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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;

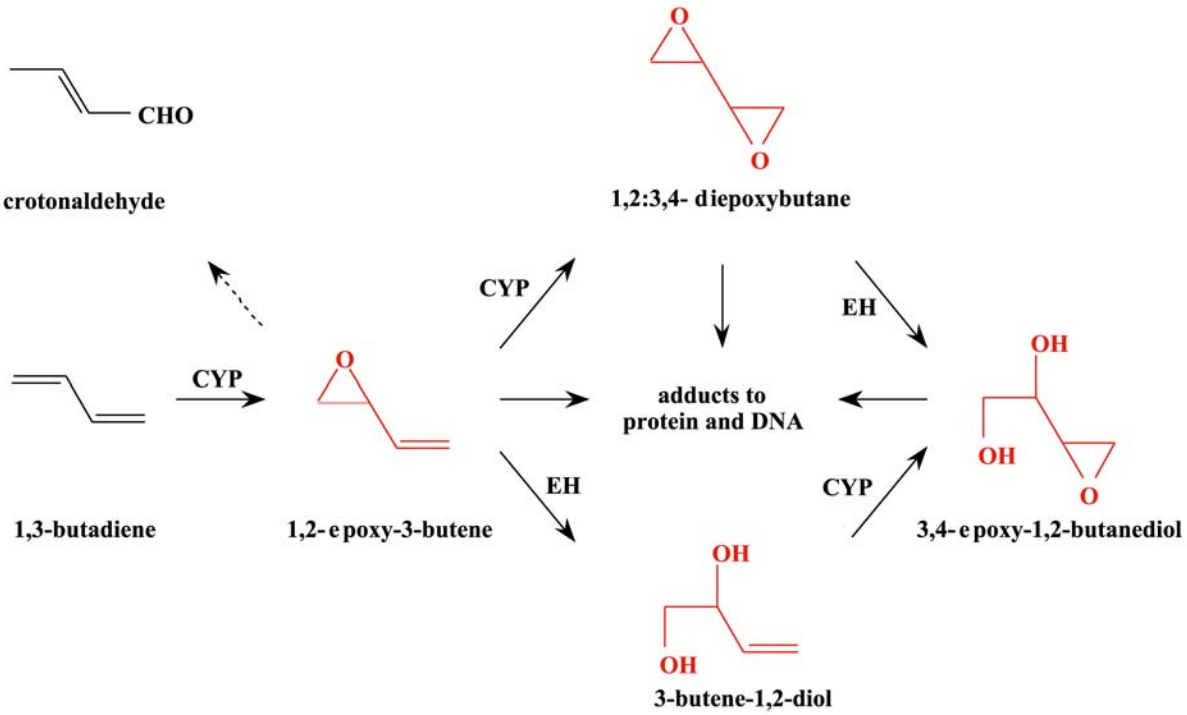
Kreuzer et al., 1991; Csanády et al., 1992) and in mouse as well as in human liver cytosol (Kreuzer et al., 1991; Csanády et al., 1992; Sharer et al., 1992b). Corresponding catabolites of the GSH conjugates with EB were detected in urine of BD exposed rodents (Sabourin et al., 1992; Sharer et al., 1992a; 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_8, 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_8, 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

$C_{150}^{gas} . \lambda$ 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.

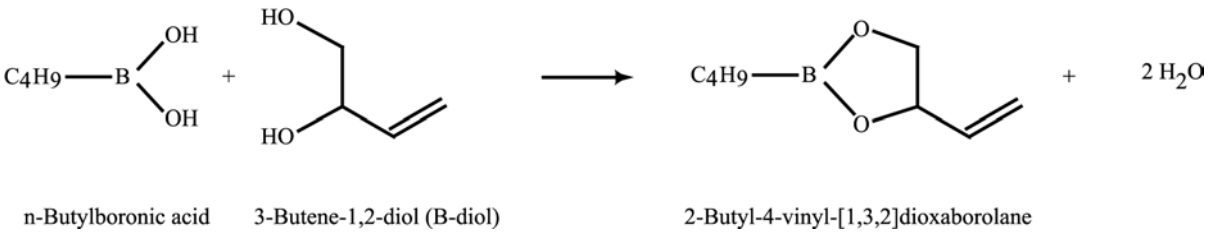


Figure 2: Reaction of B-diol with n-butylboronic acid

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.

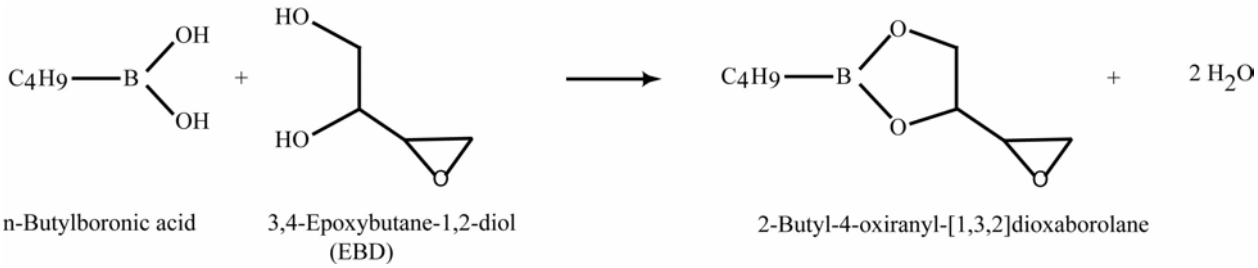


Figure 3: Reaction of EBD with n-butylboronic acid

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:

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

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

procedure was followed on different days with defined concentrations of each metabolite to measure the reproducibility between the days.

For the determination of the reproducibility of the GC/MS method within a day, a sample of a known concentration of each analyte was run thrice consecutively. This was made over a certain concentration range. For each analyte of a defined concentration, the absolute peak areas obtained from the consecutive runs were compared and their coefficient of variation was calculated from the mean and standard deviation values. The estimation of the variation of the GC/MS measurements between the days by comparing only the absolute peak areas was not plausible as the MSD sensitivity was observed to vary from day to day. Therefore, the variation of the GC/MS measurements between the days were estimated from the variations in extraction/derivatisation measurements between the days. This was carried out by comparing the corrected areas of the analyte peaks of each metabolite for a defined concentration, obtained on different days.

2.2.3 Perfusion experiments

Rat and mouse livers were isolated and perfused at 37˚C in a once-through gas-tight all-glass system according to Bitzenhofer (1993), with Krebs-Henseleit buffer (pH=7.4) containing 40 % (v/v) washed bovine erythrocytes and 1 % (w/v) bovine serum albumin (2.2.1). The perfusate was oxygenated and enriched by a constant concentration of BD before entering the liver through the vena portae. The vena cava served as outlet for the perfusate (**Figure 4**). Perfusion rates were set to 17-20 and 3-4 mL/min for rat and mouse livers, respectively. The partial pressure of O_2 was measured in the influx using the apparatus “pHOx” of NOVA Biomedical, Waltham, USA. The biochemical integrity of the livers was verified by measuring the concentration ratio of lactate to pyruvate as a measure of the redox state of the liver. The concentrations of lactate and pyruvate in the influx and efflux of the liver were determined photometrically using diagnostic kits (No. 826 and 726) obtained from Sigma-Diagnostics, Germany. Perfusion experiments were maintained up to 100 minutes.
Samples of the perfusate entering and leaving the livers were collected repeatedly at specified time points during the individual exposure experiments. In these samples the concentrations of EB, CA, DEB, B-diol, and EBD were determined as described in the previous sections.

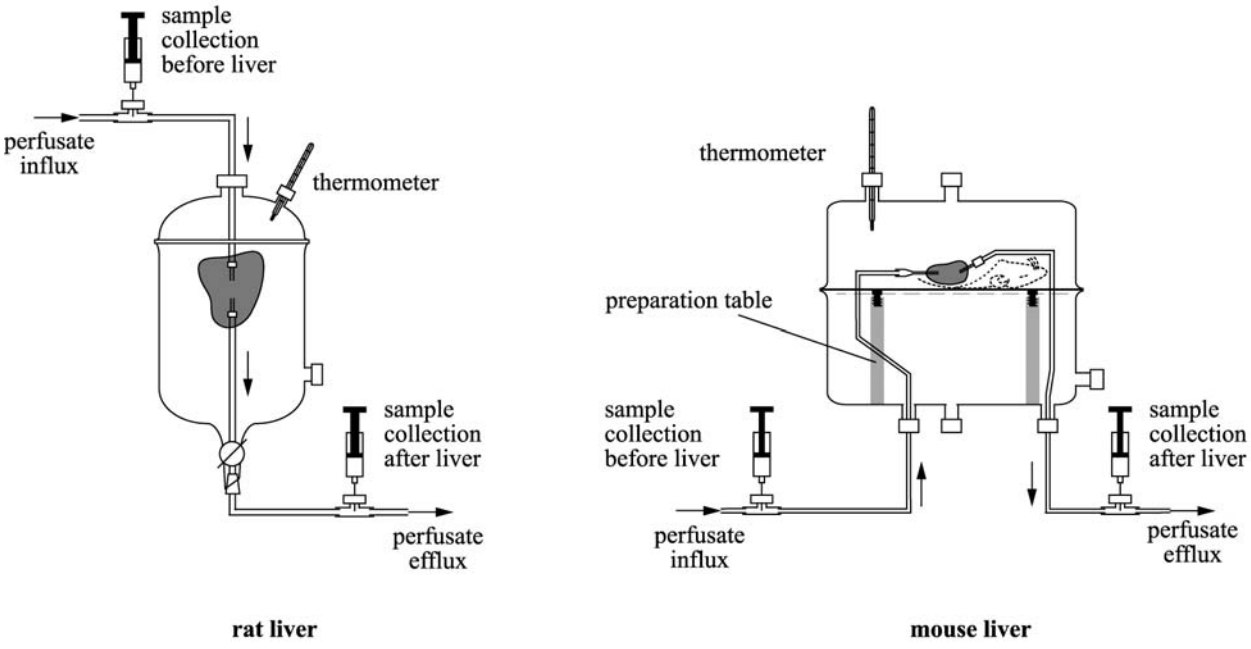


Figure 4: Diagrammatic representation of the liver perfusion systems

2.2.4 Statistics

In the present work, the following formulas were used for the calculation of the mean ($\bar{x}$) as represented in equation (14), the standard deviation as in equation (15), the standard error in case of standard calibration curves as in equation (16) and the coefficient of variation as represented in equation (17) (Geigy Documenta, 1985).

The determination of the extraction efficiency of the two analytes B-diol and EBD was carried out utilising the slopes and standard errors of the calibration curves obtained in perfusate, water and pure solvent. The standard error of this extraction efficiency determination was calculated according to the "multiplicative expression" with respect to the propagation of random errors, as expressed in equation (18) (Miller and Miller, 1993).

$$\bar{x} = \frac{\sum_{i=1}^{i=n} x_i}{n} \qquad (14)$$

$\bar{x}$ Mean value of n analyses

x_i Value of the ith sample

n Number of measurements

$$SD = \sqrt{\frac{\sum_{i=1}^{i=n}(x_i - \bar{x})^2}{n-1}} \quad (15)$$

SD Standard deviation

$\bar{x}$ Mean value of n analyses

x_i Value of the ith sample

n Number of measurements

$$SEM = \frac{SD}{\sqrt{n}} \quad (16)$$

SEM Standard Error of Mean

n Number of measurements

$$CV = \frac{SD}{\bar{x}} \times 100 \quad (17)$$

CV Coefficient of Variation [%]

SD Standard deviation

$\bar{x}$ Mean value of n analyses

100 Factor for calculation in percentage

$$SE_{efficiency} = \frac{m_I}{m_{II}} \sqrt{\left(\frac{S_I}{m_I}\right)^2 + \left(\frac{S_{II}}{m_{II}}\right)^2} \quad (18)$$

$SE_{efficiency}$ Standard error of the extraction efficiency

S_I Standard error of the ratio of the slopes of the analyte to its internal standard measured in perfusate or water. This standard error is also calculated using equation similar to equation (18) except that for this case, the S_I and S_{II} are the standard errors of the calibration curves (obtained directly by curve fit) of the analyte and the internal standard respectively in the perfusate or water.

S_{II} Standard error of the ratio of the slopes of the analytes to its internal standard measured in pure solvent. This standard error is also calculated using equation similar to equation (18) except that for this case, the S_I and S_{II} are the standard errors of the calibration curves (obtained directly by curve fit) of the analyte and the internal standard respectively in the perfusate or water.

m_I Slope ratio of the analyte to its internal standard measured in perfusate or water

m_{II} Slope ratio of the analyte to its internal standard measured in pure solvent

3 Results

3.1 Identification, quantitation, method evaluation and stability measurement of 1,3–butadiene and selected metabolites

This section deals with the identification and quantification of BD and the selected metabolites by gas chromatography equipped with either FID or MSD. The proportionality check of the analyte concentrations with the detector signal along with the sensitivity measurement, specifying the limit of detection of the instrument for each individual analyte are discussed in the following paragraphs. Method evaluation covers the calculation of the analyte recoveries from the perfusate, the reproducibilities of the GC-methods and the derivatisation/extraction procedures and finally the determination of the stabilities of the analytes in the perfusate supernatant.

3.1.1 1,3-Butadiene

GC/FID measurements

Chromatograms obtained (refer 2.2.2.1) from the calibration sample (injection of 25 µL of gaseous BD into GC/FID, taken from the desiccator containing definite BD concentrations) or from the perfusate samples containing BD (headspace method), showed a distinct peak at the retention time of 2.05 min (**Figure 5),** while the chromatograms obtained after injection of 25 µL of the simple atmospheric air contained no such peak at this particular retention time.
The calibration curve constructed by plotting absolute peak areas of BD against the concentration range was found to be linear from 0.90 to 8000 ppm of BD (**Figure 6**), while passing through the origin. The linear regression analysis performed on the absolute area versus the analyte concentration resulted in m ± SE, 256.92 ± 0.61 (m = slope; SE = standard error) and r^2 = 0.9998 (n = 24). The lowest concentration used for calibration resulted in a peak area, equivalent to three times of the background. Therefore, the detection limit was determined to be 0.9 ppm.

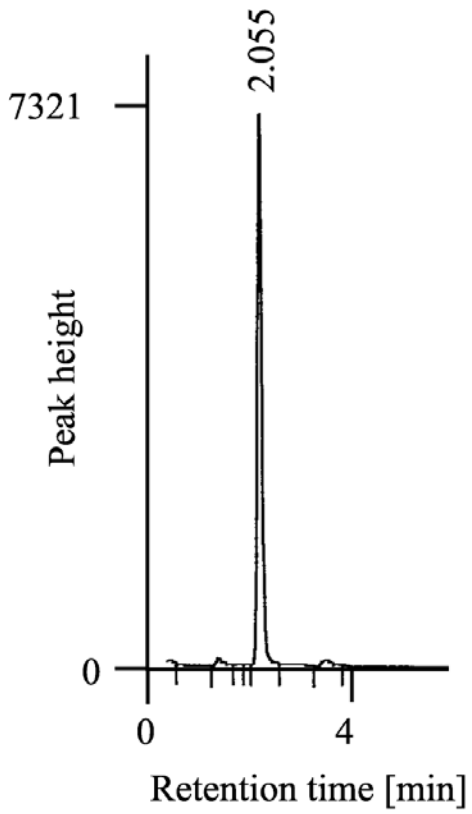


Figure 5: GC/FID chromatogram of BD obtained from a 25 µL injection volume of the gaseous sample from the desiccator containing 85 ppm of BD at 25°C (for methodology see 2.2.2.1)

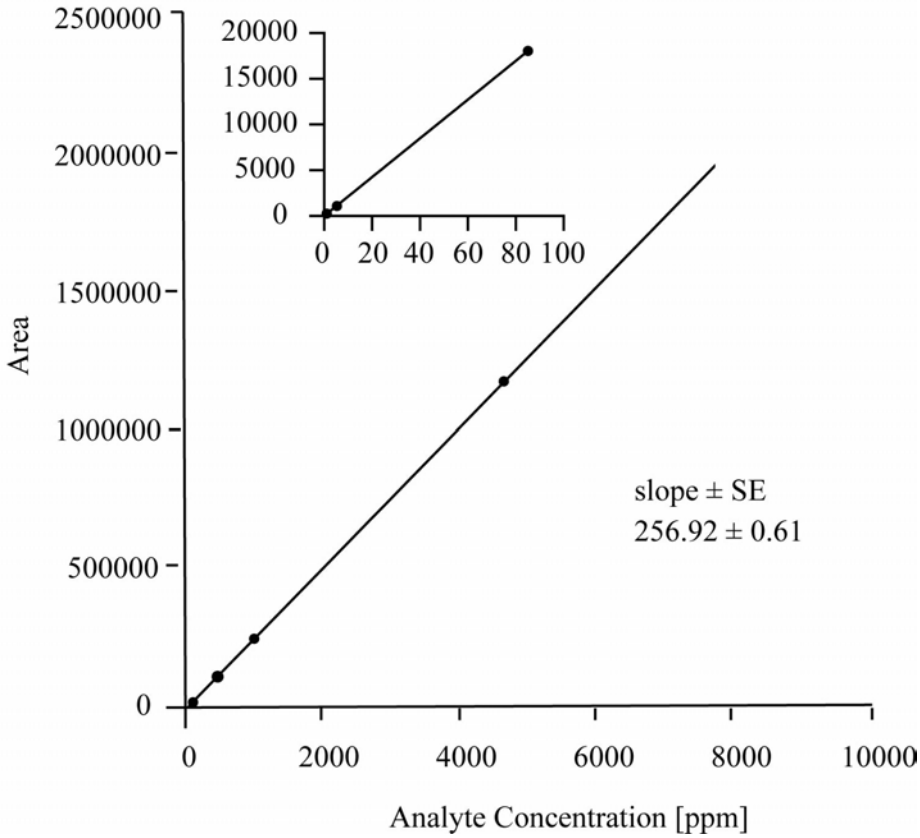


Figure 6: Calibration curve of 1,3-butadiene (BD) determined by the gas chromatograph equipped with flame ionisation detector

Analyte:	BD
SE:	standard error
Symbols (●):	measured data
Solid lines:	obtained by curve fit

For the reproducibility of the GC/FID method, 25 µL of gaseous samples of different BD concentrations were injected thrice consecutively (refer 2.2.2.1). A variation of 3.7 % (coefficient of variation) was observed in the degree of reproducibility at low concentrations, while it was about 1% at higher concentrations (**Table 5**).

