and ( $[^{13}\text{C}^{15}\text{N}]$ -VLSADDKTNIK; internal standard) were synthesized from the peptide synthesis facility core at the University of North Carolina at Chapel Hill. Trypsin (biotin-agarose from bovine pancreas) was purchased from Sigma-Aldrich. All reagents and solvents were ACS grade or higher. Amicon ultra-4 filters were obtained from Amicon Inc. (Deerfield, IL). Polyclonal antibodies against all three adducts (HB-Val, *pyr*-Val, and THB-Val) were produced by Anaspec (San Jose, CA). The N-terminal alpha chain analyte and internal standard peptides for all three adducts were synthesized based on the procedure described below and used for antibody production.

### ***Methods.***

#### *Synthesis of standard peptides*

Analyte standard peptides, internal standard peptides, and calibration standards for tuning the analytical equipment were synthesized as describe below.

#### *HB-Val (analyte) and $[^{13}\text{C}^{15}\text{N}]$ HB-Val (internal standard)*

The analyte and stable isotope-labeled rat internal standard peptides were synthesized for accurate quantitation by direct alkylation method. The *in vitro* alkylation was done by addition of EB to an aqueous solution of (1-11) peptide at molar ratio of 1:100, in 0.1 M ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) buffer pH 6.5. The concentration of the peptides and the pH of the reaction solution were optimized in order to increase the reactivity of valine, to minimize the alkylation of lysine, and to

minimize the cross-linking of peptides (data from chapter 2). The reaction was incubated at 37°C for 72 h. After incubated, the reaction was stopped with 20% formic acid and the pH was adjusted to strong acid condition (pH 2). The yield of reaction products was characterized and profiled by LC-ESI<sup>+</sup>-MS/MS.

*pyr-Val (analyte) and [<sup>2</sup>H<sub>3</sub>] pyr-Val (internal standard)*

The N-terminal alpha chain standard peptide for *pyr-Val* in rat and the stable isotope (Leu-*d*<sub>3</sub>) labeled rat standard peptide were synthesized as described previously (11).

*THB-Val (analyte) and [<sup>13</sup>C<sup>15</sup>N<sub>5</sub>] THB-Val (internal standard)*

The analyte and stable isotope-labeled rat internal standard peptides were synthesized by the direct alkylation method, similar to HB-Val standard peptides. The *in vitro* alkylation was done by addition of EB-diol to an aqueous solution of (1-11) rat peptide at a molar ratio of 1:20, in 0.1 M ammonium bicarbonate buffer pH 6.5. The concentration of the peptides and the pH of the reaction solution were optimized (data from chapter 2). The reaction was incubated at 37°C for 72 h. After incubation, the reaction was stopped with 20% formic acid and pH was adjusted to strong acid condition (pH 2). The yield of reaction products were characterized and profiled by LC-ESI<sup>+</sup>-MS/MS.

#### *Animal and Exposures*

Female F344 rats were exposed by inhalation to 0.1, 0.5, 1, 1.5, 6.25, 62.5, 200, and 625 ppm BD for 10 days (2 weeks, 5 days/week, and 6 hours/day). The exposures were performed at the Lovelace Respiratory Research Institute (LRRI,



Albuquerque, NM) as described previously in Georgieva et al. (2010) (10). All animal procedures were approved by the LRRRI Institute Animal Care and Use committee.

#### *Globin extraction and trypsin hydrolysis*

Globin was isolated according to the protocol of Mowrer et al. (1986) (12). In brief, globin was precipitated by adding 6 volumes of 50 mM of hydrochloric acid in isopropanol, followed by centrifugation for 45 min at 3,000 g at 4 °C. The supernatant was washed three times with cold ethyl acetate to precipitate globin. The precipitate was washed with pentane and dried overnight under a gentle stream of nitrogen gas in a vacuum oven. The globin was stored at -70°C until use. In sample processing, globin samples were digested with trypsin as described previously (9). Briefly, globin samples (50 mg) were dissolved in 1-2 ml of 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, pH 8 containing 2 pmol of [<sup>2</sup>H<sub>3</sub>] *pyr*-Val (1-11) peptide, 2 pmol of [<sup>13</sup>C<sup>15</sup>N<sub>5</sub>] HB-Val, and 10 pmol of [<sup>13</sup>C<sup>15</sup>N<sub>5</sub>] THB-Val as internal standard peptides. Ten µl of 10% SDS were added and the globin was digested with 50-100 µl of trypsin-biotin agarose enzyme suspension at 37°C for 24 h. Samples were filtered through Centricon-3 filters and dried by centrifugal lyophilization.

#### *Immunoaffinity (IA) enrichment*

After trypsin hydrolysis, the samples were dried and redissolved in 600 µL PBS buffer and loaded on immunoaffinity columns that had been pre-conditioned twice with PBS. The IA columns were built specifically to retain all three N-terminal peptides (HB-Val, *pyr*-Val, and THB-Val). The columns were capped and left for 4 h, washed 5 times with 7 ml of water (5×7 ml), eluted in 5% formic acid (3ml), followed by drying under reduced pressure, filtration on microspin filters, and final drying.

Samples were stored at -20°C until analysis and dissolved in 20 µl of water prior to nano-UPLC-MS/MS analysis.

#### *Liquid chromatography mass spectrometry*

A Thermo-Finnigan TSQ Quantum ultra triple quad mass analyzer (ThermoFinnigan, San Jose, CA) interfaced to Water Aquity nano-UPLC system (Waters, Milford, MA) was used in all analyses. The system utilized a 2.0 × 20 mm Symmetry C18, 5 µm column (Waters) as a “trap column” for samples loading at 15 µl/min 15 mM ammonium formate-0.7% formic acid for 1 min. After sample loading, the flow rate was reduced to 1.2 µl/min and the column exit flow was directed to a 100 µm × 100–mm BEH C18 UPLC column (Waters) for analysis. The LC solvents were 15 mM ammonium formate in 0.7% aqueous formic acid (A) and acetonitrile in 0.1% aqueous formic acid (B). A linear gradient was run from 5% B to 70% B for 15 min, at a flow rate of 1.2 µl/min. After elution, the column was washed at 90% B for 10 minutes and equilibrated at 5% B for 5 min. Under these conditions, all three adducts and internal standard eluted within the range 5.9 - 6.1 min. The MS was operated in the positive ion mode with nitrogen used as a sheath gas. Electrospray ionization was performed at a spray voltage of 1800 V and capillary temperature of 240 °C. Collision induced dissociation (CID) energy was achieved at 28 V. The MS parameters were optimized during the infusion of standard peptides solution.

#### *Method validation*

Control globins used for validation of matrix effect were obtained from female F344 rats. A three-point validation was run on three days (n=3 for each validation point for one day, n=9 for each validation point for three days). Validation samples

were prepared by spiking control globin with standard peptides. Relative recoveries in matrix effects were calculated as the response for all three adducts in a biological matrix spiked divided by the actual spiked concentration. The results are reported in % recovery for each adduct. The Limits of detection (LOD) were determined by signal to noise ratio 3:1 based on 5 µl injection volume of calibration standard. The lower limit of quantitation (LOQ) was defined as the lowest concentration to be detectable from diluted spiking of control globin. The interday and intraday precisions were expressed as % relative standard deviation (%RSD) and accuracies were expressed as % relative error (%RE).

#### *Statistical analysis*

All statistic analyses were performed using Microsoft Excel spreadsheet analysis tools. Classified precision and accuracy were estimated by simple statistics of mean and standard deviation of the estimated concentration of the determined peptides.

## **4.4 Results and discussion**

### *An improved IA-UPLC-MS/MS method*

It is well known that N-terminal globin adducts are well suited to study BD carcinogen metabolism across species (13). Specific globin adducts formed by each BD metabolite need to be identified and quantified in order to reveal important insight into species, gender, and exposure concentration differences in BD metabolism. We have recently demonstrated DEB specific adduct, *pyr-Val*, in mice and rats exposed to BD at different concentrations (10). Since our previous analyses of *pyr-Val*

adducts was labor intensive and time consuming, the objective of this study was to improve the IA UPLC-MS/MS method and simultaneously detect all three globin adducts specific for BD metabolites in a single run. We employed the same concept as the method for *pyr*-Val and were able to adapt the method for the analysis of HB-Val and THB-Val in the concurrent sample.

IA columns were produced by combining antibodies raised against all three alkylated standard peptides and used for sample purification. Control globin was spiked with different amounts of analyte and internal standard peptides to determine the reproducibility of IA column. The results showed that the percentage of recovery was lower compared to IA columns prepared with individual antibodies. To overcome this problem, we adjusted the incubation time on IA columns from 1 h to 4 h at room temperature. Using this approach, better recoveries (>70%) were obtained for all three adducts (data not shown).

The MS was performed in the selected reaction monitoring (SRM) mode by monitoring of the singly charged molecular ion ( $m/z$  817→141) for HB-Val, ( $m/z$  851→175) for THB-Val, and the corresponding transition of the [ $^{13}\text{C}^{15}\text{N}_5$ ]-label internal standard peptide ( $m/z$  823→147) for HB-Val, ( $m/z$  857→181) for THB-Val. At the same time, we monitored the doubly charged molecular ion ( $m/z$  417→158) for *pyr*-Val and the corresponding transition of the [ $^2\text{H}_3$ ]-labeled internal standard peptide ( $m/z$  418→158) (**Figure 4.2**), since this transition presented better sensitivity compare to the singly charged molecular ion.

Quantitative measurement was based on the ratio of area under the peak in the selected ion chromatogram corresponding to the analyte and internal standard

peptides. Calibration curves were constructed by analyzing the solutions containing all three adducts (50 - 500 fmol) and internal standard peptides (500 fmol), followed by regression analysis (**Figure 4.3**). All curves showed a linear response over the range of concentrations with a high coefficient of determination ( $R^2= 0.992$ ,  $R^2= 0.998$ , and  $R^2= 0.999$  for HB-Val, *pyr*-Val and THB-Val, respectively).

#### *Method validation*

##### *Limit of detection (LOD) and limit of quantitation (LOQ)*

LOD was 1 fmol for all three adducts based on 5  $\mu$ L of injection volume and a signal to noise ratio of 3:1, while the LOQ was 5 fmol on column for HB-Val and *pyr*-Val and 10 fmol on column for THB-Val. The lower LOQ for THB-Val adducts compared to the other two adducts was attributed to the lower signal on ESI<sup>+</sup>-MS/MS. This LODs and LOQs allowed detection of all three adducts in rats exposed to BD at 0.5 ppm for 10 days.

##### *Recovery*

Relative recovery values for all three adducts in matrix were as follows; 92-96% for HB-Val, 90-95% for *pyr*-Val, and 63-73% for THB-Val. The relatively low yield of THB-Val was likely due to the low titer of the THB-Val antibody, which may have cause less retention during the immunoaffinity purification step. However, despite the low yield, our method produced a highly linear calibration curve with acceptable precision and accuracy.

##### *Accuracy and Precision*

The method accuracy and precision were determined for three replicates of three concentrations of all three adducts. The validated samples were analyzed on

three separate days, the interday and intraday precision and accuracy are listed in **Table 4.1**. The RSD of both interday and intraday for all three adducts on the nano-UPLC-ESI<sup>+</sup>-MS/MS were below 7% (1.1%-6.6%). The accuracies calculated as relative error (RE) were within the range of 2%-6%. The improved quantitation method in this study is highly sensitive, accurate, and selective for all three BD adducts, allowing the detection of low amounts of adducts in 50 mg globin in rats from exposures as low as 0.5 ppm BD. However, the detection of adducts at 0.1 ppm BD exposure for 10 days was under the LOQ due to the presence of very small amounts of adducts.

#### *Application of the method to BD exposed rats*

Here, we generated a comprehensive exposure response for BD reactive epoxides in female rats exposed to BD. F344 rats were exposed by inhalation to various concentrations of BD ranging from 0.1 to 625 ppm. In perspective, ambient BD concentrations at the work place range from 0.3 to 3 ppm (14–18). Hence, the recently improved quantitative assay allowed analysis of N-terminal valine adducts after BD exposures as low as those encountered in occupational environments. The data reported herein, demonstrated for the first time the amounts of all three BD epoxide globin adducts in female F344 rats. These data will help understand important aspects of BD metabolism, carcinogenesis and mutagenicity.

The newly validated quantitative immunoaffinity nano-UPLC-MS/MS method was employed to measure the formation of three BD adducts from the same animal sample. To test the utility of our method, we determined HB-Val, pyr-Val, and THB-

Val adducts in each of the globin sample (n = 5/group). Our data (mean  $\pm$  standard deviation) are shown in **Table 4.2**. The control animals did not contain detectable amounts of HB-Val or *pyr*-Val, suggesting that these adducts are not formed endogenously. In contrast, THB-Val ( $36.8 \pm 3.9$  pmol/g globin) has been detected in control female rats with no exogenous BD exposure as a result of alkylation by threose and erythrose (19). Representative extracted ion chromatograms for nano-UPLC-ESI+-MS/MS analysis of all three BD adducts from rats exposed to BD at 6.25 ppm are shown in **Figure 4.2**. Even though *pyr*-Val is the least abundant of the three BD adducts quantified, it was 8-fold higher than the method's LOQ, ensuring accurate quantification and presenting excellent signal to noise ratio for the sample exposed at 0.5 ppm BD. Therefore, we quantified the formation of the three BD adducts in rats exposed to 0.5 - 625 ppm BD for 10 days, in order to obtain data on exposure-response relationships. The exposure response curve is shown in **Figure 4.4**, and briefly discussed below.

#### *Exposure response of HB-Val formation*

We observed a linear exposure-response curve for the HB-Val adducts (**Figure 4.4**). The formation of HB-Val increased with exposure and did not show any signs of metabolic saturation at the exposure levels studied. The slope of formation was steepest at low exposure (0.5-6.25 ppm) indicating that the rate of formation is most efficient at low exposures. These data are consistent with the study by Koc et al. (20) where the *N7*-(2-hydroxy-3-butenyl)-guanine and *N7*-(1-hydroxy-3-butenyl)-guanine (*N7*-HB-Gua), EB-specific *N7*-guanine adduct has been shown to be linear in mice and rats from the lowest exposure studied (20 ppm BD 4 weeks) to high

exposures known to induce tumorigenesis (20). Taken together, these results confirm that EB metabolism is linearly increased with the exposure.

The amounts of HB-Val were 40-fold lower compared to THB-Val at exposures less than 6.25 ppm and 5 fold lower compared to exposures greater than 6.25, demonstrating the greater efficiency of metabolism of EB, compared to EB-diol at high exposures. HB-Val formed in similar amounts to *pyr*-Val in female rats at exposures less than 200 ppm. However, HB-Val was 2-fold higher at 625 ppm BD due to the saturation of *pyr*-Val formation.

