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Cob(I)alamin for Trapping Butadiene Epoxides in Metabolism with Rat S9 and for Determining Associated Kinetic Parameters

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The reduced state of vitamin B_{12}, cob(I)alamin, acts as a supernucleophile that reacts ca. 10^5 times faster than standard nucleophiles, for example, thiols. Methods have been developed for trapping electrophilically reactive compounds by exploiting this property of cob(I)alamin. 1,3-Butadiene (BD) has recently been classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC). The carcinogenicity of BD is considered to be dependent on the activation or deactivation of the reactive metabolites of BD, that is, the epoxides (oxiranes) 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB), and 1,2-epoxy-3,4-butanediol (EBdiol). Cytochrome P450 (P450) isozymes are involved in oxidation of BD to EB and further activation to DEB. EB and DEB are hydrolyzed by epoxide hydrolases (EH) to 3,4-dihydroxy-1-butene (BDdiol) and EBdiol, respectively. EBdiol can also be formed by oxidation of BDdiol. In the present study, cob(I)alamin was used for instant trapping of the BD epoxide metabolites generated in in vitro metabolism to study enzyme kinetics. The substrates EB, DEB, and BDdiol were incubated with rat S9 liver fraction, and apparent K_m and apparent V_{max}, were determined. The ratio of conversion of EB to DEB (by P450) to the rate of deactivation of DEB by EH was 1.09. Formation of EBdiol from hydrolysis of DEB was ca. 10 times faster than that from oxidation of BDdiol. It was also found that the oxidation of EB to DEB was much faster than that of BDdiol to EBdiol. The study offers comparative enzyme kinetic data of different BD metabolic steps, which is useful for quantitative interspecies comparison. Furthermore, a new application of cob(I)alamin was demonstrated for the measurement of enzyme kinetics of compounds that form electophilically reactive metabolites.

1. Introduction

1,3-Butadiene (BD)[1] is ubiquitous in the atmosphere because of its formation during incomplete combustion of a variety of materials. The substance is found in cigarette smoke (*1*) and in emissions from automobile exhausts (*2, 3*) and arises during the burning of organic materials such as wood (*4*). BD is also a high production chemical used in the manufacture of synthetic rubber and thermoplastic resins. Epidemiological studies have shown a relationship between occupational exposure to BD and an increased risk of cancer (*5, 6*). The International Agency for Research on Cancer (IARC) recently upgraded BD from group 2A to group 1 (*7*), thus classifying BD as a "human carcinogen". Long-term inhalation experiments with BD in mice and rats (*8, 9*) showed that BD is carcinogenic in rodents and that mice are significantly more susceptible to BD than rats (*8*−*11*). This difference has been related to a species variation in the activation of BD to reactive epoxide (oxirane) metabolites versus their deactivation by epoxide hydrolases.

Scheme 1 illustrates the metabolic activation−deactivation of BD and its metabolites. BD can be activated to 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB), and 1,2-epoxy-3,4-butanediol (EBdiol) (*10*−*15*). These electrophilic epoxide metabolites can be detoxified by conjugation with glutathione (GSH) catalyzed by glutathione S-transferases (GSTs) and hydrolyzed by epoxide hydrolases (EH) (*10, 16*−*19*). Oxidation of EB to DEB is carried out primarily by cytochrome P450 (P450) 2E1, but at higher substrate concentrations, the P450s 2A6, 2C9, and 3A4 may also participate (*13, 19*). The pathway mediated by EH is thought to play a major role in the conversion of EB to 3,4-dihydroxy-1-butene (BDdiol) (*11, 20*).

It is apparent that the toxicity of BD is dependent on its metabolism. DEB is believed to play a critical role in the carcinogenicity of BD. For instance in human TK6 cells, DEB is mutagenic at a 100-fold lower concentration than EB (*12*). By comparison of different species in in vitro studies, it has been shown that enzymatic oxidation of BD to EB (*11*) and EB to DEB (*14*) is faster in mouse tissues than in rat or human tissues. Furthermore, in vivo studies have shown higher concentrations of DEB in studied tissues after BD exposure in mice compared with rats (*21*). The DEB formed can be conjugated with GSH (*16*) or hydrolyzed by EH (*22*) to EBdiol.

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[1] Abbreviations: BD, 1,3-butadiene; EB, 1,2-epoxy-3-butene; DEB, 1,2:3,4-diepoxybutane; EBdiol, 1,2-epoxy-3,4-butanediol; BDdiol, 3,4-dihydroxy-1-butene; PO, propylene oxide.

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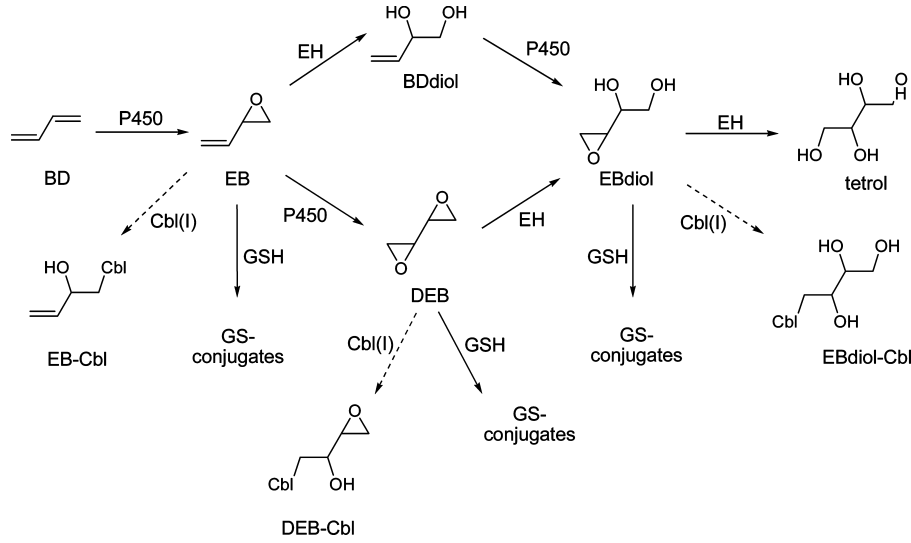
Scheme 1. Metabolism of 1,3-Butadiene Involving EH, P450, and GSH and Alkylcobalamins Formed by Trapping the Respective Epoxy Metabolites with Cob(I)alamin in Vitro (Shown by Dotted Arrows)