Sample no.	Concentration [ppm]	Absolute area	Mean area	Standard Deviation	Coefficient of Variation (%)
1	0.90	169	176	6.5	3.7
	0.90	176			
	0.90	182			
2	482	115716	115715	779	0.7
	482	114935			
	482	116493			
3	8000	1989018	1974503	21488	1.1
	8000	1949818			
	8000	1984674			

Table 5: Reproducibility of the GC/FID method for 1,3-butadiene given by the coefficient of variation (for statistical calculation see 2.2.3)

Partition coefficient (perfusate:air) of BD

The distribution of BD between the gas phase and the perfusate (on single administration of BD in the gas phase of a closed chamber) was monitored by measuring the atmospheric BD concentration in the closed system. Such a concentration time course is shown in **Figure 7**. According to the figure, two processes could describe the enrichment of BD. The first one is a rapid process describing mainly the initial enrichment of BD in the liquid phase. The second process is much slower and indicates slight elimination of BD from the system. The solid line was obtained by fitting to the measured concentration time courses and the model parameters (refer 2.2.2.1). Equations (1) to (4) (refer 2.2.2.1) were used to calculate the partition coefficient ($\lambda_{perfusate:air}$). Three experiments for the determination of the partition coefficient ($\lambda_{perfusate:air}$) was carried out and from the average of these three, $\lambda_{perfusate:air} \pm$ SD (standard deviation) was determined to be 0.63 ± 0.07.

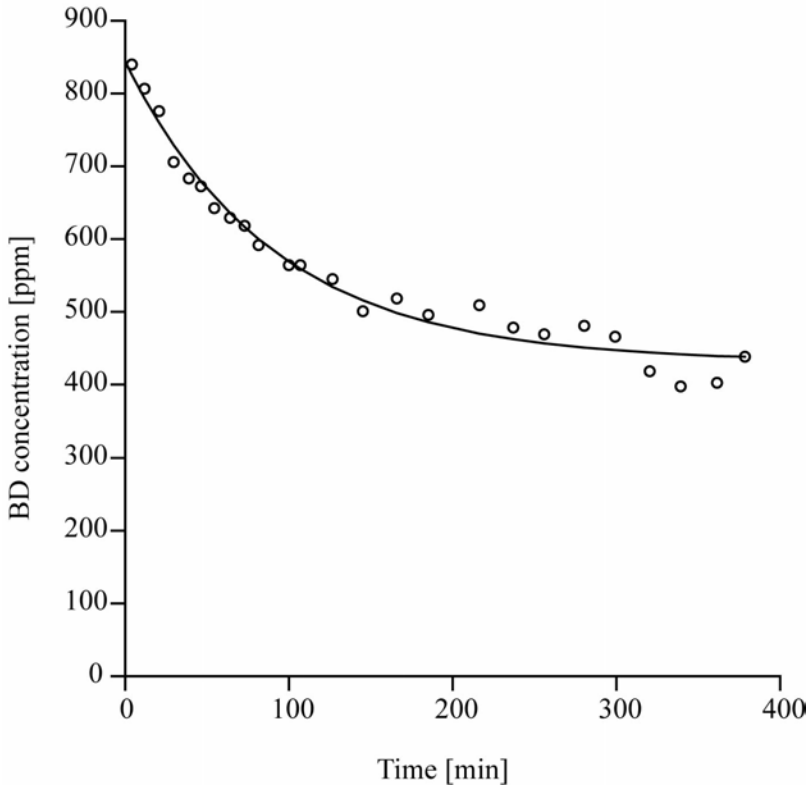


Figure 7: Concentration-time curve of BD measured in the gas phase of a desiccator (800 mL) containing 500 mL perfusate after a single administration of BD (4 mL of 61280 ppm) in the gas phase (for methodology see section 2.2.2.1). Symbols (o): measured data; Solid line: curve fit

3.1.2 1,2-Epoxy-3-butene and crotonaldehyde

GC/MS measurements

EB, CA, and their internal standard (IS) 1,2-epoxybutane were extracted from the perfusate (refer 2.2.2.2) and analysed by GC/MS in the positive chemical ionisation (PCI) mode using methane as the reagent gas. EB and CA were measured in the same chromatographical run. **Figure 8 (A and B)** show single ion chromatograms for m/z = 55 and m/z = 71 respectively. Stereoisomers of EB and the internal standard were not resolved chromatographically. However, the two isomers of CA, the (*E*)- and the (*Z*)-form were separated on the GC columns (**Figure 8**). The (*Z*)-isomer was about 4% of the (*E*)-isomer. As the portion of (*Z*)-CA was very low, quantitative analysis was only made for (*E*)-CA. Typical retention times

were 7.42 min for EB, 7.73 min for the internal standard 1,2-epoxybutane, 9.42 min for (*E*)-CA and 9.59 min for (*Z*)-CA. Under MSD conditions **(Figure 9)**, EB showed some fragmentation but the $(M+H)^+$ peak at m/z = 71 had a relatively significant abundance of 50%, where "M" represented the molecular ion (**Figure 9A**). The mass spectra of (*E*)- and (*Z*)-CA (**Figures 9B and 9C**) were nearly identical and consisted of a major $(M+H)^+$-peak at m/z = 71 and two minor peaks of the $(M+C_2H_5)^+$ and $(M+C_3H_5)^+$ adducts. 1,2-epoxybutane molecules fragmented substantially using methane as the reagent gas and the $(M+H)^+$ peak had a relatively low abundance (**Figure 9D**). For quantitative analysis of EB and (*E*)-CA m/z = 71 $(M+H)^+$ was used. For the IS 1,2-epoxybutane, the specific $(M+H)^+$-ion (m/z = 73) could not be used for quantification as a disturbance peak at the same retention time of 1,2-epoxybutane for m/z = 73 was detected in the blanks (dichloromethane extracts of perfusate containing no analyte). Therefore, the less specific 1,2-epoxybutane fragment ($C_3H_3O^+$) at m/z = 55, which had the highest abundance in the mass spectrum, was used for quantification.

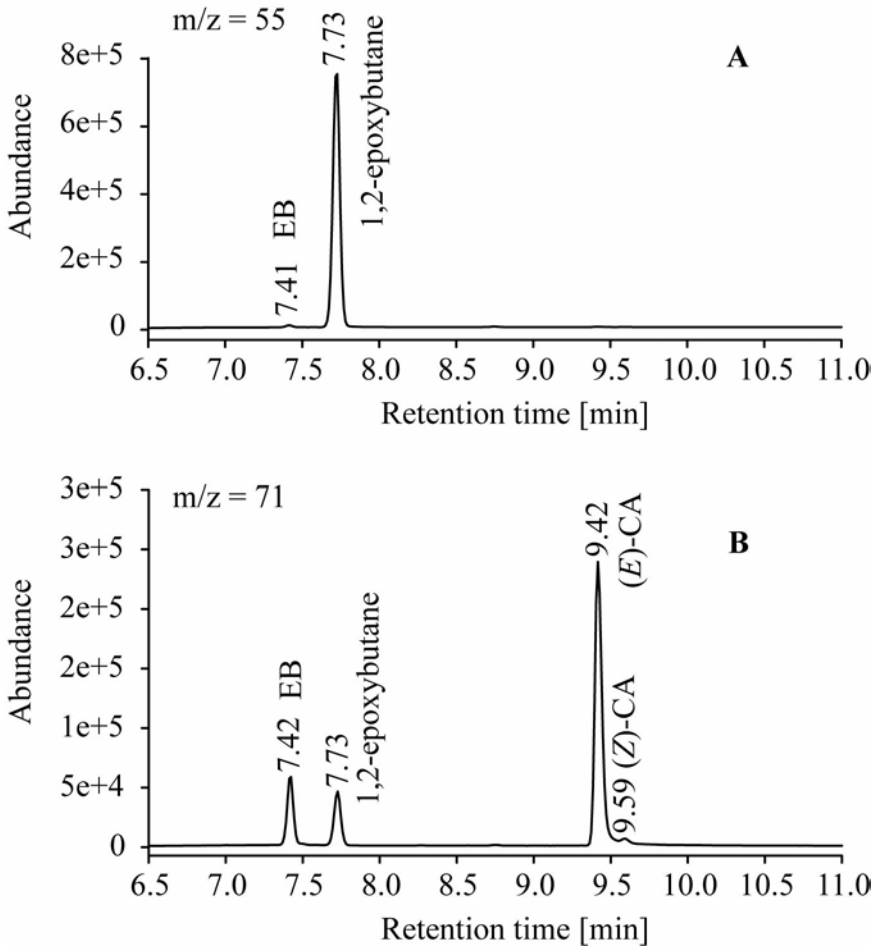


Figure 8: Single ion chromatograms of the dichloromethane extract of a perfusate sample containing 6.2 µmol/L EB, 6.1 µmol/L CA and 46.2µmol/L 1,2-epoxybutane for m/z = 55 (A) and m/z = 71 (B)

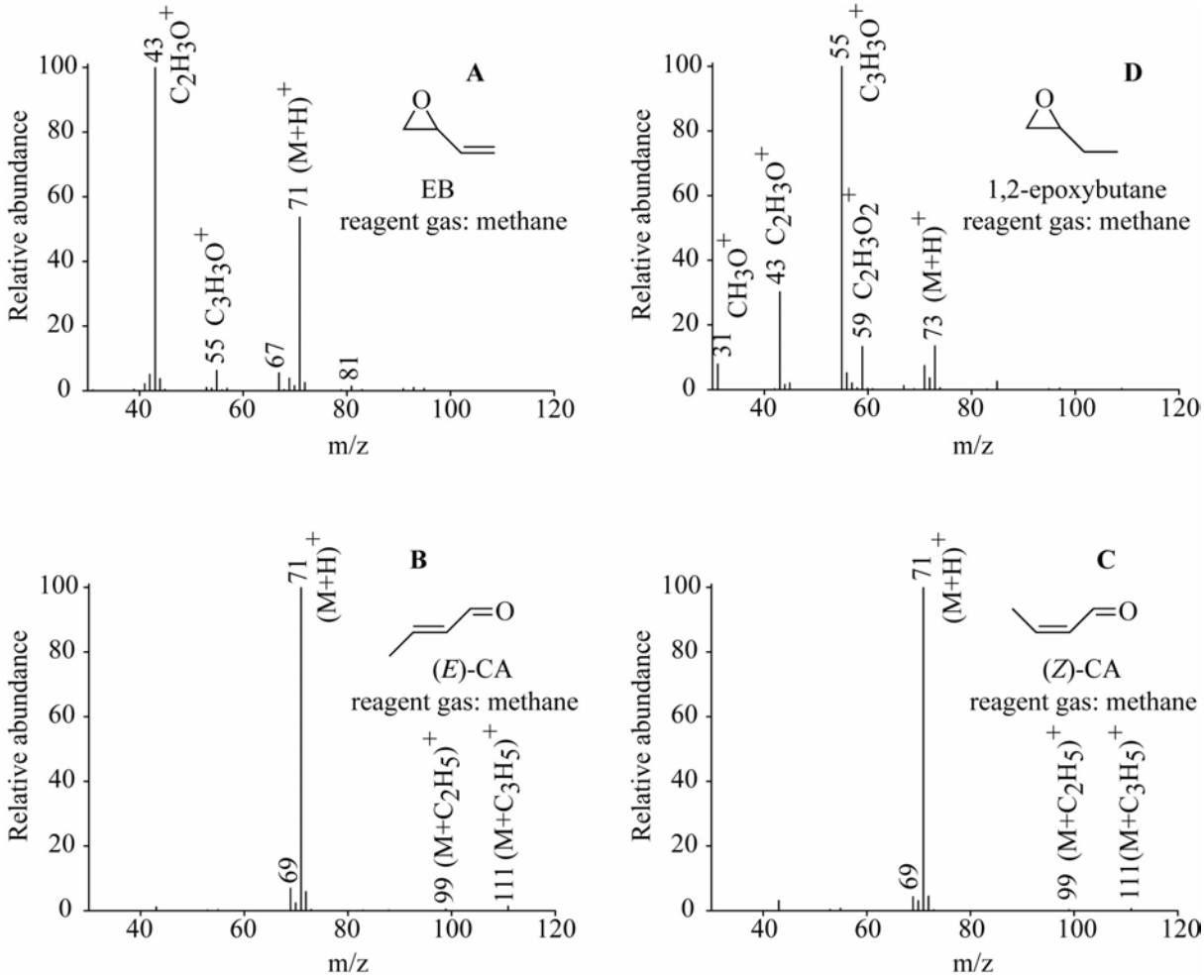


Figure 9: Normalized positive chemical ionisation mass spectra of EB, 1,2-epoxybutane, (*E*)-CA and (*Z*)-CA. The spectra were obtained from GC/MS chromatograms monitored in scan mode after background subtraction

Calibration curves obtained with 0.01- 59 µmol/L EB and 0.06 - 58 µmol/L CA in the perfusate in the presence of 46 µmol/L 1,2-epoxybutane were linear **(Figure 10)**. The linear regression analysis performed on the corrected area (refer the method section) versus the analyte concentration resulted in m ± SE as 0.011 ± 0.00005 (m = slope and SE = standard error) and r^2 = 0.9999 (n = 9) for EB and 0.040 ± 0.0012 and r^2 = 0.9992 (n = 8) for (*E*)-CA. The detection limit was 0.01 µmol/L for EB and 0.06 µmol/L for (*E*)-CA.

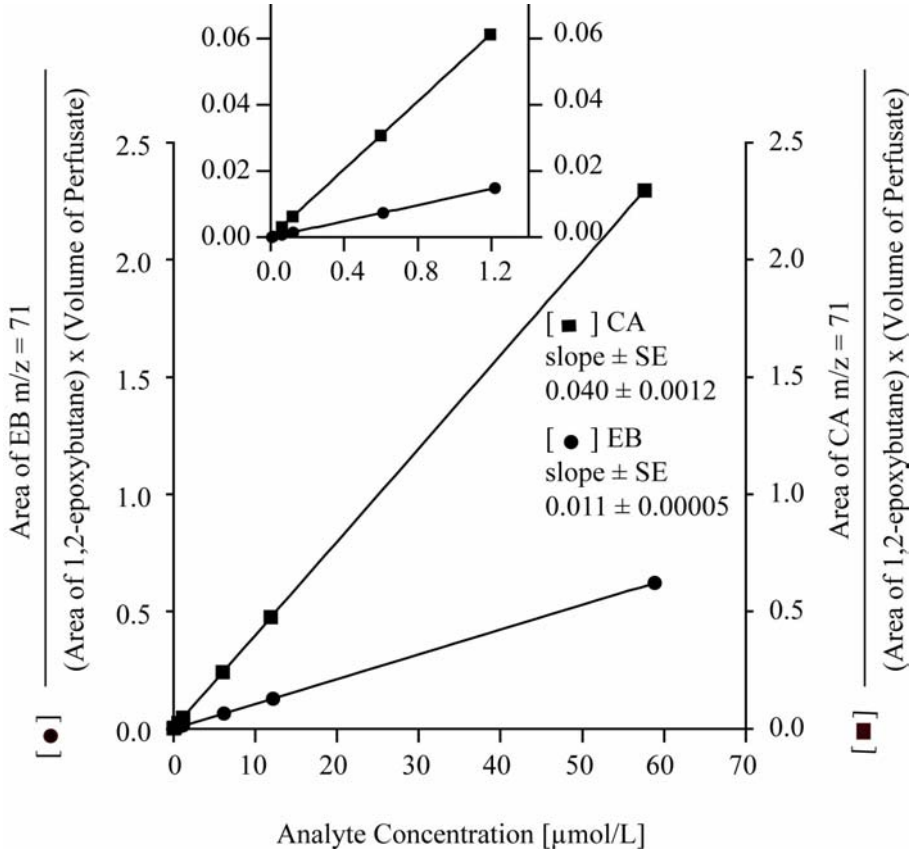


Figure 10: Calibration curves of EB [●] and CA [■] prepared in the perfusate containing 46 µmol/L 1,2-epoxybutane as internal standard (IS)

The analyte area was normalized to that of IS and corrected for the perfusate volume.

Analyte:	EB and CA
SE:	standard error
Symbols (●) and (■):	measured data
Solid lines:	obtained by curve fit

Method evaluation

For the reproducibility of the GC/MS method within a day, dichloromethane samples obtained after extraction of the perfusate containing known concentrations of the analytes (refer 2.2.2.2) were injected in triplicates (**Table 6**). The coefficient of variation was below 6 % even at low concentrations.

Analyte	Concentration [μmol/L]	Peak area	Mean	Standard Deviation	Coefficient of Variation (%)
EB	54.4	17470	17133	603	3.5
	54.4	16437			
	54.4	17493			
	8.8	3733	3608	137	3.8
	8.8	3630			
	8.8	3462			
	1.2	347	330	16	4.7
	1.2	328			
	1.2	316			
CA	53.3	139639	143997	3808	2.6
	53.3	145669			
	53.3	146684			
	8.6	24571	25057	434	1.7
	8.6	25352			
	8.6	25247			
	1.1	2649	2685	155	5.8
	1.1	2854			
	1.1	2551			

Table 6: Variation of the GC/MS measurements of dichloromethane solutions containing EB and CA at different concentrations within a day

Extraction efficiency was investigated at different concentrations. The calculated recoveries are given as percentage in **Table 7**. The mean recoveries were (% recovery ± SD) 79.8 ± 4.8 for EB, 20.5 ± 4.0 for CA and 66.4 ± 6.0 for 1,2-epoxybutane (IS).

