Anorganisch-chemisches Institut der Technischen Universität München

und

Institut für Toxikologie und Umwelthygiene

der Technischen Universität München

Development of analytical methods for the gas chromatographic determination of 1,2-epoxy-3-butene, 1,2:3,4-diepoxybutane, 3-butene-1,2-diol, 3,4-epoxybutane-1,2-diol and crotonaldehyde from perfusate samples of 1,3-butadiene exposed isolated mouse and rat livers

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1 Introduction

1.1 Objective

The gaseous olefin 1,3-butadiene (BD) is an important industrial chemical, primarily used in the production of synthetic rubber. In long-term inhalation experiments, it was a weak carcinogen in rats but a very effective one in mice. Its carcinogenic potency for humans is still under debate. Since BD is biotransformed to a series of reactive metabolites such as 1,2-epoxy-3-butene, 1,2:3,4-diepoxybutane, 3,4-epoxybutane-1,2-diol and probably crotonaldehyde, it is generally accepted that the carcinogenic potency of BD is linked to the body and tissue burden of these metabolites. Therefore a prerequisite to estimate the risk of BD to humans, which is based on available animal studies, is the knowledge of body and tissue burden in rats, mice and humans. While in rat and mouse in-vivo BD exposures can be carried out to quantify this burden, it is not possible in humans on ethical grounds. However, this burden can also be quantified in humans and rodents by using an ex vivo methodology.

Freshly prepared lungs and livers of rats and mice and pieces of lungs and livers of humans, respectively, can be perfused with artificial blood containing BD. The formation of the metabolites can be measured in the effluent perfusate. The obtained data can be incorporated in a physiological toxicokinetic model and the tissue burden of the three species can be calculated. The procedure can be validated by comparing simulated tissue burdens with the in-vivo data gained in rats and mice.

A pre-requisite of this procedure is the availability of highly sensitive quantitative methods for the detection of BD and the metabolites mentioned previously. Therefore, the objective of this work is the development of such methods using a gas chromatograph equipped with a mass spectrometric detector for the analysis of BD and the metabolites, 1,2-epoxy-3-butene, crotonaldehyde, 1,2:3,4-diepoxybutane and 3,4-epoxybutane-1,2-diol. Additionally a method is established for the determination of 3-butene-1,2-diol, being an important intermediate metabolite.

Finally the applicability of these methods is exemplified on BD perfused livers of rats and mice.

1.2 Production, properties and use of 1,3-butadiene

1,3-Butadiene (BD) is a colourless gas. The conjugated diene can form explosive peroxides upon exposure to air (Finar, 1986). Under the influence of sodium as catalyst, BD readily polymerises to a product which is used as a rubber substitute known as buna (butadiene + Na). BD is primarily produced in petrochemical industry via steam cracking of hydrocarbons (Finar, 1986). In 1989, the worldwide BD production was over 6.6 Mio tonnes (IARC, 1999). The major use of BD is found to be in the synthetic rubber industry (Bechtold et al., 1995; Finar, 1986). BD is mainly used in the production of homopolymerised or copolymerised products along with styrene and acetonitrile (Johanson and Filser, 1993), with styrene-butadiene rubber representing the largest produced synthetic rubber in the world (Otto-Albrecht, 1989; Finar, 1986). BD in the form of polybutadiene is also used in the production of car tyres, rubber bands, hoses and shoe soles (Otto-Albrecht, 1989). Copolymers with acetonitrile and styrene are found in motor vehicles and in a variety of articles of daily use, e.g. in household and office articles and cases for electrical gadgets (Otto-Albrecht, 1989; Finar, 1986). Highest BD exposure concentrations were measured at workplaces in BD producing facilities (peaks up to 100 ppm, (IARC, 1999)). Low level urban exposure may also occur through gasoline vapours, automobile exhaust and cigarette smoke (Abdel-Rahman et al., 2001; Brunnemann et al., 1990; IARC, 1999). In 1987, in USA, the yearly industrial BD emission in the atmosphere was recorded to be 4415 tonnes, which fell to 1321 tonnes till 1995 (IARC, 1999). According to IARC (1999) less than 1 to 10 ppb BD was found in urban air.

1.3 1,3-Butadiene metabolism

In mammals, BD is primarily metabolised in the liver but also in the lung resulting in the formation of different metabolites (refer **Figure 1**). Species specificity concerning the burden with BD and its epoxide metabolites was investigated in vivo and in vitro yielding considerable differences. NADPH dependent metabolism in cell fractions was demonstrated by various research groups: Schmidt and Loeser (1985) using postmitochondrial liver and lung fractions of mice, rats, monkeys and humans showed the metabolic formation of 1,2-epoxy-3-butene (EB). More specifically, BD metabolism to EB was studied in liver

microsomes of rats (Malvosin et al., 1979; Bolt et al., 1983; Wistuba et al., 1989; Csanády et al., 1992; Cheng and Ruth, 1993; Maniglier-Poulet et al., 1995) and mice (Wistuba et al., 1989; Elfarra et al., 1991; Csanády et al., 1992; Recio et al., 1992; Sharer et al., 1992a; Duescher and Elfarra, 1992; Maniglier-Poulet et al., 1995). In lung microsomes of rats and mice, BD metabolism to EB was demonstrated by Csanády et al. (1992) and Sharer et al. (1992a). Also in kidney and testis of these species, EB is produced from BD (Sharer et al., 1992a). BD metabolism to EB was also shown in human liver (Csanády et al., 1992; Duescher and Elfarra, 1994) and human lung (Csanády et al., 1992).
These findings were also established from in-vivo studies. In BD exposed rodents, EB was found in the exhaled air (rat: Filser and Bolt, 1984; Meischner, 1999; mouse: Kreiling et al., 1987; Meischner, 1999) and in blood (rat: Thornton-Manning et al., 1995a; Thornton-Manning et al., 1995b; Bechtold et al., 1995; Himmelstein et al., 1994; Himmelstein et al., 1996; Thornton-Manning et al., 1997; Thornton-Manning et al., 1998; mouse: Thornton-Manning et al., 1995a; Thornton-Manning et al., 1995b; Bechtold et al., 1995; Himmelstein et al., 1994; Himmelstein et al., 1996; Thornton-Manning et al., 1997). It was also detected in blood of BD exposed monkeys (Dahl et al., 1990; Dahl et al., 1991).

A large portion of EB is further metabolised via three pathways. One of them involves its catalytic hydrolysis mediated by epoxide hydrolase (EH) as has been demonstrated in liver microsomes of rat (Malvosin and Roberfroid, 1982; Bolt et al., 1983; Cheng and Ruth, 1993; Kreuzer et al., 1991; Csanády et al., 1992; Krause et al., 1997b), of mouse and human (Kreuzer et al., 1991; Csanády et al., 1992; Krause et al., 1997b) as well as in pulmonary microsomes of these three species (Csanády et al., 1992). The same hydrolytic pathway leading to 3-butene-1,2-diol (B-diol) was deduced from the in vivo finding of a metabolite N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine excreted in urine of BD exposed rodents (Sabourin et al., 1992; Bechtold et al., 1994; Nauhaus et al., 1996), monkeys (Sabourin et al., 1992) and also of BD exposed workers (Bechtold et al, 1994). Hydrolysis of *R*- and *S*-EB to the corresponding enantiomer of B-diol was nearly completely stereospecific in liver microsomes of rats, while in those of mice, an inversion of the configuration was observed (Nieusma et al., 1998).

The other pathways of EB involve the glutathione S-transferase (GST) catalysed conjugation with glutathione, observed in rat liver cytosol (Bolt et al., 1983; Sharer et al., 1992b;

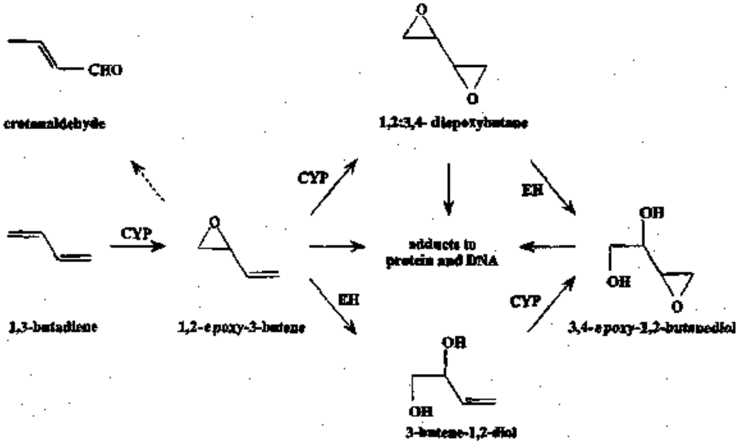
Kreuzer et al., 1991; Csanády et al., 1992) and in mouse as well as in human liver cytosol (Kreuzer et al., 1991; Csanády et al., 1992; Sharer et al., 1992b). Corresponding catabolites of the GSH conjugates with EB were detected in urine of BD exposed rodents (Sabourin et al., 1992; Sharer et al., 1992a; Elfarra et al., 1995) and of BD exposed workers (Hallberg et al., 1997).