The species differences in blood concentrations of DEB are suggested not to be due to conjugation of DEB by GSH; and as for EB, the EH-mediated hydrolysis pathway seems to have a major role in the deactivation of DEB in rats (*22*).

For the estimation of the cancer risk to humans from BD exposure, the quantitative differences in metabolism of BD and its epoxide metabolites, particularly DEB, in mice, rats, and humans are of great interest. Here we present a quantitative study of in vitro metabolism in rat with kinetic data for EB, DEB, and BDdiol as substrates. In vitro metabolizing systems have earlier been applied to study BD metabolism, for example in refs *20 and 22*−*24*. However, these studies were mostly focused on just one metabolic step, and the reported results differed, in part, because diverse methods were applied. In these earlier studies, GC-FID or GC-MS were used for quantitative analysis of the reactive metabolites, which were either analyzed directly or extracted prior to analysis. However, the analysis of BD metabolites requires a very rapid analysis, because the epoxides are unstable and volatile. In the past, reactive metabolites from other compounds have been trapped for analysis using reaction with glutathione (*25*), *p*-nitrobenzylpyridine (*26*), or 3,4-dichlorobenzenethiol (*27*). Herein we use a recently developed method using the "supernucleophile" cob(I)alamin for what is essentially instantaneous trapping and analysis of electrophiles (*28*−*31*). This nucleophile has the advantage of reacting 400 000 times faster with epoxides than other common nucleophiles (*28, 29*).

In the present metabolic study, S9 mixture was used, subjecting the substrates to multiple parallel and sequential metabolism. Cob(I)alamin (Cbl(I)) was used as a tool for accurate quantification of steps in the BD metabolism, by rapid trapping of the epoxide metabolites (Scheme 1) with the formation of relatively stable alkylcobalamins (R-Cbl), which were analyzed by LC-ESI$^+$-MS/MS. The data was used for estimation of kinetic parameters V_{max}, K_m, and rate constants for the different metabolic pathways, with EB, DEB, and BDdiol as substrates. A general objective of the study was to evaluate this new method for kinetic studies of complex metabolic pathways leading to reactive metabolites.

2. Materials and Methods

Chemicals and Other Materials. Hydroxocobalamin hydrochloride (OH-Cbl·HCl), cyanocobalamin, cobalt(II) nitrate, 3-chloroperoxybenzoic acid (MCPBA, 90%, contaminated with 3-chlorobenzoic acid), and β-nicotinamide adenine dinucleotide phosphate sodium salt (NADP) were obtained from Sigma (St. Louis, MO). *rac*-1,2:3,4-Diepoxybutane (DEB, 97%), *rac*-1,2-epoxy-3-butene (EB, 98%), *rac*-3,4-dihydroxy-1-butene (BDdiol, 99%), *rac*-propylene oxide (PO, 99%), glucose-6-phosphate, magnesium sulfate, potassium chloride, sodium borohydride, and trifluoroacetic acid were obtained from Sigma-Aldrich Sweden AB (Stockholm, Sweden). All epoxides were kept ice-cold until use to prevent hydrolysis under atmospheric conditions. All solvents used were of HPLC grade. Sprague−Dawley rat liver S9 fraction (39 mg/mL proteins) was purchased from Moltox (Boone, NC).

Caution: *EB, DEB, EBdiol, and PO are potentially carcinogenic compounds. They were handled in a ventilated hood and destroyed immediately after use by 1 M aqueous H_2SO_4.*

Thin Layer Chromatography (TLC) of Cobalamins. TLC analysis was done on silica gel plates (Merck 5554) with trifluoroethanol−dichloromethane−concentrated ammonia (5:2:0.08, v/v/v) as eluent. Hydroxo- and cyanocobalamin were used as reference standards: R_f 0.31 and 0.46, respectively.

Synthesis of 1,2-Epoxy-3,4-butanediol (EBdiol). EBdiol was synthesized according to a published method (*32*). In summary, MCPBA (2.24 g, 12 mmol) was added to 3,4-dihydroxy-1-butene (0.88 g, 10 mmol) in dichloromethane (20 mL). After 5 h at room temperature, the mixture was kept on ice for 2 h, and the white precipitate that formed was filtered. The filtrate was concentrated to ca. 10 mL and extracted with water (6 × 5 mL). The aqueous extract was freeze-dried to give racemic EBdiol (0.46 g, 44%).

^{1}H NMR [300 MHz, D_2O]: 2.70 (1 H, m, H-1), 2.79 (1 H, m, H-1′), 3.02 (1 H, m, H-2), 3.4−3.64 (3 H, m, H-3, H-4, H-4′) ppm.