Analyte	Conc. expected [µmol/L]	Amount expected [µmol]	Amount found [µmol]	Recovery (%)	Mean recovery (%)
EB	48.0	0.0480	0.0360	75.0	79.8
	47.3	0.0473	0.0353	74.5	
	48.2	0.0482	0.0372	77.2	
	12.5	0.0125	0.0101	83.3	
	11.8	0.0118	0.0101	85.6	
	12.2	0.0122	0.0110	83.3	
CA	47.2	0.0472	0.0085	18.1	20.5
	47.0	0.0470	0.0080	17.0	
	46.3	0.0463	0.0090	19.4	
	12.2	0.0122	0.0032	26.2	
	11.5	0.0115	0.0020	17.4	
	12.0	0.0120	0.0030	25.0	
1,2-epoxybutane	45.0	0.0450	0.0350	77.8	66.4
	44.9	0.0449	0.0282	62.8	
	44.2	0.0442	0.0290	65.6	
	11.4	0.0114	0.0075	65.8	
	11.0	0.0110	0.0072	65.5	
	11.7	0.0117	0.0071	60.7	

Table 7: Recoveries of EB, CA and 1,2-epoxybutane from perfusate
Conc.: concentration

The variation of the recovery within a day can be characterised by the coefficient of variation obtained from the standard deviation and the mean extraction efficiencies calculated over a certain concentration range for each analyte. The coefficient of variation is found to be 6% for EB and 19% for CA.

To obtain the day-to-day variation of the recovery, three concentrations (54 µmol/L, 11 µmol/L and 5 µmol/L) for EB and CA were selected from the calibration curves obtained on different days. The slopes of the calibration curves were determined by curve fit and for three

pre-given concentrations the corrected areas [area of the analyte / (area of the internal standard * volume of perfusate)] were calculated. These data were used to calculate the standard deviation and coefficient of variation for three concentrations and three days, considering the variation in the corrected area to be a measure for the day-to-day variation of the recovery (**Tables 8A and 8B**).

Conc.	**Corrected area**			**Mean**	**SD**	**CV (%)**
[µmol/L]	**Day 1**	**Day 2**	**Day 3**			
54.0	0.5048	0.5519	0.5526	0.5364	0.027	5.1
11.0	0.1148	0.1130	0.1281	0.1186	0.008	7.0
5.0	0.0658	0.0602	0.0701	0.0654	0.005	7.6

Table 8A: Day-to-day variation of the extraction recovery of dichloromethane solutions containing EB, calculated by means of the corrected area of calibration curves Conc.: concentration; SD: standard deviation; CV: coefficient of variation

Conc.	**Corrected area**			**Mean**	**SD**	**CV (%)**
[µmol/L]	**Day 1**	**Day 2**	**Day 3**			
54.0	1.9826	1.9472	1.7827	1.9042	0.1067	5.6
11.0	0.4071	0.4017	0.3589	0.3892	0.0264	6.8
5.0	0.1896	0.1854	0.1844	0.1865	0.0028	1.5

Table 8B: Day-to-day variation of the extraction recovery of dichloromethane solutions containing CA, calculated by means of the corrected area of calibration curves Conc.: concentration; SD: standard deviation; CV: coefficient of variation

The variations expressed by the CVs demonstrate that the extraction method was reproducible for both EB and CA. These variations are larger than those of the GC/MS measurements because the former include the variations of the latter.

Stabilities of EB and CA in the dichloromethane layer

The dichloromethane layers obtained following the extraction of perfusate samples containing defined concentrations of EB, CA and 1,2-epoxybutane were analysed immediately following preparation, after 24 h while standing at room temperature and after one week while stored at –80°C. The measured concentrations of EB and CA are given in **Table 9**.

Analyte	Concentration expected [µmol/L]	Concentrations measured [µmol/L]		
		immediately	after 24 h (RT)	after 7 days (-80°C)
EB	70.0	70.0	65.4	73.4
	70.0	73.8	50.9	70.0
	70.0	69.4	53.0	72.3
	11.0	10.7	n.d	11.3
	11.0	10.0		10.5
	11.0	9.7		12.8
CA	40.0	47.7	34.2	33.0
	40.0	33.9	29.9	35.1
	40.0	43.2	31.2	45.5
	11.0	9.0	n.d	8.3
	11.0	8.1		9.1
	11.0	8.4		7.4

Table 9: Stability determination of 1,2-epoxy-3-butene (EB) and crotonaldehyde (CA) for a certain time range and different storage conditions
n.d: not done due to MSD failure
RT: room temperature

The values of EB and CA concentrations determined immediately after preparation are very similar to those obtained after one week when the samples had been stored at –80°C. This indicates that both compounds are stable in the dichloromethane layer stored at –80°C at least up to a period of one week. But from the measurements carried out after 24h, while the samples stood at room temperature, a decrease in concentration of about 20% becomes obvious. So, care had to be taken to cool the autosampler tray at least to –4°C while the samples stood for 24h.

3.1.3 1,2:3,4-diepoxybutane

GC/MS measurements

The dichloromethane extracts were analysed by GC/MS in PCI mode using ammonia as the reagent gas. **Figure 11 (A & B)** shows single ion chromatograms for m/z = 104 and m/z = 110, which are the masses of the $(M+NH_4)^+$-adducts of DEB and DEB-D6. The stereoisomers of commercially available racemic DEB were not resolved chromatographically. The two diastereomers of DEB-D6 could be baseline separated (**Figure 11B**). The typical retention times were 8.06 min for racemic DEB and 7.93 min for meso-DEB-D6 and 8.03 min for racemic DEB-D6. In the dichloromethane solutions of the internal standard, the absolute peak areas of racemic and meso-DEB-D6 showed a 1:1 ratio. Also in the perfusate samples obtained during liver perfusions, the absolute peak areas of racemic and meso DEB were similar. To quantify meso DEB, the calibration curve of racemic DEB was utilised. With this GC/MS method no disturbing peaks were observed for blanks (dichloromethane extracts of pure perfusate containing no analyte) at the retention times of the analytes. The mass spectrum showed a major peak of $(M+NH_4)^+$ corresponding to m/z = 104 for DEB (**Figure 12A**) and 110 for DEB-D6 diastereomers (**Figure 12B & 12C**). A minor peak related to $(M+NH_4NH_3)^+$ was observed for both DEB-D6 diastereomers. For quantitative analysis, the m/z of 104 and 110 were monitored for DEB and DEB-D6 respectively.

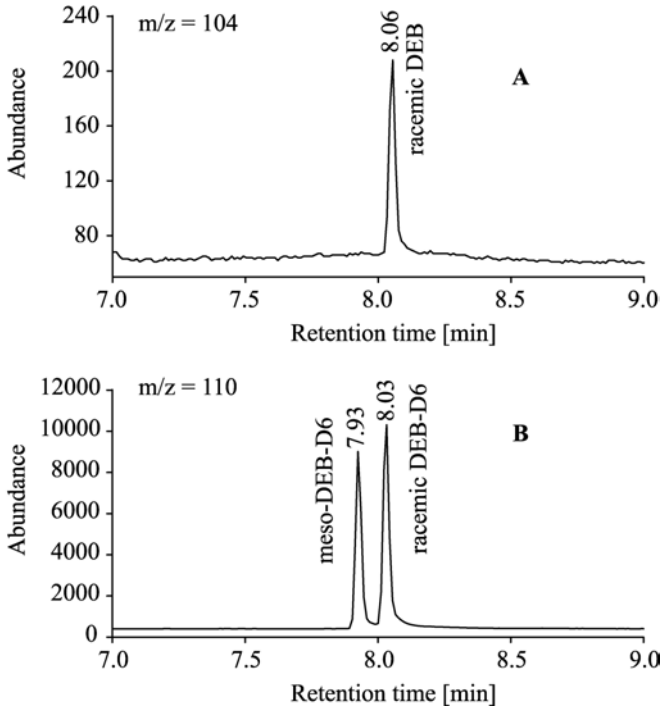


Figure 11: Single ion chromatograms of the dichloromethane extract of a perfusate sample containing 0.076 µmol/L racemic DEB and 6.4 µmol/L meso DEB-D6 and 6.4 µmol/L racemic DEB-D6

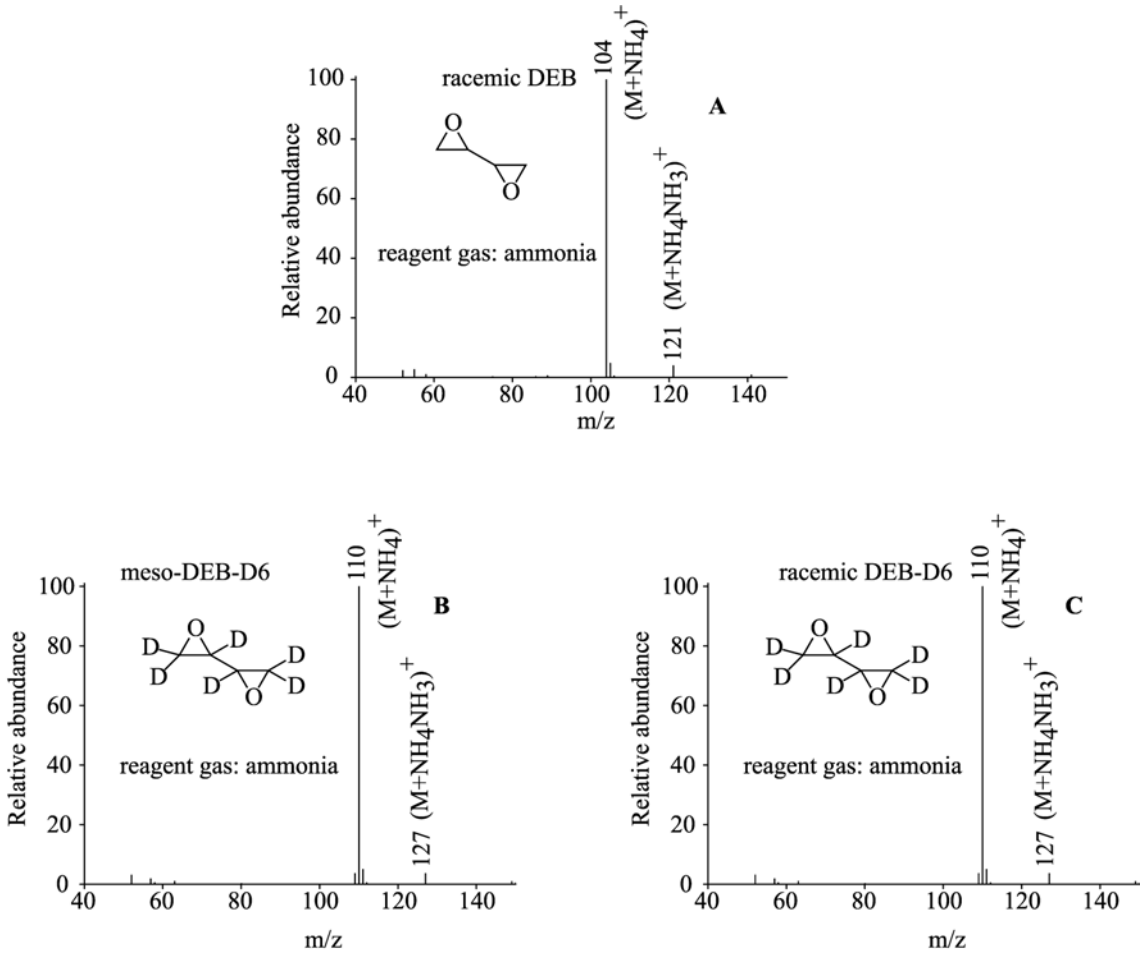


Figure 12: Normalized positive chemical ionisation mass spectra of racemic DEB, meso-DEB-D6 and racemic DEB-D6. The spectra were obtained from GC/MS chromatograms monitored in scan mode after background subtraction

Calibration curves obtained with 0.015 - 74 µmol/L racemic DEB in perfusate in the presence of 6.4 µmol/L racemic DEB-D6 were linear. The linear regression analysis performed on the corrected area (refer method section) versus the analyte concentration (**Figure 13**) resulted in m ± SE (slope ± standard error) as 0.185 ± 0.0008 and r^2 = 0.9999 (n = 8). In order to quantify meso-DEB, the slope of the calibration curve of meso-DEB was considered to be identical to that of racemic DEB. The detection limit of 0.01 µmol/L observed for racemic DEB is expected to be identical for the meso form.

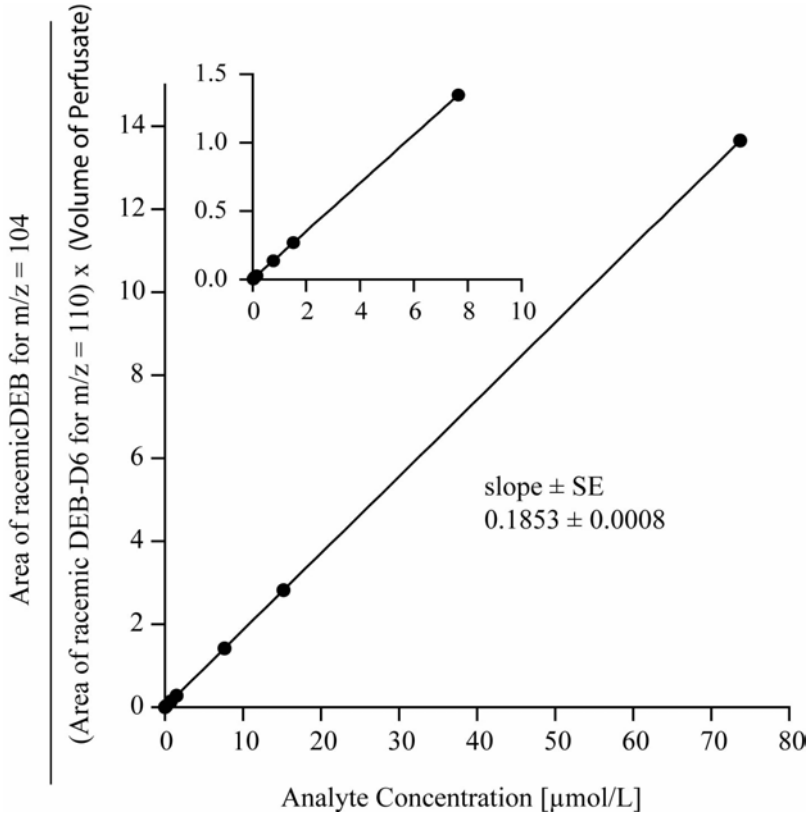


Figure 13: Calibration curve of racemic DEB prepared in perfusate containing 6.4 µmol/L racemic DEB-D6 as internal standard (IS)

The analyte area was normalized to that of IS and corrected for the perfusate volume.

Analyte:	DEB
SE:	standard error
Symbols (●):	measured data
Solid lines:	obtained by curve fit

Method evaluation

For the reproducibility of the GC/MS method within a day, dichloromethane samples obtained after extraction of perfusate containing known concentrations of the analytes were

injected in triplicates (**Table 10**). The coefficient of variation was about 10% even at low concentrations.

Concentration [µmol/L]	Corrected area	Mean	Standard deviation	Coefficient of variation (%)
0.06	0.0104	0.012	0.0014	11.4
0.06	0.0130			
0.06	0.0124			
0.80	0.1295	0.14	0.0069	5.0
0.80	0.1395			
0.80	0.1427			
12.4	2.1873	2.25	0.05	2.2
12.4	2.2744			
12.4	2.2751			

Table 10: Variation of GC/MS measurements of dichloromethane solutions containing racemic DEB at different concentrations within a day

Extraction efficiency was investigated for different concentrations and the calculated recoveries are given as percentage in **Table 11**. The mean recoveries were (% recovery ± SD) 47.8% ± 6.3 for racemic DEB, 48.1% ± 6.2 for meso-DEB-D6 and 44.3% ± 6.8 for racemic DEB.

The variation of the recovery within a day can be characterised by the coefficient of variations obtained from the standard deviations and the mean recoveries calculated over a certain concentration range for each analyte. The coefficient of variation was 13.2% for racemic DEB, 12.9% for meso DEB-D6 and 15.3% for racemic DEB-D6.

To obtain the day-to-day variation of the recovery, three concentrations (15 µmol/L, 1.5 µmol/L and 0.8 µmol/L) for racemic DEB were selected from the calibration curves obtained on different days. The slopes of the calibration curves were determined by curve fit and for three pre-given concentrations the corrected areas [area of the analyte / (area of the internal standard * volume of perfusate)] were calculated. These data were used to calculate the standard deviation and coefficient of variation for three concentrations and three days, considering the variation in the corrected area to be a measure for the day-to-day variation of the recovery (**Table 12**).

Analyte	Conc. expected [µmol/L]	Amount expected [µmol]	Amount found [µmol]	Recovery (%)	Mean recovery (%)
racemic DEB	67.5	0.0675	0.0372	55.1	
	70.1	0.0701	0.0381	54.4	
	72.0	0.0720	0.0366	50.8	
	14.0	0.0140	0.0068	48.6	47.8
	14.7	0.0147	0.0069	48.9	
	14.2	0.0142	0.0069	48.6	
	1.4	0.0014	0.0007	50.0	
	1.4	0.0014	0.0005	35.7	
	1.5	0.0015	0.0006	40.0	
racemic DEB-D6	27.9	0.0279	0.0142	50.9	
	28.9	0.0289	0.0146	50.5	
	29.7	0.0297	0.0140	47.1	
	5.8	0.0058	0.0025	43.1	44.3
	6.1	0.0061	0.0027	44.3	
	5.9	0.0059	0.0027	45.8	
	0.6	0.0006	0.0003	50.0	
	0.6	0.0006	0.0002	33.3	
	0.6	0.0006	0.0002	33.3	
meso-DEB-D6	27.9	0.0279	0.0151	54.1	
	28.9	0.0289	0.0155	53.6	
	29.7	0.0297	0.0149	50.2	
	5.8	0.0058	0.0027	46.6	48.1
	6.1	0.0061	0.0028	45.9	
	5.9	0.0059	0.0029	49.2	
	0.6	0.0006	0.0003	50.0	
	0.6	0.0006	0.0002	33.3	
	0.6	0.0006	0.0003	50.0	

Table 11: Recoveries of racemic DEB, racemic DEB-D6 and meso-DEB-D6 from perfusate

Conc.: concentration

Conc. [µmol/L]	Corrected area			Mean	SD	CV (%)
	Day 1	Day 2	Day 3			
15.0	2.6830	2.3000	2.2430	2.4087	0.239	9.9
1.5	0.2613	0.2768	0.2443	0.2608	0.016	6.1
0.8	0.1270	0.1403	0.1280	0.1318	0.007	5.6

Table 12: Day-to-day variation of the extraction recovery of dichloromethane solutions containing DEB, calculated by means of the corrected area of the calibration curves
Conc.: concentration SD: standard deviation
CV: coefficient of variation

From these CVs it follows that the extraction method was reproducible for racemic DEB between the days. These variations were larger than those of the GC/MS measurements because here variations of both, extraction and GC/MS measurements are included.