The first step of another pathway of EB represents the CYP450 catalysed formation of 1,2:3,4-diepoxybutane (DEB), which has been shown in EB exposed liver microsomes of rats (Seaton et al., 1995; Krause and Elfarra, 1997a), mice (Seaton et al., 1995; Krause et al., 1997a) and humans (Csanády et al., 1992; Seaton et al., 1995; Krause et al., 1997a). The same product was determined in blood of mice (Himmelstein et al., 1994; Himmelstein et al., 1995; Bechtold et al., 1995; Thornton-Manning et al., 1995a, Thornton-Manning et al., 1997) and also in blood of rats (Bechtold et al., 1995; Thornton-Manning et al., 1995a; Thornton-Manning et al., 1997; Thornton-Manning et al., 1998) following exposure of both rodent strains to BD.

Crotonaldehyde (CA) was formed NADH-dependently as a minor metabolite of BD in microsomes obtained from liver, lung or kidney of male B6C3F1 mice (Sharer et al., 1992a) and from human liver (Duescher and Elfarra, 1994).

The formation of 3,4-epoxybutane-1,2-diol (EBD) which could be generated by the oxidation of B-diol or by hydrolysis of DEB has been tentatively found in BD exposed rat liver microsomes (Cheng and Ruth, 1993).

There were drastic species differences in the formation rates of these metabolites, not all of them being detectable in every species investigated. Especially, DEB was by far less in rats as compared to mice, (comparatively summarised in IARC, 1999).



CYP: Cytochrome P450 dependent monooxygenases

EH: Epoxide hydrolase

Figure 1: Overview of pathways of 1,3-butadiene metabolism

1.4 Mutagenic and carcinogenic properties of 1,3-butadiene and its selected metabolites

1,3-Butadiene is an indirect mutagen. Metabolic activation of BD is required to exert mutagenic effects. All three epoxides of BD, namely EB, DEB and EBD, were mutagenic (summarised in IARC, 1999). Mutagenicity is probably linked with the capability of these compounds to form adducts to macromolecules, especially to DNA, as has been demonstrated by several working groups (reviewed and summarised in IARC, 1999). Of these epoxides, DEB having two reactive sites was shown to be able to form DNA-DNA (Ristau et al., 1990) and DNA-protein (Costa et al., 1997) cross-links. These findings might explain the by far higher mutagenic potency of this compound compared to the others.

Concerning the carcinogenic potency of BD, a drastic species difference between mice and rats was observed. In inhalation studies carried out with the former species, BD was a highly effective carcinogen (Huff et al., 1985; Irons et al., 1989; Melnick et al., 1990). It evoked dose dependent tumours at several sites. After 2 years of exposure, lung tumours were increased already at the lowest concentration of 6.25 ppm BD (Melnick et al., 1990). Contrastingly, in the only long-term carcinogenicity study carried out in rats so far, BD was only a weak carcinogen (Owen et al., 1987). Animals had been exposed to 0, 1000 and 8000 ppm BD (6h/day, 5day/week). Tumours occurred only in the highest dose group.

For humans, too, BD has been concluded to be carcinogenic (DFG, 1998) based on the outcome of epidemiological studies (summarised in IARC, 1999) and considering its metabolism to epoxides, which had been demonstrated in vitro (see above). However, based on the same data IARC 1999 evaluated BD only as "probably carcinogenic to humans". However, concerning the carcinogenic potency of BD it has been speculated that humans behave more like rats than like mice (Bond et al., 1995), which means BD would be by far less effective in the human species than it is in the mouse.

From the findings that the mutagenic activity of BD resulted from epoxides formed in the BD catabolism, it was concluded that the BD induced carcinogenicity could originate from the tissue burden by these intermediates (Malvosin and Roberfroid, 1982; Filser and Bolt, 1984; Schmidt and Loescher, 1985; Kreiling et al., 1986; Kreuzer et al., 1991), several of which being proven carcinogens.

For EB, only one single animal study is available. A dose of 100 mg, 3days/week over the entire lifetime was administered epicutaneously to 300 male mice. There was a small increase in skin tumours compared to the controls (Van Duuren et al., 1963).

DEB was also tested for carcinogenicity in mice by skin painting (Van Duuren et al., 1963; Van Duuren et al., 1965). Compared to the controls, a significant increase of skin tumours was observed at both administered doses (3 and 10 mg per week for life). In a second study, the same authors administered DEB subcutaneously to rats for more than one year (1 mg/animal/w). At the injection site, a high induction of malignant tumours was observed in contrast to the controls, which were negative (Van Duuren et al., 1966). In another study, DEB was intraperitoneally administered to mice (12 injections thrice weekly), yielding total doses of 1.7 to 192 mg/kg. The compound led to an increase of the incidence of diverse lung tumours (*Shimkin et al., 1966, cited in IARC, 1999*).

The carcinogenic potency of EBD has not been investigated.

1.5 Quantitative determination of 1,3-butadiene metabolism

In order to get information on the human burden with BD and its metabolically produced epoxides, several physiological toxicokinetic models have been developed (Csanády et al., 1992; Johanson and Filser, 1993; Filser et al., 1993; Kohn and Melnik, 1993; Johanson and Filser, 1996 and Csanády et al., 1996). Such models describe the toxicokinetic behaviour of a compound in an organism respecting physiological parameters as blood flow, tissue volumes etc. The most advanced ones for BD enable to simulate concentrations of BD, EB and DEB in tissues of mice, rats and humans resulting from various exposure conditions (Johanson and Filser, 1996; Kohn and Melnik, 1993; Csanády et al, 1992). For comparable conditions of BD exposure, these models predict distinctly lower burdens with both epoxides for humans than for both rodent species rats and mice. Also, adducts of EB to the N-terminal valine of haemoglobin (Tretyakova et al., 1996), which can be used to estimate the EB burden of blood resulting from BD exposure, indicate for humans a by far lower EB burden. This burden can be expressed by the "haemoglobin binding index", defined as pmol adducts / (g globin x ppm x h). Haemoglobin binding indices have been determined to be 0.3 - 0.5 in mice and 0.09 - 0.3 in rats but only 0.0005 - 0.004 in humans (Tretyakova et al., 1996). Only limited data are reported on adducts of the intermediate EBD on the N-terminal valine of haemoglobin. No findings have been published on DEB in humans. Due to these gaps of knowledge a rational, on mechanistic data based estimate of the carcinogenic human risk (the dose dependent probability of getting cancer) from BD is not yet possible.

In order to obtain the required knowledge on the BD related tissue burden by EB, DEB and EBD, the isolated, once through perfused liver can be used according to Bitzenhofer (1993). Livers of rodents can easily be gained and also fresh liver pieces of human donors can be obtained occasionally from medical explantations. The perfused liver system has several advantages over other in-vitro systems; it can be considered as an ex-vivo system. BD and its metabolites can be delivered to the liver similar to the in-vivo situation. The highly complex kinetic interactions of BD and its metabolites (several of them being metabolised simultaneously by the same enzymes) can proceed as in vivo and the resulting mixture leaving the liver can be directly determined from the effluent. The obtained data can be incorporated into a physiologic toxicokinetic model for BD and the resulting body and tissue burden by the investigated metabolites can be predicted for different situations of in-vivo exposure to BD. However, in order to determine quantitatively all of the BD-metabolites mentioned above from small perfusate samples of BD perfused livers, highly sensitive, selective and easy to handle methods are required.

1.6 Available analytical methods for determining 1,3-butadiene and selected metabolites

Several analytical methods have been published describing the quantitative determination of EB, CA, DEB, B-diol and EBD in biological material. These methods are shortly described below.

Various analytical procedures are available for the determination of BD. Bechtold et al. (1995) described a sensitive and selective method for BD determination from blood samples of rats and mice exposed to low BD concentrations. In order to achieve sufficient sensitivity, the blood samples were vacuum distilled (so that BD and its volatile metabolites were separated from the non-volatile ones) and the gaseous distillate was condensed in a cold trap held at −196°C by liquid nitrogen. Analysis was carried out by using a gas chromatograph equipped with a mass spectrometric detector (GC/GC/MS). BD was also detected from blood (Sato and Nakajima, 1979; Himmelstein et al., 1994; Himmelstein et al., 1996), rodent lung and liver samples (Himmelstein et al., 1995; Himmelstein et al., 1996) and from simulated saliva in contact with chewing gum (Abrantes et al., 2000) by using simple headspace methods and gas chromatographs equipped with flame ionisation detectors (GC/FID). To determine the average contamination levels of airborne BD in factories processing rubber and plastics containing BD, Yoshida et al. (2002) collected air samples for 10 minutes with a charcoal tube and a portable small pump adjusted to a certain flow rate. The samples were subjected to GC/MS after thermal desorption from the charcoal. Meischner (1999) determined actual atmospheric concentrations of BD by directly collecting air samples with disposable polypropylene syringes and injecting the gas samples onto the column of a GC/FID via a gas loop.