Synthesis of 2,3,4-Trihydroxybutylcobalamin (EBdiol-Cbl). Cyanocobalamin (100 mg, 0.07 mmol) and a catalytic quantity of cobalt(II) nitrate (2 mg, 0.01 mmol) in water−ethanol (8:2 v/v, 10 mL) at room temperature were deoxygenated by bubbling a slow stream of nitrogen for 25 min. Sodium borohydride (20 mg, 0.55 mmol) in water (1 mL) was added causing a color change from red to blackish-green and the evolution of hydrogen. All further manipulations were carried out in the dark or where needed under a dim red light. After 5 min, EBdiol (100 μL) was added, and stirring under nitrogen was continued for 30 min, after which EBdiol-Cbl was observed by TLC analysis. Finally, the reaction was quenched by addition of acetone (0.1 mL). The solution of EBdiol-Cbl was transferred to a separating funnel and extracted with 1-propanol in ethyl acetate (1:1 v/v, 10 × 5 mL). The organic layer was washed with water (2 × 5 mL) and diluted with ethyl acetate (10 times the original volume of the organic layer). The alkylcobalamin was re-extracted into water (8 × 5 mL), and

the aqueous extract was washed with ethyl acetate (3 × 10 mL). The aqueous layer was dried by rotary evaporation, and the residual liquid was freeze-dried to give EBdiol-Cbl (85 mg, 71%). Prior to NMR analysis, a sample (ca. 20 mg) was taken up in D_2O (0.6 mL) and, after removal of the solvent, was redissolved in D_2O.

^{1}H NMR [300 MHz, D_2O]: 0.34 (C2−H), 0.52 (C1−H), 1.52 (C1−H′), 3.05 (C3−H), 3.22 (H-13), 3.3−3.62 (C4−H, C4−H′), 3.72 (H-8), 4.18 (H-19), 4.23 (H-3), and other resonances. ESI-MS: m/z 1435 $[M + H]^+$, 718.2 $[M + 2H]^{2+}$, 665.5 $[M - C_4H_8O_3 + 2H]^{2+}$. R_f 0.54.

Trapping Method for Epoxides (EB, DEB, EBdiol, and PO) Using Cob(I)alamin To Form Alkylcobalamins. The method using cob(I)alamin to trap epoxide metabolites has been validated earlier by our group (*31*). A similar method has been used here whereby hydroxocobalamin (5 mM in water, 200 μL) and cobalt(II) nitrate (25 mM in water, 10 μL) were added to an amber vial sealed with a septum. The solution was degassed with argon for 10 min. Aqueous sodium borohydride (150 mM, 50 μL) was added, and the mixture was agitated by argon bubbling. After about 2 min, cob(I)alamin formation was judged to be complete. Aliquots from the incubation samples containing epoxy metabolites (EB, DEB, and EBdiol) either as substrate or as product were added to an ice-cold solution of the internal standard (PO), and the whole was added to the reduced cobalamin to form the alkylcobalamins (EB-Cbl, DEB-Cbl, EBdiol-Cbl, and PO-Cbl). After 10 min, air was passed into the reaction mixture for 5 min to oxidize any remaining reduced cobalamin. The analytes were frozen until analysis by LC-MS/MS.

Nonenzymatic Hydrolysis of EB, DEB, and EBdiol. The epoxide (EB or DEB, 1 mM) in CO_2-free water (10 mL) was incubated at 37 °C, pH 7. Aliquots of 200 μL were removed from this solution over time intervals and added to ice-cold internal standard, PO (0.3 mM in methanol, 200 μL). A volume of 100 μL of the resulting solution was added to cob(I)alamin (see above for procedure) to form the respective alkylcobalamins. The analytes were frozen until analysis by LC-MS/MS. For EB and DEB, the time interval was 0−225 h. Because EBdiol is formed by hydrolysis of DEB, to estimate EBdiol hydrolysis the reaction system started with DEB and was continued for 600 h, within which time EBdiol hydrolysis was measured. Duplicate samplings were done for each time point.

Preparation of Calibration Curves for EB, DEB, and EBdiol. To rat liver S9 fraction (150 μL, 5.9 mg proteins) at 4 °C in a 2 mL vial with a screw-tight cap were added aqueous KCl (0.15 M, 300 μL), $MgSO_4$ (80 mM, 150 μL), and glucose-6-phosphate (50 mM, 150 μL), and the volume was made up to 1.5 mL with pH 7.5 phosphate buffer. To this mixture was added six to eight different concentrations (2 μM to 2 mM) of the respective epoxide (EB, DEB, or EBdiol). Two hundred microliters of this mixture was removed and mixed with 200 μL of ice-cold PO solution, used as internal standard. The mixture was centrifuged at 3000 rpm to precipitate the proteins. Three hundred microliters of the supernatant was added to cob(I)alamin solution to form the respective alkylcobalamins. To generate the calibration curve, the initial concentration of the epoxide was plotted against the ratio of the peak area of the alkylcobalamin formed from EB, DEB, or EBdiol to the peak area of the alkylcobalamin from PO, the internal standard. Quantification was done by LC-MS/MS using the "multiple reaction monitoring (MRM)" mode. A suitable concentration of the internal standard in methanol was found to be 0.3 mM PO for reactions with DEB as substrate and 3 μM PO for reactions with EB and BDdiol as substrates. The trapping efficiency of the internal standard was estimated by comparing responses of spiked S9 fraction samples at 3 μM and 0.3 mM of PO in methanol with the matrix-free samples.

Metabolism Experiments over a Time Period and Enzyme Kinetics with EB, DEB, and BDdiol. To rat liver S9 fraction (150 μL, 5.9 mg proteins) at 4 °C in a 2 mL vial with a screw-tight cap were added aqueous KCl (0.15 M, 300 μL), $MgSO_4$ (80 mM, 150 μL), glucose-6-phosphate (50 mM, 150 μL), and NADP (40 mM, 150 μL), and the volume was made up to 1.5 mL with pH 7.5 phosphate buffer. The mixture was incubated for 5 min at 37 °C prior to the addition of the substrate (EB, DEB, or BDdiol).