Stability of DEB in the dichloromethane layer

The dichloromethane layer obtained following the extraction of perfusate samples containing a defined concentration of racemic DEB and a constant concentration of IS was analysed immediately, after 24 hr while standing at room temperature and after one week while stored at –80°C. The measured concentrations of racemic DEB are given in **Table 13**.

Concentration expected [µmol/L]	Concentration measured [µmol/L]		
	immediately	after 24 h (RT)	after 7 days (–80°C)
1.0	0.99	0.90	1.06
1.0	0.96	0.87	0.99
1.0	0.81	0.83	1.09

Table 13: Stability determination of racemic DEB for a certain time range
RT: room temperature

The above data reflects that racemic DEB is relatively stable in the dichloromethane layer at least over a period of one week when stored at –80°C and also while standing at room temperature for ~24h.

3.1.4 3-Butene-1,2-diol

GC/MS measurements

The ethyl acetate extracts of the boron esters of B-diol (2-butyl-4-vinyl-[1,3,2]dioxaborolane) and B-diol-D8 obtained after derivatisation with 4 mg/mL n-butylboronic acid (refer 2.2.2.4) were analysed by GC/MS in PCI mode in the same chromatographical run (**Figure 14A & B)**. The figure shows single ion chromatograms of m/z = 109 for derivatised B-diol and of m/z = 161 for derivatised B-diol-D8. The reasoning for the choice of different masses is explained below. The typical retention time for both derivatised B-diol and B-diol-D8 was 7.45 min. The mass spectra of the derivatised B-diol (**Figure 15A**) consisted of a major $(M+H)^+$ peak of m/z = 155 along with a peak of m/z = 99 $(M - C_4H_7)^+$ and a fragment of m/z = 109. The peak of m/z = 109 was utilized for quantification of B-diol, being better in shape and without any disturbance in the blank (ethyl acetate extracts of pure perfusate containing no analyte). The peak at m/z = 155 was used only for qualitative identification since there was a disturbance peak at m/z = 155. The boron ester of B-diol-D8 produced a similar fragmentation pattern as that of B-diol. Here, the $(M+H)^+$ peak of m/z = 161 **(Figure 15B**) was utilized for quantification of derivatised B-diol-D8 while the fragment peak of m/z = 115 was utilized for identification. In all these cases, a quantifiable analyte peak was considered to have three times the area of the base line noise.

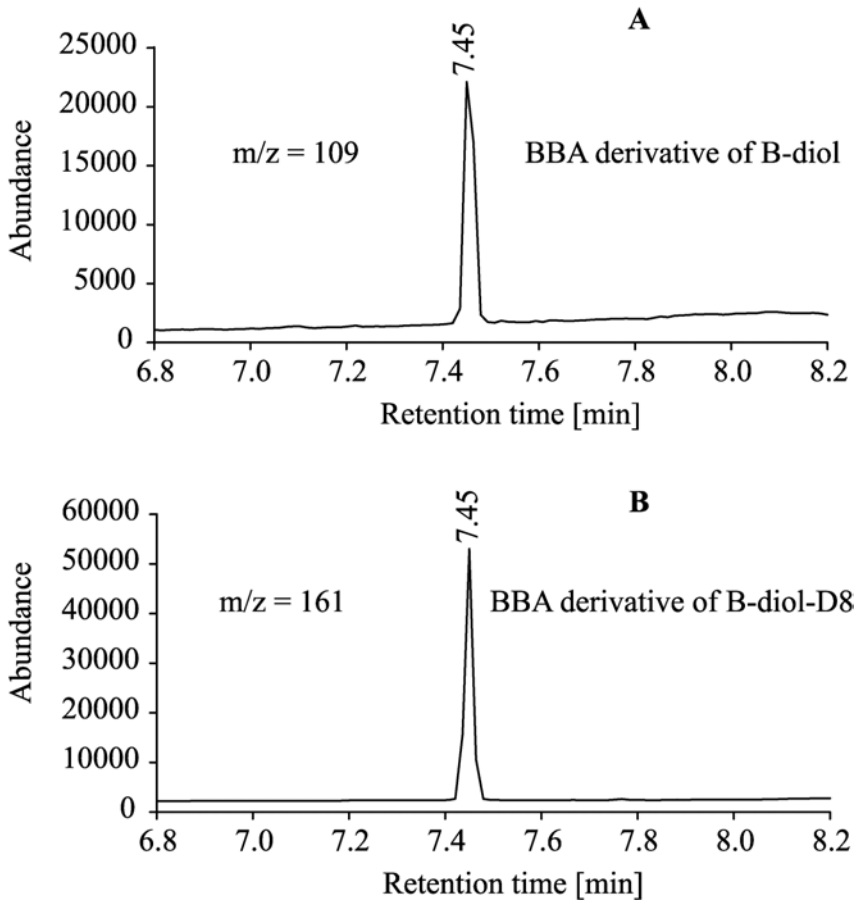


Figure 14: Single ion chromatograms showing a perfusate sample containing 6.7 µmol/L B-diol and 9.8 µmol/L B-diol-D8 after derivatisation with n-butylboronic acid and extraction with ethyl acetate
BBA: n-butylboronic acid

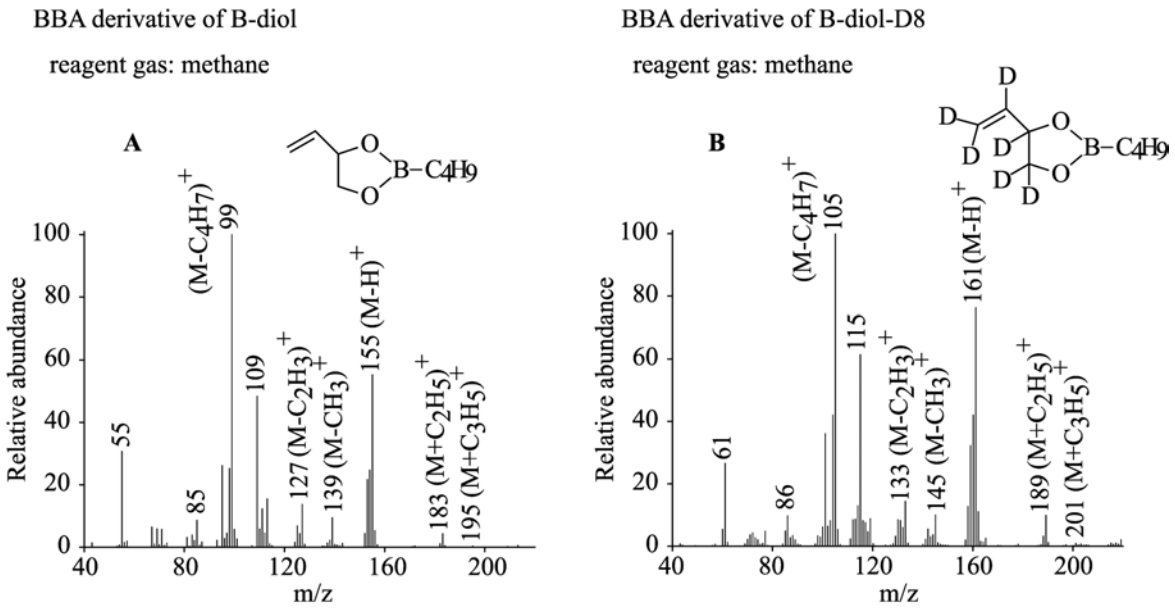


Figure 15: Normalized positive chemical ionisation mass spectra of the butylboron esters of B-diol (2-butyl-4-vinyl-[1,3,2]dioxaborolane) and B-diol-D8. The spectra were obtained from GC/MS chromatograms monitored in scan mode after background subtraction
BBA: n-butylboronic acid

The calibration curve obtained with 0.02 - 97 µmol/L B-diol in perfusate in the presence of 6 µmol/L of B-diol-D8 was linear. The linear regression analysis performed on the corrected area versus the analyte concentration **(Figure 16)** resulted in m ± SE as 0.0354 ± 0.0001 (m = slope; SE = standard error) and r^2 = 1.000 (n= 9). The detection limit of 0.02 µmol/L was found for B-diol.

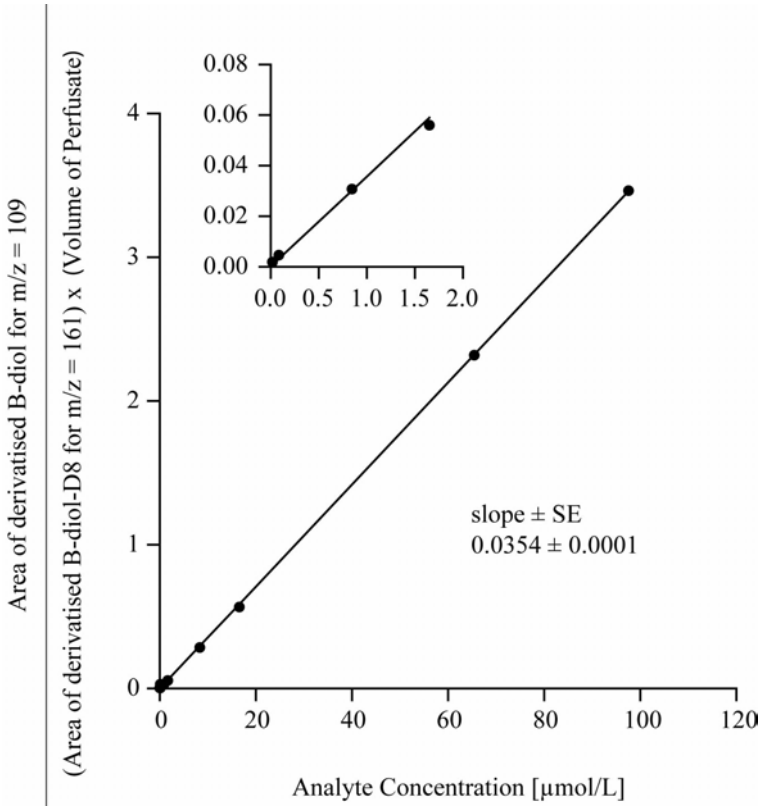


Figure 16: Calibration curve of B-diol prepared in perfusate containing 6 µmol/L B-diol-D8 as the internal standard (IS)

The analyte area was normalised to that of IS and corrected for the perfusate volume.

Analyte:	B-diol
SE:	standard error
Symbols (●):	measured data
Solid lines:	obtained by curve fit

Method evaluation

For testing the reproducibility of the GC/MS method within a day (refer 2.2.2.4), ethyl acetate extracts obtained after derivatisation of perfusate samples with n-butylboronic acid containing known concentrations of the analytes were injected in triplicates (**Table 14**). The coefficients of variation were below 10 % even at low concentrations.

Concentration [μmol/L]	Area	Mean	Standard deviation	Coefficient of variation (%)
43.7	19577	20609	1921	9.3
43.7	19425			
43.7	22825			
9.7	5047	5161	310	6.0
9.7	4925			
9.7	5512			

Table 14: Variation within a day in the GC/MS measurements of ethyl acetate extracts containing derivatised B-diol

Efficiency of the extraction of the derivatised product from the perfusate was calculated using the slopes of the calibration curves of derivatised B-diol relative to that of the derivatised B-diol-D8, obtained in perfusate ($m_p = 0.9054$), water ($m_w = 0.9474$) and acetone ($m_a = 0.8263$) as discussed in the method section (2.2.2.4). The extraction factor (%) ± $SE_{efficiency}$ (standard error) from perfusate and water was found to be 72.5% ± 2.0 and 75.9% ± 3.0 respectively.

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

During the perfusion experiments the samples were measured within the time frame of one week from the day of the experiment while the samples were stored at –80°C. So it was necessary to check the stability of the analytes in the perfusate supernatant for this time period. The method of standard addition was followed to determine the stabilities of B-diol and B-diol-D8 in the perfusate supernatants over a period of three weeks (refer 2.2.2.4). The result (**Table 15**) indicates them to be quite stable at –80°C over this time period. This satisfied our requirement.

Analyte	Concentration added initially (µmol/L)	Concentration obtained (µmol/L)		
		1st week	2nd week	3rd week
B-diol	22.8	21.6	21.8	19.4
	9.7	10.7	10.6	10.1
B-diol-D8	24.3	19.8	17.5	17.0
	4.9	3.6	3.9	3.9

Table 15: Stability determination of B-diol and B-diol-D8 in the perfusate supernatant stored at –80°C over a period of three weeks

3.1.5 3,4-Epoxybutane-1,2-diol

GC/MS measurements

The ethyl acetate extracts (refer 2.2.2.5) containing butylboron esters of the diastereomeric EBD (2-butyl-4-oxiranyl-[1,3,2]dioxaborolane) and its internal standard EBD-D6 were analysed by GC/MS in PCI mode using ammonia as the reagent gas. The diastereomeric forms of the EBD and EBD-D6 boron esters could be baseline separated. **Figure 17A** shows a single ion chromatogram for m/z = 188 with typical retention times of 8.61 min and 8.70 min for the boron ester adducts $(M+NH_4)^+$ of the EBD diastereomers. **Figure 17B** shows a single ion chromatogram for m/z = 194 with typical retention times of 8.61 min and 8.69 min for the boron ester adducts $(M+NH_4)^+$ of the EBD-D6 diastereomers. **Figure 18 (A & B)** depicts the mass spectra consisting of major peaks reflecting the boron ester adducts $(M+NH_4)^+$ of diastereomeric EBD (m/z = 187 and 188) and of diastereomeric EBD-D6 (m/z = 193 and 194). The intensity ratio of m/z = 187 to m/z = 188 is similar to that of the deuterated counterpart (m/z = 193 to m/z = 194) and corresponds to the isotope distribution of naturally occurring boron isotopes (^{10}B 15.9% and ^{11}B 80.1%).

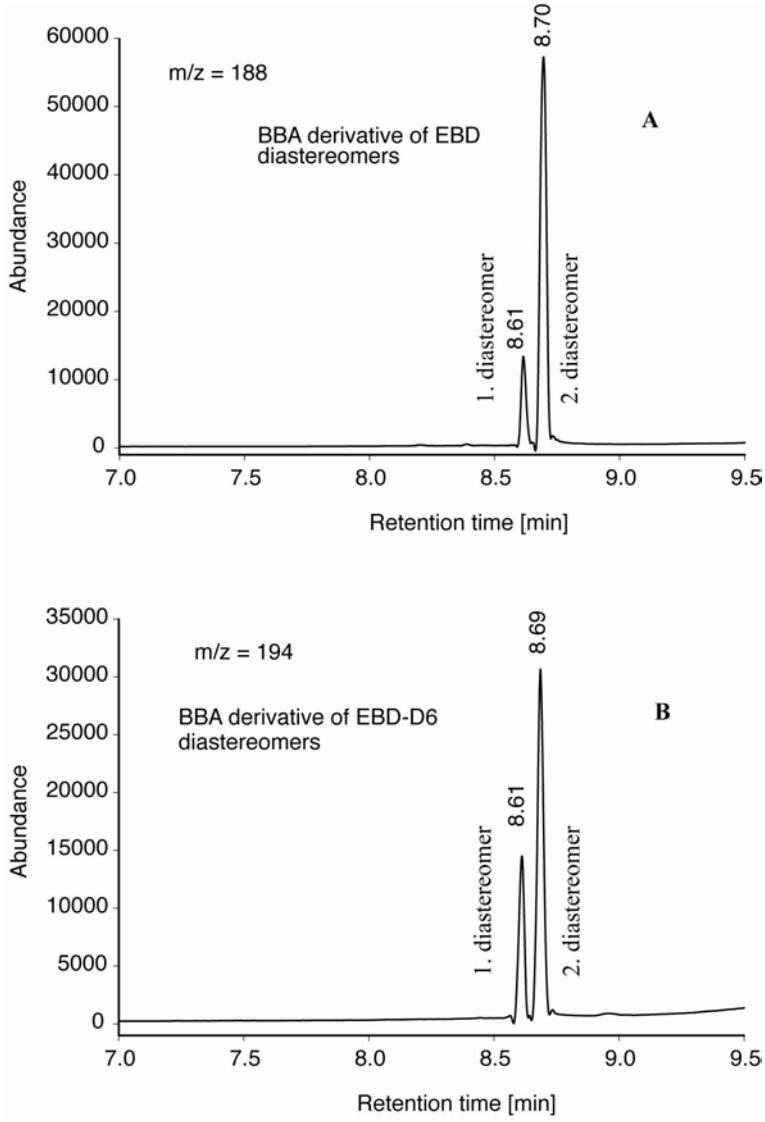


Figure 17: Single ion chromatograms showing a perfusate sample containing 13.2 µmol/L diastereomeric EBD and 25 µmol/L diastereomeric EBD-D6 after derivatization with n-butylboronic acid and extraction with ethyl acetate. As the stereochemical structures of the diastereomeric EBD derivatives were not known, they are described as 1. diastereomer (with lower retention time) and 2. diastereomer (with higher retention time).
BBA: n-butylboronic acid