For determination of EB from rodent blood (Himmelstein et al., 1994; Himmelstein et al., 1996; Leavens et al., 1996) and from rodent lung and liver (Himmelstein et al., 1995; Himmelstein et al., 1996), samples were extracted with dichloromethane and analysed using GC/MS running in the electron impact (EI) ionisation mode. Elfarra et al. (1991) analysed EB and CA from toluene extracts of rat liver microsomes exposed to BD by GC/FID. Cheng and Ruth (1993) determined the same compounds from microsomal incubations by headspace analysis with GC/FID. For EB determination from rodent tissues Thornton-Manning et al. (1995a) used a vacuum line-cryogenic distillation followed by GC/GC/MS analysis. A similar procedure was used by Bechtold et al. (1995) for the determination of EB from blood samples.

Like EB, similar procedures have been reported for the determination of DEB from blood (Himmelstein et al., 1994; Himmelstein et al., 1996; Leavens et al., 1996) and from rodent lung and liver samples (Himmelstein et al., 1995; Himmelstein et al., 1996). The samples were extracted with dichloromethane and analysed by GC/MS in the EI mode. Krause and Elfarra (1997a) and Cheng and Ruth (1993) published a GC/FID method for DEB determination from liver microsomes after extraction with ethyl acetate. DEB was also detected by a more complicated method from blood samples (Bechtold et al., 1995) and from rodent tissues (Thornton-Manning et al., 1995a) exposed to BD. The samples were subjected to vacuum distillation where the volatile BD metabolites were separated from the non-volatile ones. The gaseous distillates were then condensed into a cold trap held at –196°C by liquid nitrogen and analysed using a GC/GC/MS.

B-diol was determined from rat urine following extraction with i-propanol and analysis by GC/FID (Anttinen-Klemetti.T. et al., 1999). Kemper et al. (1998) published a method for the determination of B-diol from blood samples by solid phase extraction with ethyl acetate followed by derivatisation with N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane and analysed by GC/MS. Cheng and Ruth (1993) specified a direct extraction method with ethyl acetate followed by detection of B-diol with GC/FID.

For our purpose, all of these methods are either not selective or not sensitive enough.

For EBD, no reliable analytical method has been published though its detection as a BD metabolite in liver microsomal incubations have been claimed by Cheng and Ruth (1993) (for further explanation see discussion). This epoxide could represent a toxicological important not investigated metabolite of BD, since in a binding study in rats and in few humans exposed to BD, high levels of adducts to N-terminal valine of haemoglobin (N-(2,3,4-trihydroxybutyl)valine) were determined, which could result from EBD (Perez et al., 1997; Begemann et al., 2001). There were 30 times (rats) and about 70 times (humans) higher binding levels of this adduct than of those resulting from EB.

1.7 Aim

The following work is part of a bigger study aimed to quantify the body and tissue burden of BD exposed mice, rats and humans by several mutagenic BD metabolites (EB, CA, DEB, EBD) and by the metabolic intermediate B-diol with the final goal to elaborate the carcinogenic risk of BD exposed humans based on the outcome of long-term animal exposure experiments.

The focus of the present work is to develop simple, fast, selective and sensitive analytical methods for the detection of BD and the selected metabolites in micro amounts (picogram) from perfusate samples of isolated, perfused rat and mouse livers.

At first, a headspace method had to be developed using GC/FID to measure BD concentration in the perfusate of perfused livers. Furthermore, selective and sensitive analytical methods had to be developed for each of the selected metabolites, EB, CA, DEB, B-diol and EBD, which allow to determine their concentrations in the liver perfusate.

For the somewhat lipophilic compounds EB, CA and DEB, the analytical procedures ought to be based on the direct extraction of the analytes from the perfusate with dichloromethane followed by GC/MS analysis. The highly hydrophilic compounds B-diol and EBD were intended to be derivatised from the perfusate as boron esters and analysed by GC/MS after extraction with ethyl acetate.

Finally, the established methods had to be applied to the perfused liver system, thus enabling the quantitation of BD together with the selected metabolites.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

The purity of the chemicals, if not specifically mentioned, is of highest analytical grade

Acetone PESTANAL®	Riedel-de Haën, Seelze
Acetonitrile, Chromasolv	Riedel-de-Haën, Seelze
Albumin, Fraction V, No. A-2153, 96%	Sigma-Aldrich,Deisenhofen
Ammonia, 3.6	Linde, München
1,3-Butadiene,2.5	Linde, München
(R,S)-3-Butene-1,2-diol, 98 %	Emka-Chemie, Neufahrn
3-Butene-1,2-diol-D_6, 98 %	Synthon, Augsburg
n-Butylboronic acid, 97 %	Sigma-Aldrich, Steinheim
Calcium chloride	Merck, Darmstadt
Calcium chloride dihydrate	Merck, Darmstadt
Chloroform, SupraSolv®	Merck, Darmstadt
Crotonaldehyde, 99.5 %, *E* / *Z* isomer = 1:25	Fluka, Steinheim
racemic 1,2:3,4-Diepoxybutane ,99.5 %,	Aldrich, Steinheim
1,2:3,4-Diepoxybutane-D6, 98%, meso / racemic = 1:1	Synthon, Augsburg
Diethyl maleate, 95 %	Merck, Darmstadt
2,2'-Dimethoxypropane	Sigma-Aldrich, Steinheim
N,N-dimethylformamide , > 99.8 %	Fluka, Steinheim
Dimethylsulphoxide, 99.7 %	Fluka, Steinheim
1,2-Epoxybutane, 99 + %,	Sigma-Aldrich, Steinheim
3,4-Epoxy-1,2-butanediol, 98%, meso / racemic (R:S) = 1:1:1	Synthon, Augsburg

3,4-Epoxy-1,2-butanediol-D_4, 98 %, meso / racemic (R:S) = 1:1:1	Synthon, Augsburg
1,2-Epoxy-3-butene ,98 %	Sigma-Aldrich, Steinheim
meso-Erythritol, 99 %	Sigma-Aldrich, Steinheim
Ethanol	Merck, Darmstadt
Ethyl acetate	Riedel-de Haën, Seelze
Forene® (isoflurane)	Abbott, Wiesbaden
Glucose monohydrate	Merck, Darmstadt
Helium 5.0	Linde, München
Hexane PESTANAL®	Riedel-de-Haën, Seelze
Hydrochloric acid,~ 10 mol/L	Merck, Darmstadt,
Hydrogen 5.0	Linde, München
N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid, HEPES	Sigma-Aldrich, Steinheim,
Krebs–Henseleit-Buffer-Powder	Sigma, Deisenhofen
Liquemin® N 25000	Hoffmann- La Roche, Grenzach-Wyhlen
Magnesium sulfate, anhydrous	Merck, Darmstadt
Methane, 4.5	Linde, München
Methanol	Merck, Darmstadt
Methylenchloride	Riedel de Haën, Seelze
Multi-Enzyme, LIN-TROL®	Sigma Diagnostics Inc., St. Louis, USA
Nitrogen 5.0	Linde, München
Perchloric acid	Aldrich, Steinheim
Potassium carbonate	Merck, Darmstadt
Potassium chloride	Merck, Darmstadt
Potassium phosphate, monobasic	Merck, Darmstadt
2-Propanol, SupraSolv®	Merck, Darmstadt,
Sodium bicarbonate	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium hydroxide	Merck, Darmstadt

Sodium thiocyanide, >98.0 %,	Sigma-Aldrich, Steinheim
Synthetic Air	Linde, München
Test-Kit 826-A "Lactate"	Sigma Diagnostics Inc., St. Louis, USA
Test-Kit 726-UV "Pyruvate"	Sigma Diagnostics Inc., St. Louis, USA
D,L-Threitol (D,L-THB), 97 %	Sigma-Aldrich, Steinheim
Trifluroacetic acid, >99.06 %	Fluka, Steinheim
bis(Trimethylsiliyl)trifluoroacetamide / Trimethylchlorsilane, 99/1,	Sigma-Aldrich, Steinheim
Water, deionized	GSF, Neuherberg
Xylitol, 99 %	Sigma-Aldrich, Steinheim

Handling of all chemicals during different sample preparations was carried out under the hood as far as possible.