#### *Exposure response of pyr-Val formation*

The formation of *pyr*-Val in female F344 rats exposed to BD is plotted in **Figure 4.4**. The amounts of *pyr*-Val increased with exposure, but with lower efficiency shown by decreasing slopes between 1.5 ppm and 6.25 ppm BD. In contrast to the linear slope of HB-Val, the *pyr*-Val response curve reached a plateau at exposures greater than 200 ppm. This suggests that the formation of DEB saturates at high exposures in rats, which may saturate the capacity of the P450 enzymes in the later steps of BD metabolism (10). In addition to reaching a plateau, the exposure-response in rats also showed evidence of decreases in slope for *pyr*-Val formation similar to our previous observations in the same animals (10).

#### *Exposure response of THB-Val formation*

The exposure response curve for the THB-Val adduct is illustrated in **Figure 4.4**. The decreases in slope were observed at concentrations greater than 6.25 ppm



BD, showing a supralinear exposure-response curve in the formation of THB-Val adducts in female rats. This result suggests that the metabolic activation of BD to EB-diol and DEB reach saturation. The amounts of THB-Val adducts were approximately 5-40 fold higher than HB-Val and *pyr*-Val adducts. The saturation curve for THB-Val in rats is not surprising, since formation of THB-Gua adducts have been shown to reach a plateau at exposures greater than 62.5 ppm BD for 20 days. (20, 21). These data are in agreement with the direct comparison of THB-Val to THB-G adduct formation in animals from the same exposures studied. The EB-diol specific DNA adduct (THB-G) was the most abundant and exhibited saturation of metabolic activation at 62.5 ppm BD.

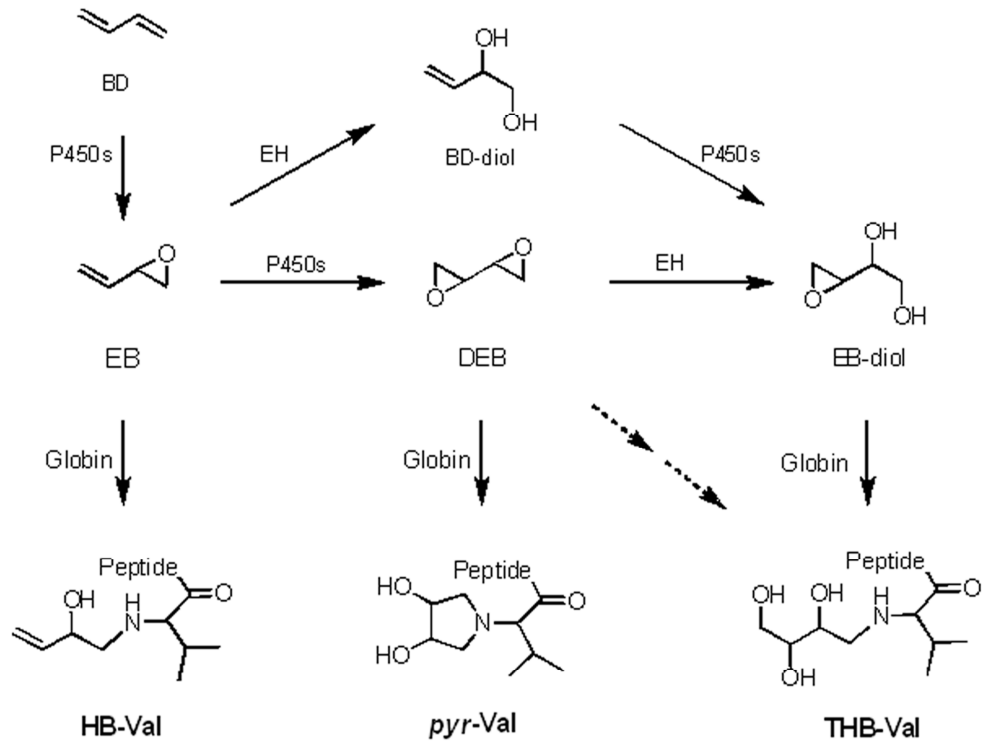
#### **4.5 Conclusion**

In conclusion, we developed a novel immunoaffinity nano-UPLC-ESI<sup>+</sup>-MS/MS combined method for the simultaneous measurement of all three BD-derived epoxide N-terminal globin adducts in female rats exposed to BD by inhalation. The present method allows selective and sensitive detection of multiple adducts from the same sample. In addition, this study is the first report of the measurement all three BD adducts using one analysis method in rats exposed to BD. This improved method can be further developed to investigate BD globin adduct formation in mice and in humans. The current investigation greatly extends our mechanistic knowledge that will be important for science-based risk assessment of BD.

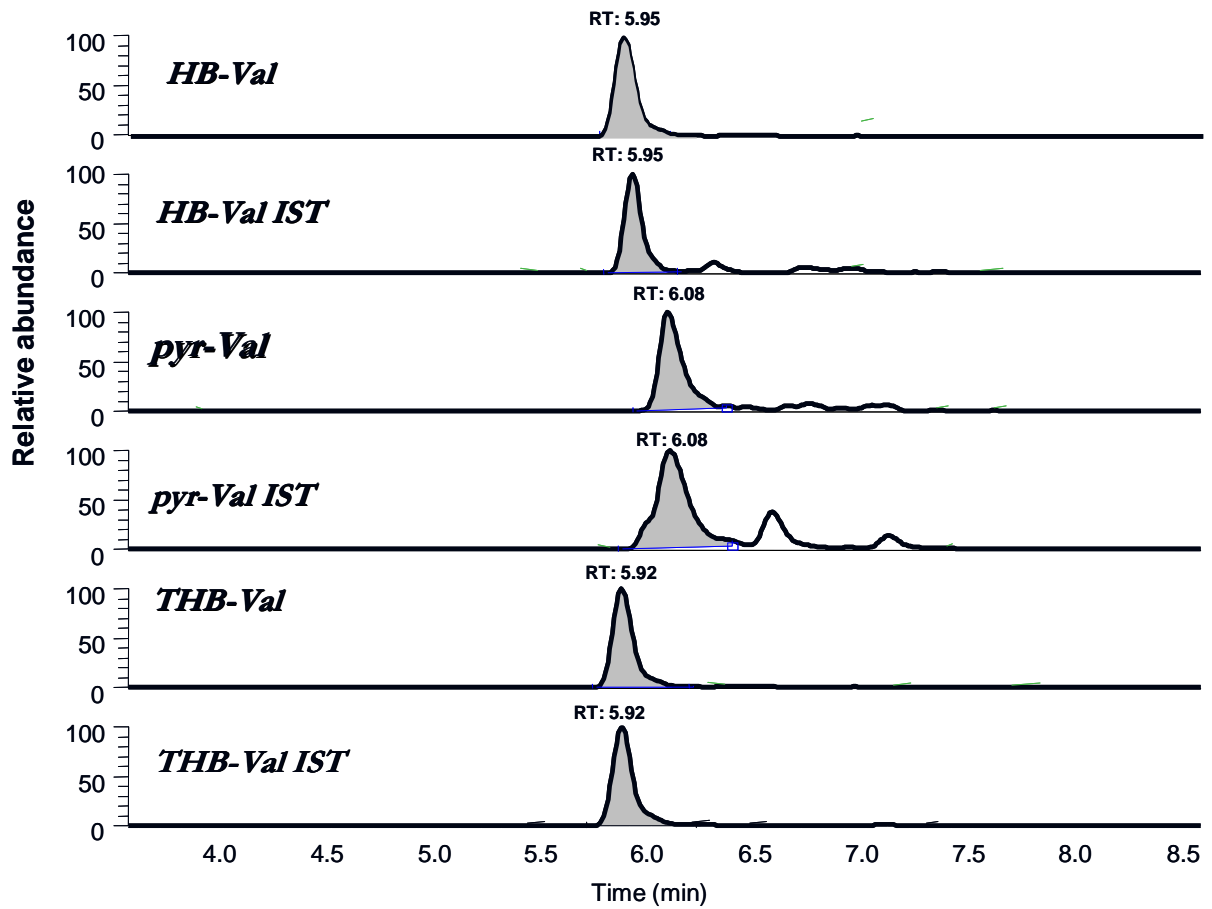
## **4.6 Acknowledgements**

We thank the Biomarker Mass Spectrometry facility for helpful suggestions on UPLC and nano-ESI<sup>+</sup>-MS/MS method development and Valeriy Afonin for the isolation of all the globin samples. This work is supported by grants from the NIH (1 R01 ES012689, 5 P30-ES10126), the Health Effects Institute (agreements 99-5 and 05-12) and the American Chemistry Council.

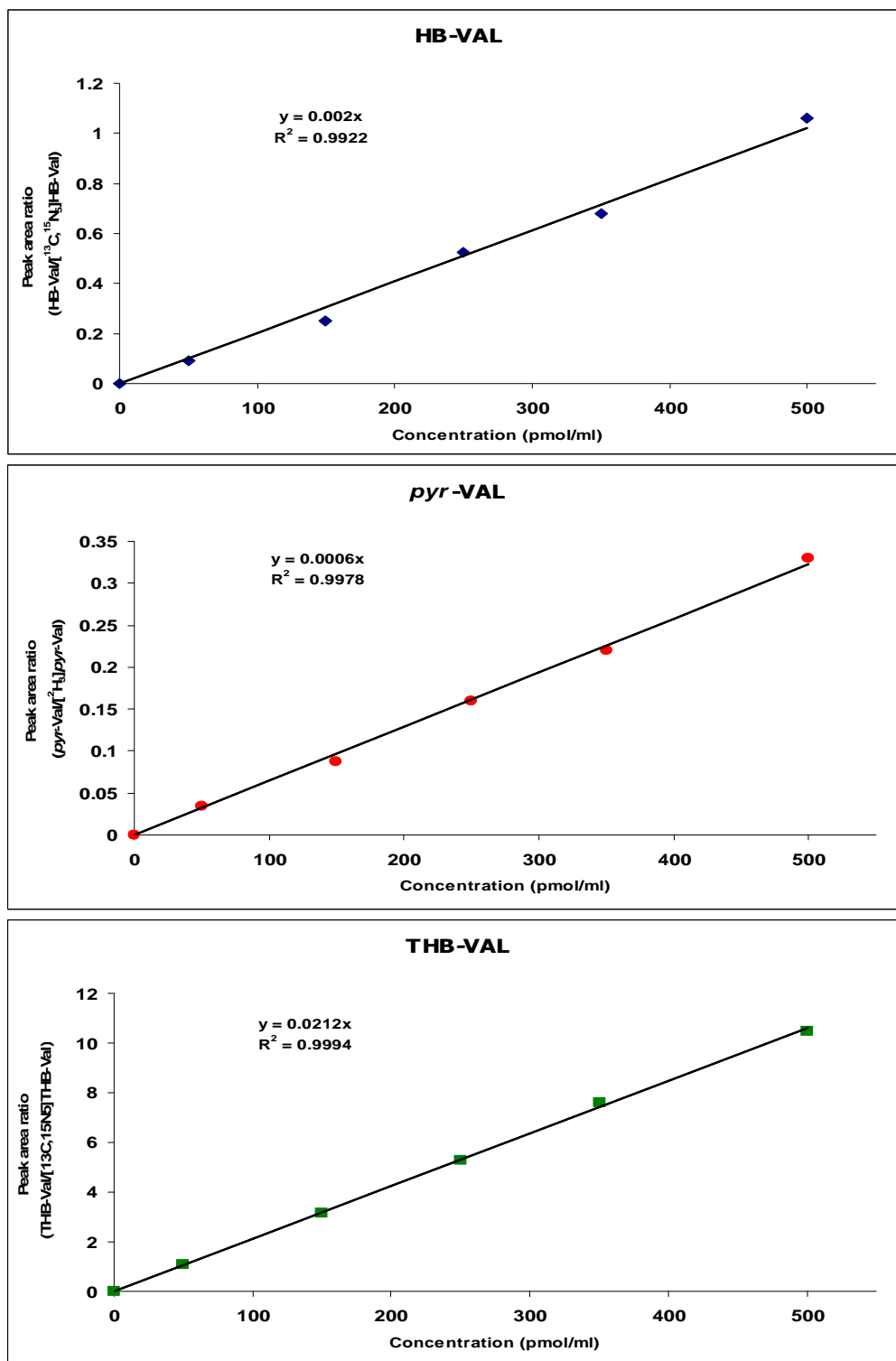
## FIGURES



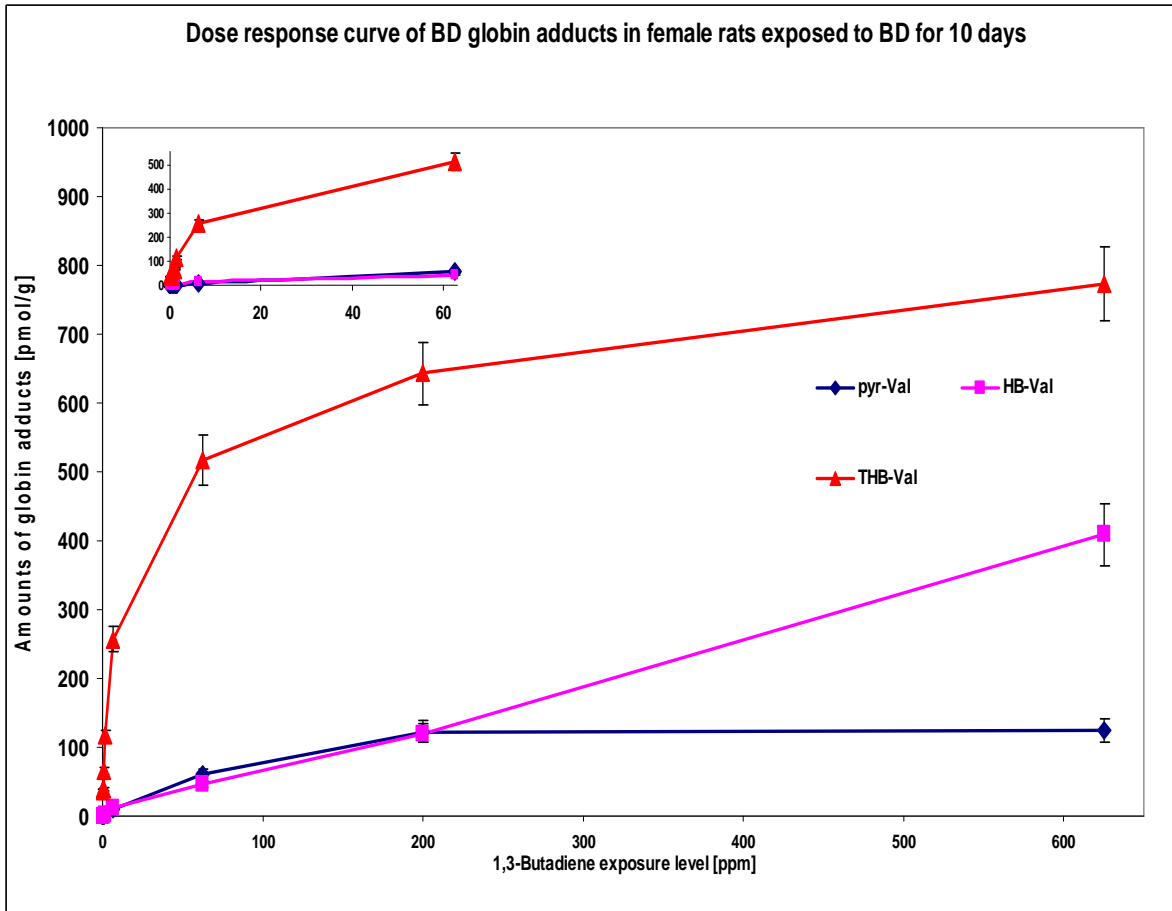
**Figure 4.1** BD metabolism and formation of *N*-terminal valine adducts