In the metabolic time studies, the initial concentration of the substrate was 1 mM in the incubation mixture. Two hundred microliters of the mixture was taken out at different time points (0−80 min) and mixed with 200 μL of ice-cold PO solution with concentration similar to that noted above for the preparation of calibration curves. The mixture was centrifuged at 3000 rpm to precipitate the proteins. Three hundred microliters of the supernatant was added to cob(I)alamin solution.

In a kinetic study, different starting concentrations of the substrate (20 μM to 2 mM; seven or eight concentrations, double samples) were added to the incubation mixture. An aliquot (200 μL) was taken from each mixture after 15 min, and the analysis was performed as described in the preceding paragraph. In control experiments, incubations were performed by the same method, but in the absence of substrate.

With EB as the substrate, the formation of DEB and EBdiol and disappearance of EB were followed. With DEB as the substrate, the formation of EBdiol and disappearance of DEB were followed. With BDdiol as the substrate, the formation of EBdiol was followed.

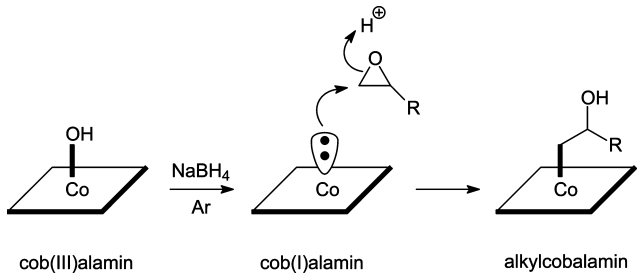
Formation or disappearance of the epoxides for all of the studies was determined by measuring the amount of alkylcobalamin formed. The concentrations of the epoxides could then be estimated from calibration studies. The parameters, V_{max} and K_m, were estimated using Lineweaver−Burk plots.

The S9 fraction from an uninduced male rat (Sprague−Dawley strain) was used. Protein concentration was determined as 39 mg/mL, according to the assay method described by Lowry et al. (*33*). Tests for P450 activity, presence of adventitious agents, and promutagen activation were provided by the supplier (Moltox). Specific forms of P450s were not distinguished, because the initial objective was to obtain an overall view of the metabolic pathways mentioned above in an S9 system and not to look for specific isozyme activity.

Liquid Chromatography. A Shimadzu Prominence LC-system coupled to an API 3200 Q trap triple quadrupole mass spectrometer (Applied Biosystems) was used. The alkylcobalamins were analyzed using an ACE 5 C18, 10 mm × 1 mm precolumn, which was joined to an automated switching valve connected with an ACE 5 C18, 150 mm × 1 mm analytical column (both columns purchased from Scantec Lab AB, Sweden), which was coupled to the mass spectrometer. The analytes were eluted with gradient conditions using two mobile phase systems referred to as solution A and solution B. Solution A contained 5% acetonitrile in water and solution B contained 70% acetonitrile in water with 0.1% trifluoroacetic acid in each solution. A linear gradient was used from 0% to 10% solution B in 5 min, which was increased to 50% in 12 min, followed by 50% to 100% in 1 min. The injection volume was 10 μL with a flow rate of 0.1 mL/min. To start with, the switching valve was in the loading position for 5 min, which caused the excess OH-Cbl and other unwanted waste from the injected sample to pass the precolumn directly to the waste. After 5 min of injection, the valve was switched to the eluting position, and the flow system entered the analytical column, where the different alkylcobalamins were separated.

Mass Spectrometry. The mass spectrometer was operated using an electrospray ionization source in the positive ion mode (ESI^+). Acquisition and data processing from the mass spectrometer were performed using the Analyst software, version 1.4.1, from Applied Biosystems. The ion source temperature was set to 100 °C with collision energy of 30 eV. The other settings of the ESI instrument for declustering potential, entrance potential, collision cell exit potential, curtain gas, collision gas, ion spray voltage, and ion source gas were 25 V, 7.5 V, 25 V, 10, 5, 5500 V, and 15, respectively. The numbers in those settings without units were arbitrary values from the Analyst software. The mass spectrometer was operated first on full scan mode, and then product-ion scan was done to observe specific alkylcobalamin fragmentation. Finally, MRM of the analytes was done for quantitative purposes. The MRM precursor ion to product ion transitions for EB-Cbl, DEB-Cbl,

Scheme 2. Method of Trapping an Epoxide by Cob(I)alamin



EBdiol-Cbl, and PO-Cbl were *m/z* 701.2, 709.2, 718.2, and 695.2, respectively, to 665.5.

3. Results and Discussion

Formation and Identification of Alkylcobalamins. In the trapping method, hydroxocobalamin was reduced under argon with sodium borohydride in the presence of a catalytic amount of cobalt(II) nitrate (*31, 34*). Cob(I)alamin was generated during the reduction, and the epoxide was added to give an alkylcobalamin (Scheme 2). Alkylcobalamins from EB, DEB, EBdiol, and PO were thus formed under the experimental conditions. We also synthesized the alkylcobalamin EBdiol-Cbl (Figure 1) for use as a reference compound, according to the same method.

The alkylcobalamins were characterized by LC-MS/MS. The MS spectra of alkylcobalamins in the full scan positive ion mode showed a doubly protonated molecule $[M + 2H]^{2+}$ along with the major fragment, 665.5, which is a doubly protonated fragment from the loss of the alkyl ligand. The singly charged protonated molecule $[M + H]^{+}$ was also seen. Figure 2 shows the doubly protonated $[M + 2H]^{2+}$ for EB-Cbl, DEB-Cbl, and EBdiol-Cbl, along with the 665.5 fragment, in the centroid view. The MRM (MS/MS) ion chromatogram of the different alkylcobalamins is exemplified in Figure 3.