2.1.2 Instruments

Analytical systems:

Gas Chromatograph with Flame Ionization Detector	
GC-8A Gaschromatograph	Shimadzu, Duisburg
Integrator, C-R5A Chromatopac	
Stainless steel column packed with Tenax TA, 60-80 mesh, 3,5 m x 1/8" x 2 mm SS (T_{max} = 375 °C)	Chrompack, Frankfurt/M
Gas Chromatograph with Mass Spectrometer	
Gas chromatograph, HP 6890	Agilent Technologies,
Mass Selective Detector, HP 5973	Waldbronn

Hewlett Packard Special Performance Capillary Column: HP-5MS, (Crosslinked 5% Ph Me Siloxane), 30 m x 0.25 mm x 1.0 µm	Agilent Technologies, Waldbronn
Zebron ZB-5 fused silica capillary column, 30 m x0.25 mm ID, film thickness of 1.00 µm	Phenomenex, Aschaffenburg
Pre-column, fused Silica Rohr 0.32 mm, ID: 5m	ThermoQuest, Egelsbach
Column Connector, press-fit	Agilent Technologies, Waldbronn
Chemical Ionization Gas Purifier	
MS Gas Purifier	
Ionization Gauge Controller	
HP Chemstation B.02.05	
O_2 and H_2O purifier: OMI®1	Supelco, Bellefonte, PA, U.S.A.
Oil Pump Filter	Edwards, Kirchheim,
Spectrophotometer Lambda 5	Perkin Elmer, Überlingen

Computers and Softwares

Computers

Hewlett Packard, Kayak XA	Agilent Technologies, Waldbronn
Apple Macintosh, G-3	Apple Inc, Cupertino, USA
Macintosh Powerbook 540c	Apple Inc, Cupertino, USA

Printers

HP laser Jet 4000 PCI 5e	Hewlett Packard, Waldbronn
HP laser Jet IIP	Hewlett Packard, Waldbronn

Software	
Adobe Illustrator 9.0.2	Adobe Systems Incorporated, USA
CSC Chemdraw 3.1	Cambridge Scientific Computing Inc, USA
Endnote 4.0.1	ISI Reasearch Soft, USA
Mac Draw Pro 1.5 Dv2	Claris Corp.,Santa Clara,, USA
Microsoft Office 2001	Microsoft Corporation, USA
Nist Standard Reference Database IA NIST/EPA/NIH mass Spectral Libraray (NIST'98) and NIST mass Spectral Search Programme 1.6	National Institute of Standards and Technology, USA
Sigma Plot 4.17	Jandel, Erkrath, USA
Exposure system	
Liver chamber, rat liver, glass, 463 mL	Kuglstatter, Garching
Liver chamber, mouse liver, glass, 2062 mL	Kuglstatter, Garching
Oxygenator, glass	Kuglstatter, Garching
Desiccator, glass,6430 mL,800 mL	
Large round bottom flask, 67655 mL	
Pumps	
-Diaphragm pump, N 726.3 FT.18 with teflon-coated membrane	KNF Neuberger, Freiburg
-Peristaltic pump	Roth, Karlsruhe
Surgical Instruments	
Dissecting instruments	Aesculap, Tuttlingen
Indwelling cannula Introcan®-W, 24 G, 3/4", 0.7 x 19 mm	B.Braun, Melsungen

Preparation table for rats	GSF-Workshop, Neuherberg
Sewing Cotton Silkam®, metric 3, USP 2/0,	B. Braun Surgical, Melsungen
Silicone tubes, different diameters	K&K Laborbedarf, München
Laboratory instruments	
Beaker, glass, 100 mL, 300 mL, 5000 mL	Duran, Schott, Mainz
Bluko-bottles, glass, 1000 mL	ASID• Veterinär Vertriebs, Unterschleißheim
Centrifuges	
Biofuge B	Haraeus Sepatech, Osterode
Variofuge 20	Heraeus Sepatech, Osterode
EBA 12R	Hettich, Tuttlingen
Sigma 4K10	Sigma, Osterode
Cryostate, RM6 T	Lauda-Königshohen
Cuvets , suprasil®, glass, 1.8 mL, thickness 1.0 cm	Perkin Elmer, Überlingen
Eppendorf-cups, 1.5 mL, 2mL	Eppendorf, Hamburg
Filter paper, dia: 150 & 270 mm; 595 1/2 & 597 1/2	Schleicher & Schuell, Dassel
Flowmeter D10A3239N	Fischer-Porter, Göttingen
Flask, glass, conical, 5000 mL	Duran Schott, Mainz
Funnel, glass, diameter 14.5 cm	GSF-Store, Neuherberg
Inserts, glass, conical microinserts, 6 mm, 0.1 ml, 15 mm tip	K & K Laborbedarf, München
Magnetic Fish	Bohlender, Lauda
Magnetic Stirrer, M5 CAT	M.Zipperer, Staufen-Etzenbach

Manual crimper for 11 mm caps	Agilent Technologies, Waldbronn
Measuring cylinder, glass, 10, 50, 2000 mL	Schott, Mainz
Needle, disposable, 0.90 x 40 mm, 0.45 x 12 mm, 1.20 x 40 mm	B. Braun, Melsungen
Pasteur pipettes, lengh 23 cm	GSF-store, Neuherberg
pH meter, Knick 646	Knick, Berlin
pH- Electrode In lab®	Mettler Toledo, Urdorf, Switzerland
Pipe for Bluko bottles; 1 Donafix	B.Braun Melsungen
Pipetting devices	
Pipette, 0.5-10 µL, 200 µL and 1000 µL	Eppendorf, Hamburg
Standardtips,10 µL, 200 µL, 1000 µL	Eppendorf, Hamburg
Polyethylene Bottles , Kautex®, 2L	GSF- store, Neuherberg
Reagent bottle, glass, 500 mL, 5000 mL	Schott, Mainz
Sample concentrator	Alltech, Unterhaching
Septa CS, silicone, teflon coated, GL 14	SGE, Weiterstadt
Syringes	
Omnifix-F Solo, disposable, 1,3 & 10 mL	Braun, Melsungen
HP autosampler syringe, 5 and 10 µL, with tapered needle for 0.32 mm ID column	Agilent Technologies, Waldbronn, Germany
Hamilton syringe, gas tight, 10 and 100 and 1000 µL	Hamilton, Darmstadt
Test tubes, 16 x 160 mm	GSF-store, Neuherberg
Thermometer	K&K Laborbedarf, München
Thermostat	Haake / Fisons, Karlsruhe
Thermostat M3, for spectrophotometer	Lauda, Königshofen
Ultrasonicator, Sonorex TK 30	Bandelin Electronics, Berlin

Vortex, VF2	Janke & Kunkel, Staufen
Vials	
Centrifuge vials, glass, round bottom, 6ml and 45 ml, with thread for screw caps, 6 ml/GL 14 and 45 ml/GL 25	K&K Laborbedarf, München
Headspace vials, glass, 7.5-7.9 mL, with rim for clamp caps, butyl / PTFE septum, 20 mm	K&K Laborbedarf, München
Injection vials, glass, clear, 2 mL, with crimp caps (aluminium) 11 mm with teflon/silicon septa	Wicom, Heppenheim
Sample vials, glass, clear, 16 mL, 17 x 21 mm, thread 18-400 with screw caps	K&K Laborbedarf, München
Sample vial, glass, brown, 1 mL, screw caps with butyl-teflon septa	K&K Laborbedarf, München
Water bath, GFL 1083	GFL, Burgwedel
Weighing balance, A 210-P and BL 600	Sartorius, Göttingen

Animals

For the preparation of the isolated perfused liver, male Sprague-Dawley rats (200-300 g) and male B6C3F1 mice (22 and 24 g) from Charles River, Sulzfeld, Germany, were used. The animals were housed in macrolon cages Typ III, at a constant room temperature (23°C) and a relatively constant humidity of 50 %. A light and dark cycle of 12 hours was maintained. In a cage either two rats or five mice were kept at a time. The cages were placed in the Top Flow System IVC of Tecniplast, Buguggiate, Italy. Animals were fed with standard diet 1324 of Altromin, Lage, and tap water ad libitum.

2.2 Methods

2.2.1 Preparation of perfusate

The perfusate was required for the perfusion experiments (2.2.4) and for sample preparations (2.2.2.1 to 2.2.2.6). The perfusate was prepared according to (Bitzenhofer, 1993). The erythrocytes were isolated from bovine blood, which was collected from the slaughter house in Munich. To prevent blood clotting, the blood was added to Liquemin N25000 (5000 I.E. heparin/mL), resulting in a heparin concentration of 30 I.E./mL blood. Thereafter, the blood was centrifuged with 2940 rpm (2000 x g) for 11 min at 20°C using a Variofuge 20RS centrifuge. The residual erythrocyte suspension was washed thrice with Krebs-Henseleit buffer (9.55 g/L Krebs-Henseleit buffer powder, sodium bicarbonate 2.1 g/L (0.25 mol/L), 0.373 g/L (0.025 mol/L) calcium chloride dihydrate, adjusted to a pH of 7.4) using a buffer to erythrocyte ratio of 2 to 1 and the clear supernatants were discarded. In order to prepare the perfusate, the total volume of the required Krebs-Henseleit buffer (usually 1000 ml) was divided equally into two parts. To one part, the washed residual erythrocyte suspension was added (usually 666 mL) and to the other part albumin was added while being magnetically stirred, till everything got dissolved. The two parts were then mixed and the pH of the solution was adjusted to 7.4. This perfusate comprised of solution with 40% (v/v) washed residual erythrocyte suspension and 1% (w/v) albumin, with the physiological pH of 7.4.

2.2.2 Determination of 1,3-butadiene and selected metabolites

This section deals with the analytics of BD and selected metabolites, (EB, CA, DEB, B-diol and EBD). Sample preparation from the perfusate, gas chromatographic methods adopted for their identification and the procedures followed for establishing the proportionality between the analyte concentration and the detector signal along with its sensitivity check are dealt with here. The methods for determination of the recovery of the analytes from the perfusate, the reproducibility of the GC/MS and the extraction/derivatisation methods and also the methods adopted for the stability measurements of the analytes in the perfusate are discussed in this section.

BD being a gas and its metabolites being volatile and reactive with mutagenic and carcinogenic charactarestics were always handled under the hood.