**Figure 4.2** Ion chromatograms N-terminal valine adducts in F344 rat samples exposed to 6.25 ppm



**Figure 4.3** Calibration curve for N-terminal valine adducts (HB-Val, *pyr*-Val, and THB-Val)



**Figure 4.4** Exposure response curves of HB-Val, *pyr*-Val, and THB-Val adducts formation in female F344 rats exposed to BD for 10 days

**TABLES**

**Table 4.1** Accuracy and precision of IA-nanoUPLC-MS/MS method

<b>Adduct</b>	<b>Intraday (n=3)</b>				<b>Interday (n=3)</b>			
	Conc. (μM)	Determined Conc. (μM)	Precision (%RSD)	Accuracy (%RE)	Conc. (μM)	Determined Conc. (μM)	Precision (%RSD)	Accuracy (%RE)
<b>HB-Val</b>	1.0	0.98 ± 0.02	2	2	1.0	0.98 ± 0.07	6.6	2
<b>Pyr-Val</b>	1.0	0.94 ± 0.01	1.1	6	1.0	0.96 ± 0.06	6.6	4
<b>THB-Val</b>	2.0	1.91 ± 0.10	5.1	4.5	2.0	1.88 ± 0.03	1.3	6

**Table 4.2** Hemoglobin adducts from female rats exposed to butadiene for 10 days

<b>Dose (ppm)</b>	<b>HB-Val (pmol/g)</b>	<b><i>Pyr</i>-Val (pmol/g)</b>	<b>THB-Val (pmol/g)</b>
<b>0</b>	ND	ND	36.8 ± 3.9
<b>0.1</b>	NQ	NQ	NQ
<b>0.5</b>	0.9 ± 0.1	0.8 ± 0.1	2.4 ± 0.3
<b>1</b>	1.6 ± 0.5	1.4 ± 0.2	29 ± 3
<b>1.5</b>	2.5 ± 0.4	2.0 ± 0.5	59 ± 6
<b>6.25</b>	8.1 ± 0.7	6.7 ± 0.7	220 ± 19
<b>62.5</b>	60.0 ± 3.0	46.5 ± 8.3	481 ± 53
<b>200</b>	148 ± 19	123 ± 8	606 ± 75
<b>625</b>	410 ± 15	124 ± 11	737 ± 74

**ND: not detected, NQ: not quantified**  
**Endogenous THB-Val was subtracted in all groups using data from control rats (36.8 pmol/g)**



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## CHAPTER V

### INTERNAL DOSE AND GENOTOXIC POTENCY OF 1,3-BUTADIENE: APPLICATION TO A MULTIPLICATIVE RISK MODEL

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*[Manuscript]*

#### 5.1 Abstract

Butadiene (BD) carcinogenicity shows gender, species and concentration dependency, making the extrapolation of animal results to humans complex. BD is a multi-species multi-site carcinogen, with mice being a much more sensitive species than rats. This is considered to be related to the metabolism of BD to its epoxy metabolites, 1,2-epoxy-3-butene (EB), 1,2;3,4-diepoxybutane (DEB) and 1,2-epoxy-butanediol (EB-diol). The mutagenic potency of individual epoxides varies up to 200-fold, with DEB being the most mutagenic metabolite. For accurate risk assessment it is important to elucidate species differences in the internal formation of the individual epoxides in order to assign the relative risks associated with their different mutagenic potencies.

N-terminal globin adducts have been widely used for measurements of the formation of BD derived epoxides. In this study, the internal adduct dose of each of the epoxides was evaluated in globin samples from both genders of mice and rats exposed to BD from 0.1 to 625 ppm by inhalation for 10 days. The number of globin adducts was then converted into EB dose equivalents. These were calculated on the basis of the combined internal dose (globin adducts) and the relative genotoxic potency of the respective epoxides inferred from the efficiency of inducing mutations at the *Hprt* locus. Then, the multiplicative cancer risk model was applied to quantitatively estimate tumor incidence by using the EB dose equivalent and long-term cancer bioassay data. Based on the EB dose equivalent, higher exposures formed lower amounts per ppm BD. This indicates that metabolism of BD to epoxides is most effective at low exposures (0.5-1.5 ppm). The EB equivalent for mice was about 40-fold higher than that of rats at same exposures levels. No gender differences were noted in globin adducts of mice or rats at all exposures. Predicted tumor incidences from the cancer risk model correlated well with the earlier observed tumor incidences in cancer test. As such, EB dose equivalents provide quantitative data on biomarkers of exposure that can be extended to a scientific basis for BD risk assessment.

## **5.2 Introduction**

1,3-Butadiene (BD) is a high-volume industrial chemical used in the production of plastics and rubber (1). It is also an environmental pollutant present in

automobile exhaust and cigarette smoke (2, 3). BD is classified as a human carcinogen based on laboratory animal data linking it to the formation of cancer. Human epidemiology data are revealing increased incidences of leukemia and lymphohematopoietic cancer in workers occupationally exposed to BD. Genotoxicity data derived from laboratory animal studies demonstrated the induction of point mutations, large deletions, and chromosomal aberrations following exposure to BD (4– 8).

Bioactivation to yield reactive epoxides of BD in animal and human tissues has been suggested to be an important determinant of the mutagenic and carcinogenic effects of BD (9). BD is metabolized by cytochrome P450, forming reactive epoxides: 1,2-epoxybutene (EB), 1,2:3,4-diepoxybutane (DEB) and 3,4-epoxy-1,2-butanediol (EB-diol) (10). Of critical importance is the fact that BD-derived epoxides differ in their mutagenic potencies by up to 200-fold, with DEB being the most potent mutagenic metabolite due to its capacity as a bifunctional electrophile (11, 12). All three epoxides are known to form DNA and protein adducts. EB produces *N*-(2-hydroxy-3-buten-1-yl)-valine (HB-Val), and DEB forms *N,N*-(2,3-dihydroxy-1,4-butadiyl)-valine (*pyr*-Val) and 1,2,3-trihydroxybutyl-valine (THB-Val). The later adduct is also formed from EB-diol and is present in unexposed animals and humans from endogenous sources. A simplified pathway for metabolic and globin adduct formation of BD is shown in **Figure 5.1**

BD inhalation cancer studies in mice and rats revealed that it was carcinogenic in both species, but with a striking difference in sensitivity and tissue specificity. Mice developed tumors following exposures as low as 6.25 ppm BD (7),

Rats were studied at 0, 1000, 8000 ppm BD and no lower exposure levels were tested for carcinogenicity. Tumors were observed at the exposure reached 1,000 ppm BD (13). Furthermore, the target tissues for BD-induced cancer in mice (lung, heart, and hematopoietic system) were different from those in rats (thyroid, pancreas, testis, and uterus) (8). Therefore, BD is a potent carcinogen in mice and considerably weaker in rats. The increased susceptibility of mice to the carcinogenicity of BD may result from a more efficient metabolic activation of BD in this species (11). Mice formed 10- to 60-fold more *pyr*-Val compared to rats at similar exposures (14). In addition, concentrations of racemic *bis*-N7G-BD, a DEB specific DNA cross-link, were 4- to 10-fold higher in mice than in rats at all exposure levels. (15). These studies provide a possible explanation for the strong tumorigenic response to BD in mice.

We have recently developed a quantitative ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI<sup>+</sup>-MS/MS) method for quantifying *pyr*-Val and HB-Val adducts (14, 16). In our approach, the N-terminal valine adducts are released from the globin chain by trypsin hydrolysis, enriched by immunoaffinity chromatographic (IA) and analyzed by nano UPLC-ESI-MS/MS using the corresponding [<sup>13</sup>C<sup>15</sup>N]-labeled adducts as internal standards. Using nano-UPLC-MS allowed analysis of *pyr*-Val in mice and rats exposed to BD as low as 0.1 and 0.5 ppm BD, respectively, and demonstrated significant differences in the amounts of *pyr*-Val formed. The study analyzed *pyr*-Val in rodent tissues in an attempt to explain the observed interspecies differences in carcinogenic response. We observed that the lower susceptibility of rats to BD-

induced carcinogenesis due to greatly reduced formation of DEB following exposure to BD. Additional data for HB-Val and THB-Val adduct formation are needed to better understand the species susceptibility and dose dependence of BD mutagenesis and carcinogenesis.

Research has shown that BD is genotoxic in rodents. Differences in *Hprt* Mf were significantly higher in both species when exposed to 1250 ppm BD for 2 weeks compared to controls (3.5-fold, rats/control) (9). The lowest exposure to BD that produced a significant increase in *Hprt* Mf was 3 ppm in mice and 62.5 ppm in rats (9). Since DEB has been shown to be a much more potent inducer of mutations than EB and EB-diol, the interspecies difference in the potency of genotoxic effects may be related to the differences in the rate of formation and detoxification of DEB (14). Recent *in vitro* studies have exposed TK6 cells to nine different stereochemical configurations of BD epoxides and assayed them by multiplex PCR for both *Hprt* and *TK* genes (17). These studies demonstrated that the mutagenic potency of EB was 100-fold less than the average equimolar effect of DEB, and was 5-fold greater than the average equimolar effect of EB-diol. In addition, marked differences were observed in cytotoxicity and mutagenicity among the four stereoisomers of EB-diol. (2*R*,3*S*)-EB-diol was the most mutagenic monoepoxide stereoisomer, being 30 times more mutagenic than the other 3 forms of EB-diol and 5-10-fold less mutagenic than DEB (17).

Cancer risk estimation on the basis of the internal dose of genotoxic chemicals represents an important factor for the assessment of cancer risk. An improved approach for risk estimation, the multiplicative risk model, is being



developed based on experiences from studies of ionizing radiation (18, 19). Evaluations of animal cancer tests using this approach indicate that a multiplicative cancer risk model could be applied to genotoxic chemicals (20). Accordingly, the cancer risk increment is proportional to the internal dose of the causative genotoxic agent, and its genotoxic potency specific for the studied species, strain and gender. In addition, the relative risk increment, according to this model, is common for different responding sites. Therefore, the model has been shown to provide a way of predicting tumor incidence in animal cancer tests with genotoxic agents. This model can be used for extrapolation to human cancer risk, giving an approximate risk coefficient for different species (19). Moreover, the applicability of the multiplicative risk model to cancer test data for ethylene oxide (20), acrylamide (21), and 1,3-butadiene (22) has been evaluated. It has been shown that the internal dose of the genotoxic agent and the observed tumor incidence in animal cancer tests are well correlated with the predicted incidences from the multiplicative risk model. Furthermore, the model can be applied to genotoxic agents and is useful for the interspecies extrapolation. The major factor that is not incorporated into this model is the role of endogenous DNA damage in determining low dose risk assessments (23, 24).

A dose concept for genotoxic electrophiles has been developed where the dose is defined as the time integral of the concentration of the genotoxic compound, which is the same as the “area under the concentration-time curve” (AUC). For the purpose of internal dosimetry of the genotoxic chemical, the internal dose of the reactive epoxide metabolites, EB, DEB, and EB-diol will be calculated from the

measurement of globin adducts at the N-terminal site using immunoaffinity UPLC-MS/MS method. While endogenous THB-Val is formed, no endogenous BD DNA adducts are formed, making this data set appropriate for the risk assessment model.

The evaluation of BD cancer studies is a challenge because of large species differences in sensitivity, and because all three epoxide metabolites are genotoxic and may contribute to tumor formation. Therefore, this study provides a comprehensive data set for the exposure-response of globin adduct formation due to BD-derived reactive epoxides in male and female mice and rats exposed to various concentrations of BD by inhalation. Exposures ranged from 0.1 to 625 ppm BD for 10 days. The multiplicative risk model is then used to assess the BD cancer bioassay data and internal adduct doses.

### **5.3 Materials and Methods**

#### *Long-term inhalation exposure to BD in cancer bioassays*

Data from two chronic cancer bioassays that used inhalation of BD in Sprague-Dawley rats and B6C3F<sub>1</sub> mice are used for the evaluations in this study (13, 25). The animals were treated for 6 h per day, 5 days per week for approximately 2 years. Duration of exposures for the different groups were: 3090 h (female and male mice), 3150 h (female rats) and 3330 h (male rats). Five groups of 50 mice were exposed to 0, 6.25, 20, 62.5, 200, or 625 ppm BD and three groups of 100 rats were exposed to 0, 1000, or 8000 ppm BD. All exposure doses and all tissues showing a significant response in the cancer bioassays, as reported in the review of NTP (25), were used in the evaluation. Since the BD exposures in the carcinogenicity bioassay

in mice were matched with that of the animals in the globin adducts study, only data from mice were applied to the multiplicative model. **Table 5.1** summarizes the target organs and types of neoplasia observed.

#### *Animals and exposures in globin adducts studies*

The exposures were performed at the Lovelace Respiratory Research Institute (Albuquerque, NM) according to protocols approved by the Institutional Animal Care and Use Committee. B6C3F1 mice (male and female) and F344 rats (male and female) were exposed by inhalation to 0.1, 0.5, 1, 1.5, 6.25, 62.5, 200, or 625 ppm BD for 10 days (2 weeks, 5 day/week, and 6 h/day). At the end of the last exposure, animals were killed by exsanguination under CO<sub>2</sub> anesthesia and blood samples were collected by cardiac puncture within 2 h of the last exposure. Red blood cells were isolated, washed twice with 0.9% saline, diluted 2x in distilled water, and stored at –80°C before extraction of globin.

#### *Analysis of Hemoglobin adducts*

##### *Globin sample processing*

Globin adducts from BD derived reactive epoxides were quantified by measuring, HB-Val, pyr-Val, and THB-Val based on an improved IA-UPLC-MS/MS method using peptide standards that had been quantified accurately as described by Bordeerat *et al.* (2009) (26). In brief, globin isolation was performed according to Mowrer *et al.* (1986) (27). Globin samples of 10–50 mg (depending on the species and the exposures) were dissolved in 1.5 ml of 0.1 M NH<sub>4</sub>HCO<sub>3</sub> and 2 pmol of [<sup>2</sup>H<sub>3</sub>]

*pyr*-Val, 2 mol [<sup>13</sup>C<sup>15</sup>N<sub>5</sub>] HB-Val, and 10 mol [<sup>13</sup>C<sup>15</sup>N<sub>5</sub>] THB-Val internal standard (1–11) peptide were added. Samples were digested for 24 h at 37°C with 50–100 µl of trypsin-biotin-agarose suspension (washed in advance twice with 0.1 M NH<sub>4</sub>HCO<sub>3</sub>). After Centricon-3 filtration, samples were dried and redissolved in 600-µl PBS buffer, loaded on IA columns, and left capped for 4 h. After extensive washing with distilled water (5 × 7 ml) and elution in 3 ml of 5% formic acid, followed by sample drying, filtration on Microspin filters, and final drying, samples were stored at –20°C until analysis by nano-ultra high pressure liquid chromatography-tandem-mass spectrometry (nano-UPLC-MS/MS).