The characterization by mass spectrometry of alkylcobalamins formed from the epoxy metabolites of BD have also been described by our group (*30*) showing singly charged ions in the positive and negative ion mode. One significant difference here is that doubly charged ions were detected in the *m/z* range of 600−800, where the resolution of the mass spectrometer is better than that in the higher *m/z* range (*31, 35*). The relative molecular masses of the alkylcobalamins EB-Cbl, DEB-Cbl, and EBdiol-Cbl are 1400, 1416, and 1434, respectively.

A new method was employed for the isolation of the synthesized EBdiol-Cbl, avoiding the use of hazardous phenol as in the standard extraction method (details on improving the extraction method of alkylcobalamins to be published elsewhere,

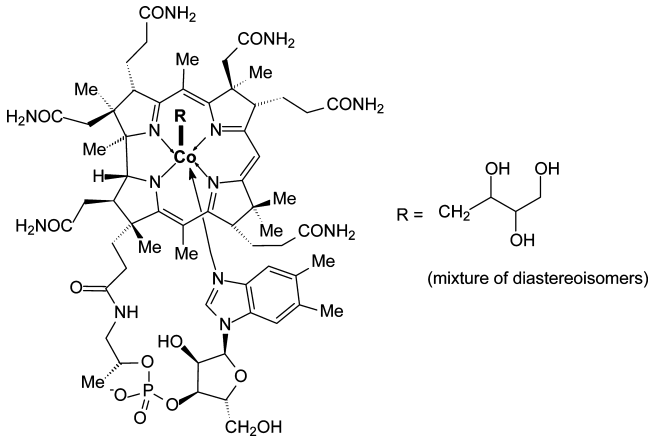


Figure 1. EBdiol-Cbl structure.

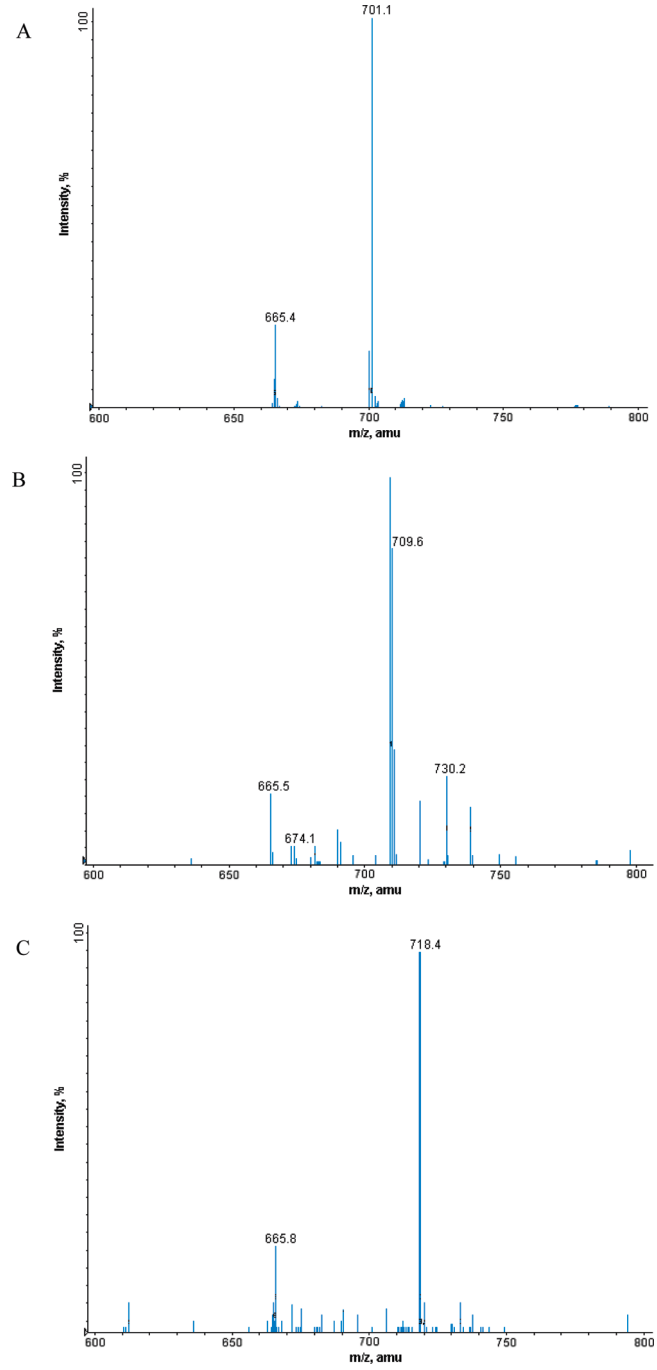


Figure 2. LC-ESI^{+}-MS spectra of (A) EB-Cbl, (B) DEB-Cbl, and (C) EBdiol-Cbl.

by Motwani, H. V., et al.). In addition to characterization by LC-MS/MS, the synthesized EBdiol-Cbl was validated by ^{1}H NMR spectroscopy (*36*). Signals observed in the high field region were characteristic for the protons of the alkyl ligand on the cobalamin. In EBdiol-Cbl, the C-2 protons resonate at higher field (i.e., lower δ value) than the C-1 protons, because the C-2 protons are closer to the shielding region of corrin system, even though further from the cobalt atom. Protons on the third or subsequent carbon atoms were, however, shielded to a lesser extent, exhibiting chemical shifts similar to those for protons in simple aliphatic compounds.