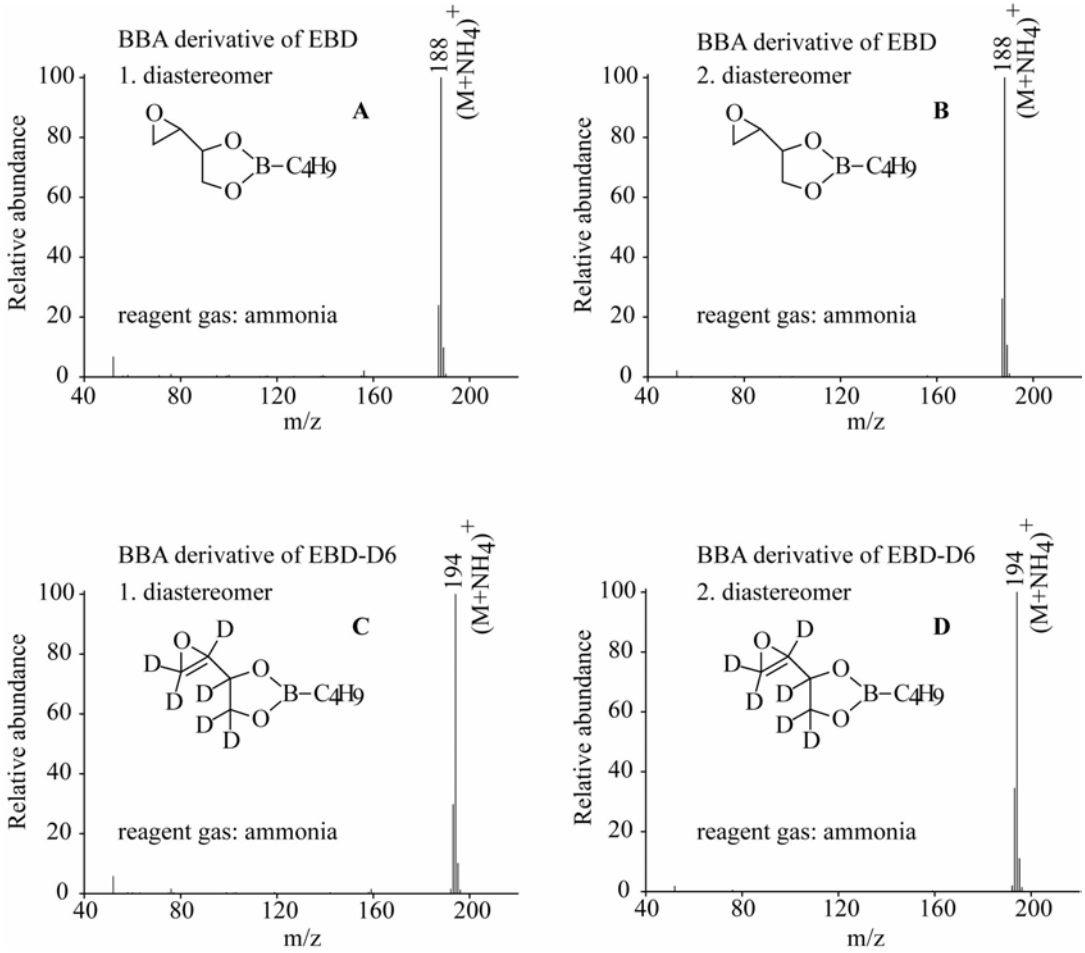


Figure 18: Normalized positive chemical ionization mass spectra of the butylboron esters of diastereomeric EBD (2-butyl-4-oxiranyl-[1,3,2]dioxaborolane) and EBD-D6. The spectra were obtained from GC/MS chromatograms monitored in scan mode after background subtraction.
BBA: n-butylboronic acid

In order to construct calibration curves for EBD, concentrations from 0.04 to 92 µmol/L diastereomeric EBD in perfusate were prepared in presence of 25 µmol/L diastereomeric EBD-D6. Assuming a 1:1 ratio of the diastereomers, the concentration for each of them ranged from 0.02 to 46 µmol/L, each containing 12.5 µmol/L of the respective EBD-D6 diastereomer. The calibration curves obtained were passed through the origin and were linear (**Figure 19**). Regression analysis performed on the areas of each diastereomeric EBD to the corresponding diastereomeric EBD-D6 area (corrected area) versus the analyte concentrations resulted in m ± SE of 0.0128 ± 0.0002 and $r^2 = 0.9973$ (n = 8) for the first diastereomer of

EBD and 0.0163 ± 0.0003 and r^2 = 0.9982, (n = 8) for the second diastereomer of EBD. The detection limit was 0.02 µmol/L for each EBD diastereomer.

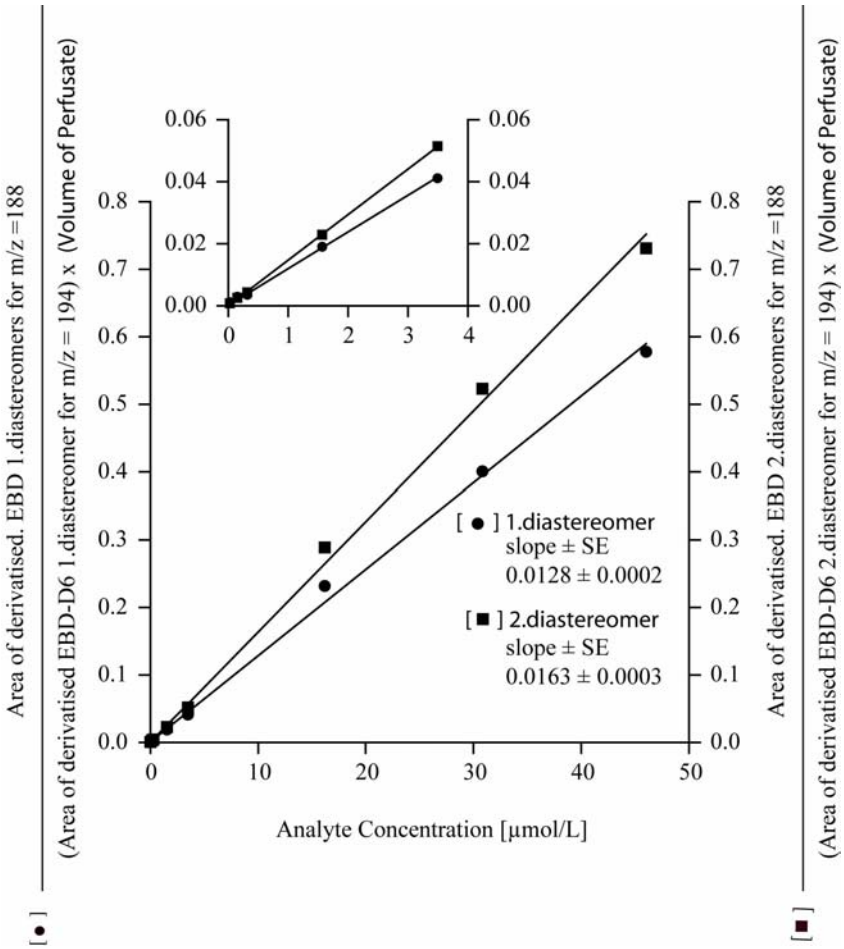


Figure 19: Calibration curves of EBD diastereomers [1 (●) and 2 (■)] prepared in perfusate containing 12.5 µmol/L of each diastereomeric EBD-D6 as the internal standard (IS)

The areas of the analytes were normalized to that of the IS and corrected for the perfusate volume.

Analyte:	EBD
SE:	standard error
Symbols (●) and (■):	measured data
Solid lines:	obtained by curve fit

Method validation

For the reproducibility of the GC/MS method within a day, ethyl acetate extracts obtained after derivatisation of perfusate samples with n-butylboronic acid containing known concentrations of the diastereomeric EBD and EBD-D6 were injected in triplicates (**Table 16**). The coefficient of variation was below 3 % even at low concentrations.

Analyte	Concentration [µmol/L]	Corrected area	Mean	Standard deviation	Coefficient of variation (%)
EBD (dia.1)	30.8	0.408	0.406	0.0057	1.5
	30.8	0.411			
	30.8	0.400			
	3.5	0.0421	0.042	0.0006	1.5
	3.5	0.0422			
	3.5	0.0411			
EBD (dia.2)	30.8	0.5051	0.507	0.003	0.6
	30.8	0.5104			
	30.8	0.5055			
	3.5	0.0515	0.052	0.0006	1.2
	3.5	0.0525			
	3.5	0.0513			

Table 16: Variation within a day in the GC/MS measurements of ethyl acetate extracts containing the EBD diastereomers 1 and 2, respectively

The relative percentage recovery of derivatised diastereomeric EBD from the perfusate and water was calculated by comparing the slopes of the calibration curves of EBD relative to that of EBD-D6, obtained in the perfusate (m_p : 1.diastereomer = 0.8454, 2.diastereomer = 0.9103), water (m_w: 1.diastereomer = 0.7370, 2.diastereomer = 0.9281), and in acetone (m_a: 1.diastereomer= 1.0373, 2.diastereomer = 1.0612) as described in (2.2.2.5). The relative percentage recovery ± $SE_{efficiency}$ (standard error) from perfusate was 81.5% ± 11.5 and 85.8% ± 25.7 for 1.diastereomer and 2.diasteromer and from water 71.1% ± 2.4 and 85.8% ± 28.3 for 1.diastereomer and 2.diasteromer respectively.

Stabilities of EBD and EBD-D6 in the perfusate supernatant

Stability determination of EBD and EBD-D6 (refer 2.2.2.5) was carried out according to the method of standard addition (**Table 17**). The scatterings in the data indicate the analytical problems, which might be related to the standard addition method. The analyte seems to be stable for at least one week at –80°C, during which the analysis of the perfusion samples are usually carried out. After the period of one week a decrease is observed.

Analyte	**Concentration added initially (µmol/L)**	**Concentration obtained (µmol/L)**		
		1st week	**2nd week**	**3rd week**
EBD (dia.1)	24.7	23.2	12.2	n.d
	12.7	12.9	13.2	6.9
EBD (dia.2)	24.7	23.2	12.2	n.d
	12.7	12.9	13.2	6.9

Table 17: Stability determination of EBD diastereomers over a period of three weeks
n.d.: not done

3.2 Perfusion experiments

In the perfusion experiments (refer 2.2.3), the liver was perfused with constant flows which were chosen to agree with conditions in vivo where the corresponding flows are reported to be 21 and 4 mL/min (Arms and Travis, 1988) for rats and mice respectively. BD concentration in the perfusate entering the liver was kept constant. BD concentration in the perfusate leaving the liver appeared to approach a steady-state value, which remained almost constant after 10 min and 40 min of perfusion of rat and mouse livers respectively, up to the end of the exposure as exemplarily shown in **Figure 20** and **Figure 21**.

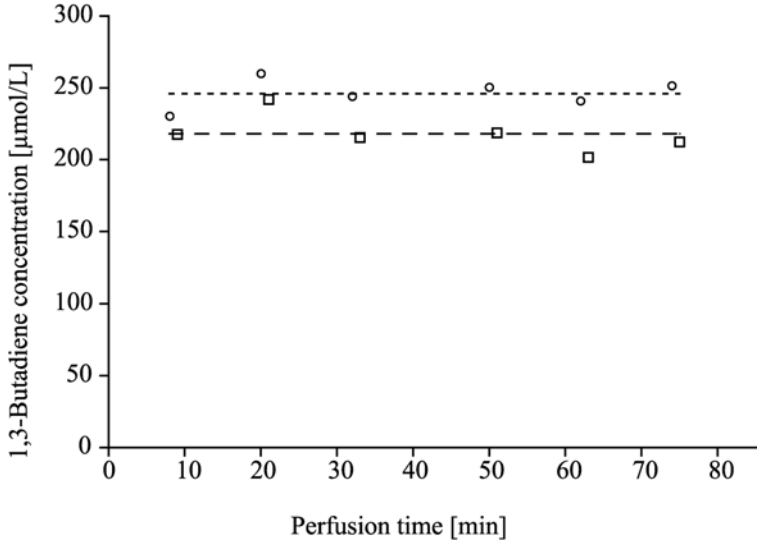


Figure 20: Concentration-time courses of 1,3-butadiene in the perfusate entering (O) and leaving (□) a once-through perfused rat liver during exposure to 1,3-butadiene

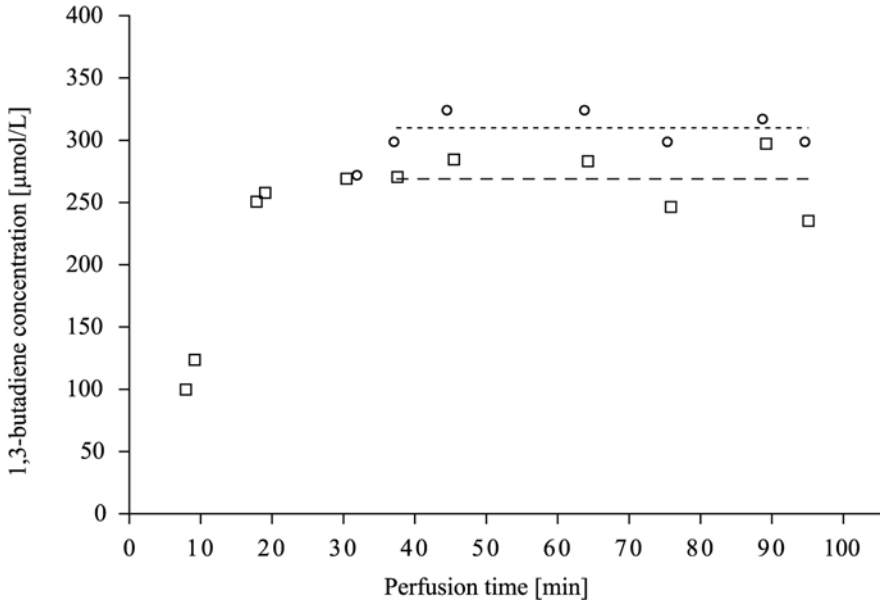


Figure 21: Concentration-time courses of 1,3-butadiene in the perfusate entering (O) and leaving (□) a once-through perfused mouse liver during exposure to 1,3-butadiene

In case of **Figure 21** data points for BD in the influent perfusate is observed only after ~30 min of perfusion. This was due to negligence in sample collection for BD measurement in the influent perfusate from the beginning of the perfusion for this particular experiment.

Figure 22 and **Figure 23** give typical concentration-time courses of EB and B-diol measured before and after the liver of a rat and a mouse respectively. A significant difference between the mean values in the effluent perfusates and those in the influent perfusates was observed, the effluent being of much higher value than the influent. The EB concentrations before the liver might result from BD oxidation by the erythrocytes as has been reported for styrene (Bitzenhofer, 1993; Belvedere and Tursi, 1981). Some hydrolysis of EB to B-diol is probably catalyzed by the perfusion medium too. Otherwise it would not have been detected, since EB is rather stable in Tris-buffer at pH 7.4 (37°C) with a half-life of 13.7 h. (Gervasi et al., 1985). The slower perfusion rate of the mouse liver leading to a longer stay of BD in the medium is probably the cause of the higher concentrations of EB and B-diol in the perfusate entering the liver as observed in **Figure 23**. The concentrations in the effluent perfusate parallel the time axes indicating that steady states were reached.

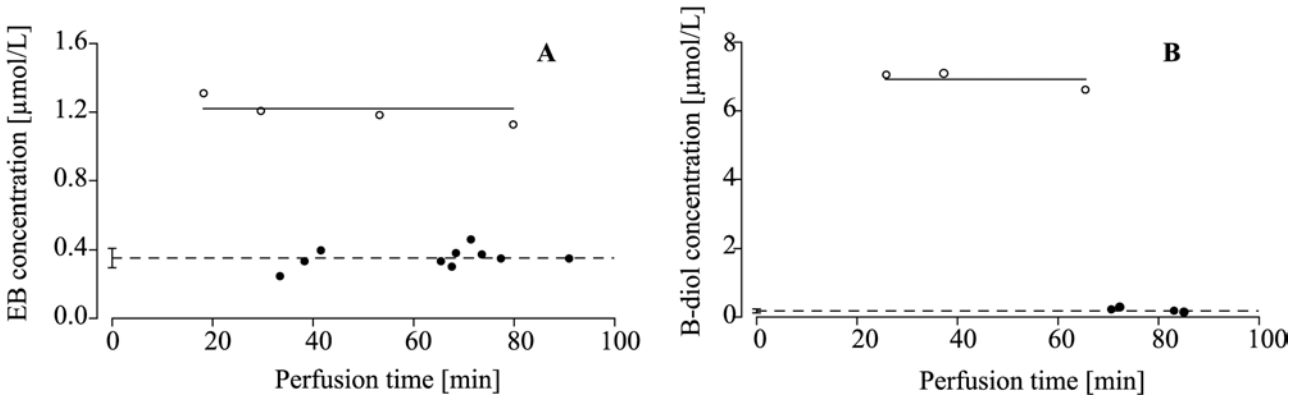


Figure 22: Concentrations of EB (A) and B-diol (B) in the once-through perfusate during exposure of a rat liver to BD (280 µmol/L perfusate). (●): influent perfusate (mean ± S.D.; n = 10 for EB and n=4 for B-diol; determined in different experiments at the given time points); (O): effluent perfusate

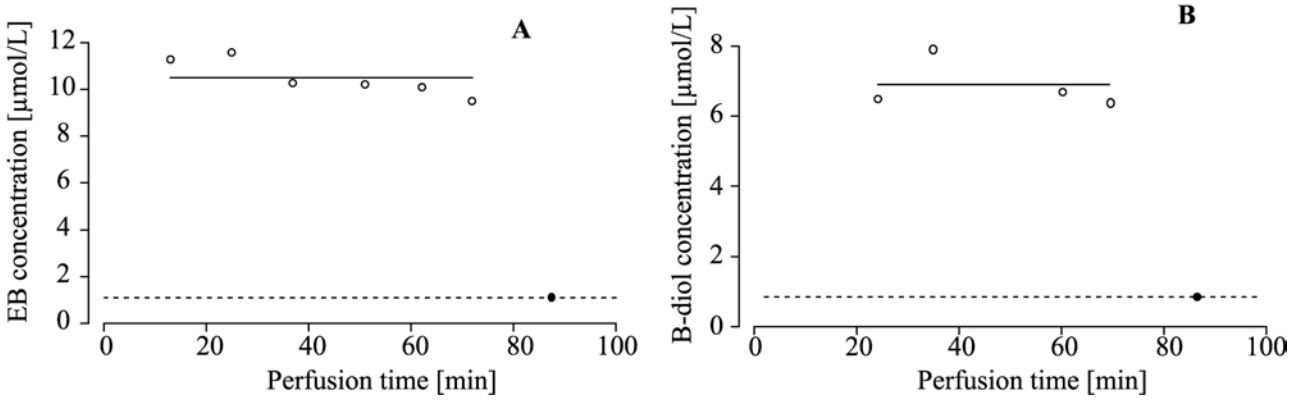


Figure 23: Concentrations of EB (A) and B-diol (B) in the once-through perfusate during exposure of a mouse liver to BD (400 µmol/L perfusate).
(●): influent perfusate; (O): effluent perfusate

DEB and EBD concentrations during BD perfusion of a mouse liver are depicted in **Figure 24**. In BD perfused livers of rats, both metabolites were not detectable.