2.2.2.1 1,3-Butadiene

Sample preparation and headspace analysis of BD

BD was measured from the perfusate by using the headspace method. For this purpose 1 mL of perfusate sample was injected to a gastight 7.5-7.9 mL headspace vial through the teflon coated rubber septum with a disposable Braun syringe (2 mL syringe with a 0.55 x 25 needle). To avoid an overpressure in the vials, a syringe needle was put through the septum of each vial immediately before the injection of the perfusate and drawn out together with the sample syringe right after the sample injection. The exact weight of the sample was measured and its volume was calculated. The vial was then shaken in a water bath at 37°C until equilibrium was reached between the gas and the liquid phase after 150 min. The BD concentration in the gas phase was determined by injecting 25 µL of the gas phase into the GC/FID. The BD concentration in the liquid phase at equilibrium was calculated via the partition coefficient ($\lambda_{perfusate:air}$). The sum of the BD amount in the perfusate and in the air at equilibrium gave the initial BD amount in the sample. The following equations are used for the determination of initial BD concentration in the perfusate.

Initial amount of BD in the perfusate = Amount of BD in the perfusate at equilibrium + Amount of BD in the gas phase at equilibrium

$$V_{per} \times C_{in}^{per} = (V_{per} \times C_{150}^{per}) + (V_{gas} \times C_{150}^{gas}) \quad (1)$$

$$C_{in}^{per} = C_{150}^{per} + (C_{150}^{gas} \times \frac{V_{gas}}{V_{per}}) \quad (2)$$

$$C_{in}^{per} = (C_{150}^{gas} \times \lambda) + (C_{150}^{gas} \times \frac{V_{gas}}{V_{per}}) \quad (3)$$

C_{in}^{per} initial BD concentration in the perfusate expressed in ppm

C_{150}^{gas} BD concentration in the gas phase after incubation of the headspace vials for 150 minutes expressed in ppm

C_{150}^{per} BD concentration in the perfusate after incubation of the headspace vials for 150 minutes expressed in ppm

λ partition coefficient perfusate:air at 37°C = 0.63 ± 0.07 (see 3.1.1)

$$\lambda = \frac{C_{150}^{per}}{C_{150}^{gas}}$$

V_{gas} volume of gas phase in mL

V_{per} volume of perfusate in mL

$C_{150}^{gas} \cdot \lambda$ BD concentration in the perfusate at equilibrium in ppm

Determination of the partition coefficient ($\lambda_{perfusate:air}$) of BD

For determination of the partition coefficient an 800 mL desiccator was filled with about 500 mL perfusate and 4 mL of 61280 ppm gaseous BD (prepared in an 816 mL desiccator after introduction of 50 mL pure gaseous BD from BD gas cylinder with a 50 mL disposable syringe through a teflon coated rubber septum) was added to the desiccator with a 5 mL disposable syringe, yielding an initial BD concentration of 800 ppm. While the dessicator was shaken in a water bath at 37°C for about 6.5 h, the BD concentration in the gas phase was monitored by analysing 25µL air samples with the GC/FID. The value of the partition coefficient was calculated in analogy to the two compartment model as described in Filser et al. (1992). In this model, one compartment represented the gas phase and the other, the perfusate, which was enriched with BD from the gas phase over the time period till equilibrium was reached. Curve fit of BD concentration against the time range was carried out according to the following equation:

$$c_{(t)} = C_1 e^{-kt} + C_2 \qquad (4)$$

$c_{(t)}$: BD concentration at time point (t) in ppm

k: rate constant in h^{-1}

C_2: BD concentration at equilibrium in ppm

C_1: Difference between the initial BD concentration and concentration at equilibrium in ppm

Quantitation of BD by GC/FID

BD was analysed using a GC-8A gas chromatograph equipped with a flame ionisation detector (Shimadzu, Duisburg, Germany) according to Johanson and Filser (1993). Of the gas phase, 25 µL (from the headspace vials) and 200 µl (from the liver chamber and from the environment) of the gaseous samples were injected via an on-column injection port and separated on a stainless steel column (3.5 m x 1/8" x 2 mm) packed with Tenax TA, 60-80 mesh (Chrompack, Frankfurt a. M.). The carrier gas was nitrogen 5.0 and the flow-rate was kept at 26.5 mL/min with a column pressure of 3.75 kg/cm^2. The oven temperature was maintained constant at 120 °C. The injector and the detector were heated to 200°C. Hydrogen 5.0 (pressure: 0.6 kg/cm^2) and synthetic air (pressure: 0.6 kg/cm^2) were used as detector gases. The GC polarity was adjusted positive with range 10 and with attenuation 1. The detector signal was recorded with a C-R5A integrator (Shimadzu, Duisburg).

Calibration of BD

Calibration curves were obtained by plotting the absolute peak area (obtained on injecting 25 µL gas samples of known BD concentrations in the GC) against the BD concentrations. For this purpose, various BD concentrations were adjusted in desiccators (see equation 5) by injecting definite volumes of pure gaseous BD obtained from a BD gas cylinder into the desiccators with disposable syringes.

$$\text{Resulting BD concentration in the desiccator [ppm]} = \frac{\text{volume of BD [mL]}}{\text{volume of desiccator [mL]}} \times 10^6 \quad (5)$$

10^6: multiplication factor for conversion to ppm

Homogenous atmosphere in the desiccator containing BD was achieved with the help of a magnetic stirrer. Gas samples were withdrawn from the desiccators through the teflon coated rubber septum using Hamilton syringes and were injected directly onto GC column. Sample analysis was carried out as described previously. The calibration curves were inspected for linearity and sensitivity. The detection limit was defined as a signal to noise ratio of 3 to 1.

On each day of liver perfusion, a one-point calibration was carried out using a desiccator containing a defined BD concentration. Each concentration was analysed thrice. The mean of the three determinations was calculated. A multiplication factor was calculated by dividing concentration of BD (expressed in ppm) in the desiccator with the mean of peak area or height. This multiplication factor was utilized to calculate the BD concentration in the gaseous phase of the headspace vials taking into consideration the peak area or height according to the following equation:

BD concentration in ppm = (multiplication factor) x (peak area/height) (6)

2.2.2.2 1,2-Epoxy-3-butene and crotonaldehyde

Sample preparation

Perfusate samples were taken from the liver perfusion system with a 1 mL disposable syringe (connected to a 0.45 x 12 mm needle), which was filled before with 10 µL of the 1 mol/L diethyl maleate solution, resulting in a final concentration of 10 mmol/L diethyl maleate in a 1 mL perfusate sample. The diethyl maleate depleted the GSH in the erythrocytes of the perfusion medium in the syringe. By this procedure the conjugation of GSH with EB could be avoided. The 1 mL vial was crimp capped immediately after the perfusate was transferred into it. Thereafter, the exact perfusate volume was determined from the measured perfusate weight.

The stock solutions of EB, CA and their internal standard 1,2-epoxybutane in ethanol were prepared fresh on each day of the perfusion experiments. With a 1000 µL Hamilton syringe, 1000 µL of ethanol was added to a 2 mL injection vial and closed immediately with a crimp cap. Via the septum 10 µL of pure analytes were added with a 10 µL gastight Hamilton syringe to achieve the required concentration in the stock solutions. This procedure was followed taking into consideration the relatively high volatility of the analytes. Calibration samples in the perfusate were prepared by spiking 1 mL of perfusate (already containing 10 µL of 1 mol/L diethyl maleate) with definite volumes of defined concentrations of ethanolic analyte solutions obtained on dilution of the stock solutions.

The liver perfusion and the calibration samples were spiked with 10 µL of 4600 µmol/L ethanolic 1,2-epoxybutane solution via the septum using a 10 µL Hamilton syringe. After shaking, the perfusate samples were taken out of the vials with 1 mL disposable syringes (connected to a 0.45 x 12 mm needle). For pressure compensation a second needle (1.20 x 40 mm) was passed simultaneously through the septum. The samples were transferred into 1.5 mL Eppendorf cups, which contained 200 µL dichloromethane and were closed immediately. The mixtures were vortexed for about 1 min and centrifuged for 4 min at 21910 x g with the centrifuge EBA12R, Hettich, Germany. The Eppendorf cups were then cooled to 0°C on ice for two minutes. Then, about 100 µL of the dichloromethane layers (lower layer) were transferred into injection bottles equipped with 0.1 mL glass inserts using 100 µL Hamilton syringes. The injection bottles were closed immediately with crimp caps (silicon septum) and the samples were analysed by GC/MS.