#### *Liquid chromatography and mass spectrometry analysis*

The quantitative analysis of the globin adducts from BD derived reactive epoxides by nano-LC-MS/MS was performed with a nano-UPLC (Waters, Milford, MA) coupled to a TSQ-Quantum Ultra triple quad mass analyzer (ThermoFinnigan, San Jose, CA). The system utilized a 2.0 × 20 mm Symmetry C18, 5 µm column (Waters) as a “trap column” for samples loading at 15 µl/min 15 mM ammonium formate-0.7% formic acid for 1 min. After sample loading, the flow rate was reduced to 1.2 µl/min and the column exit flow was directed to a 100 µm × 100–mm BEH C18 UPLC column (Waters) for analysis. A linear gradient was run from 5% acetonitrile/15 mM ammonium formate in 0.7% aqueous formic acid for 5 min then to 70% acetonitrile/15 mM ammonium formate in 0.7% aqueous formic acid for 10 min at a flow rate of 1.2 µl/min. Under these conditions, all three adducts and internal standard eluted within the range 5.9 - 6.1 min. The retention times for the analyte

and internal standard were determined with authentic standards, and all adducts in the sample were detected in selected reaction monitoring mode. The electrospray conditions were spray voltage of 1800 V and capillary temperature of 240°C. Collision energy was 28 V.

*Calculation of internal adduct dose from levels of Hemoglobin (Hb) adducts*

The internal adduct dose ( $D$ ; in mMh·ppm $^{-1}$ ) was calculated from the measured number of Hb adducts ( $A$ ; in mol·g $^{-1}$ ) and the reaction rate constant of the electrophilic agent towards the nucleophile,  $k_{val}$  in [L·g $^{-1}$ ·h $^{-1}$ ] as shown in **Equation 1**.

**(Equ. 1)** 
$$D = (1 \times A) \div k_{val}$$

In situations when  $A$  is measured after a period of exposure (10 days), the life span of erythrocytes ( $t_{er}$ ) has to be considered for adjustment of the accumulation and disappearance of Hb adducts. The number of accumulated adducts ( $A_{cc}$ ) is related to the daily adduct increment ( $a$ ) according to **Equation 2**. The  $t_{er}$  values used were 40 days for mouse (28) and 60 days for rat (29).

**(Equ. 2)** 
$$\text{at } t < t_{er}; \quad A_{cc} = a \times t (1 - t/2t_{er})$$

The rate constant for the reaction of the epoxy metabolites of BD were used as reported in Fred et al. (2008) (22). The different values of  $k_{val}$  used in calculations for each epoxide were as follows: EB 4.0×10 $^{-5}$  L·g $^{-1}$ ·h $^{-1}$ , DEB 5.5×10 $^{-5}$  L·g $^{-1}$ ·h $^{-1}$  and

EB-diol  $2.3 \times 10^{-5} \text{ L}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  for rat; EB  $2.9 \times 10^{-5} \text{ L}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , DEB  $4.7 \times 10^{-5} \text{ L}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  and EB-diol  $2.1 \times 10^{-5} \text{ L}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  for mouse.

#### *Calculation of EB dose equivalent*

The  $D$  of the genotoxic and potential cancer-initiating agent is one parameter in the multiplicative risk model (20). The cancer bioassay results for BD have to be evaluated with regard to the  $D$  of the genotoxic epoxide metabolites (EB, DEB and EB-diol) at different exposures. Moreover, the differences in the mutagenic potency of the epoxides have to be considered.

In a study using human TK6 cells it was shown that EB, DEB, and EB-diol were effective at inducing mutations in *Hprt* loci. Meng *et al.* (17) reported that the initial treatment concentrations of individual epoxides inducing a doubling of the mutation frequency were 200  $\mu\text{M}$ , 2  $\mu\text{M}$ , and 1,000  $\mu\text{M}$  for EB, DEB and EB-diol, respectively. We used this reference as our estimate for the relative mutagenic potencies, with EB as a reference having a value of 1, DEB having a value of 100 and EB-diol having a value of 0.2. The EB dose equivalent was then calculated by multiplying the internal hemoglobin adduct dose ( $D$ ) of the respective epoxide with the corresponding mutagenic potency (**Equation 3**).

**(Equ. 3)**                      Dose-equivalent =  $D_{\text{EB}} \times 1 + D_{\text{DEB}} \times 100 + D_{\text{EB-diol}} \times 0.2$

Equation 3 is expressed as EB dose equivalents (nMh) per exposure dose of BD (ppmh).

### *Application to multiplicative risk model*

This model known as the excess relative risk model, often applied in radiation research, was fitted to the tumor incidence data. The model implies that the fraction of animals with tumors in target tissue ( $i$ ), i.e.  $P_i(D)$ , can be described by the following equation:

$$\text{(Equ. 4)} \quad P_i(D) = 1 - e^{-P_i^0(1+\beta D)}$$

$D$  denotes the EB-equivalent dose in target tissue. In the present evaluation of cancer bioassays for BD, the dose denotes the total dose in the blood of the genotoxic BD epoxides. For specific organs the dose in the blood has to be related to target dose.  $P_i^0$  represent the cumulative hazard for tumor formation in the target tissue.  $i$  represent tumor incidence among unexposed animals.  $\beta$  is the common relative excess risk per unit of dose.

The parameters of this nonlinear function were estimated by a binomial regression model with 95% confidence intervals. The fit of the model is presented as observed versus predicted tumor incidence.

## **5.4 Results and discussion**

### *N-terminal valine globin adducts in rodents exposed to BD*

N-terminal hemoglobin adducts have been used as markers for BD exposure and the formation of individual epoxides in rodent models. These studies are critical for understanding the mechanisms of BD carcinogenesis and mutagenesis, because the epoxides exhibit significantly different mutagenicity. Although analyses of *pyr*-Val

in a previous report (14) have greatly enhanced our knowledge of BD metabolism across species, information on HB-Val and THB-Val formation are needed to provide better understanding of species, gender and concentration dependencies in BD metabolism. This report describes the first analysis of all three BD-derived epoxide adducts bound at N-terminal valine in mice and rats after 1BD inhalation exposure at concentrations ranging from those known to induce tumors in rodents and to those approaching human occupational exposures. Hemoglobin adducts from rats and mice exposed to butadiene for 10 days are given in **Tables 5.2** and **Tables 5.3**, respectively.

#### *HB-Val adducts in rats and mice*

The first step in BD metabolism is oxidation by P450 2E1 to EB, which forms the HB-Val adduct. Using nano-UPLC-MS/MS increased sensitivity and allowed detection of HB-Val in mice and rats exposed to BD as low as 0.1 and 0.5 ppm, respectively. The amounts of HB-Val in rats exposed to 0.1 ppm were below the limit of detection. The exposure-responses of HB-Val formation in F344 rats and B6C3F1 mice are plotted in **Figure 5.2**. After 10 days of exposure, female mice formed  $10 \pm 4.4$  more EB, measured as HB-Val formation, than rats at same exposure levels. Thus, mice were shown to be more efficient in EB formation than rats, providing insight into possible mechanisms responsible for the observed species differences in tumor susceptibility. The formation of HB-Val in mice and rats increased with exposure, exhibiting curvilinear dose response curves with no evidence of metabolic saturation. (**Figure 5.2**) These data are in agreement with the study by Troutman *et. al.* (unpublished data) where the EB specific DNA adduct N-7-



(2-hydroxy-3-buten-1-yl)guanine (HB-G I), and N-7-(1-hydroxy-3-buten-2-yl)guanine (HB-G II) formation in the liver were shown to be curvilinear in the same group of mice exposed to BD for 10 days.

*pyr-Val adducts in rats and mice*

The reaction of DEB with Hb produces an N-terminal valine adduct that undergoes an intra-molecular ring closing reaction to form the pyrrolidine adduct, *pyr-Val* (30). Cross-linking with other Hb sites and hydrolysis to THB-Val have also been reported; however, they are minor products compared to *pyr-Val* formation (31). To examine the formation of *pyr-Val*, we used a nanospray-UPLC-MS/MS approach. Similar to HB-Val, this method allowed the detection of *pyr-Val* in mice and rats exposed to BD as low as 0.1 and 0.5 ppm, respectively. The exposure-responses of *pyr-Val* formation in F344 rats and B6C3F1 mice are plotted in **Figure 5.3**. The formation of *pyr-Val* in female mice increased with exposure, and the slopes of formation in mice was steepest at exposures  $\leq 1.5$  ppm (**Figure 5.3 insert**). In female rats exposed for 10 days, progressive decreases in the slope of formation of *pyr-Val* were found at exposures between 1.5 and 62.5 ppm BD with a plateau in formation observed at exposures to 200 ppm BD and above (**Figure 5.3**). The presented exposure-response curves for *pyr-Val* clearly establish a change in the slope at exposures below 1.5 and 6.25 ppm in mice and rats, respectively (see inserts in **Figure 5.3**). In mice, exposures below 1.5 ppm BD produced about 26 pmol/g globin/ppm BD, while at exposures greater than 1.5 ppm, this rate decreased as much as 10-fold at higher exposures, reaching 2.5 pmol/g globin/ppm BD at 625 ppm. These data demonstrate that formation of *pyr-Val* is highest at low exposures

(0.5-1.5 ppm). The exposure-response curve reached a plateau in rats at exposures of 200 ppm or greater, providing evidence for the saturation of metabolic activation pathways in rats. In addition, mice formed  $10 \pm 2.9$  more DEB than rats at similar exposures. These data confirm that mice form much higher amounts of *pyr*-Val than rats, suggesting metabolic differences as a cause for the species differences in cancer susceptibility.

#### *THB-Val adducts in rats and mice*

THB-Val is primarily formed from EB-diol, however, small amounts can be formed by one of the epoxy groups of DEB binding to the N-terminal valine, followed by hydrolysis (31). THB-Val in several species, including rats, mice, monkeys, horses, dogs, and humans has been detected without exogenous BD exposure. The source of this endogenous THB-Val remains unknown, but some studies suggest it may arise from the alkylation reactions of threose and erythrose (32). The exposure response curve for THB-Val adduct in F344 rats and B6C3F1 mice is illustrated in **Figure 5.4**. The formation of THB-Val in mice increased with exposure, and the slopes of formation in mice were steepest at exposures  $\leq 6.25$  ppm (**Figure 5.4 insert**). The exposure-response curves were linear and did not show any signs of metabolic saturation. In contrast to mice, the exposure-response curve in rats was supralinear and decreased in slope at greater than 6.25 ppm BD. This result suggested the metabolic activation of BD to EB-diol becomes less efficient as the exposure increases.

The amounts of THB-Val adducts were approximately 5–40-fold higher than HB-Val and *pyr*-Val adducts in both rats and mice. These data are in agreement with

the direct comparison of THB-Val to THB-G adduct formation in mouse tissue from the same exposures studied by Troutman *et. al.* (unpublished data). The EB-diol specific DNA adduct (THB-G) was the most abundant and exhibited supralinear metabolic activation at 6.25 ppm BD.

#### *Species differences in globin adduct formation*

The data presented suggest that species differences observed in globin adduct formation are directly related to differences in the extent of BD metabolism to its epoxides. BD has been shown to be a potent carcinogen in mice, inducing neoplasms at multiple sites. In contrast, rats were less susceptible, developing neoplasms only at high BD concentration. The data from long term studies in mice and rats are in agreement with the studies on globin adduct formation. Determination of all three adducts (HB-Val, *pyr*-Val, and THB-Val) in rodents demonstrated that mice form much higher amounts of the adducts (6–10- fold) than rats under identical exposure conditions ranging from 0.1 – 6.25 ppm BD. The data from this study point to DEB as the key metabolite, producing the observed species diversity in BD susceptibility. The formation of *pyr*-Val increases with exposure in mice and saturates in rats at exposures greater than 200 ppm BD, clearly demonstrating different kinetics in DEB formation. These findings are consistent with *in vitro* metabolism data from Filser *et al.* (2007), reporting higher amounts of DEB formation in mice compared to rats after EB exposure (33). Furthermore, these data are in agreement with the study of Walker and Meng *et al.* (34-35), demonstrating greater formation and potentially increased mutagenicity of DEB in mice compared to rats.

### *Gender differences*

Multiple BD studies have revealed gender differences in the development of neoplasia, *Hprt*, and amounts of DNA-DNA cross-links formed. Mutagenicity studies measuring BD-induced *Hprt* gene mutations in mice and rats have demonstrated gender differences in sensitivity, with rat and mouse females having 2.3–3-fold greater *Hprt* Mfs than their male counterparts (9, 36). Furthermore, Thornton-Manning et al. (1997) reported an approximately 6-fold greater formation of DEB in the blood of female mice after a single 6 h inhalation exposure (37).

By comparison, female rats and mice had more *bis*-N7G-BD, racemic and meso DNA-DNA cross-links than males at high exposures (200 ppm and 625 ppm) (15). However, our study showed no significant gender differences for the three BD epoxide adducts at the exposure concentrations studied in both rats and mice (**Figure 5.5a and Figure 5.5b**). In addition, there were no gender differences in the amount of N-7 guanine monoadducts in mice. HB-G and THB-G indicate that there is no difference in BD metabolism in female and male rats and mice at multiple exposure concentrations and durations (Troutman *et. al.*; unpublished data). This comprehensive data set strongly suggests that a deficiency in DNA cross-link repair in females is the most plausible hypothesis for the gender differences in mutagenic and carcinogenic susceptibility.

### *Internal adduct dose and EB dose equivalent*

The present globin adduct data provide valuable insight into cross-species comparisons and represents the most comprehensive data set available for mice

and rats. The internal adduct dose of epoxy metabolites of BD ( $D$ ; in  $\text{nMh}\cdot\text{ppmh}^{-1}$ ) was calculated from the measured Hb adducts level ( $A$ ; in  $\text{mol}\cdot\text{g}^{-1}$ ). These were then used to calculate the total genotoxic dose, express as EB dose equivalent ( $D_{EB}$ ), considering the genotoxic potency of the individual epoxide metabolites. The internal adduct doses of all three epoxy metabolites of BD, and the EB dose equivalents for mice and rats are presented in **Table 5.3**. Generally, the internal adduct dose of the epoxide metabolites decreased with exposure (per ppmh) in both mouse and rat. For all three epoxides the highest internal adduct doses per exposure dose were observed at 0.5 ppm. This indicates a more effective metabolism to epoxides at lower exposure levels. A predominant difference between the mouse and the rat was observed with regard to the internal adduct dose of DEB, which was higher in the mouse than the rat by about 30 – 50 times. In addition, the internal dose of DEB in the rat showed saturation at 200 ppm and above. The species differences in DEB formation represent the major cause of the dissimilarity in the calculated EB dose equivalent.