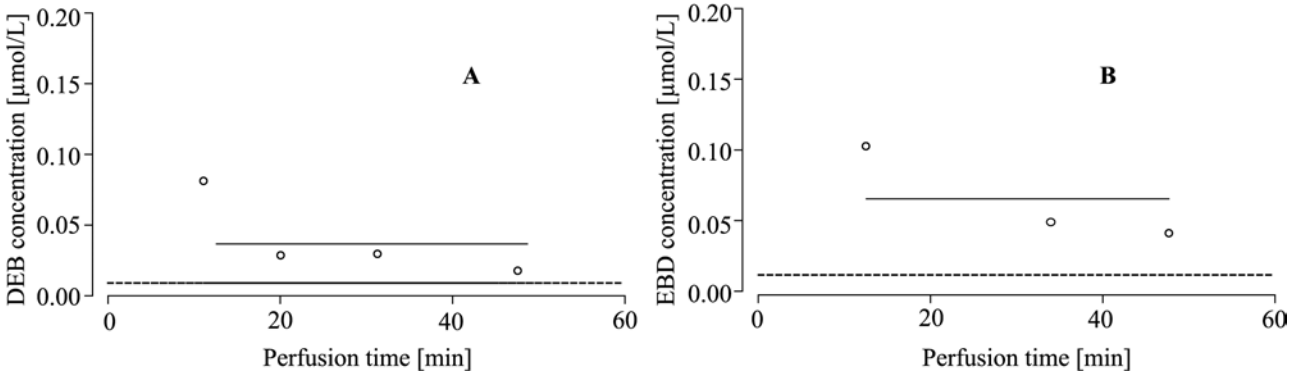


Figure 24: Concentration-time courses of DEB (A) and EBD (B) in the perfusate leaving (O) a once-through perfused mouse liver during exposure to BD (400 µmol/L perfusate). The dashed lines depict the limits of detection. Neither DEB nor EBD was detectable in the perfusate entering the liver

CA was not detectable. There was no peak observed at the retention time where CA should be found.

4. Discussion

4.1 Determination of 1,3-butadiene and selected metabolites

The present work dealt with identification and quantitation of BD and a series of metabolites (EB, CA, DEB, B-diol, and EBD) by means of gas chromatography. Methods were developed to extract the analytes from the perfusion medium (refer 2.2) before chromatographical analysis. For gaining the metabolites from the perfusate, several techniques were investigated: solid-phase extraction, liquid-liquid extraction, and derivatisation followed by solvent extraction. The gas chromatographic analyses of the metabolites were carried out by mass spectrometric detection (GC/MS) using internal standards. For ionisation of the compounds the PCI mode was used due to its higher selectivity compared to the EI mode, which results from less fragmentation in the PCI mode (Martz et al., 1983; Harison, 1992). In comparison to the EI mode, the PCI mode showed equal sensitivity. Both methane and ammonia were used as reagent gases in the PCI mode. Various column materials were investigated for the optimum separation: from highly polar wax columns to non-polar ones. The non-polar HP-5MS gave the best separation. A film thickness of 1.0 µm was used, as columns with such dimensions are most suitable for separation of compounds, which elutes best between 100 to 200°C (Böcker, 1997; Grob, 1995).

4.1.1 1,3-Butadiene

Various references are found for analysis and detection of BD. Himmelstein et al. (1994) determined BD from blood by the headspace technique using a gas chromatograph equipped with flame ionisation detector as also used by Sato and Nakajima (1979). Similar procedures of headspace analysis of BD in simulated saliva in contact with chewing gum was carried out by GC/FID by Abrantes et al. (2000). 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. Bechtold et al. (1995) also determined BD from blood by the method of headspace analysis but using a gas chromatograph equipped with a mass selective detector. GC/FID procedures to measure BD and other gaseous compounds on a Tenax column were also published by Filser et al.

(1983), Bolt et al. (1983) and Johanson and Filser (1993). Considering all the above cases, in the present case the BD determination and quantification from the perfusion medium was based on the principles of the headspace analysis along with the GC/FID procedure. The detection limit in the present work was 0.9 ppm (0.04 μmol/L) being identical to that of Himmelstein et al. (1994). Bechtold et al. (1995) achieved a detection limit of 0.1 μmol/L (100 pmol/mL). Detection limits of 0.07 μmol/L and of 0.04 μmol/L (1 ppm) were reported by Abrantes et al. (2000) and by Meischner (1999), respectively. A low variability of about 3% in the reproducibility measurement of the present GC/FID method and the achievement of the fairly low detection limit compared to the other published methods, satisfied the sensitivity requirement for BD measurement during the perfusion experiments carried out in the present work.

4.1.2 1,2-Epoxy-3-butene and crotonaldehyde

Several publications indicate various methodologies used for the detection of EB and CA. Himmelstein et al. (1994) and Leavens et al. (1996) reported a determination of EB from blood after being extracted with dichloromethane using GC/MS in EI mode. Isolation of the volatile BD metabolite EB from rat and mouse tissue by cryogenic vacuum distillation followed by GC/GC/MS analysis was reported by Thornton-Manning et al. (1995a). Sharer et al. (1992a) and Elfarra et al. (1991) extracted EB and CA from microsomes with toluene and carried out GC/FID analysis. Cheng and Ruth (1993) reported a headspace GC/FID analysis of these metabolites from rat liver microsomes.

In trying to establish a method for EB and CA determination, all of the above cited procedures except that of the Thornton-Manning et al. (1995a) were investigated for applicability. The GC/FID methods were not selective and sensitive enough to distinguish between EB, CA and their internal standard 1,2-epoxybutane peak. Different types of solvent (ethanol, methanol, ethyl acetate, acetonitrile and dichloromethane) with varying polarity were tested for direct analyte extraction from the perfusate. Ethyl acetate, which was used as the extraction solvent by Cheng and Ruth (1993) for the analysis of EB, was futile may be due to the following reasons. A direct ethyl acetate extraction from perfusate was not feasible as the retention gap deteriorated quickly from run to run. This might have been caused by the lower lipophilicity

of ethyl acetate compared to that of dichloromethane, which could have increased the extraction of hydrophilic impurities. Removing the erythrocytes by centrifugation before extracting with ethyl acetate solved this problem, but the extraction of the perfusate supernatant twice with ethyl acetate and reducing the volume of the pooled organic layer under a stream of nitrogen resulted in a very low analyte recovery. With the other solvents either many disturbance peaks were observed along with the main analyte peak or the extraction recovery was too low. The efficiency of toluene as the extracting solvent as used by Sharer et al. (1992a) and Elfarra et al. (1991) was not tested. Among the investigated solvents, dichloromethane was found to be most suitable for EB, CA and 1,2-epoxybutane extractions, as in this case highly polar impurities were not extracted along with the main analytes. In the present work, a headspace method using GC/MS equipped with PTI/TCT CP4010 injector (Purge and Trap Injector/Thermal Desorption Cold Trap Injector) from Chrompack was also tried for quantification of EB and CA. But due to the tedious and time consuming procedure and also due to difficulty in the leak proof installation of TCT test tubes in the injector before individual analysis could be carried out, this method failed. Finally, EB and CA were extracted directly from the perfusion medium using dichloromethane and quantified by GC/MS. No prior protein precipitation was required, which made the procedure very simple and less time consuming.

EB and CA along with their internal standard 1,2-epoxybutane were analysed by GC/MS in the PCI mode using methane as reagent gas. Before analysing these compounds in the PCI mode their identification was done in the EI mode following the method of Himmelstein et al. (1994) and Leavens et al. (1996) by comparing their library searched mass spectra from the NIST'98 mass spectral library. But the EI mode was not sensitive enough for the analysis from liver perfusate, as it led to too much fragmentation. As the mass spectra with ammonia as the reagent gas are expected to show less fragmentation compared to methane (Harison, 1992), ammonia was also tested as reagent gas for the PCI analysis of EB and 1,2-epoxybutane. Since the use of ammonia deteriorates the functioning of the diffusion pump over time, GC/MS analysis was finally performed with methane as the reagent gas.

A detection limit of 0.01 µmol/L for EB and 0.06 µmol/L for (*E*)-CA was achieved in the perfusate considering the analyte peaks to be at least three times higher in abundance compared to the disturbance peaks. For EB, Himmelstein et al. (1994) published a detection

limit of 0.03 µmol/L and Leavens et al. (1996) of 0.15 µmol/L. In both cases methylene chloride extracts of blood were analysed with GC/MS in the EI mode. Thornton-Manning et al. (1995a) reported a detection limit of 0.03 µmol/kg for EB detected from tissues using a self constructed vacuum line-cryogenic distillation followed by GC/GC/MS analysis. Elfarra et al. (1991), carrying out GC/FID analysis of toluene extracts, published a detection limit of 3 µmol/L for EB and 2 µmol/L for CA. A somewhat higher sensitivity of 1 µmol/L for EB was claimed by Cheng and Ruth (1993). Thus, it becomes obvious that the detection limit obtained for EB and CA after direct extraction with dichloromethane from perfusate and analysis in the PCI mode using methane as the reagent gas proved to be more sensitive than the other published methods.

Extraction efficiency for EB reported by Himmelstein et al. (1994) was greater than 70 % using a volume ratio of methylene chloride to blood of 5 to 1. Using the same solvent Leavens et al. (1996) achieved an extraction efficiency greater than 95 % with a volume ratio of 3 to 1. Both authors did not measure CA. In the present study recoveries ± SD were 79.8% ± 4.8% for EB and 20.5% ± 4.0% for CA were obtained using a much smaller volume ratio of 1 to 5 in order to avoid a concentrating step, which could have led to an unpredictable loss of the volatile EB. Neither Himmelstein et al. (1994) nor Leavens et al. (1996) mentioned standard deviations of extraction measurements. Also reproducibility and efficiency of extraction recovery was not reported. The variability of 5% for the GC/MS measurements within a day for both CA and EB and also the variability of about 7 % for extraction efficiencies of both analytes, both within and between the days, validated the robustness of the analytical method developed in the present case.

The concentrations of EB, CA and also DEB (see below) in perfusate were determined within one week after finishing the perfusion experiments. Within this time period the concentrations of the solvent extract remained constant if stored at –80°C as was confirmed by stability experiments (3.1.2 and 3.1.3). Because of their volatility longer store periods were avoided.

4.1.3 1,2:3,4-Diepoxybutane

DEB is less volatile than EB. Due to its structural similarity with EB Himmelstein et al. (1994), Leavens et al. (1996), Thornton-Manning et al. (1995a) and Cheng and Ruth (1993) had used the same procedures for the determination of DEB as cited in section 4.1.2 for EB. For DEB quantitation from blood samples, Bechtold et al. (1995) published a headspace analysis using GC/FID. In the present work, the methods of Himmelstein et al. (1994), Leavens et al. (1996), Cheng and Ruth (1993) and also further analytical procedures including direct extraction of DEB from the perfusate using dichloromethane with 1 to 1, 1 to 2 and 1 to 5 perfusate to solvent volume ratios in the presence and absence of NaCl (for better phase separation) were tried out. The extraction procedure of Cheng and Ruth (1993) using ethyl acetate proved to be ineffective in contrast to that using dichloromethane. Additionally tested solid phase extraction of DEB with a medium polar RP-18e phase matrix using acetonitrile as solvent proved futile. Though with the more polar NH_2/diol phase and dichloromethane as solvent, DEB could be extracted, but the whole procedure of solid phase extraction proved to be too time consuming. Quantitation of DEB with GC/FID and GC/MS using different injectors (KAS-Kaltaufgabesystem or on column) with capillary columns of both polar and non polar nature were also investigated. Finally, the direct DEB extraction from the perfusate using dichloromethane with 1 to 5 solvent to perfusate volume ratio and quantification by GC/MS according to the same principles as described above in the case of EB proved to be the most easy and sensitive procedure to be carried out.

The use of PCI was necessary as in the EI mode a disturbing peak was detected to occur at the same retention time as that of racemic DEB. Furthermore, DEB showed substantial fragmentation in the EI mode, with no single relatively high abundance characteristic compound peak. Unfortunately, the PCI mode using methane as the reagent gas was not selective enough to distinguish between the disturbance and the analyte peak appearing at very close retention times. However, with ammonia as the reagent gas, the PCI mass spectrum showed much less fragmentation than using methane. The racemic DEB and racemic DEB-D6 consisted mainly of a major $(M+NH_4)^+$-peak and a minor $(M+NH_4NH_3)^+$-peak, leading to clear peak identification.

A detection limit of 0.01 µmol/L for DEB was obtained in the present method. With the exception of Bechtold et al. (1995), who reported the same value, other published methods are less sensitive. Himmelstein et al. (1994) obtained a detection limit of 0.13 µmol/L and Leavens et al. (1996) of 0.30 µmol/L after GC/MS analysis in the EI mode. Krause and Elfarra (1997a) and Cheng and Ruth (1993) published a detection limit of 1.3 µmol/L and 1 µmol/L, respectively, after GC/FID analysis of incubation mixtures containing liver microsomes.

Himmelstein et al. (1994) and Leavens et al. (1996) reported the same extraction efficiency for DEB as they have reported for EB (greater than 70% and 95% respectively). In the present study a recovery ± SD of 48% ± 6.3% for racemic DEB was obtained using a much smaller volume ratio of 1 to 5 in order to avoid the concentrating step, which could lead to an unpredictable loss of the volatile DEB. Variability within 10% for the GC/MS measurements within a day and variability within 15% for the extraction method both within a day and between the days, indicated good reproducibility for both methods. Neither Himmelstein et al. (1994) nor Leavens et al. (1996) mentioned standard deviations of extraction measurements. Also reproducibility and efficiency of extraction recovery was not reported.

4.1.4 3-Butene-1,2-diol

The current work characterises the isolation of B-diol from the perfusate, its derivatisation and the quantitative analysis by GC/MS. Concerning the isolation of B-diol no comparable method is published. The derivatisation of B-diol to its corresponding boron ester in the aqueous phase was carried out according to the method described for ethylene glycol by McCurdy and Everett (1982) and followed by Bogusz et al. (1986). Thereafter, the boron ester was extracted with ethyl acetate. This procedure results in a more efficient extraction than the reversed one (Knapp, 1979), which was used by Needham et al. (1982) and Balikova and Kohlícek (1988) for 1,2- and 1,3-diols. These authors carried out extraction prior to derivatisation. The derivatisation of diols with n-butylboronic acid to their n-butylboronate esters for enhancement of volatility is well known (Knapp, 1979).

Published methods of liquid-liquid extraction followed by subsequent gas chromatographic analysis of B-diol from urine (Anttinen-Klemetti et al., 1999) and direct extraction of B-diol from microsomes by ethyl acetate followed by GC/FID analysis (Cheng and Ruth, 1993) were also tested in the present work. Carrying out direct extraction of B-diol from perfusate with various solvents with different polarity as methanol, ethyl acetate (according to Cheng and Ruth (1993) and Nieusma et al. (1998)) and acetonitrile proved to be futile as B-diol could not be extracted reproducibly and separated distinctly from other disturbing substances. The procedure of solid phase extraction of B-diol from blood followed by derivatisation with N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane and GC/MS analysis carried out by Kemper et al. (1998) was also tried out. The solid phase extraction with partially polar RP-18e phase matrix and acetonitrile, or with more polar NH_2/diol matrix and acetonitrile resulted either in non-reproducible extraction efficiencies or inefficiency to detect the analyte. When B-diol analysis of GC/MS using a KAS injector was carried out, the peaks showed strong tailing and not reproducible retention times. May be, the vaporisation in the KAS injector was incomplete. Summarising, all of these methods were less suitable for B-diol analysis than the direct n-butylboronic acid esterification followed by ethyl acetate extraction.

The ethyl acetate extracts were analysed by GC/MS in PCI mode using methane as reagent gas. Ammonia was also tested as reagent gas to reduce fragmentation of B-diol and its IS B-diol-D8. The resulting mass spectra for boron esters of B-diol and B-diol-D8 contained a very low abundance compound peak for $(M+NH_4)^+$ and $(M+H)^+$ ions, compared to the peaks obtained with methane. Also big disturbance peaks from other mass numbers were observed at the same retention times as the analyte peaks. Since ammonia has a negative influence on the oil pump, too, GC/MS analysis was restored with methane as the reagent gas.

The limit of detection for B-diol was 0.02 µmol/L. Anttinen-Klemetti et al. (1999) and Kemper et al. (1998) published much higher detection limits of 0.9 µmol/L and 0.7 µmol/L, respectively. Cheng and Ruth (1993) claimed a detection limit of 1.0 µmol/L for B-diol after GC/FID analysis. The achievement of a low detection limit in the present case in comparison to the other published methods, validates and emphasises the high sensitivity of the current method along with it's simple methodology. It also fulfils the requirement of B-diol analysis in micro amounts as became obvious during the perfusion experiments.

The extraction efficiency ± $SE_{efficiency}$ from perfusate and water was 72.5% ± 2.0% and 75.9% ± 3.0%, respectively. Similar values in both matrices indicate that perfusate exerts no additional effect on derivatisation and the extraction procedure. The low standard deviations demonstrate the reproducibility and robustness of the method.

4.1.5 3,4-Epoxybutane-1,2-diol

As has been mentioned in the introduction, only one method is published for the analysis of EBD. Cheng and Ruth (1993) claimed to have extracted EBD from BD treated microsomes after evaporation to dryness of the microsomal suspension followed by dilution in ethyl acetate and analysis in the GC/FID mode. Reports of N-terminal valine adducts to haemoglobin of BD exposed rats (Perez et al., 1997) and humans (Begemann et al., 2001), presumably resulting from EBD, hint to the quantitative relevance of this mutagenic metabolic intermediate of BD. The detection of the N7-guanine adduct of EBD formed from the 2*S*-3*R* enantiomer in lung tissues of BD exposed mice is reported by Koivisto and Peltonen (2001).