Gas chromatography

EB, CA and 1,2-epoxybutane were analysed using a gas chromatograph HP 6890 (Agilent Technologies, Waldbronn) equipped with a HP 5973 mass spectrometric detector (Agilent Technologies, Waldbronn). The compounds were analysed in the positive chemical ionization (PCI) mode. The analytes were separated on a Zebron ZB-5 fused silica capillary column (30 m x 0.25 mm ID, film thickness of 1.00 µm) or alternatively, on a HP-5MS crosslinked 5% Ph Me Siloxane capillary column (30 m x 0.25 mm x 1.00 µm), which were protected by a pre-column (fused silica capillary, deactivated). The parameters of the method used for sample analysis are stated in **Table 1**. The ions m/z = 43, 55, 71 and 73 were measured in the single ion monitoring (SIM) mode. For quantification of EB and CA, ion m/z = 71 was utilized, while ion m/z = 55 was used to quantify 1,2-epoxybutane. For quantitative analysis, the peak ratio of EB or CA to 1,2-epoxybutane (corrected area) was used according to the following formula:

EB:

$$\text{Corrected area} = \frac{(\text{area of EB for m/z} = 71)}{(\text{area of 1,2} - \text{epoxybutane for m/z} = 55) \times (\text{volume of perfusate in mL})} \quad (7)$$

CA:

$$\text{Corrected area} = \frac{(\text{area of CA for m/z} = 71)}{(\text{area of 1,2} - \text{epoxybutane for m/z} = 55) \times (\text{volume of perfusate in mL})} \quad (8)$$

To maintain exactly the same concentration of the internal standard in each of the samples so that the peak ratios of the EB or CA to 1,2-epoxybutane followed a steady relationship in each sample, the exact perfusate volume in the denominator of the above equation was essential. The calibration curves were inspected for linearity and sensitivity. The detection limit was defined as a signal to noise ratio of 3 to 1.

Method evaluation

Determination of the extraction factors of EB, CA and 1,2-epoxybutane from perfusate

Since EB, CA and 1,2-epoxybutane were extracted from the perfusate with the help of dichloromethane, a set of experiments were carried out to investigate the extraction factors of these analytes from the perfusate.

Calibration samples were prepared directly in dichloromethane. EB concentration ranged from 0.012 to 49.2 µmol/L, CA from 0.012 to 48.2 µmol/L and that of 1,2-epoxybutane from 0.012 to 46 µmol/L. To 1 mL of perfusate known amounts of ethanolic EB, CA and 1,2-epoxybutane solutions were added. Groups of ~48 µmol/L, ~12 µmol/L, and blank (only with 1 mL of perfusate) of EB and CA and ~45 µmol/L, 11 µmol/L and blank (only with 1 mL of perfusate) of 1,2-epoxybutane were prepared. These perfusate samples were then processed in the similar manner as described above by extracting the analytes with 200 µL dichloromethane. All experiments were carried out in triplicates. The dichloromethane layer of these samples and the calibration samples were then analysed by the method described in **Table 1**. The calibration curves were used to calculate the concentrations of the analytes in the perfusate samples. Thereafter, the extraction factors were obtained by dividing the calculated amounts by the initially spiked ones.

Stabilities of EB and CA in the dichloromethane layer

Analysis of EB, CA and 1,2-epoxybutane was started on the perfusion day. During analysis, the samples remained on the autosampler tray for up to one day at room temperature. Due to the volatility of the compounds, it was necessary to know their stability in dichloromethane at room temperature and whether the dichloromethane layer could also be stored at –80°C for later analysis. The following experiments were carried out.

Calibration samples containing EB and CA were prepared in 1 mL perfusates with defined constant concentration of 1,2-epoxybutane in all of them. The concentrations of EB and CA varied from 0.006 to 54 µmol/L with ~40 µmol/L of 1,2-epoxybutane. After extraction with dichloromethane, samples were subjected to GC/MS analysis.

For the stability experiments, a vial of about 15 mL perfusate containing 150 µL diethyl maleate (1 mol/L) was prepared. To this a certain amount of analytes were added with nearly a constant concentration of 1,2-epoxybutane (30 µmol/L). The solution was distributed equally in 1 mL aliquots into 1.5 mL Eppendorf cups containing 200 µL dichloromethane, which were immediately closed. Extraction was carried out as described before. The organic layer was divided in three groups consisting of 3 Eppendorf cups each. The first group was measured immediately together with the calibration samples. The second group was measured 24 h after standing at room temperature. The third group stored at –80°C was measured after a week. These experiments were repeated 2 times.

Parameters	EB , CA and 1,2-epoxybutane
Column	ZB5 or HP-5MS (30 m x 0.25 mm x 1.0 µm)
Gas flow	Mode: constant flow: 0.7 mL/min; Nominal initial pressure: 0.247 bar Average velocity:30cm/sec
Oven temperature program	5 min at 35 °C, 5 °C/min to 100 °C, held 0.1 min, 25 °C/min to 225 °C
Inlet temperature	Oven track mode (3°C above the oven temperature)
Injection mode	Cool-on-column
Injection volume	3 µL
Transfer line temperature	250 °C
Reagent gas for positive chemical ionisation (PCI)	Methane (flow rate 20%)
Electron energy	150 eV
Ions measured in single ion mode (SIM)	55, 71, 73
Ions used for quantification in SIM	71 (EB and CA), 55 (1,2-epoxybutane)

Table 1: GC/MS operational conditions for EB, CA and 1,2-epoxybutane

2.2.2.3 1,2:3,4-Diepoxybutane

Purity determination of synthesised perdeuterated 1,2:3,4-diepoxybutane

The deuterated 1,2:3,4-diepoxybutane (DEB-D6; 98%) used as the internal standard for the analysis of DEB was synthesised by SYNTHON (Augsburg, Germany). The compound was identified by GC/MS in comparison with the library searched mass spectrum of the non-deuterated counterpart. In addition, ^{13}C NMR analysis carried out on a DMX 500 NMR-Spectrometer (Brucker, Germany) in the laboratory of Dr. Hertkorn, (GSF, Neuherberg), confirmed the chemical structure and revealed no apparent impurity.

Sample preparation

The sample preparation for DEB and DEB-D6 was similar to that of EB and CA, the only difference being the use of Eppendorf cups in this case instead of close crimp cap vials for the sample preparation. Eppendorf cups could be used since the volatility of DEB is by far less than that of the two other chemicals. To the 1 mL perfusate samples in 2 mL Eppendorf cups (both calibration and samples obtained from liver perfusion), containing DEB and 10 mmol/L diethyl maleate, 10 µL of the 1000 µmol/L ethanolic DEB-D6 solution was added using a 10 µL Hamilton syringe. The Eppendorf cups were immediately closed after the addition. The exact perfusate volumes were determined from the measured perfusate weight. After shaking, exactly 200 µL dichloromethane was added to the samples with the help of a Hamilton syringe. The mixtures were vortexed for about 1 min and centrifuged for 4 min at 11000 rpm and 0°C. A 100 µL Hamilton syringe was used to transfer about 100 µL of the dichloromethane layers (lower layer) into injection vials equipped with 0.1 mL glass inserts. The injection bottle was closed with a crimp cap (silicon septum) and stored at –80°C for GC/MS analysis in the following days.

Gas chromatography

The GC/MS analysis of the diepoxides was carried out in the positive chemical ionisation (PCI) mode using ammonia as the reagent gas. Refer **Table 2** for the parameters of the GC/MS method. The cool on-column injector was used in oven track mode. The analytes were separated on a Zebron ZB-5 fused silica capillary column (30 m x 0.25 mm ID, film thickness of 1.00 μm) or alternatively, on a HP-5MS crosslinked 5% Ph Me Siloxane capillary column (30 m x 0.25 mm x 1.00 μm), which were protected by a pre-column (fused silica capillary, deactivated). The DEB-D6 used for spiking the perfusate was assumed to be 1:1 mixture of two diastereomeric forms, the meso form and the racemate. For DEB, only the racemate is commercially available. Therefore, quantitative analysis could only be made for racemic DEB. Due to their structural similarity, the racemic DEB and racemic DEB-D6 had nearly the same retention time. They were distinguished by their different masses. The ions m/z = 104 and 110 were measured in the single ion monitoring (SIM) mode and were further used for the quantification of the analytes. The $(M+NH_4)^+$ ion of DEB had the mass 104 while that of DEB-D6 had the mass 110. For quantitative analysis, the relative peak area of the racemic DEB (corrected area) was used. It was calculated according to the following equation:

$$\text{Corrected area} = \frac{(\text{area of racemic DEB for } m/z = 104)}{(\text{area of racemic DEB} - \text{D6 for } m/z = 110) \times (\text{volume of perfusate})} \qquad (9)$$

volume of perfusate is expressed in mL

The calibration curves were inspected for linearity and sensitivity. The detection limit was defined as a signal to noise ratio of 3 to 1.

Parameters	DEB and DEB-D6
Column	ZB5 or HP-5MS (30 m x 0.25 mm x 1.0 μm)
Gas flow	Mode: constant flow: 0.7 mL/min; Nominal initial pressure: 0.247 bar Average velocity:30cm/sec
Oven temperature program	1 min at 40 °C, 5 °C/min to 60 °C, 30 °C/min to 150 °C, held 1 min, 30 °C/min to 220 °C
Inlet temperature	Oven track mode (3°C above the oven temperature)
Injection mode	Cool-on-column
Injection volume	4 μL
Transfer line temperature	250 °C
Reagent gas for positive chemical ionisation (PCI)	Ammonia (flow rate 20%)
Electron energy	150 eV
Ions measured in single ion mode (SIM)	55, 104, 110
Ions used for quantification in SIM	104 (DEB), 110 (DEB-D6)

Table 2: GC/MS operational conditions for DEB and DEB-D6

Method evaluation

Determination of the extraction factors of DEB and DEB-D6 from the perfusate

For the determination of the extraction factors of DEB and DEB-D6 from the perfusate, the same procedure as described in the cases of EB and CA was followed. Extraction factors were determined for the concentration of about 68, 14 and 1.4 µmol/L for DEB and of about 58, 12 and 1.2 µmol/L for DEB-D6 along with a blank (only 1 mL of perfusate) in each case. The set of calibration samples prepared in dichloromethane varied in the concentration range of 0.015 to 74 µmol/L for DEB along with 0.012 to 61 µmol/L for DEB-D6.