Based on the EB dose-equivalent, the highest dose per ppm exposure was observed at sub-part per million exposures. This demonstrates that metabolism of BD to epoxides is most effective at low exposures. Comparing the EB dose-equivalents between species, mice clearly differ from rats, being ~30 times greater at similar exposures. A quantitative difference in metabolism between the two species suggests that DEB is the major contributor to the EB dose equivalent in the mouse, while in the rat DEB represents a minor fraction of the EB dose equivalent. Therefore, it may be concluded that DEB is the predominant cause of tumor

formation in mouse, and EB together with EB-diol are the causative agents in the rat. This provides important information relevant to susceptibility issues and species differences for BD.

#### *Relative genotoxic potency*

In the present study the relative *in vitro* mutagenic potencies of the three epoxide metabolites were used to calculate the EB dose equivalent from internal adduct doses of epoxides. The EB dose equivalent was then applied as a parameter in a multiplicative risk model. The estimations of mutagenic potencies of the epoxides were calculated from the initial treatment concentration of individual epoxides inducing a doubling of the mutation frequency in *Hprt* loci of human TK6 cells. This procedure might be associated with potential bias, since the relative genotoxic potencies were estimate from *in vitro* data. It would have been desirable to perform *in vivo* mutation tests in parallel with internal adduct dose measurements of the reactive metabolites, but these data are not available. However, the observed mutation frequency of BD and 3-butene-1,2-diol (B-diol), a precursor of EB-diol, in rodent samples (9) is in agreement with the estimated relative mutagenic potency as used in the present study.

#### *EB dose equivalent and mutagenic efficiency*

Direct comparisons of EB dose equivalent to mutagenic efficiency in female mice can be made, since animals from the same exposure have been studied. Walker and Meng et al. determined the *Hprt* mutant frequencies in T-cells from mice

after 10 days of inhalation exposures to 3, 20, 62.5, 200, and 625 ppm BD (9, 38). The induction of *Hprt* mutations in T lymphocytes in BD exposed mice was highest at the lowest exposure (3 ppm BD), similar to the EB dose equivalent. These data demonstrate that metabolism of BD to epoxides and BD mutagenic efficiency are most effective at low exposures. The shape of the dose–response curve for the EB dose equivalent was very similar to the shape of the dose–response curve for the induction of *Hprt* mutations in T lymphocytes in BD exposed mice (**Figure 5.6**). The figure demonstrates that EB dose equivalent and the induction of *Hprt* mutations drops rapidly between 3 and 62.5 ppm BD. Comparison between the biomarker of exposure (EB dose equivalent) and biomarker of effect (*Hprt* mutant frequency) were highly correlated ( $r^2=0.97$ ) (**Figure 5.7**). Additional analyses are needed to fill gaps in the EB dose equivalent and *Hprt* mutant frequency data for male mice and both genders of rats. However, these data in female mice suggest that EB dose equivalent can be a highly predictive biomarker for BD-induced mutations and cancer risk.

#### *Multiplicative cancer risk model*

The EB dose equivalents obtained in the short-term exposure to BD were used to quantitatively estimate the percentage of tumor incidence in the long-term BD cancer test. In this study according to the multiplicative risk model, the data for sites with significant response were evaluated together, that is, all sites, doses, and genders. The survival-adjusted neoplasm rates for B6C3F<sub>1</sub> mice used for fitting the model are presented in **Table 5.1**. The long-term cancer test data from F344 rats

were not evaluated in this study, since the BD exposure levels were higher (1000 ppm and 8000 ppm) than the short-term experimental data available, and lower exposures have not been tested for carcinogenicity.

The fit of the model (**equ.3**) was displayed as the relationship between the percentages of observed and predicted numbers of animals with tumors at any target site (**Figure 5.8**). The goodness of fit of the model demonstrated a significant correlation ( $p < 0.001$ ) between observed tumor incidence and predicted values based on internal adduct doses. In addition, the quantitative estimations of the percentage tumor incidence specific for each BD exposure level in male and female mice are shown in the **Figures 5.9** and **5.10**, respectively. The percentage of tumor incidence increased with exposure as follows: 5% for 200 ppm BD, 15% for 625 ppm BD in male mice; and 4% for 6.25 ppm BD, 15% for 62.5 ppm BD, 15% for 200 ppm BD, 22% for 625 ppm BD in female mice. This approach can be applied to genotoxic agents (or metabolites) when doses in the target tissue can be estimated. The model provides a reasonable result for applying internal adduct doses in estimation of tumor incidence from long-term inhalation exposure to BD.

There is some potential bias in our study. The inclusion of all target sites showing a significant increase in tumor incidence may lead to an over-estimation of the relative potency since the spontaneous lymphomas of aging B6C3F<sub>1</sub> mice were observed and included in all exposure groups. The second source of bias is related to the fact that exposure decreases the overall survival. Exposure-related mortalities first occurred during week 23, mainly in mice exposed to 625 ppm, and were due primarily to the induction of fatal neoplasms and their associated lesions. No female



mice exposed to 200 and 625 ppm or male mice exposed to 625 ppm survived to the end of the study. Survival decreased for males and females exposed to concentrations of 20 ppm or above. The decrease in survival was dose related. However, this problem has been corrected using the survival-adjusted neoplasm percentage as a parameter for fitting the model.

*Application of measurements of butadiene hemoglobin adducts in occupationally exposed workers*

The utility of hemoglobin adducts of BD was investigated in molecular epidemiology studies by Hayes et al. (39) and Albertini et al. (40). The Hayes et al. study examined workers in a BD latex plant for the urinary metabolite (M-1 mercapturic acid butanediol formation), THB-Val, *hprt* mutations, sister chromatid exchanges (SCE), glycophorin A mutations and chromosomal aberrations. THB-Val showed a significant correlation (Spearman's  $\rho = 0.40$ ,  $P = 0.03$ ) with exposure to butadiene, which averaged 1–3 ppm for exposed workers. Urinary M-1 metabolites were also correlated with exposure. None of the genetic toxicity endpoints exhibited any increase in exposed individuals compared to the unexposed. The results indicate that THB-Val is a useful biomarker of biologically effective dose.

The Albertini et al. study was the first to examine both THB-Val and HB-Val. This study had extensive industrial hygiene measurements of BD exposures in the work environment, including individual breathing-zone monitoring during 60 workdays. THB-Val exhibited excellent correlation with exposure responses ( $r^2 = 0.74$ ). Biomarkers for genotoxicity were examined and no increases were associated

with BD exposure. While both hemoglobin adducts and urinary metabolites were good surrogates for exposure, genotoxicity endpoints had no correlation with exposure (40)

Recent BD exposure-biomarker study by Swenberg et al. presented the comprehensive data set available for the three species (rat, mouse, and human) (41). The average air exposures in human studies were 0.29 ppm and 0.81 ppm in monomer workers and polymer workers, respectively. In rodents, the exposures were 0.5 -625 ppm BD for 10 days. The period of exposure in humans was 5.3 times longer than in the rodent studies. Comparing the internal hemoglobin-adducts dose between the three species, the rat appears to be a more representative animal model to study human effects following BD exposure than the mouse. Surprisingly, EB-diol appears to pose the greatest risk for mutations in humans. This provides important information relevant to susceptibility issues and species differences in BD exposure assessment.

Smoking is considered to be an important confounder in occupationally exposed workers. The BD-intake caused by smoking can be calculated assuming 3.5 ppm BD/cigarette for mainstream smoke (42). Accordingly, the median exposure level of 14 ppm BD corresponds to smoking of about 3–4 cigarettes per day whereas the highest BD exposure level of 90 ppm measured in the plant corresponds to smoking of about 22 cigarettes per day (43). Therefore, moderate smoking provides an important contribution to the amount of BD inhaled. The relationship of hemoglobin adducts (THB-Val) and smoking was studied by Begemann et al. (44) and Vacek et al. (45). Accordingly, mean THB-Val

concentrations were significantly higher in BD exposed smokers (567.5 pmol/g globin) than in BD exposed non-smokers (367.2 pmol/g globin). In addition, among unexposed males, smokers had a higher mean THB-Val concentration than did unexposed non-smokers (502 pmol/g globin versus 179 pmol/g globin). Therefore, smoking is identified an important source of BD-exposure and should be accounted for in human BD exposure assessment studies.

## **5.5 Conclusion**

The data presented clearly demonstrate the results of internal dose formation following 10 days exposure from low to high concentrations of BD in rodents. The application of the internal adduct dose equivalent provides valuable insights for cross species comparisons. Mice were shown to be more efficient in metabolism of BD to epoxides than rats, suggesting possible mechanisms responsible for the observed species differences in tumor susceptibility. Furthermore, the carcinogenic potency of BD between species can be quantitatively explained by this approach, using internal adduct doses combined with relative genotoxic potency, and a multiplicative risk model. The model provided estimation of tumor incidence data that are in reasonable agreement with observed numbers in long-term cancer bioassays. These data will be very helpful in science-based risk assessment.

## **5.6 Acknowledgements**

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## Tables

**Table 5.1** Survival-adjusted neoplasm percentage for B6C3F<sub>1</sub> mice and F344 rats in the long-term inhalation studies of 1,3-Butadiene [NTP 1993 TR 434].

Mice			Exposure concentration (ppm)					
Sex	Neoplastic lesion	Target organ	0	6.25	20	62.5	200	625
Males								
	Initial number <sup>a</sup>		70	70	70	70	70	70
	Number of survivors		35	39	24	22	3	0
-	Malignant lymphomas	All	4 (9%) <sup>b</sup>	3 (4%)	8 (10%)	11 (15%) <sup>c</sup>	9 (27%) <sup>c</sup>	69 (97%) <sup>c</sup>
-	Haemangiosarcoma	Heart	0 (0%)	0 (0%)	1 (3%)	5 (14%) <sup>c</sup>	20 (64%) <sup>c</sup>	6 (53%) <sup>c</sup>
-	Alveolar/bronchiolar carcinoma	Lung	22 (48%)	23 (49%)	20 (45%)	33 (75%) <sup>c</sup>	42 (88%) <sup>c</sup>	12 (45%) <sup>c</sup>
-	Papilloma and carcinoma	Forestomach	1 (2%)	0 (0%)	0 (0%)	5 (3%)	12 (29%) <sup>c</sup>	13 (54%) <sup>c</sup>
-	Adenoma and adenocarcinoma	Harderian gland	6 (14%)	7 (15%)	11 (22%)	24 (51%) <sup>c</sup>	33 (81%) <sup>c</sup>	7 (64%) <sup>c</sup>
-	Hepatocellular carcinoma	Liver	31 (45%)	27 (48%)	35 (65%)	32 (62%)	40 (86%)	12 (61%)
Females								
	Initial number <sup>a</sup>		70	70	70	70	70	70
	Number of survivors		37	33	24	11	0	0
-	Malignant lymphomas	All	10 (13%)	14 (27%)	18 (27%)	10 (20%)	19 (40%) <sup>c</sup>	43 (86%) <sup>c</sup>
-	Haemangiosarcoma	Heart	0 (0%)	0 (0%)	0 (0%)	1 (3%)	20 (72%) <sup>c</sup>	26 (83%) <sup>c</sup>

Mice			Exposure concentration (ppm)					
Sex	Neoplastic lesion	Target organ	0	6.25	20	62.5	200	625
-	Alveolar/bronchiolar carcinoma	Lung	4 (9%)	15 (33%)	19 (47%)	27 (61%) <sup>c</sup>	32 (81%) <sup>c</sup>	25 (83%) <sup>c</sup>
-	Papilloma and carcinoma	Forestomach	0 (0%)	0 (0%)	3 (8%)	4 (6%)	7 (23%) <sup>c</sup>	28 (83%) <sup>c</sup>
-	Adenoma and adenocarcinoma	Harderian gland	9 (18%)	10 (23%)	7 (17%)	16 (41%)	22 (71%) <sup>c</sup>	7 (58%) <sup>c</sup>
-	Hepatocellular carcinoma	Liver	17 (33%)	20 (30%)	23 (36%)	24 (51%) <sup>c</sup>	20 (65%) <sup>c</sup>	3 (22%)
-	Adenocarcinoma	Mammary gland	0 (0%)	2 (5%)	2 (10%)	6 (33%) <sup>c</sup>	13 (57%) <sup>c</sup>	13 (67%) <sup>c</sup>
-	Benign and malignant granulo cell	Ovarian	1 (2%)	0 (0%)	2 (3%)	9 (26%) <sup>c</sup>	11 (44%) <sup>c</sup>	6 (47%)

Rats			Exposure concentration (ppm)		
Sex	Neoplastic lesion	Target organ	0	1,000	8,000
Males					
Initial number <sup>a</sup>			110	110	110
Number of survivors			55	50	69
-	Exocrine adenoma	Pancreas	3 (3%) <sup>b</sup>	1(1%)	10 (10%)
-	Glial cell	Brain	1 (1%)	4 (4%)	5 (5%)
-	Leydig cell tumor	Testis	0 (0%)	3 (3%)	8 (8%) <sup>c</sup>

Sex	Rats		Exposure concentration (ppm)		
	Neoplastic lesion	Target organ	0	1,000	8,000
Females					
Initial number <sup>a</sup>			110	110	110
Number of survivors			60	68	76
• Sarcoma		Uterus	1 (1%)	4 (4%)	5 (5%)
• Follicular cell adenoma		Thyroid gland	0 (0%)	2 (2%)	10 (10%) <sup>c</sup>
• Benign or malignant		Mammary gland	50 (50%)	79 (79%)	81 (81%) <sup>c</sup>
• Carcinoma		Zymbal gland	0 (0%)	0 (0%)	4 (4%)

From Melnick et al (1990); National Toxicology Program (1993); Owen (1987); Owen (1993)

<sup>a</sup>Initial numbers include animals removed from study for interim sacrifices.

<sup>b</sup>Mortality-adjusted tumor rates are given in parentheses.

<sup>c</sup>p < 0.05, based on regression analysis with adjustment for intercurrent mortality.