Quantification of Epoxides. Calibration curves for epoxide concentrations used in the kinetic studies were generated from samples where the epoxides had been added to the incubation mixture in different concentrations (2 μM to 2 mM) and immediately recovered by trapping with cob(I)alamin. The area

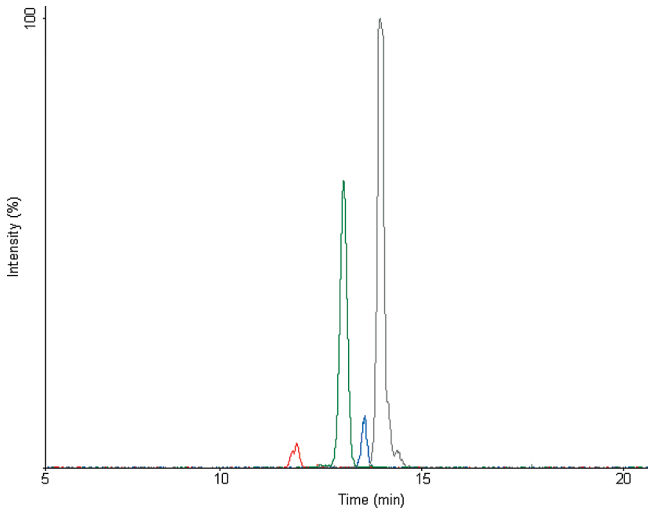


Figure 3. Example of an LC-ESI$^+$-MRM chromatogram recorded from EB incubation. Peaks from left to right: EBdiol-Cbl, DEB-Cbl, PO-Cbl, and EB-Cbl.

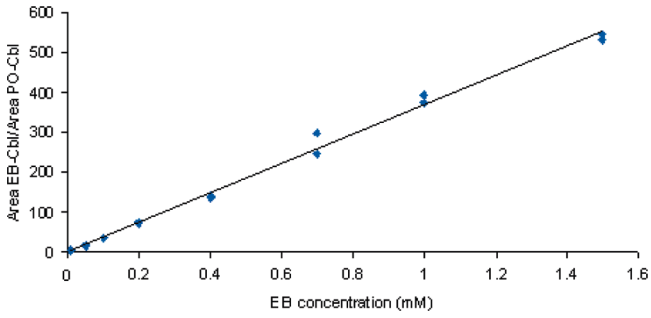


Figure 4. Calibration curve for EB.

ratios obtained from MRM (LC-MS/MS) analysis of the alkylcobalamin from the epoxide to the alkylcobalamin from internal standard were plotted against the concentration of epoxide added. Reproducibility of the method was verified by doing several calibration curves for each epoxide. An example of a calibration curve for EB is given in Figure 4, wherein the number of samples was 8 × 2 and the correlation coefficient (r^2) was 0.994. The calibration curve was also a linearity test for trapping and analysis of EB as EB-Cbl. Similar results were obtained with DEB and EBdiol, with $n = 8 \times 2$ and high r^2 (>0.98). After the calibration curves were established, the concentration of the epoxides present at the time of trapping with cob(I)alamin could be determined.

The trapping efficiency, tested with the internal standard (PO) with and without S9, as described in Materials and Methods, was estimated to be more than 90%. This result is also supported by an earlier study on recovery of epoxides (PO and DEB) using the cob(I)alamin method (*31*). The internal standard adjusted for variations in the trapping method and for variations in the LC-MS/MS analysis.

Nonenzymatic Hydrolysis. The rate constants for the nonenzymatic hydrolysis of EB, DEB, and EBdiol were determined by measuring the disappearance of each epoxide in an aqueous solution. After certain time intervals of incubation in water at 37 °C, the epoxides were trapped by cob(I)alamin. This process is essentially instantaneous and prevents any further hydrolysis. The alkylcobalamin were measured by LC-MS/MS, and the area ratio of the respective analyte to the internal standard analyte was plotted against time to give an exponential curve.

The hydrolysis rate of DEB was measured by following the disappearance of DEB. While hydrolysis of DEB was measured, the formation of EBdiol and disappearance of EBdiol through further hydrolysis were also followed. After nearly 6 half-lives of DEB (ca. 200 h), when most of the DEB had hydrolyzed, the disappearance of the EBdiol formed was measured for another 400 h to estimate the hydrolysis of EBdiol. An advantage of this method is that the hydrolysis of both DEB and EBdiol were measured in the same experimental setup, resembling the situation in these studies of in vitro metabolism when kinetic constants for metabolic hydrolyses were determined.

Table 1. Nonenzymatic Hydrolysis Rates for EB, DEB, and EBdiol at 37 °C, pH 7[a]

epoxide	k'_{H_2O} (h^{-1}) × 10^2	$t_{1/2}$ (h)	r^2	n
EB	3.5 (5.0 (*37*))	20	0.976	32
DEB	2.1 (3.17 (*22*); 0.68 (*38*))	33	0.995	32
EBdiol	0.85	82	0.806	18

[a] Literature values are given in parentheses.

All of the hydrolysis data fitted pseudo-first-order kinetics. Rate constants (k'_{H_2O}), the corresponding $t_{1/2}$ for the hydrolysis of the epoxides, and the r^2 and n for the experiment are given in Table 1. Among the three epoxides, the hydrolysis rate for EB and DEB has been reported earlier. As shown in Table 1, the rates of chemical hydrolysis for the BD epoxides were relatively slow: $t_{1/2}$ was 20, 33, and 82 h for EB, DEB, and EBdiol, respectively. This suggests that the loss of these substances by nonenzymatic hydrolysis in S9 incubations as substrates or products plays only a minor role during the enzyme-mediated reactions described below.