In the present work, several techniques of direct extraction of EBD from perfusate using solvents of different polarity including ethyl acetate according to Cheng and Ruth (1993) as well as solid phase extraction with medium polar RP-18e phase and more polar NH_2/diol both with acetonitrile as the eluent were tried, but all of them were unsuccessful. By these methods EBD was not extractable in substantial amounts from the perfusate so that it could be detected in micro amounts on GC/MS analysis. For analysis with GC/MS both on column and KAS injection was tried out. In case of KAS injection either no distinct EBD peak appeared or there was an overlap with a disturbance peak. Apart from certain small changes, the best method so far achievable for quantitation of EBD in micro amounts from the perfusate was according to Kimura et al. (1976), where the epoxides as 1,2:5,6-dihydrogalactitol bearing vicinal OH groups had been determined from plasma by derivatisation with boronic acid. In the current method (refer 2.2.2.5), diastereomers of EBD and its deuterated IS diastereomeric EBD-D6 were extracted from perfusate with a 1:10 volume ratio of i-propanol/chloroform (9/1) followed by derivatisation with n-butylboronic acid. Extractability was enhanced by the addition of potassium carbonate to the reacting medium. As the epoxide was relatively

unstable in potassium carbonate saturated perfusate supernatant, isopropanol/chloroform was first mixed with the supernatant and the biphasic system was then generated by addition of the salt. To minimise the effect of co-extraction of undesired contaminants from the perfusate by the highly polar i-propanol, chloroform was added to decrease its polarity. Also to remove contamination by erythrocytes, the perfusate was centrifuged to obtain a clear supernatant before carrying out the extraction. To increase the volatility of the molecule and to reduce its interaction with the stationary support phase of the GC-column, the hydroxyl groups were derivatised with n-butylboronic acid yielding the boron esters.

The boron esters of the diastereomeric EBD were analysed by GC/MS in the PCI mode using ammonia as the reagent gas. The use of the PCI mode was necessary as in the EI mode the distinction between the EBD derivative and its IS EBD-D6 derivative was not sensitive enough. The PCI mode using methane as the reagent gas was not selective enough to distinguish between the disturbance and the analyte peaks appearing at very close retention time intervals of the two diastereomers. In contrast to this, with ammonia as the reagent gas, the PCI mass spectrum of diastereomeric boron esters of EBD and EBD-D6 consisted only of a major $(M+NH_4)^+$-peak along with a less relative abundance peak for the isotopic boron ester of $(M+NH_4)^+$.

In the present work, the detection limit was 0.02 μmol/L for each EBD diastereomer, indicating the method to be sensitive enough for the liver perfusion experiments.

Similar extraction efficiencies (refer 3.1.5) from perfusate and water indicated that no major interference from the perfusate constituents in the whole derivatisation and extraction process took place. The day-to-day variability for the GC/MS measurements were below 2% emphasizing the robustness of the concerned method.

4.2 Perfusions of rat and mouse liver

The gas-tight all-glass liver perfusion system was used to investigate quantitatively the oxidative and hydrolytic metabolism of BD during its first passage through the liver of mouse and rat. In both species, immediate formation of EB and its hydrolysis product B-diol was

demonstrated. Although B-diol might be a highly relevant BD metabolite because of its probable further metabolism to EBD (see 1.3) which forms characteristic trihydroxybutyl adducts to macromolecules in rodents and also in humans (see above and 1.5), the B-diol production from BD has never been demonstrated in a situation in-vivo or close to in-vivo. This is directly shown for the first time in this work. The same holds true for EBD, which was detected in mouse liver perfusate, since only characteristic adducts have been reported. Whereas the B-diol concentrations were similar in the effluents of the livers of both species, there was a drastic increment in the EB concentration in mouse compared to that in rat.

Concerning EB, the ex-vivo findings can be compared with in vivo data published by Himmelstein et al. (1994) and Meischner (1999). At the same BD exposure concentration in the influent perfusate, the EB concentrations in the effluent perfusate of rat livers was about 7 times smaller than in that of mouse. In vivo, at high BD exposure concentrations, the respective factors in blood were 6.6 (Himmelstein et al., 1994) and 8.3 (Meischner, 1999) being in agreement with the results obtained here. This comparison demonstrates that the difference in the EB burden between mouse and rat can be solely related to the liver. The metabolic share of other organs, which has been discussed as possibly relevant for the total body clearance of BD (Evalo et al., 1993; Medinsky et al., 1994) seems to be negligible for the EB blood burden. Furthermore, it follows that in the mouse much more EB is available for further biotransformation to the highly mutagenic and carcinogenic DEB than in the rat. This agrees with the observation that the tumorigenic potency of BD is much higher in the mouse (1.4).

Compared to the corresponding EB concentrations, B-diol in the effluent perfusates were 7 times higher in rat liver and 0.9 times smaller in mouse liver. This points towards the relevance of the epoxide hydrolase (EH) mediated biotransformation of EB, a detoxification step, which is obviously of minor importance in mice than in rats. This could also explain that DEB and EBD could be found only in mouse liver effluent being already formed during the passage of BD through the liver.

CA was neither found in the effluent of rat livers nor in that of mouse livers. The negative findings in rat livers were expected considering that also in BD exposed rat liver microsomes no CA could be determined (Sharer et al., 1992a). Since these authors reported CA to have

been detected in mouse liver microsomes in a ratio of 1 to 50 in comparison to EB, a CA concentration of about 0.16 µmol/L was vainly expected to be found in the mouse liver effluent. Considering that the perfused liver represents a near to in vivo situation, one has to conclude this assumed direct oxidation product of BD to be of minor importance for the BD metabolism, if of any.

These first results prove the analytical procedures developed to be sensitive enough to measure the BD metabolites in BD perfused livers.

Outlook

The analytical methods established in this work will be used to quantify at a broad concentration range not only the first pass metabolism of BD and also that of its metabolites in perfused livers of rodents and in pieces of human livers. The gained data will then be incorporated in a "physiological toxicokinetic model" in order to calculate the burden of diverse tissues by BD and its metabolites species-specifically. Based on the comparison of this burden among mouse, rat and human, an estimation of the BD related carcinogenic risk for humans will be carried out respecting the results of the long-term studies in BD exposed mice and rats.

5. Summary

Mutagenicity and carcinogenicity of 1,3-butadiene (BD) highly probably results from epoxide metabolites as 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB) and 3,4-epoxybutane-1,2-diol (EBD). A further metabolite crotonaldehyde (CA) has also been discussed to be relevant. So far, in BD exposed rodents only EB and DEB concentrations had been quantified. However, the methods used were either not very sensitive or instrumentally expensive. Therefore, the goal of the present work was to establish simple analytical methods selective and sensitive enough to determine all of these compounds and a further secondary BD intermediate, 3-butene-1,2-diol (B-diol), in BD exposed rodent livers. The once-through perfused liver system was chosen for testing the applicability of the methods to be developed, since it enables BD exposures of this quantitatively most relevant metabolising organ near to the in-vivo situation.

All the metabolites were extracted from the aqueous perfusion medium and analysed using a gas chromatograph equipped with a mass selective detector (GC/MS) in the PCI mode.
The epoxides EB and DEB as well as the aldehyde CA were directly extracted from the perfusion medium with dichloromethane and analysed by GC/MS. For the calibration of EB and CA, 1,2-epoxybutane was used as the internal standard and perdeuterated DEB was used for DEB. The detection limits achieved for EB and DEB were 0.01 µmol/L and that for CA was 0.06 µmol/L. The recoveries ± SD were 79.8 % ± 4.8 % for EB, 20.5 % ± 4.0 % for CA and 48 % ± 6.3 % for racemic DEB. The variabilities of the GC/MS measurements within a day were 6 % for EB and CA and 10 % for DEB. The variabilities of the extraction efficiencies between the days were 7 % for EB and CA and 15 % for DEB. These analytes were confirmed to be stable at –80°C for at least one week.
The highly hydrophilic glycols, B-diol and EBD were derivatised within the aqueous phase with n-butylboronic acid to the corresponding esters and then extracted with ethyl acetate. The ethyl acetate extracts were subjected to GC/MS analysis. For B-diol and EBD calibration, B-diol-D8 and EBD-D6 were used as the internal standards. The detection limit was 0.02 µmol/L for B-diol and for both EBD diastereomers, respectively. The extraction efficiency (recovery ± $SE_{efficiency}$) of B-diol from perfusate was 72.5% ± 2.0% and from water was 75.9% ± 3.0%. The extraction efficiencies ± $SE_{efficiency}$ of the first and the second diasteromer of EBD were 81.5% ± 11.5 and 85.8% ± 25.7 from perfusate and from water 71.1% ± 2.4 and

85.8% ± 28.3 respectively. The day-to-day variability for the GC/MS measurements laid below 10% and 2% for B-diol and EBD respectively. The analytes were confirmed to be stable at –80°C for at least one week.
For all of these compounds neither such sensitive nor such robust methods have been published before.

These methods were applied to quantify BD metabolites in perfusates of BD perfused rat and mouse livers. Freshly prepared livers of mice and rats were perfused with Krebs-Henseleit buffer (pH 7.4) containing 40% washed bovine erythrocytes and BD concentrations of 0 (controls) and about 240 µmol/L (rat) and 330 µmol/L (mice), respectively. Samples from the perfusate were analysed for BD using a headspace method according to published procedures and were tested for the metabolites mentioned. In the perfusate of the controls, no analyte was detectable. In the perfusate of BD exposed mouse livers, BD and all the investigated metabolites (EB: 9.4 µmol/L; DEB: 0.06 µmol/L; EBD: 0.07 µmol/L; B-diol: 8.1 µmol/L) could be determined, with the exception of CA. Only BD together with EB (1.1 µmol/L) and B-diol (6.9 µmol/L) were found in perfusate of BD exposed rat livers. The mean rates of BD transformation were about 0.014 and 0.055 mmol/h/liver of a mouse and a rat respectively, being similar to the values expected from in-vivo measurements.
From these results the relevance of a species-specific intrahepatic first-pass metabolism for metabolic intermediates of BD becomes obvious. Immediately after their production in the liver the first metabolite EB (mouse and rat) as well as the secondary metabolic intermediate B-diol (mouse) undergo further transformation before leaving this organ.

The investigations have demonstrated that the perfused liver system in combination with the highly sensitive analytical methods is an excellent tool to explore quantitatively the species-specific BD metabolism, which probably causes the difference between mice and rats in the carcinogenic potency of BD.

6. Abbreviations

Amt	Amount
b.d.	Below detection limit
BBA	n-Butylboronic acid
BD	1,3-Butadiene
B-diol	3-Butene-1,2-diol
B-diol-D8	Perdeuterated 3-butene-1,2-diol
CA	Crotonaldehyde
Conc.	Concentration
CV	Coefficient of variation
CYP 2E1	Isoenzyme 2E1 of CYP450
CYP450	Cytochrome P-450 dependent monooxygenases
DEB	1,2:3,4-Diepoxybutane
DEB-D6	Perdeuterated 1,2:3,4-diepoxybutane
DEM	Diethyl maleate
DMF	N,N,-Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EB	1,2-Epoxy-3-butene
EBD	3,4-Epoxybutane-1,2-diol
EBD-D6	3,4-Epoxy-[1,1,2,3,4,4-$^{2}H_{6}$]butane-1,2-diol
EH	Epoxide hydrolase
EI	Electron Impact
eV	Electron volt
FID	Flame ionisation detector
GC	Gas chromatograph
GSH	Glutathione
GST	Glutathione S-transferase
h	Hour
IARC	International Agency for Research on Cancer (WHO)
ID	Internal diameter
λ	Partition coefficient, perfusate:air at 37°C

m/z	Mass-to-charge ratio
MAK	Maximum Workplace Concentration (Maximale Arbeitsplazkonzentration) refer Deutsche Forschungsgemeinschaft, List of MAK and BAT values 2001, Report No.37, P-9
min	Minute
Mio	Million
MSD	Mass spectrometric detector
n	Number of experiments
NMR	Nuclear magnetic resonance
PCI	Positive chemical ionisation
Ph	Phenyl
ppb	Parts per billion
ppm	Parts per million
r^2	Coefficient of regression
Rec.	Recovery
RT	Room temperature
SD	Standard deviation
SE	Standard error
TFA	Trifluroacetic acid
THB	Tetrahydroxybutane
TIC	Total ion chromatogram
x_i	Concentration of the ith sample
$\bar{x}$	Mean concentration of n samples
WHO	World Health Organisation

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Naunyn-Schmiedeberg's Arch. Pharmacol., 361, Suppl. 4, R 140, 2000

Andreas Schuster, **Swati Bhowmik**, Thomas H. Faller, György A. Csanády, and Johannes G. Filser
Quantification of the selected butadiene metabolites 1,2-epoxy-3-butene, 1,2:3,4-diepoxybutane and 3-butene-1,2-diol in the effluent of isolated perfused livers of rats and mice
Toxicol Lett, Suppl 1/110, 57, 2000

Johannes Georg Filser, Thomas Herbert Faller, **Swati Bhowmik**, Andreas Schuster, Winfried Kessler, Christian Pütz and György András Csanády
First pass metabolism of 1,3-butdiene in once-through perfused livers of rats and mice
Chemico-Biological Interactions, 135-136, 249-265, 2001

Thomas H. Faller, **Swati Bhowmik**, Andreas Schuster, György A. Csanády, Matthew W. Himmelstein, Johannes G. Filser
Metabolism of 1,3-butadiene in once-through perfused livers of rats and mice
Naunyn-Schmiedeberg's Arch. Pharmacol., 363, Suppl. 4, R 138, 2001

Thomas H. Faller, **Swati Bhowmik**, Supatta Midpanon, Vimal Veereshwarayya, Matthew W. Himmelstein, Johannes G. Filser
Dose dependency of metabolism of 1,3-butadiene in once-through perfused rat liver
Naunyn-Schmiedeberg's Arch. Pharmacol., 365, Suppl. 1, R 127, 2002

7. Literature

Abdel-Rahman, S.Z., Ammenheuser, M.M. and Ward, B.J.J.
Human sensitivity to 1,3-butadiene: role of microsomal epoxide hydrolase polymorphisms
Carcinogenesis, 22, 3, 415-423 (2001)

Abrantes, S., Dossantos, T.C. and Simoes, G.
Headspace gas chromatographic determination of 1,3-butadiene in simulated saliva
JAOAC Int., 83, 4, Jul-Aug, 929-932 (2000)

Anttinen-Klemetti, T., Vaaranrinta, R. and Peltonen, K.
Gas chromatographic determination of 3-butene-1,2-diol in urine samples after 1,3-butadiene exposure
J.Chromatogr. B. Biomed. Sci. Appl., 730, 257-264 (1999)

Arms, A. and Travis, C.
Reference physiological parameters in pharmacokinetic modeling
EPA/600/6-88-004, (1988)

Balíková, M. and Kohlícek, J.
Rapid determination of ethylene glycol at toxic levels in serum and urine
Journal of Chomatography, 434, 469-474 (1988)

Bechtold, W.E., Strunk, M.R., Chang, I., Ward, J.J.B. and Henders, R.F.
Species differences in urinary butadiene metabolites: comparisons of metabolite ratios between mice, rats and humans
Toxicol. Appl. Pharmacol., 127, 44-49 (1994)

Bechtold, W.E., Strunk, M.R., Thornton-Manning, J.R. and Henderson, R.F.
Analysis of butadiene, butadiene monoepoxide, and butadiene dioxide in blood by gas chromatography/mass spectroscopy
Chem. Res. Toxicol., 8, 182-187 (1995)

Begemann, P., Upton, P.B., Ranasinghe, A., Swenberg, J.A., Soleo, L., Vimercetti, L., Gelormini, A., Fustinoni, S., Zwirner-Baier, I. and Neumann, H.G.
Hemoglobin adducts as biomarkers of 1,3-butadiene in occupationally low exposed Italian workers and a few diesel-exposed miners
Chemico-Biological Int., 135-136, 675-678 (2001)

Belevdere, G. and Tursi, C.
Reference physiological parameters in pharmokinetic modelling
EPA/ 600/6-88-004, (1998)

Bitzenhofer, U.N.
Entwicklung einer gasdichten Apparatur zur Untersuchung von flüchtigen Fremdstoffen mit der isoliert perfundierten Rattenleber- Kinetik von Styrol und seinem Metaboliten Styrol-7,8-oxid
GSF-Bericht 24/93, ISSN 0721-1694
GSF-Institute of Toxicolgy, (1993)

Böcker, J.
Chromatographie, Instrumentelle Analytik mit Chromatographie und Kapillarelektrophorese
Vogel Verlag und Druck GmbH & Co., Würzburg (1997)

Bogusz, M., Bialka, J., Gierz, J. and Klys, M.
Rapid determination of ethylene glycol in biological material
Zeitschrift fuer Rechtsmedizin, 96, 23-26, (1986)

Bolt, H.M., Schmiedel, G., Filser, J.G., Rolzhäuser, H.P., Lieser, K., Wistuba, D. and Schurig, V.
Biological activation of 1,3-butadiene to vinyl oxirane by rat liver microsomes and expiration of the reactive metabolite by exposed rats
J. Cancer Res. Clin. Oncol., 106, 112-116 (1983)

Bond, J.A., Recio, L. and Andjelkovich, D.
Epidemiological and mechanistic data suggest that 1,3-butadiene will not be carcinogenic to humans at exposures likely to be encountered in the environment or workplace
Carcinogenesis, 16, 2 (Feb), 165-171 (1995)

Brunnemann, K.D., Kagan, M.R., Cox, J.E. and Hoffmann, D.
Analysis of 1,3-butadiene and other selected gas-phase components in cigarette mainstream and sidestream smoke by gas chromatography-mass selective detection
Carcinogenesis, 11, 1863- 1868 (1990)

Cheng, X. and Ruth, J.A.
A simplified methodology for quantitation of butadiene metabolites
Application to the study of 1,3-butadiene metabolism by rat liver microsomes
Am. Soc. Pharmacol. Exp.Therap., 21, 1, 121-124 (1993)

Costa, M., Zhitkovich, A., Harris, M., Paustenbach, D. and Gargas, M.
DNA-protein crosslinks produced by various chemicals in cultured human lymphoma cells
J. Toxicol. Environ. Health, 50, 433-449 (1997)

Csanády, G.A., Guengerich, F.P. and Bond, J.A.
Comparison of the biotransformation of 1,3-butadiene and its metabolite, butadiene monoepoxide, by hepatic and pulmonary tissues from humans, rats and mice
Carcinogenesis, 13, 7, 1143-1153 (1992)

Csanády, G.A., Kreuzer, P.E., Baur, C. and Filser, J.G.
A physiological toxicokinetic model for 1,3-butadiene in rodents and man: blood concentrations of 1,3-butadiene, its metabolically formed epoxides, and of haemoglobin adducts - relevance of glutathione depletion
Toxicology, 113, 300-305 (1996)