**Table 5.2** Hemoglobin adducts from rats and mice exposed to 1,3-Butadiene for 10 days

Species/ Duration	BD exposure	EB		DEB		EB-diol	
	[ppm]	[pmol/g]	Adduct/ppm BD	[pmol/g]	Adduct/ppm BD	[pmol/g]	Adduct/ppm BD
<b>10 days Female Rats (F344)</b>	0	ND	ND	ND	ND	36 ± 4	
	0.5	0.9 ± 0.1	1.8	0.8 ± 0.1	1.8	2.4 ± 0.3	4.8
	1	1.6 ± 0.5	1.6	1.4 ± 0.2	1.4	29 ± 3	29
	1.5	2.5 ± 0.4	1.7	2.0 ± 0.5	1.3	59 ± 6	39
	6.25	8.1 ± 0.7	1.3	6.7 ± 0.7	1.1	220 ± 19	35
	62.5	60 ± 3	1	47 ± 8	0.8	481 ± 53	7.7
	200	148 ± 19	0.7	123 ± 8	0.6	606 ± 75	3.0
625	410 ± 15	0.7	124 ± 11	0.2	737 ± 74	1.2	
<b>10 days Male Rats (F344)</b>	0	ND	ND	ND	ND	34 ± 4	
	0.5	0.8 ± 0.1	1.8	0.7 ± 0.2	1.4	1.1 ± 0.2	2.2
	1.5	2.3 ± 0.4	1.5	2.0 ± 0.4	1.3	70 ± 3	46
	200	171 ± 0.4	0.9	120 ± 11	0.6	585 ± 7	3.0
625	394 ± 8	0.6	117 ± 12	0.2	796 ± 84	1.3	
<b>10 days Female Mice (B6C3F1)</b>	0	ND	242	ND	ND	35 ± 8	
	0.1	2.3 ± 0.8	23	2.1 ± 1.4	21	15 ± 8	150
	0.5	11 ± 1	22	11 ± 3	22	152 ± 43	304
	1	16 ± 1.2	16	20 ± 2	20	352 ± 34	352
	1.5	30 ± 5	20	38 ± 2	25	525 ± 160	350
	6.25	59 ± 9	9	76 ± 10	12	1008 ± 235	161
	62.5	364 ± 55	6	419 ± 63	7	1448 ± 445	23
200	751 ± 23	4	859 ± 62	4	2587 ± 238	13	
625	3644 ± 534	6	1532 ± 170	2	4490 ± 467	7	
<b>10 days Male Mice (B6C3F1)</b>	0	ND	ND	ND	ND	38 ± 5	
	0.1	2.3 ± 0.5	23	3.0 ± 1.0	30	19 ± 5	190
	0.5	11 ± 1	22	15 ± 2	30	142 ± 17	284
	1.5	32 ± 3	21	50 ± 9	33	260 ± 15	173
	200	1165 ± 112	6	859 ± 126	4	2324 ± 212	12
625	3644 ± 520	6	1980 ± 195	3	4383 ± 373	7	

Endogenous THB-Val was subtracted in all group using data from control animal; 36 pmol/g globin in female rats, 34 pmol/g globin in male rats, 35 pmol/g globin in female mice and 38 pmol/g globin in male mice

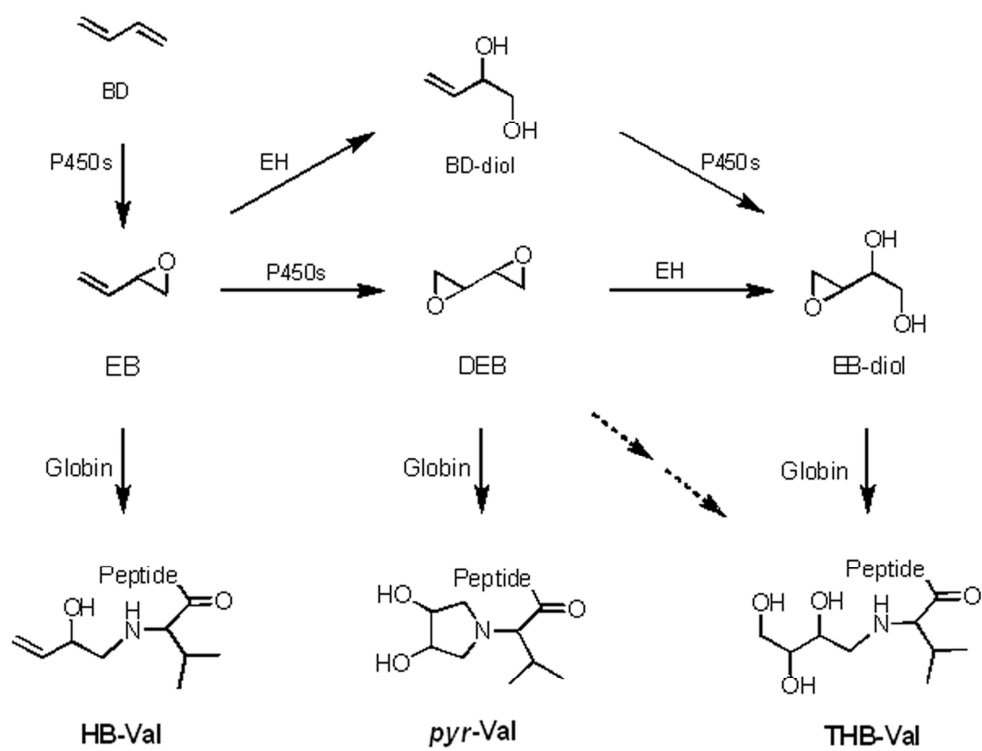


**Table 5.3** Internal adduct dose and EB dose equivalent from rats and mice exposed to 1,3-Butadiene for 10 days

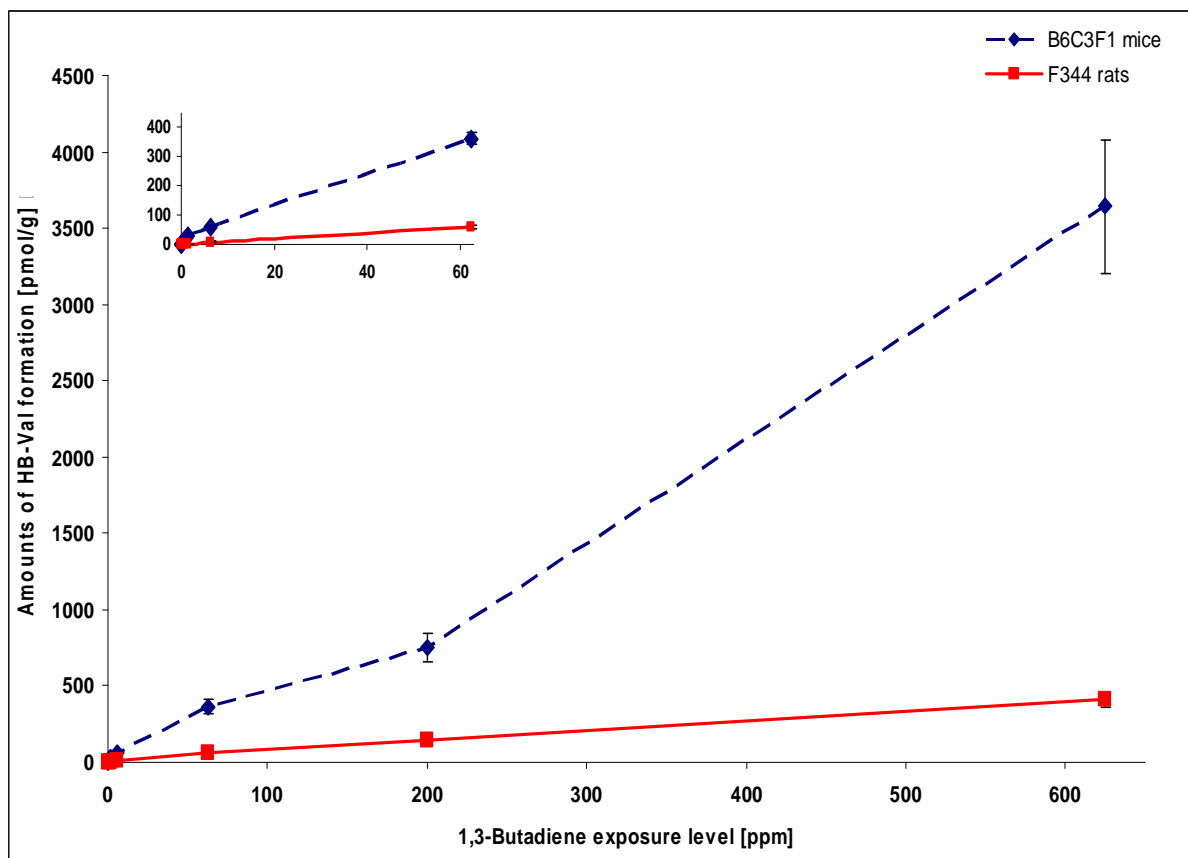
Species/ Duration	BD exposure		Internal adduct dose [mMh/ppmh]			
	ppm	ppmh	EB	DEB	EB-diol	D <sub>EB</sub> -equi.
<b>10 days</b> <b>Female Rats</b> <b>(F344)</b>	0.5	30	0.73	0.48	3.5	49
	1	60	0.67	0.42	21.2	47
	1.5	90	0.64	0.40	28.3	46
	6.25	375	0.54	0.32	26.7	38
	62.5	3750	0.31	0.29	5.6	30
	200	12000	0.25	0.18	2.2	19
	625	37500	0.27	0.06	0.9	6
<b>10 days</b> <b>Male Rats</b> <b>(F344)</b>	0.5	30	0.67	0.42	3.0	47
	1.5	90	0.63	0.40	20.0	43
	200	12000	0.25	0.12	2.2	13
	625	37500	0.23	0.06	0.9	6
<b>10 days</b> <b>Female Mice</b> <b>(B6C3F1)</b>	0.5	30	12	10	242	1060
	1	60	9	7	280	765
	1.5	90	11	9	278	967
	6.25	375	5	4	128	431
	62.5	3750	3	2	18	207
	200	12000	2	2	10	204
	625	37500	3	1	7	105
<b>10 days</b> <b>Male Mice</b> <b>(B6C3F1)</b>	0.5	30	13	11	225	1158
	1.5	90	12	12	137	1240
	200	12000	3	2	9	205
	625	37500	3	1	6	104

Endogenous THB-Val was subtracted from EB-diol prior to calculating EB dose equivalent.

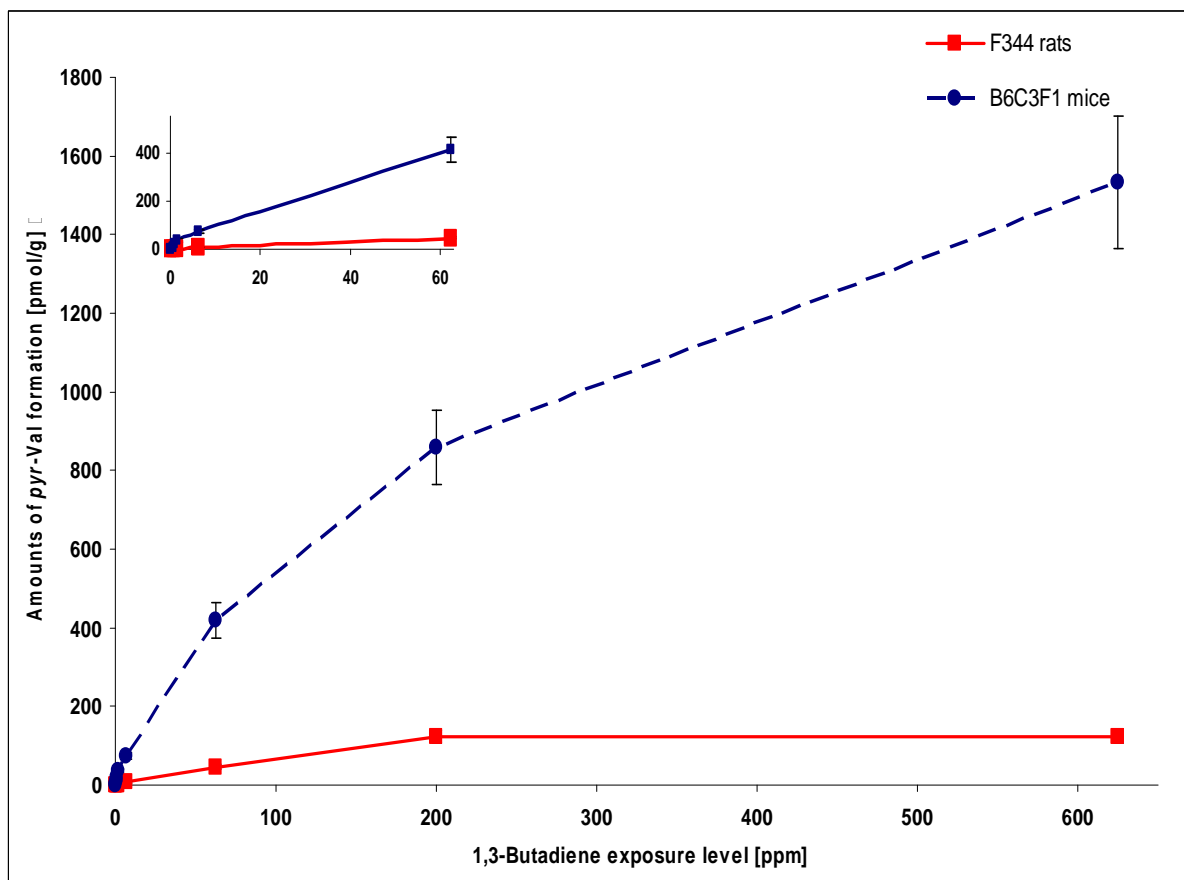
## Figures



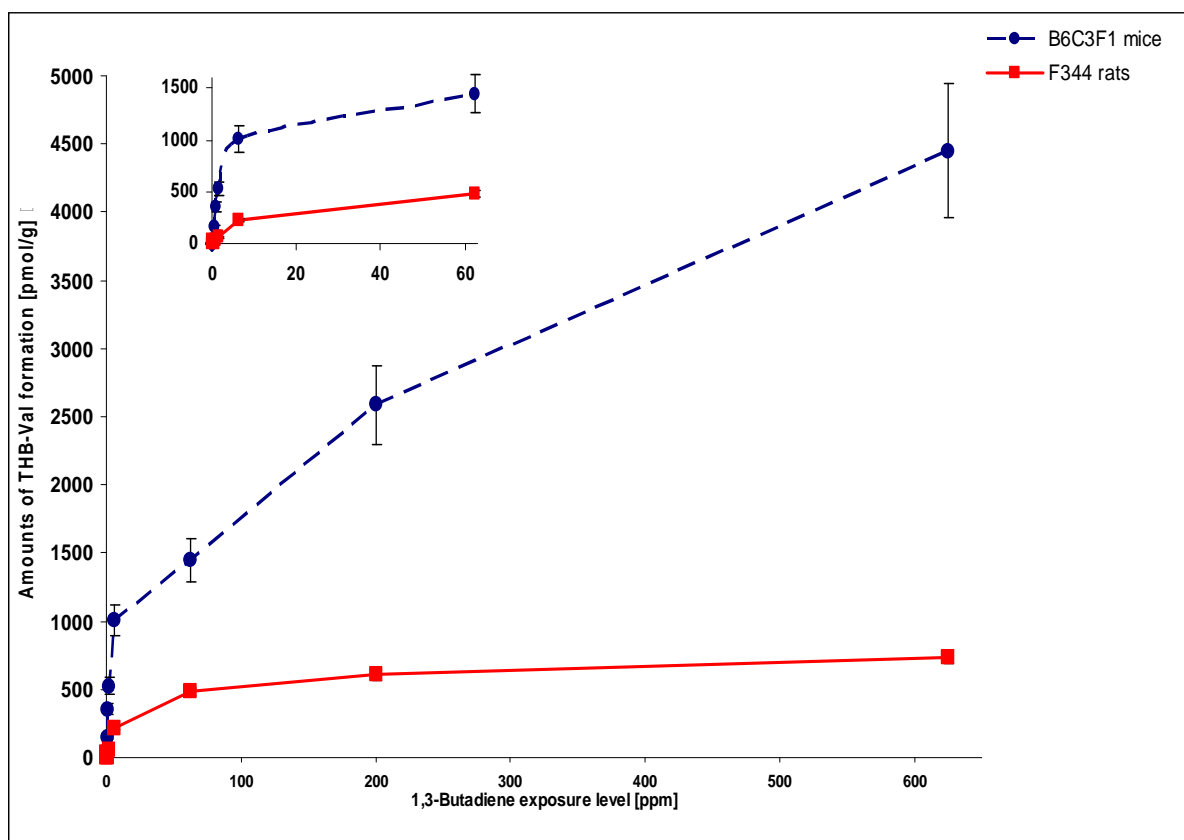
**Figure 5.1** BD metabolism and formation of *N*-terminal valine adducts