Enzyme Kinetics. The in vitro model system using the uninduced S9 mixture described here aims to resemble closely the in vivo conditions in mammalian liver cells, in this case rat, which was used in BD cancer tests (*21, 39*). The use of cob(I)alamin gave us the advantage that the epoxides were trapped instantly once removed from the S9 mixture. Scheme 1 shows the epoxides (EB, DEB, and EBdiol) that were trapped with cob(I)alamin to form stable alkylcobalamins. In our study, the racemic form of the three epoxides was quantified. The quantification was done by LC-MS/MS using calibration curves for each epoxide as described above. The BDdiol and tetrol metabolites, without an epoxy group, could not be trapped and analyzed by cob(I)alamin.

From time trend studies of the metabolic activities of the enzymes up to 80 min, a linear relationship of the enzymatic activity against time was observed at 15 min, and this time point was further used for the kinetic studies. With EB as a substrate, we could follow the formation of DEB and EBdiol but not the formation of BDdiol (Scheme 1). During the first 5 min after incubation with EB, EBdiol formation was negligible. This is because EBdiol formation is the second step when the substrate is EB. In the experiments when DEB and BDdiol were used as the substrates, we again measured formation of EBdiol.

For the substrates EB, DEB, and BDdiol, the enzyme-mediated hydrolysis or oxidation and the sum of the reactions causing disappearance (including, for example, conjugations with glutathione) were well described by Lineweaver−Burk kinetics. Figure 5A−C illustrates the Lineweaver−Burk plots for EB to DEB, DEB to EBdiol, and BDdiol to EBdiol, respectively. The apparent K_m and V_{max} values (obtained from the plots) for different metabolic reactions are shown in Table 2. Since K_m and V_{max} were not determined independently in this study and also because in vivo concentrations of the substrate will be several orders of magnitude lower than the apparent K_m, the rates for the enzymatic reactions are best judged as the enzyme efficiency that can be measured as the ratio V_{max}/K_m,

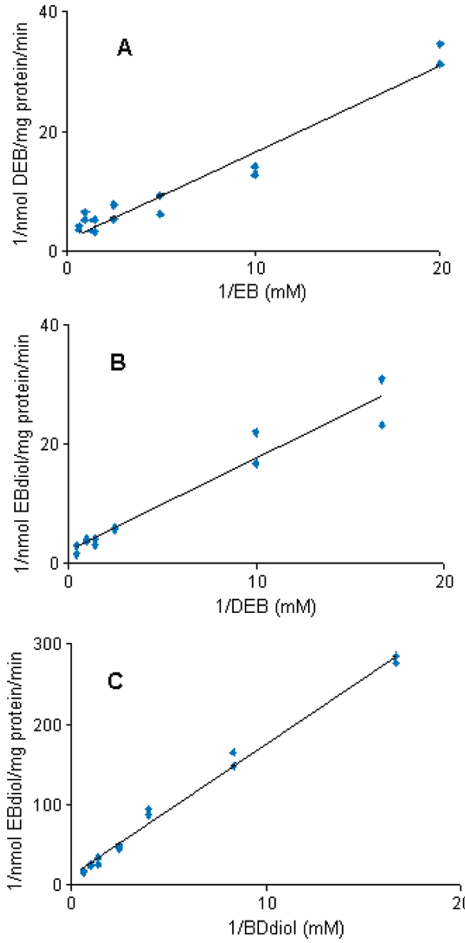


Figure 5. Lineweaver−Burk plots for (A) EB, (B) DEB, and (C) BDdiol as substrates when incubated in rat S9 for 15 min at 37 °C.

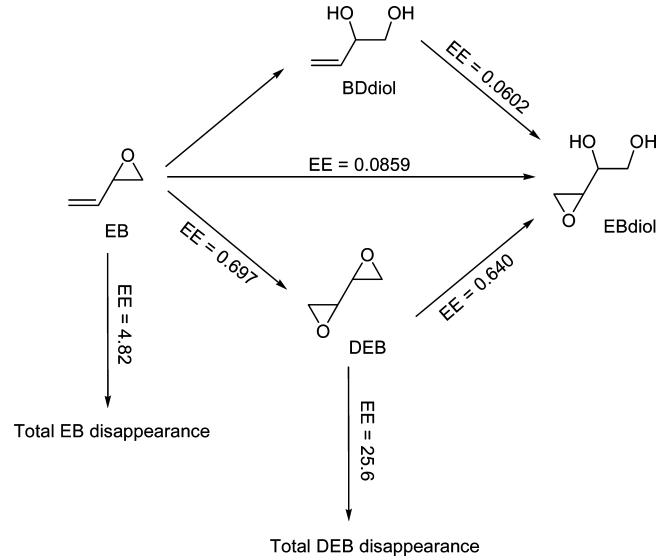
Table 2. Enzyme Kinetic Constants for the Different Pathways in BD Metabolism by Rat S9

metabolic reaction	n	r^2	V_{max} (nmol/(mg protein·min))	K_m (mM)
EB to DEB	16	0.952	0.426	0.611
EB to EBdiol	16	0.555	0.0122	0.142
EB disappearance	16	0.975	3.86	0.801
DEB to EBdiol (DEB hydrolysis)	14	0.950	0.491	0.767
DEB disappearance	14	0.964	7.44	0.290
BDdiol to EBdiol	16	0.992	0.0944	1.57

corresponding to a second-order rate constant (L/mg protein/min). The enzyme efficiencies are described below with respect to activation−deactivation steps of BD metabolism examined herein. Scheme 3 gives a convenient comparison between the enzyme efficiencies of the different metabolic pathways studied.