Dahl, A.R., Bechtold, W.E., Bond, J.A., Henderson, R.F., Mauderly, J.L., Muggenburg, B.A., Sun, J.D. and Birnbaum, L.S.
Species differences in the metabolism and disposition of inhaled 1,3-butadiene and isoprene
Environ. Health Perspect., 86, 65-69 (1990)

Dahl, A.R., Sun, J.D., Birnbaum, L.S., Bond, J.A., Griffith, W.C.J., Mauderly, J.L., Muggenburg, B.A., Sabourin, P.J. and Henderson, R.F.
Toxicokinetics of inhaled 1,3-butadiene in monkeys: comparison to toxicokinetics in rats and mice
Toxicol. Appl. Pharmacol., 110, 9-19 (1991)

DFG, Deutsche Forschungsgemeinschaft:
MAK- und BAT-Werte-Liste 1998
Senatskommision zur Prüfung gesundheitsschädlicher Arbeitsstoffe, Mitteilung 29, VCH Verlagsgesellschaft mbH, Weinheim (1998)

Duescher, R.J. and Elfarra, A.A.
1,3-butadiene oxidation by human myeloperoxidase
Role of chloride ion in catalysis of divergent pathways
J. Biol. Chem., 267, 28, 19859-19865 (1992)

Duescher, R.J. and Elfarra, A.A.
Human liver microsomes are efficient catalysts of 1,3-butadiene oxidation: evidence for major roles by cytochromes P450 2A6 and 2E1
Arch. Biochem. Biophys., 311, 2, 342-349 (1994)

Elfarra, A.A., Duescher, R.J., Pasch, C.M. and Christoph, M.
Mechanisms of 1,3-butadiene oxidations to butadienemonoepoxide and crotonaldehyde by mouse liver microsomers and chloroperoxidase
Arch. Biochem. Biophys., 286, 1 (April), 244-251 (1991)

Elfarra, A.A., Sharer, J.E. and Duescher, R.J.
Synthesis and characterisation of N-acetyl-L-cysteine S-conjugates of butadiene monoxide and their detection and quantitation in urine of rats and mice given butadiene monoxide
Chem. Res. Toxicol., 8, 68-76 (1995)

Evalo, C.T.A., Oostendrop, J.G.M., Ten Berge, W.F. and Borm, P.J.A.
Physiologically based toxicokinetic modelling of 1,3-butadiene lung metabolism in mice becomes more important at low doses
Environ. Health Perspect., 101, 496-502 (1993)

Filser, J.G., Bolt, H.M., Muliawan, H. and Kappus, H.
Quantitative evaluation of ethane and n-pentane as indicators of lipid peroxidation in vivo
Arch. Toxicol., 52, 135-147 (1983)

Filser, J.G. and Bolt, H.M.
Inhalation pharmacokinetics based on gas uptake studies.VI. Comparative evaluation of ethylene oxide and butadiene monoxide as exhaled reactive metabolites of ethylene and butadiene in rats
Arch. Toxicol., 55, 219-223 (1984)

Filser, J.G., Altthaler, B., Welter, H.F. and Johanson, G.
Metabolism of 1,3-butadiene in microsomes from livers of mouse, rat and man
33rd Spring Meeting - Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl.4, (1992)

Filser, J.G., Johanson, G., Kessler, W., Kreuzer, P.E., Stei, P., Baur, C. and Csanády, G.A.
A pharmacokinetic model to describe toxicokinetic interactions between 1,3-butadiene and styrene in rats: predictions for human exposure
IARC Sci. Publ., 127, 65-78 (1993)

Finar, I.L.
Organic Chemistry, The fundamental priciples
ELBS Longmann group Ltd. (1986)

Geigy Documenta
Wissenschaftliche Tabellen
Georg Thieme Verlag, Stuttgart (1985)

Gervasi, P.G., Citti, L., Del Monte, M., Longo, V. and Benetti, D.
Mutagenicity and chemical reactivity of epoxidic intermediates of the isoprene metabolism and other structurally related compounds
Mutat. Res., 156, 77-82 (1985)

Grob, R.L.
Modern Practice of Gas Chromatography
Wiley-Interscience, John Wiley & Sons Inc., New York (1995)

Hallberg, L.M., Bechtold, W.E., Grady, J., Legator, M.S. and Au, W.W.
Abnormal DNA repair activities in lymphocytes of workers exposed to 1,3-butadiene
Mut. Res., 383, 213-221 (1997)

Harison, A.G.
Chemical Ionization Mass Spectrometry
CRC Press, UK (1992)

Himmelstein, M.W., Turner, M.J., Asgharian, B. and Bond, J.A.
Comparison of blood concentrations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation
Carcinogenesis, 15, 8, 1479-1486 (1994)

Himmelstein, M.W., Asgharian, B. and Bond, J.A.
High concentrations of butadiene epoxides in livers and lungs of mice compared to rats exposed to 1,3-butadiene
Toxicol. Appl. Pharmacol., 132, 1723-1731 (1995)

Himmelstein, M.W., Turner, M.J., Asgharian, B. and Bond, J.A.
Metabolism of 1,3-butadiene: inhalation pharmacokinetics and tissue dosimetry of butadiene epoxides in rats and mice
Toxicology, 113, 306-309 (1996)

Huff, J.E., Melnick, R.L., Solleveld, H.A., Haseman, J.K., Powers, M. and Miller, R.A.
Multiple organ carcinogenicity of 1,3-butadiene in B6C3F1 mice after 60 weeks of inhalation exposure
Science, 227, 548-549 (1985)

IARC
Re-evaluation of some carcinogenic chemicals, hydrazine and hydrogen peroxide
IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man
71, 1, 109-225 (1999)

Irons, R.D., Cathro, H.P., Stillman, W.S., Steinhagen, W.H. and Shah, R.S.
Susceptibility to 1,3-butadiene-induced leukemogenesis correlated with endogenous ecotropic retroviral background in the mouse
Toxicol. Appl. Pharmacol., 101, 170-176 (1989)

Johanson, G. and Filser, J.G.
A physiologically based pharmacokinetic model for butadiene and its metabolite butadiene monoepoxide in rat and mouse and its significance for risk extrapolation
Arch. Toxicol., 67, 151-163 (1993)

Johanson, G. and Filser, J.G.
PBPK model for butadiene metabolism to epoxides: quantitative species differences in metabolism
Toxicology, 113, 40-47 (1996)

Kemper, R.A., Elfarra, A.A. and Myers, S.R.
Metabolism of 3-butene-1,2-diol in B6C3F1 mice
Evidence for involvement of alcohol dehydrogenase and cytochrome P450
Drug Metab. Dispos., 26, 914-920 (1998)

Kimura, T., Sternson, L., A., and Higuchi, T.
Gas-chromatographic analysis for 1,2:5,6-dianhydrogalactitol, an anti-neoplastic agent, in plasma
Clin. Chem., 22, 10, 1639-1643 (1976)

Knapp, D.R.
Handbook of Analytical Derivatization Reactions
John Wiley & Sons, New York (1979)

Kohn, M.C. and Melnik, R.L.
Species differences in the production and clearance of 1,3-butadiene metabolites: a mechanistic model indicates predominantly physiological, not biochemical, control
Carcinogenesis, 14, 4, 619-628 (1993)

Koivisto, P. and Peltonen, K.
N7-guanine adducts of the epoxy metabolites of 1,3-butadiene in mice lung
Chemico-Biological Int., 135-136, 363-372 (2001)

Krause, R.J. and Elfarra, A.A.
Oxidation of butadiene monoxide to meso- and (±)-diepoxybutane by cDNA-expressed human cytochrome P450s and by mouse, rat and human liver microsomes: Evidence for preferential hydration of meso-diepoxybutane in rat and human liver microsomes
Arc. Biochem. Biophys., 337, 2 (January15), 176-184 (1997a)

Krause, R.J., Sharer, J.E. and Elfarra, A.A.
Epoxide hydrolase-dependent metabolism of butadiene monoxide to 3-butene-1,2-diol in mouse, rat, and human liver
Drug Metab. Dispos., 25, 8, 1013-1015 (1997b)

Kreiling, R., Laib, R.J., Filser, J.G. and Bolt, H.M.
Species differences in butadiene metabolism between mice and rats evaluated by inhalation pharmacokinetics
Arch. Toxicol., 58, 235-238 (1986)

Kreiling, R., Laib, R.J., Filser, J.G. and Bolt, H.M.
Inhalation pharmacokinetics of 1,2-epoxybutene-3 reveal species differences between rats and mice sensitive to butadiene-induced carcinogenesis
Arch. Toxicol., 61, 7-11 (1987)

Kreuzer, P.E., Kessler, W., Welter, H.F., Baur, C. and Filser, J.G.
Enzyme specific kinetics of 1,2-epoxybutene-3 in microsomes and cytosol from livers of mouse, rat, and man
Arch. Toxlicol., 65, 59-67 (1991)

Leavens, T.T., Moss, O.R., Turner, M.J., Janszen, D.B. and Bond, J.A.
Metabolic interactions of 1,3-butadiene and styrene in male B6C3F1 mice
Toxicol. Appl. Pharmacol., 141, 2 (Dec), 628-636 (1996)

Malvoisin, E., Lhoest, G., Poncelet, F., Roberfroid, M. and Mercier, M.
Identification and quantitation of 1,2-epoxybutene-3 as the primary metabolite of 1,3-butadiene
Journal of Chomatography, 178, 419-425 (1979)

Malvoisin, E. and Roberfroid, M.,
Hepatic microsomal metabolism of 1,3-butadiene
Xenobiotica, 12, 2, 137-144 (1982)

Maniglier-Poulet, C., Cheng, X., Ruth, J.A. and Ross, D.
Metabolism of 1,3-butadiene to butadiene monoxide in mouse and human bone marrow cells
Chemico-Biological Int., 97, 119-129 (1995)

Martz, R.M., Reutter, D.J. and Lassewell, L.D.
A comparison of ionization techniques for gas chromatography/mass spectroscopy analysis of dye and lachrymator residues from exploding bank security devices
J. Forensic Sci., 28, 1, 200-207 (1983)

McCurdy, H.H. and Everett, T.S.
An improved procedure for the determination of ethylene glycol in blood
J. Anal. Toxicol., 6, September/October, 253-254 (1982)

Medinsky, M.A., Leavens, T.L., Csanády, G.A., Gargas, M.L. and Bond, J.A.
In vivo metabolism of butadiene by mice and rats: a comparison of physiological model predictions and experimental data
Carcinogenesis, 15, 7, 1329-1340 (1994)

Meischner, V.
Kinetik von 1,3-Butadiene und Belastung durch den Metaboliten 1,2-Epoxy-3-buten bei Ratten, Mäusen und Mäusen ohne Cytochrome P450 2E1
Dissertation, Technische Universität, ISBN 3-89675-679-6, (1999)

Melnick, R.L., Huff, J., Chou, B.J. and Miller, R.A.
Carcinogenicity of 1,3-butadiene in C57BL/6 x C3H F1 mice at low exposure concentrations
Cancer Res., 50, 6592-6599 (1990)

Miller, J.C. and Miller, J. N.
Statistics for analytical Chemistry
Propagation of random errors
Prentice Hall, Chichester (1993)

Nauhaus, S.K., Fennell, T.R., Asgharian, B., Bond, J.A. and Sumner, S.C.J.
Characterization of urinary metabolites from Sprague-Dawley rats and B6C3F1 mice exposed to (1,2,3,4-^{13}C)butadiene
Chem. Res. Toxicol., 9, 764-773 (1996)

Needham, L.L., Hill, R.L.J., Orti, D.L., Felver, M.E. and Liddle, J.A.
Electron-capture, capillary column gas chromatographic determination of low-molecular-weight diols in serum
Journal of Chromatography, 233, 10 (Dec), 9-17 (1982)

Nieusma, J.L., Claffey, D.J., Maniglier-Poulet, C., Imiolczyk, T., Ross, D. and Ruth, J.A.
Stereochemical aspects of 1,3-butadiene metabolism and toxicity in rat and mouse liver microsomes and freschly isolated rat hepatocytes
Toxicol. Sci., 43, 102-109 (1998)

Otto-Albrecht, N.
Römpps Chemie-Lexikon
Frank'sche Verlagshandlung, Stuttgart (1989)

Owen, P.E., Glaister, J.R., Gaunt, I.F. and Pullinger, D.H.
Inhalation toxicity studies with 1,3-butadiene.
3 Two year toxicity/carcinogenicity study in rats
Am. Ind. Hyg. Assoc. J., 48, 407-413 (1987)

Perez, H.L., Lähdeite, J., Landin, H.H., Kilpeläinen, I., Koivisto, P., Peltonen, K. and Osterman-Golkar, S.
Haemoglobin adducts of epoxybutanediol from exposure to 1,3-butadiene epoxides
Chemico-Biological Int., 105, 181-198 (1997)

Recio, L., Osterman-Golkar, S., Csanády, G.A., Turner, M.J., Myhr, B., Moss, O., Bond, J.A.,
Determination of mutagenicity in tissues of transgenic mice following exposure to 1,3-butadene and N-ethyl-N-nitrosourea
Toxicol. Appl. Pharmacol., 117, 58-64, (1992)

Ristau, C., Deutschmann, S., Laib, R.J. and Ottenwalder, H.
Detection of diepoxybutane induced DNA-DNA crosslinks by cesium trifluoracetate (CsTFA) density-gradient centrifugation
Arch. Toxicol., 64, 343-344 (1990)

Sabourin, P.J., Burka, L.T., Bechtold, W.E., Dahl, A.R., Hoover, M.D., Chang, Y. and Henderson, R.F.
Species differences in urinary butadiene metabolites: identification of 1,2-dihydroxy-4-(N-acetylcysteinyl)butane, a novel metabolite of butadiene
Carcinogenesis, 13, 1633-1638 (1992)

Sato, A. and Nakajima, T.
Partition coefficients of some aromatic hydrocarbons and ketones in water, blood and oil
Br. J. Ind. Med., 36, 231-234 (1979)

Schmidt, L. and Loeser, E.
Species differences in the formation of butadiene monoepoxide from 1,3-butadiene
Arch. Toxicol., 57, 222-225 (1985)

Seaton, M.J., Follansbee, M.H. and Bond, J.A.
Oxidation of 1,2-epoxy-3-butene to 1,2:3,4-diepoxybutane by cDNA-expressed human cytochromes P450 2E1 and 3A4 and human, mouse and rat liver microsomes
Carcinogenesis, 16, 10, 2287-2293 (1995)

Sharer, J.E., Duescher, R.J. and Elfarra, A.A.
Formation, stability and rearrangements of the glutathione conjugates of butadiene monoxide: evidence for the formation of stable sulfurane intermediates
Chem. Res. Toxicol., 4, 430-436 (1991)

Sharer, J.E., Duescher, R.J. and Elfarra, A.A.
Species and tissue differences in the microsomal oxidation of 1,3-butadiene and glutathione conjugation of butadiene monoxide in mice and rats
Drug Metab. Dispos., 20, 658-664 (1992a)

Sharer, J.E. and Elfarra, A.A.
S-(2-hydroxy-3-buten-1-yl)glutathione and S-(1-hydroxy-3-buten-2-yl)glutathione are in vivo metabolites of butadiene monoxide: detection and quantitation in bile.
Chem. Res. Toxicol., 5(6), Nov-Dec, 787-790 (1992b)

Thornton-Manning, J.R., Dahl, A.R., Bechtold, W.E., Griffith, J.W.C. and Henderson, R.F.
Disposition of butadienemonoepoxide and butadienediepoxide in various tissues of rats and mice following a low-level inhalation exposure to 1,3-butadiene
Carcinogenesis, 16, 8, 1723-1731 (1995a)

Thornton-Manning, J.R., Dahl, A.R., Bechtold, W.E., Griffith, W.C.J., Pei, L. and Henderson, R.F.
Gender differences in the metabolism of 1,3-butadiene in Sprague-Dawley rats following a low level inhalation exposure
Carcinogenesis, 16, 2875-2878 (1995b)

Thornton-Manning, J.R., Dahl, A.R., Bechtold, W.E., Griffith, W.C.J., Pei, L. and Henderson, R.F.
Comparison of the disposition of butadiene epoxides in Sprague-Dawley rats and B6C3F1 mice following a single and repeated exposures to 1,3-butadiene via inhalation
Toxicology, 123, 125-134 (1997)

Thornton-Manning, J.R., Dahl, A.R., Bechtold, W.E., Griffith, W.C.J., Pei, L. and Henderson, R.F.
Disposition of butadiene epoxides in Sprague-Dawley rats following exposures to 8000 ppm 1,3-butadiene: comparison with tissue epoxide concentrations following low level exposures
Toxicol. Sci., 41, 167-173 (1998)

Tretyakova, N.Y., Lin, Y.P., Upton, P.B., Sagaiah, R. and Swenberg, J.A.
Macromolecular adducts of butadiene
Toxicology, 113, 70-76 (1996)

Van Duuren, B.L., Nelson, N.N., Orris.L., Palmes, E.D. and Schmitt. F.L.
Carcinogenicity of epoxides, lactones and peroxy compounds
J. Natl. Cancer Inst., 31, 41-55 (1963)

Van Duuren, B.L., Orris.L. and Nelson.N.
Carcinogenicity of epoxides, lactones, and peroxy compounds. II
J. Natl. Cancer Inst., 35, 707-717 (1965)

Van Duuren, B.L., Langoeth, L., Orris, L., Teebor, G. and Nelson, N., Kuschner, M.,
Carcinogenicity of epoxides, lactones and peroxy compounds, IV. Tumor response in epithelial and connective tissue in mice and rats
J. Natl. Cancer Inst., 37, 825-838 (1966)

Wistuba, D., Nowotny, H.P., Träger, O. and Schurig, V.
Cytochrome P-450 catalyzed asymmetric epoxidation of simple prochiral and chiral aliphatic alkenes. Species dependence and effect of enzyme induction on enantioselective oxirane formation
Chirality, 1, 127-136 (1989)

Yoshida, T., Tainaka, H., Matsunaga, I. and Goto, S.
The airborne 1,3-butadiene concentrations in rubber and plastic processing plant
Sangyo Eiseigaku Zasshi, 44, 2(March), 56-63 (2002)