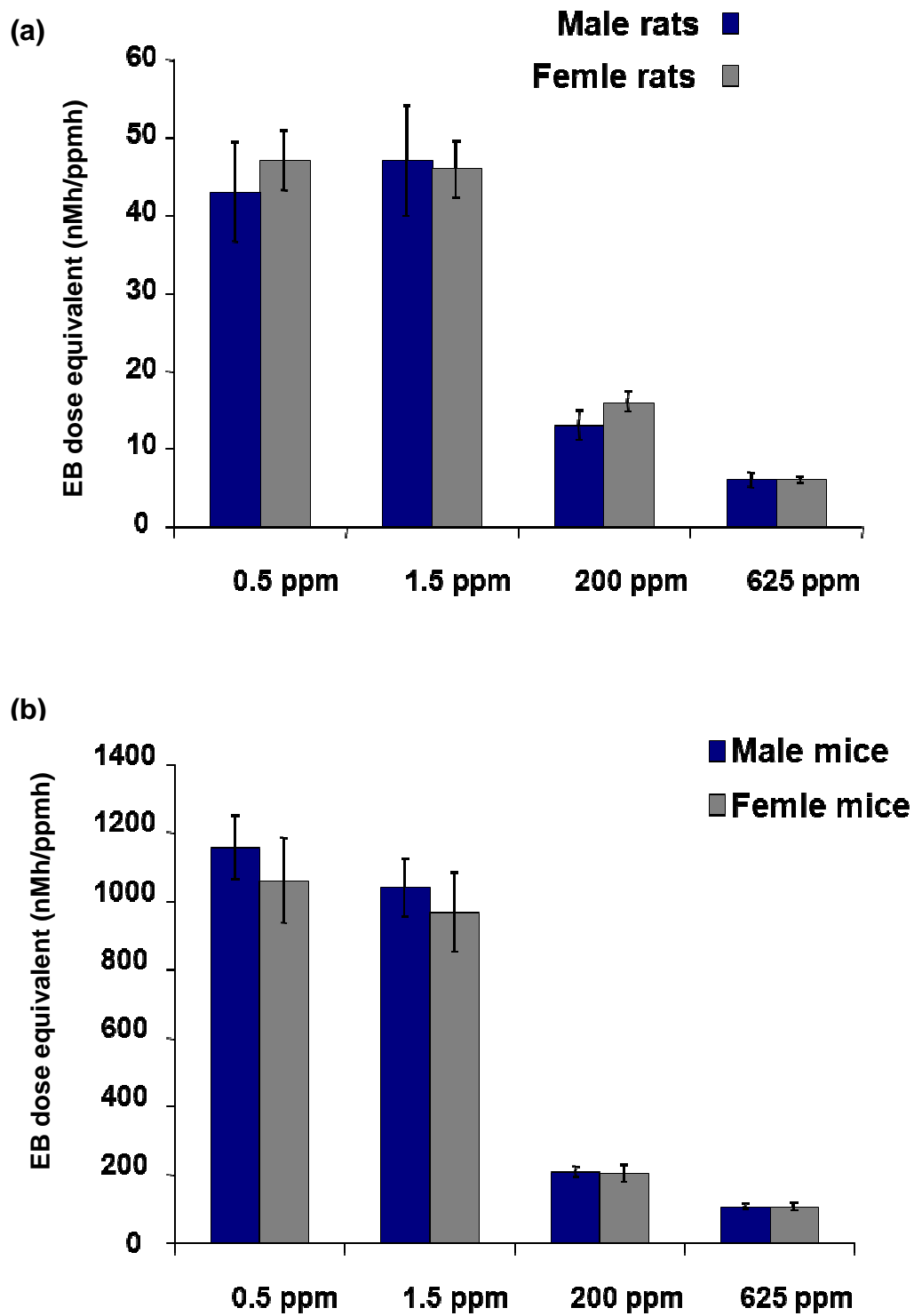
**Figure 5.2** Exposure-response of HB-Val in female B6C3F1 mice and F344 rats



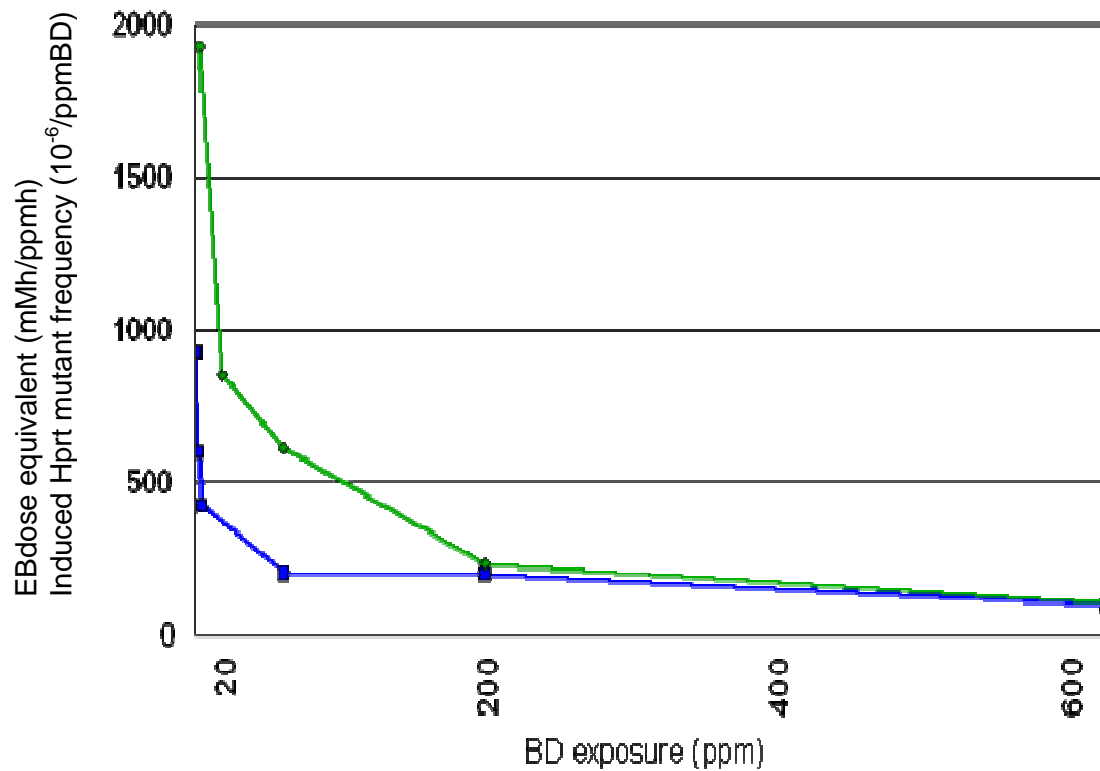
**Figure 5.3** Exposure-response of *pyr*-Val in female B6C3F1 mice and F344 rats



**Figure 5.4** Exposure-response of THB-Val in female B6C3F1 mice and F344 rats



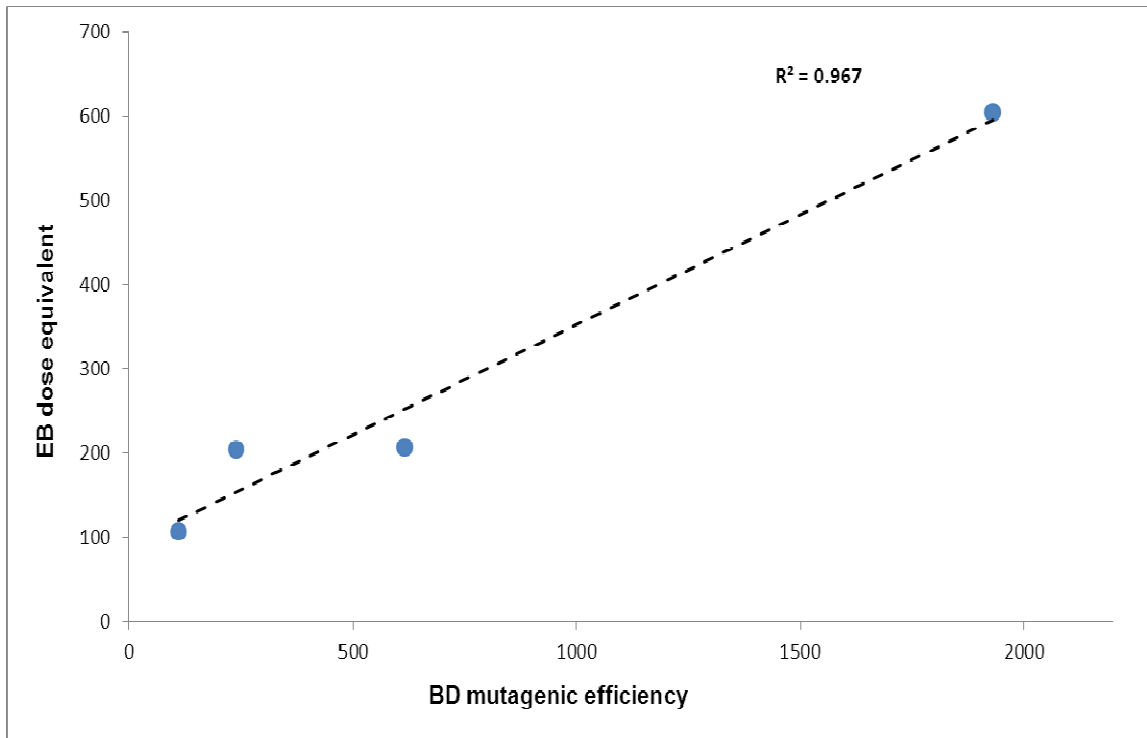
**Figure 5.5** Gender differences in F344 rats (a) and B6C3F1 mice (b) exposed to BD for 10 days



**Figure 5.6** Exposure-response curves of EB dose equivalent

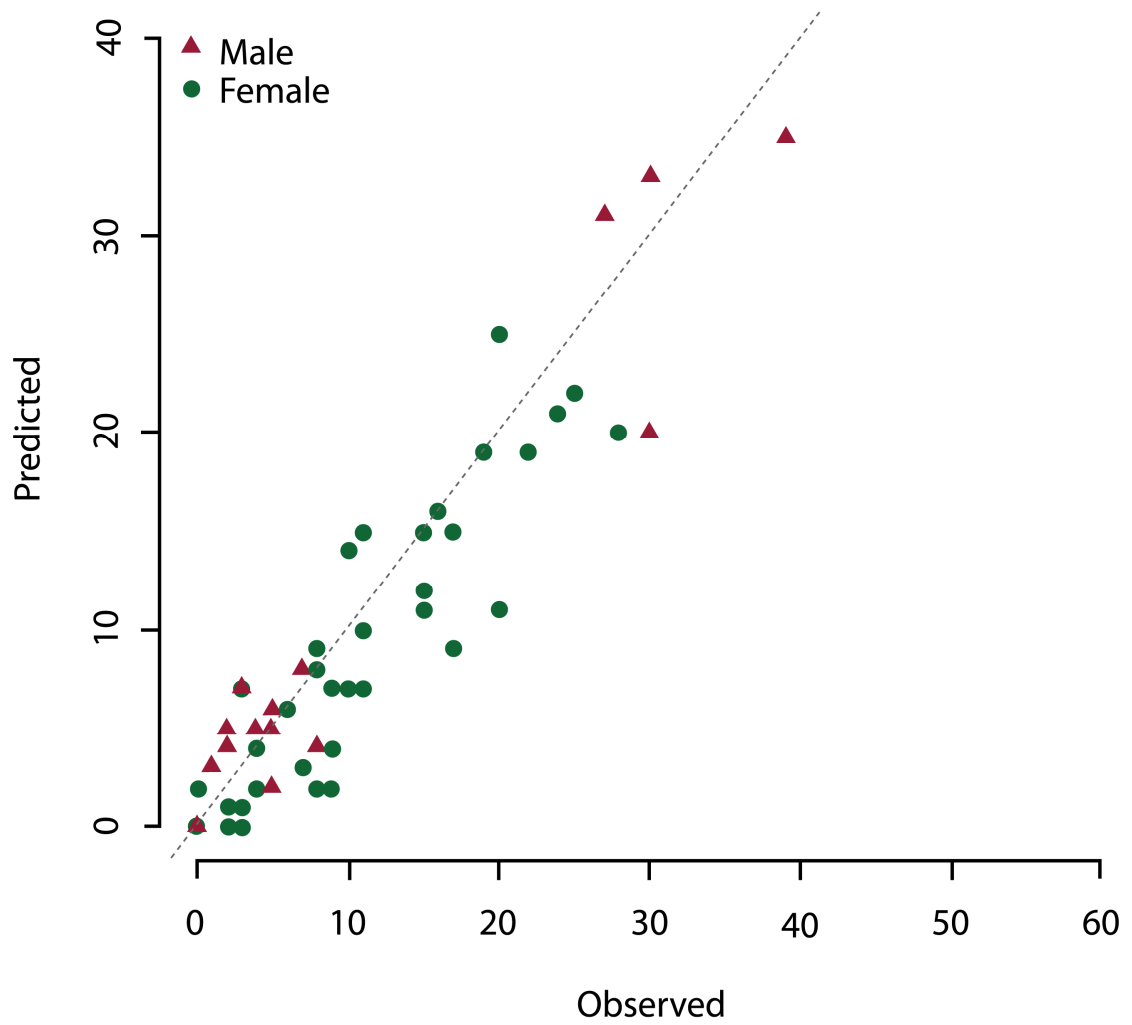
(blue curve) versus BD mutagenic efficiency (induced Hprt mutant frequency/ppm