The activation steps evaluated, which are oxidations in the BD metabolic pathway, were EB to DEB and BDdiol to EBdiol. It was shown that the oxidation of EB to DEB (0.697 μL/(mg protein·min)) was ca. 10 times faster than that of BDdiol to EBdiol (0.0602 μL/(mg protein·min)) (cf. Table 2 and Scheme 3). BDdiol can also be converted by alcohol dehydrogenase to 1-hydroxy-2-butanone, 1-hydroxy-3-butene-2-one, and 2-hydroxy-3-butenal (*15, 40*), which could not be followed by our method. The enzyme efficiency determined for the oxidation of EB to DEB (Scheme 3) is between those of two earlier results, that is, 2.8 (*14*) and 0.21 (*23*) μL/(mg protein·min). These studies, however, differed with respect to incubation methods, cellular fractions, enzyme lots, buffer systems, reaction vials, analytical techniques, etc. A comparison of the different

Scheme 3. Enzyme Efficiency (EE) in μL/(mg protein·min) of the Different BD Metabolic Pathways Measured



metabolic pathways within the same experimental setup is hence more reliable, as performed in our work.

The deactivation steps evaluated concern the hydrolysis of EB and DEB. The enzyme efficiency for the hydrolysis of DEB to EBdiol was 0.64 μL/(mg protein·min). The ratio of the activation to the deactivation (by EH) of DEB was thus 1.09. The formation of EBdiol from hydrolysis of DEB (0.64 μL/(mg protein·min)) was ca. 10 times faster than that from oxidation of BDdiol (0.0602 μL/(mg protein·min)). DEB hydrolysis measured was only due to deactivation by EH. The measured total DEB disappearance, 25.6 μL/(mg protein·min), is in accordance with the estimate in an earlier study of rat liver microsomes, 19.2 μL/(mg protein·min) (*22*).

The activation−deactivation process leading to EBdiol formation with EB as a substrate was also followed. EB could first be hydrolyzed to BDdiol, which then is oxidized to EBdiol, or EBdiol could be formed from EB via DEB (Scheme 3). This resulted in a low r^2 (0.555) from the Lineweaver−Burk plot for EB to EBdiol. The enzyme efficiency for this step was 0.086 μL/(mg protein·min).

The rate for EB hydrolysis to BDdiol has to be lower than the rate for total EB disappearance (4.8 μL/(mg protein·min)). Kemper et al. have showed that when Sprague−Dawley rat hepatocytes were incubated with EB (5, 25, or 250 μM) up to 45 min, the BDdiol area under curve (AUC) was more than 10-fold higher than DEB AUC at all EB concentrations (*19*). We therefore assume the enzyme efficiency for EB to BDdiol to be considerably higher than 0.697 μL/(mg protein·min) (the enzyme efficiency for EB to DEB). Thus we estimate that the enzyme efficiency for EB to BDdiol is between 0.7 and 4.8 μL/(mg protein·min).

As mentioned above, there are low values of V_{max}/K_m in the EBdiol formation from both EB and BDdiol, 0.086 and 0.0602 μL/(mg protein·min), respectively. Together with the relatively high estimate of the enzyme efficiency for the formation of BDdiol, these indicate that the oxidation of BDdiol to EBdiol could be considered as the rate-determining step in the EB metabolism to EBdiol via BDdiol. An explanation of slow conversion of BDdiol could be that the conditions in the enzymatic system are less suitable for polar molecules like BDdiol, which has two hydroxyl groups, and because of the substrate specificities of the enzymes involved in the process (*15*).

4. Conclusions

Cob(I)alamin was proven to be an efficient analytical tool for trapping epoxide metabolites of BD from in vitro metabolism and to enable determination of enzyme kinetics. DEB is the epoxide metabolite of BD with the highest genotoxic potency and has a crucial role in the carcinogenicity of BD; therefore the activation-to-deactivation ratio of DEB is of interest. It was found that the rate of formation of DEB versus detoxification of DEB via EH was almost the same with the ratio 1.09. Oxidation of EB to DEB was slower than the estimated hydrolysis of EB to BDdiol. Furthermore, there is a faster rate of formation of EBdiol from DEB than from BDdiol. It can thus be estimated that the concentration of BDdiol at any time will be higher than that for DEB. This is probably also the case for BD metabolism.

Different research groups have performed studies on rats and mice exposed to BD or its metabolites to assess the importance of the different epoxide metabolites for the observed difference in sensitivity to BD in cancer tests (*21*). Concentrations of the epoxide metabolites in tissues and levels of adducts to hemoglobin and DNA have been measured. Recent studies, enabling analysis of hemoglobin adducts of DEB, have given results useful for quantitative comparisons of the BD metabolism in the mouse and the rat (*41*). Using this data, we have earlier calculated the in vivo doses (AUC) of the different epoxides in the mice and the rats in the cancer tests with BD (*39*). This showed a relatively higher AUC of the BD monoepoxides for the rat, particularly of EBdiol, than the AUC of DEB. The results of the present study, showing a faster disappearance of DEB relative to that of EB, as well as the demonstration that the activation-to-deactivation (by EH) ratio is close to 1 for DEB, give support to our earlier results concerning AUC in vivo.

Similar in vitro studies using cob(I)alamin will be performed for determination of enzyme kinetics in BD metabolism for the mouse and human to make quantitative interspecies comparisons. The results will be compared with the AUC measurements of the BD epoxide metabolites in rats and mice. This has the broader aim to evaluate in vitro metabolism studies combined with cob(I)alamin for trapping short-lived electrophiles for quantitative prediction of in vivo metabolism. The BD metabolism to three epoxides offers the complexity to enable an evaluation.

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