Stability of DEB in the dichloromethane layer

A similar procedure as described for EB, CA and 1,2-epoxybutane was followed in case of DEB for the determination of its stability in the dichloromethane extract. On each day of the stability measurements, calibration samples were prepared in the perfusate, ranging from 0.006 to 62 µmol/L of DEB with a constant concentration of 11 µmol/L of DEB-D6 in each of them. Stability of DEB was determined for the defined concentrations of 0.1, 1.0 and 12 µmol/L in perfusate, each of them being spiked with a constant concentration of 11 µmol/L DEB-D6.

2.2.2.4 3-Butene-1,2-diol

Synthesis and purity determinations of B-diol and its internal standard perdeuterated B-diol

B-diol (99%) was synthesized by EMKA Chemie (Neufahrn, Germany). Synthesis of the internal standard perdeuterated B-diol (B-diol-D8; 99 %) was carried out by Synthon (Augsburg, Germany). The compounds B-diol and B-diol-D8 were identified by GC/MS in comparison with the library searched mass spectrum of the non-deuterated B-diol. In addition, ^{13}C NMR analysis carried out on a DMX500 NMR-Spectrometer (Brucker) in the laboratory of Dr. Hertkorn, (GSF, Neuherberg), confirmed the chemical structures and revealed no apparent impurity.

Sample preparation

About 6 mL perfusate samples were taken from the liver perfusion system with 10 mL disposable syringes (connected to a 0.45 x 12 mm needles) containing 60 µL of the 1 mol/L diethyl maleate solution. The perfusate samples were added immediately to 6 mL screw capped glass centrifugation vials, which were closed immediately thereafter. Then, the exact perfusate volume was determined from the measured perfusate weight.

The stock solutions of B-diol and B-diol-D8 in acetone were prepared fresh on each day of the perfusion experiments using 10 µL and 1000 µL Hamilton syringes. Calibration samples were prepared by spiking 6 mL of perfusate (containing 60 µL of 1 mol/L diethyl maleate) with definite volumes of defined concentrations of acetonic analyte solutions obtained on dilution of the stock solutions.

To 6 mL perfusates containing defined concentrations of B-diol (considered for calibration or obtained during liver perfusion experiments) a constant concentration of acetonic B-diol-D8 (internal standard) was added (resulting concentration of 5 µmol/L for rat liver and 17 µmol/L for mouse liver). The samples were centrifuged at 4044 x g and 0°C for 5 min using a Sigma 4K10 centrifuge. The supernatants were stored at –80 °C. On the day of the measurement, 250 µL of the frozen samples were thawed and treated at room temperature with 250 µL of a solution of 0.04 mol/L n-butylboronic acid in acetone, which led to the formation of the corresponding cyclic butylboron esters (**Figure 2**). The derivatives were extracted from the acetonic-aqueous solution with 500 µL ethyl acetate. This derivatisation procedure was carried out according to the method described by McCurdy and Everett (1982) and also followed by Bogusz et al. (1986) and for GC analysis of ethylene glycol. The ethyl acetate-acetone mixture was then centrifuged with 16250 x g at 0°C for 5 min using centrifuge Sigma 4K10 to separate from the residual water. The organic layer (upper layer) was transferred into an autosampler vial and subjected to GC/MS analysis. The derivatisation was necessary for GC separation of these rather non volatile compounds.

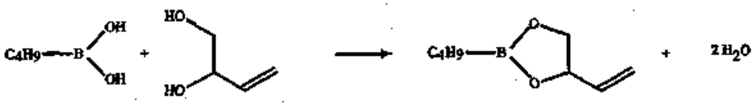


Figure 2: Reaction of B-diol with n-butylboronic acid

Gas chromatography

The ethyl acetate extracts (4 µL) containing both derivatised B-diol and B-diol-D8 were injected on-column into the GC/MSD. The analytes were separated on a Zebron ZB-5 fused silica capillary column (30 m x 0.25 mm ID, film thickness of 1.00 µm) or alternatively, on a HP-5MS crosslinked 5% Ph Me Siloxane capillary column (30 m x 0.25 mm x 0.25 µm), which were protected by a pre-column (fused silica capillary, deactivated). The details of the GC method are listed in **Table 3**. Mass spectrometric analysis using the positive chemical ionisation mode with methane as the reagent gas was carried out for the detection of the analytes. The $(M+H)^+$ ions of derivatised B-diol (m/z =155) and of B-diol-D8 (m/z = 161), along with the less specific ion of m/z = 109 for B-diol were chosen for their identification and quantification. The m/z = 109 for derivatised B-diol was observed to have a good abundance and when extracted, no disturbing peaks were observed as it was the case of m/z = 155. Thus the m/z ion chosen for B-diol quantification was 109, while in case of B-diol-D8 it was 161. For quantitative analysis, the relative peak area of B-diol was calculated according to the following equation:

$$\text{Corrected area} = \frac{(\text{area of B}-\text{diol derivative for m/z} = 109)}{(\text{area of B}-\text{diol}-\text{D8 derivative for m/z} = 161) \times (\text{volume of sample})} \qquad (10)$$

The volume of perfuste was expressed in mL

The calibration curves were inspected for linearity and sensitivity. The detection limit was defined as a signal to noise ratio of 3 to 1.

Parameters	**Cyclic butylboron esters of B-diol and B-diol-D8**
Column	ZB5 or HP-5MS (30 m x 0.25 mm x 1.0 μm)
Gas flow	Mode: constant flow: 0.7 mL/min; Nominal initial pressure: 0.349 bar Average velocity: 31 cm/sec
Oven temperature program	1 min at 65 °C, 5 °C/min to 75 °C, 30 °C/min to 270 °C
Inlet temperature	Oven track mode (3°C above the oven temperature)
Injection mode	Cool-on-column
Injection volume	4 μL
Transfer line temperature	280 °C
Reagent gas for positive chemical ionisation (PCI)	Methane
Ions measured in single ion mode (SIM)	109, 115, 155, 161
Ions used for quantification in SIM	109 (B-diol), 161 (B-diol-D8)

Table 3: GC/MS operational conditions for derivatised B-diol and B-diol-D8

Method evaluation

Determination of the extraction factor of B-diol from the perfusate

Perfusate samples and water samples (6 mL) containing known concentrations of B-diol and B-diol-D8 (0, 0.6, 6, 12, and 54 µmol/L) were prepared in triplicates. In order to determine the extraction efficiency, these samples were compared to those, which were prepared in pure acetone containing both B-diol and B-diol-D8 (varied from 0 to 120 µmol/L). For the derivatisation, 250 µL of water, perfusate supernatant and acetone samples (containing defined concentrations of B-diol and B-diol-D8) were taken and derivatised with n-butylboronic acid and extracted with ethylacetate as described previously.

A dilution factor was determined, since the water and perfusate samples with ethyl acetate resulted in two phases (aqueous and organic phase) whereas acetonic samples gave a single organic layer. The volume of the organic phase obtained from the water and perfusate samples was determined to be 662 µL by a Hamilton syringe.
The extraction factor of B-diol from water and perfusate was calculated using the slopes of the calibration curves of B-diol relative to that of B-diol-D8, obtained in the perfusate (m_p), water (m_w) and acetone (m_a) by considering their respective dilution factor according to the following equations.

$$\text{Extraction factor}_{\text{water}} = \frac{662 \times m_w}{1000 \times m_a} \qquad (11)$$

$$\text{Extraction factor}_{\text{perfusate}} = \frac{662 \times m_p}{1000 \times m_a} \qquad (12)$$

Due to some analytical problems, the extraction method followed here is different from those followed in case of EB, CA and DEB. Instead of considering single values of concentration for the extraction efficiency determination, for B-diol the slopes obtained over a wide concentration range were considered.

Stabilities of B-diol and B-diol-D8 in the perfusate supernatant

The stabilities of the analytes B-diol and B-diol-D8 were investigated by the method of standard addition. For this purpose, four perfusate samples (6 mL) were prepared. Two of them contained B-diol and the other two B-diol-D8. The samples comprised of 23 µmol/L and 10 µmol/L of B-diol and 24 µmol/L and 5 µmol/L of B-diol-D8. The samples were subjected immediately to centrifugation at 4044 x g and 0°C for 5 min using a Sigma 4K10 centrifuge. A part of the supernatant of these samples was frozen at –80°C and analysed in the following 1, 2 and 3 weeks. From the remaining part, four sets of Eppendorf cups were prepared each containing 250 µL of the supernatant from the four samples. These solutions were spiked with a defined concentration of the corresponding acetonic B-diol or B-diol-D8 solution to have added concentrations of 0x, 1x, 2x and 3x of the initial concentrations. Then, they were derivatised with n-butylboronic acid followed by extraction with ethyl acetate and analysed by GC/MS. The peak area vs. added concentration of analyte resulted in a straight line. The intercept on the x-axis indicated the actual concentration of the analyte in the unspiked sample. The intercept values of the curves obtained during these three weeks were compared.