BD × 100; green line) in female B6C3F1 mice exposed for 10 days to BD

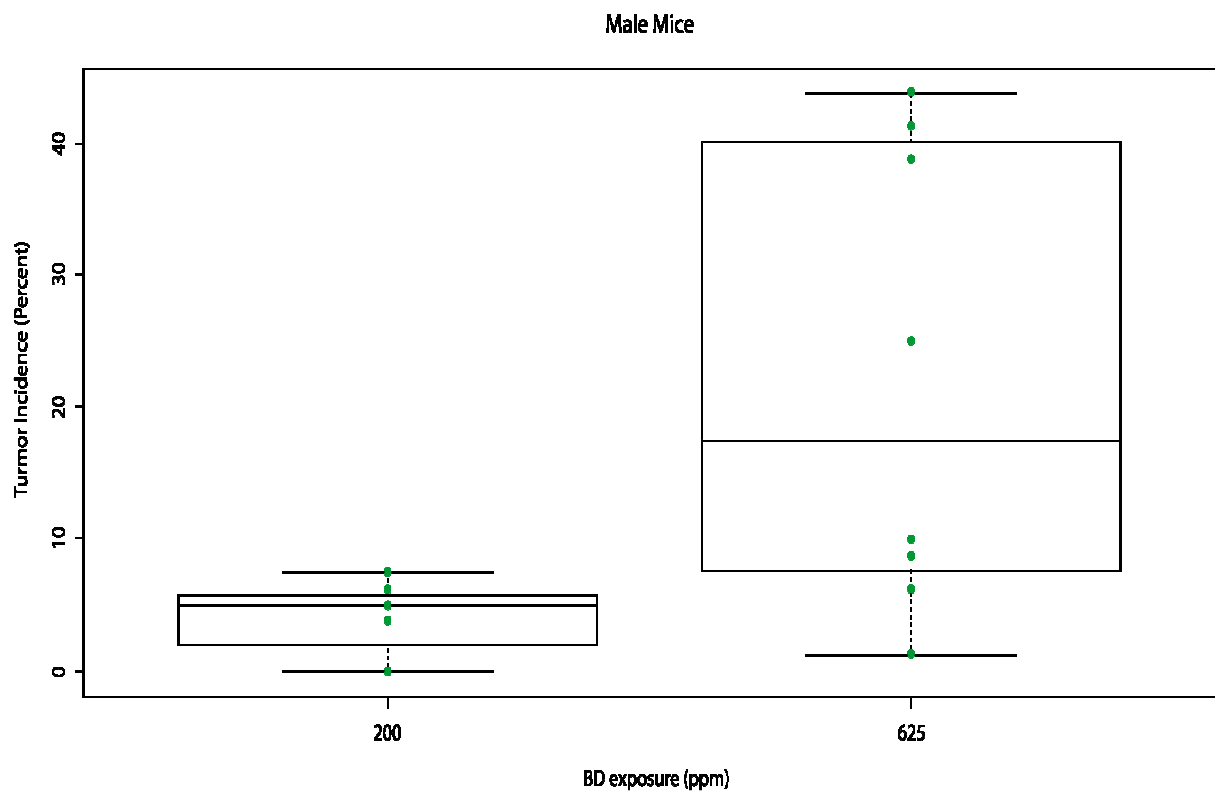


**Figure 5.7** EB dose equivalent (mMh/ppmh) versus BD mutagenic efficiency(induced Hprt mutant frequency/ppm BD  $\times$  100) in female B6C3F1 mice exposed for 10 days to BD

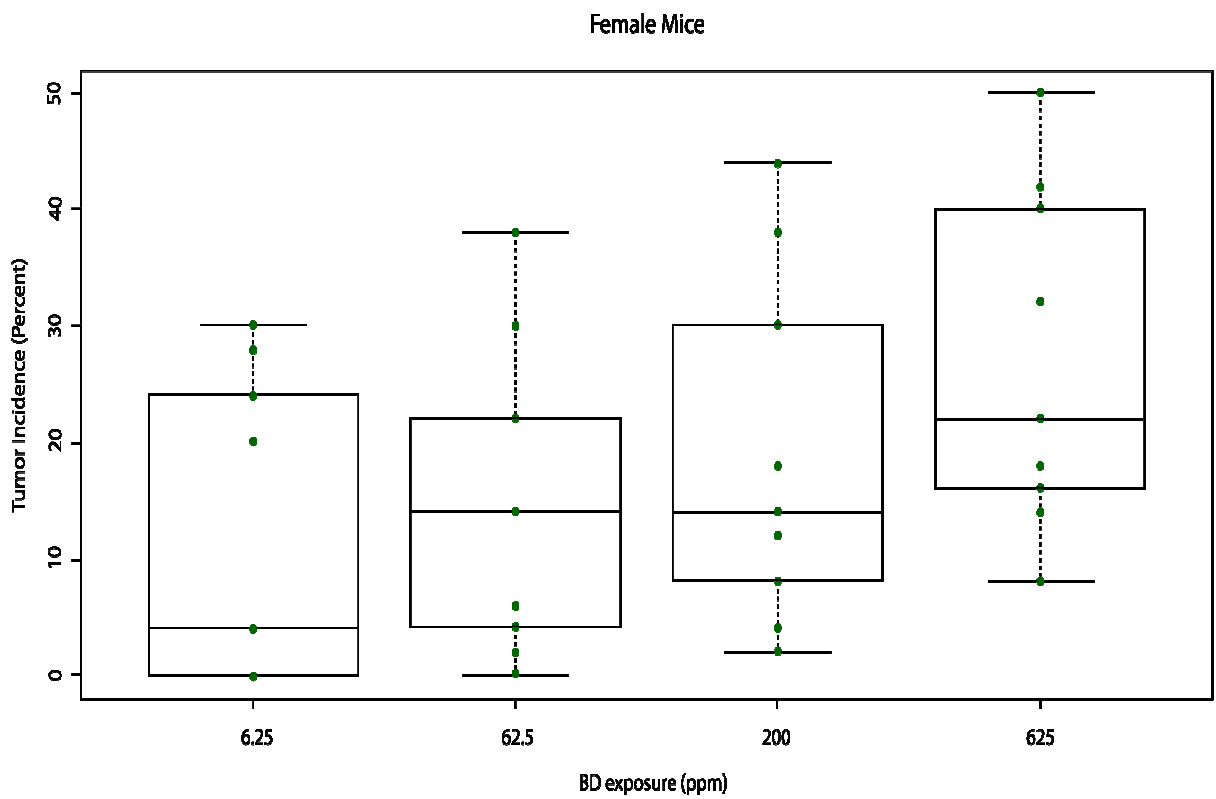




**Figure 5.8** The percentage of predicted versus observed tumor incidence in mice from multiplicative cancer risk model



**Figure 5.9** The estimated percentage of tumor incidence in male mice at different BD exposure levels



**Figure 5.10** The estimated percentage of tumor incidence in female mice at different BD exposure levels

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## CHAPTER VI

### CONCLUSIONS AND FUTURE DIRECTIONS

#### 6.1 Conclusions

1,3-Butadiene (BD) is a known animal and human carcinogen (1, 2). BD inhalation cancer bioassays demonstrated striking interspecies and gender differences, where mice were much more susceptible to tumor formation than rats, complicating human risk assessment (3, 4). A gender difference in susceptibility was also demonstrated in mice, with female mice developing tumors at lower exposures than males. To date, the complexity of BD carcinogenesis largely has been attributed to species-dependent differences in metabolic activation of BD to reactive electrophilic metabolites (5). Species and gender differences related to BD metabolism can be examined by measuring suitable biomarkers. Hemoglobin adducts have been widely used as surrogate biomarkers for exposure. Measurement of globin adducts provides useful information on exposure-specific internal formation of individual epoxide metabolites. In addition, gender differences in mutation induction and DNA repair may further expand important differences between exposed male and female rats and mice.

In this dissertation, we have investigated the internal adduct dose of the individual epoxides to assign the relative risk for each metabolite and to improve our understanding on the molecular mechanisms responsible for major species differences in BD carcinogenicity. This dissertation is presented in four related chapters (Chapters 2-5). Each chapter contains research that makes significant contributions to improving BD exposure and risk assessment.

In chapter 2, we described the methodology for synthesis and characterization of epoxide alkylated peptides to serve as analyte and internal standards for globin adduct quantification in rodents exposed to BD. To synthesize the standard peptides, (1-11) globin peptides specific for rat and mouse were reacted with butadiene monoepoxides (EB and EB-diol) using a direct alkylation method. The peptides were purified by HPLC and characterized by their fragmentation patterns obtained from tandem mass spectrometry (MS/MS). Use of isotope labeled [ $^{13}\text{C}^{15}\text{N}_5$ ] amino acids on N-terminal valine in the oligopeptide synthesis provided the required isotopic labeled peptide for use as an internal standard. This strategy enabled us to obtain pure and well characterized alkylated analyte standard peptides in adequate quantity for quantification of globin adducts in rodent samples exposed to BD.

In chapter 3, we developed a standard peptide quantitation method based on the analysis of their amino acid components after acid hydrolysis, using ultra performance LC-MS/MS (UPLC-MS/MS). Accurate quantitation of the standard peptides was challenging because the amounts of standard peptides available are usually so small that accurate weights cannot be obtained. Additionally, peptides are hygroscopic and easily absorb moisture from the air, and the presence of water and salts leads to overestimation of peptide amounts. To

overcome these limitations, this approach accurately quantitates micromolar solutions of standard peptides, significantly reduces the amounts needed and allows for high throughput quantitative analysis of peptide standards.

In chapter 4, we have developed a simultaneous analysis of all three adducts formed by BD epoxides at the N-terminal valine of globin alpha chain in female F344 rats exposed to 1,3-butadiene via inhalation. Our laboratory has previously developed quantitative immunoaffinity (IA) LC-ESI<sup>+</sup>-MS/MS method for two adducts of EB-specific adduct, HB-Val, and DEB-specific adduct, *pyr*-Val. Recently, the method has been applied with UPLC-ESI<sup>+</sup>-MS/MS. This method allow the measurement of adducts over a wide range of samples from BD exposed mice and rats. However, the application of the method is labor intensive requiring extensive sample processing time since the analysis of all three adducts could not be measured in a single run. Therefore, a simultaneous analysis of all three adducts in a single run represents a great advance in the analysis of N-terminal globin adducts. In this study, IA columns were produced that combined all three antibodies raised against the alkylated standard peptides for sample purification. The sample incubation period had to be adjusted from 1 h to 4 h in order to obtain acceptable recoveries of the method. The improved quantitation method was highly sensitive, accurate, and selective for all three BD adducts, allowing the detection of low amounts of adducts in 50 mg globin in rats from exposure as low as 0.5 ppm BD.

We further applied this method to generate a comprehensive dose response for BD reactive epoxide in female rats exposed to BD. F344 rats were exposed by inhalation to various concentrations of BD ranging form 0.1 to 625 ppm. The control animals did not contain detectable amounts of HB-Val and *pyr*-

Val. In contrast, THB-Val ( $36.8 \pm 3.9$  pmol/g globin) has been detected in control female rats with no exogenous BD exposure as a result of endogenous THB-Val formation. The formation of BD-epoxide adducts increased with increasing exposures. It was most efficient with regards to formation per ppm BD at low exposures. The amounts of *pyr*-Val and THB-Val formation saturated at exposures greater than 200 ppm for 10 days. In contrast, HB-Val linearly increased with exposure. The data reported herein, demonstrated for the first time the formation of all three BD-epoxide globin adducts in female F344 rats. These data will be very helpful for understanding the mechanisms of BD carcinogenesis and mutagenicity.

After our extensive work on method development of all three BD derived epoxides globin adduct, in chapter 5, we applied the improved method to analyze globin samples from both genders of rats and mice exposed to BD. The number of globin adducts were then converted into EB dose equivalents. These were calculated on the basis of the combined internal dose (globin adducts) and the relative genotoxic potency of the respective epoxides inferred from the efficiency of inducing mutations at the *Hprt* locus. Then, the multiplicative cancer risk model was applied to quantitatively estimate tumor incidence using the EB dose equivalent and long-term cancer bioassay data. The evaluation of the cancer data for BD is a challenge because of large species differences in sensitivity, and because all three epoxide metabolites are genotoxic and may contribute to carcinogenesis.

Based on the EB equivalent, higher exposures formed lower amounts per ppm BD. This indicates that metabolism of BD to epoxides is most effective at low exposures. The EB equivalent for mice was about 40-fold higher than that of rats

at similar exposures. No gender differences were noted in globin adducts of mice or rats at all exposures. The application of internal dose equivalents has the potential to provide valuable insight for cross species comparisons. Mice were shown to be more efficient in metabolism of BD to epoxides than rats, demonstrating important mechanisms responsible for the observed species differences in tumor susceptibility. The tumor incidences predicted with the multiplicative cancer risk model correlated well with the earlier observed tumor incidences in cancer bioassays. As such, internal dose equivalents provide quantitative data on biomarkers of exposure that can extend the scientific basis for improved risk assessment.

## **6.2 Future directions**

As mention earlier, we have generated data to address critical species differences in BD metabolism and carcinogenesis. The results, we believe substantially improve our ability to conduct BD risk assessments. However, there are still some critical issues to be further investigated, which will bridge the current gaps of our understanding on BD.

Our study has focused on the quantitative analysis of globin adducts in rodent samples exposed to BD. However, BD metabolism and globin adduct formation in humans remains to be fully elucidated. Presently, We have conducted extensive exposure-biomarker studies on mice, rats and humans (6). Using low exposures that range from current occupational levels to human exposures from tobacco smoke has provided evidence that mice are very different from humans, with mice forming ~200 times more DEB than humans at exposures to 0.1-1.5 ppm BD (6). The studies utilized Edman chemistry, followed

by GC-MS analysis for HB-Val and THB-Val and IA UPLC-MS for *pyr*-Val. Therefore, we are simultaneously quantifying all three BD epoxide globin adducts in workers exposed to BD by using improved IA-UPLC-MS/MS method. We expect to generate extensive exposure-response data valuable insight for cross-species comparisons at low exposures and present the most comprehensive data set available for the three species.

Additional research will be necessary to identify the mechanism(s) responsible for gender differences in mutagenesis and cancer sensitivity. The globin data demonstrated that there were no gender differences in metabolism [(7) and data in Chapter 5], yet female rats and mice have been shown to have increased amounts of DNA cross-links and *Hprt* mutations. The collective data strongly suggests that a deficiency in DNA cross-link repair in females is the most plausible hypothesis for the gender differences in mutagenic and carcinogenic susceptibility. In addition, unpublished preliminary results from our laboratory demonstrate that homologous recombination and nucleotide excision repair are critical pathways for DEB. Therefore, more studies are needed in order to develop data based on gender differences in repair of DNA cross-links. This can be accomplished by exposing both genders of BRCA1 *-/-* mice and wild-type mice to DEB and measuring the *bis*-N7G-BD adducts in tissues over different periods of time post exposure using HPLC-MS/MS method. Such studies are clearly required to establish gender differences in DNA repair pathway. It will also be critical to determine if these gender differences are present in humans, as that would suggest greater susceptibility of females in humans exposed to BD and possible to many other bifunctional chemicals, including many cancer chemotherapeutics.

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