2.2.2.5 3,4-Epoxybutane-1,2-diol

Synthesis and purity determination of EBD and its internal standard 3,4-Epoxy-[1,1,2,3,4,4-$^{2}H_6$]butane-1,2-diol

Synthesis of both EBD (98%) and its deuterated internal standard 3,4-Epoxy-[1,1,2,3,4,4-$^{2}H_6$]butane-1,2-diol (EBD-D6; 98%) was carried out by Synthon (Augsburg, Germany). The compounds were identified by GC/MS in the electron impact ionisation mode by comparing the spectra of the non-deuterated analyte with its deuterated counterpart. In addition ^{13}C NMR analysis carried out on a DMX 500 NMR-Spectrometer (Brucker) in the laboratory of Dr. Hertkorn, (GSF, Neuherberg), confirmed the chemical structure and revealed no apparent impurity. It was assumed that in both EBD and EBD-D6, the diastereomers exist in 1:1 ratio.

Sample Preparation

Apart from certain small changes, the sample preparation of EBD and EBD-D6, concerning their extraction from the perfusate supernatant, evaporation of the extract and their derivatisation with n-butylboronic acid was carried out according to the method of Kimura et al. (1976) for GC analysis of 1,2:5,6-dianhydrogalactitol.

Perfusate samples of about 6 mL were taken from the liver perfusion system with 10 mL disposable syringes (connected to 0.45 x 12 mm needles) containing 60 µL of the 1 mol/L diethyl maleate solution. The perfusate samples were added immediately to 6 mL screw capped glass centrifugation vials, which were closed immediately thereafter. Then, the exact perfusate volume was determined from the measured perfusate weight.

The stock solutions of EBD and EBD-D6 in acetone were prepared fresh on each day of the perfusion experiments using 10 µL and 1000 µL Hamilton syringes. Calibration samples were prepared by spiking 6 mL of perfusate (containing 60 µL of 1 mol/L diethyl maleate) with definite volumes of defined concentrations of acetonic analyte solutions obtained on dilution of the stock solutions.

To perfusate samples of 6 mL containing defined concentrations of EBD (considered for calibration or obtained during liver perfusion experiments) a constant concentration of the acetonic EBD-D6 (internal standard) was added (resulting concentration of 1 µmol/L for both rat liver and mouse liver). The samples were centrifuged at 4044 x g and 0°C for 5 min using a Sigma 4K10 centrifuge and the supernatants were stored at –80 °C. On the day of the measurement, from each sample, 3 mL of the frozen supernatant was thawed and poured into a 45 mL centrifugation tube at room temperature. 30 mL of i-propanol/chloroform (90/10, v/v) was added and the mixture was shaken for 2 min (the i-propanol/chloroform solution was prepared fresh on each day of measurement). Potassium carbonate (3 g) was added and the mixture was again shaken vigorously for 5 min by hand. Thereafter, it was centrifuged (2910 x g at 0°C for 5 min using a Sigma 4K10 centrifuge) to produce two clearly separated layers. The upper organic layer was transferred into a test tube and evaporated to dryness under a stream of nitrogen at 45°C in the sample concentrator. The dry residue was treated with 600 µl of a solution of 0.04 mol/L n-butylboronic acid in acetone, which led to the formation of the corresponding cyclic butylboron esters (**Figure 3**). The mixture was vortexed for 1 min and after standing at room temperature for 5 min, it was transferred to a 2 mL Eppendorf cup. Thereafter, 500 µL of ethyl acetate was added to the mixture and centrifuged (16249 x g at 0°C for 5 min using a Sigma 4K10 centrifuge). The clear supernatant obtained after centrifugation was transferred into an autosampler vial and subjected to GC/MS analysis.

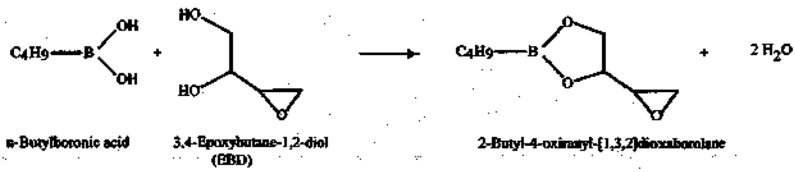


Figure 3: Reaction of EBD with n-butylboronic acid

Gas Chromatography

The ethyl acetate extracts (4 µL) containing derivatised EBD and EBD-D6 were injected on-column into the GC/MS. The analytes were separated on a Zebron ZB-5 fused silica capillary column (30 m x 0.25 mm ID, film thickness of 1.00 µm) or alternatively, on a HP-5MS crosslinked 5% Ph Me Siloxane capillary column (30 m x 0.25 mm x 0.25 µm). Both columns, were protected by pre-columns (fused silica capillary, deactivated). The details of the GC method are listed in **Table 4**. The cyclic butylboron esters of EBD and EBD-D6 were analysed in the positive chemical ionisation mode (PCI mode) using ammonia as reagent gas. The diastereomeric EBD derivatives had nearly the same retention time as the corresponding EBD-D6 ones. They were distinguished by their different masses. The $(M+NH_4)^+$ ion of the isotopic boron esters of EBD (m/z = 188) and that of EBD-D6 (m/z = 194) were used for quantification while the ions, m/z = 187 for EBD derivative and m/z = 193 for EBD-D6 derivative were characteristic ion peaks used only for their identification. For quantitative analysis, the relative peak areas of both diastereomers of EBD were used. They were calculated according to the following equation:

$$\text{Corrected area} = \frac{(\text{area of EBD derivative for } m/z = 188)}{(\text{area of EBD} - \text{D6 derivative for } m/z = 194) \times (\text{volume of sample})} \tag{13}$$

The volume of perfusate was expressed in mL.

The calibration curves were inspected for linearity and sensitivity. The detection limit was defined as a signal to noise ratio of 3 to 1.

Parameters	**Cyclic butylboron esters of EBD and EBD-D6**
Column	ZB5 or HP-5MS (30 m x 0.25 mm x 1.0 μm)
Gas flow	Mode: constant flow: 0.7 mL/min; Nominal initial pressure: 0.349 bar Average velocity: 31cm/sec
Oven temperature program	1 min at 65 °C, 5 °C/min to 75 °C, 30 °C/min to 270 °C
Inlet temperature	Oven track mode (3°C above the oven temperature)
Injection mode	Cool-on-column
Injection volume	4 μL
Transfer line temperature	280 °C
Reagent gas for positive chemical ionisation (PCI)	Ammonia (Flow rate 20%)
Ions measured in single ion mode (SIM)	187, 188, 195, 194
Ions used for quantitation in SIM	188 (EBD), 194 (EBD-D6)

Table 4: GC/MS operational conditions for derivatised EBD and EBD-D6

Method evaluation

Determination of the extraction factor of EBD from the perfusate

For the determination of the extraction factor of EBD from the perfusate, the same procedure as described for B-diol was followed. Samples in perfusate, water and pure solvent (acetone) were prepared containing EBD (43 µmol/L, 23 µmol/L, 10 µmol/L, 1.0 µmol/L and 0 µmol/L) and EBD-D6 (47 µmol/l, 25 µmol/l, 10 µmol/l, 0.9 µmol/l and 0 µmol/L). These samples were then processed, derivatised and analysed by GC/MS, as explained in the sample preparation and gas chromatography section. The slopes of the calibration curves prepared in perfusate (m^*_p), water (m^*_w) and ethyl acetate (m^*_a) were calculated. For further calculations, the relative slope of EBD to EBD-D6 was used (m_p, m_w, m_a). The extraction efficiency from water was obtained as the ratio of m_w to m_a and that from perfusate was obtained as the ratio of m_p to m_a (see also 2.2.2.4).

Stabilities of EBD and EBD-D6 in the perfusate supernatant

Stability determinations of the EBD and EBD-D6 in the perfusate supernatant were determined over the period of three weeks using the method of standard addition, as described for B-diol. Stability determinations were carried out for 50 µmol/L and 25 µmol/L EBD and 50 µmol/L EBD-D6.

2.2.2.6 Reproducibility of the extraction/derivatisation and the GC/MS methods

In order to investigate the reproducibility of the extraction/derivatisation methods of all of the previously mentioned BD analytes (EB, CA, DEB, B-diol, and EBD), certain sets of perfusate samples with defined analyte concentrations were prepared in triplicates within a day. They were subjected to conditions of extraction/derivatisation as mentioned in the respective sample preparation sections of the different analytes. For each analyte, the absolute peak areas obtained from identically prepared concentrations were compared. Thereafter, their mean, standard deviation (SD) and coefficient of variation (CV) were calculated. The same