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Cobalamin as an analytical tool for analysis of oxirane metabolites of 1,3-butadiene: Development and validation of the method

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Abstract

The reduced form of vitamin B12 [cob(I)alamin] is known to be a supernucleophile, with the ability to react 10^5 times faster than standard nucleophiles. Procedures have been developed where cob(I)alamin is used as an analytical tool for the trapping of electrophilically reactive compounds. In the present work, a sensitive and accurate method for determination of reactive metabolites produced in vitro has been developed and validated. Diepoxybutane (DEB), a metabolite of 1,3-butadiene, was used as a model compound. The intermediate precursor 1,2-epoxybutene (EB) was incubated in a mouse liver S9 metabolic system and the formation of DEB was studied. Samples were taken at different times from the incubation mixture and added to the cob(I)alamin. The alkyl–cobalamins (alkyl–Cbl) formed were directly analysed by a miniaturized LC–MS/MS method and column switching. The assay was linear over the concentration range of 1.5–500 μM with acceptable precision and accuracy.

Keywords: Cobalamin; Diepoxybutane; Butadiene; In vitro metabolism

1. Introduction

Electrophilically reactive compounds/intermediates could, through their ability to react with nucleophilic sites in DNA, give rise to genotoxic effects already at low doses. Accurate analysis of electrophiles is difficult because of their inherent reactivity. For in vivo studies this has been solved through the analysis of stable reaction products (adducts) with proteins and DNA. For studies of the potential formation of genotoxic metabolites it is desirable, due to ethical reasons, to reduce animal experiments and instead perform studies on the formation and disappearance of reactive metabolites in vitro.

In vitro metabolic studies are conducted by incubating a precursor in a metabolising system, such as microsomes or liver suspension (S9-fraction). The formation of reactive metabolites has to be detected and quantified by rapid and accurate measurements, which can be difficult, particularly as regards low-molecular weight, hydrophilic compounds with high reactivity. Gas chromatography–mass spectrometry (GC–MS) or gas chromatography–flame ionisation detection (GC–FID) have been used as analytical techniques for the detection and quantification of reactive epoxy-metabolites of different low molecular weight compounds, formed in in vitro metabolism (such as isoprene, chloroprene or 1,3-butadiene (BD)). GC analyses were performed directly by either head-space analysis [1] or solvent extraction [2]. However, these procedures were not ideal since hydrophilic low molecular weight compounds are not easily extracted with organic solvents or analysed by head-space analysis. To overcome the analytical problem, the use of different nucleophiles, which are able to trap electrophilic compounds, has been suggested. For instance, 3,4-dichlorobenezenethiol was previously tried as a trapping agent of reactive metabolites for studies of in vitro metabolism of vinyl chloride [3]. After trapping, the metabolic products were analysed by GC–MS. Recently, in order to avoid the lengthy procedures related to GC, the peptide angiotensin II was suggested for trapping of reactive low molecular weight compounds, to form a complex suitable for LC–MS/MS analysis [4]. Angiotensin II has a low nucleophilic reactivity, which requires long trapping times and is therefore less suitable for metabolic studies, where the formation over time is followed.

In the present study, the supernucleophile cob(I)alamin is used as an analytical tool for characterization of reactive

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metabolites produced in vitro. Cob(I)alamin, formed by reduction of cob(III)alamin, is a compound with high nucleophilic strength which can react about 10^5 times faster than other standard nucleophiles [5,6]. Previous reaction-kinetic studies of oxiranes have already shown the ability of cob(I)alamin to instantly trap and thereby stabilize electrophilically reactive compounds with the formation of alkyl–cobalamin (alkyl–Cbl) complexes [7,8]. The alkyl–cbls formed have high molecular weight and are polar complexes. These properties favour their analysis by liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC–ESI–MS/MS) [9].

In the present work, a sensitive and accurate method for detection and quantification of electrophilically reactive metabolites produced in vitro in mouse liver S9 metabolic system has been developed and validated. Diepoxybutane (DEB), a metabolite of BD, which is of interest due to its high genotoxic potency, was chosen as the model compound. BD occurs in the environment (e.g. automobile emissions, urban air and cigarette smoke) and in certain occupational settings [10–12]. BD is bioactivated by cytochrome P450 to epoxybutene (EB), which is further metabolized to DEB. DEB is electrophilically reactive and has relatively low molecular weight (MW = 86.1 Da).

In a previous paper, we described that DEB could be instantly trapped by cob(I)alamin and be characterized as diepoxybutane–alkylated cobalamin (DEB–Cbl) by LC–MS/MS analysis [13]. This was a preliminary work, using synthetic metabolites, which forms a basis for the present work where a validation of a method for quantitative measurement of DEB formed in vitro is described. With the present method DEB, formed by in vitro metabolism of EB, is instantly trapped by cob(I)alamin to form DEB–Cbl and analysed by LC–MS/MS.

In order to correct for reduction and trapping variability and any drift in detector response during the LC–MS/MS analysis, an internal standard (IS), the chemically related compound propylene oxide, was added to the samples prior to the trapping. The alkyl–Cbls formed, denoted DEB–Cbl and IS–Cbl, were analysed by capillary LC–MS/MS and column switching. The method described in this work fulfils the criteria for bioanalytical method validation.

2. Experimental

2.1. Chemical and reagents

Mouse hepatic S9 fractions (30.0 mg/ml proteins) were purchased from Moltox (Boone, NC, USA). Diepoxybutane (DEB), epoxybutene (EB), propylene oxide (PO), hydroxocobalamin (OH–Cbl), glucose-6-phosphate, magnesium sulfate, β-nicotinamide adenine dinucleotide phosphate (NADP), potassium chloride, sodium borohydride ($NaBH_4$), trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich Sweden AB (Stockholm, Sweden). All solvents used were of HPLC grade. *Caution*: The oxiranes are alkylating agents and therefore hazardous. The compounds should be handled carefully in a hood or in closed systems and destroyed as earlier described [14].

2.2. Liquid chromatography

A capillary LC-system (CapLC, Micromass, Manchester, UK) coupled to a Quattro II triple quadropole mass spectrometer (Micromass, Manchester, UK) was used for the analyses.

The alkyl–cobalamins denoted DEB–Cbl and IS–Cbl were analysed using a pre-column (300 μm × 5 mm, PepMap, 5 μm; LC-Packings, Amsterdam, The Netherlands) coupled by a 10-port automated switching valve to an analytical column (300 μm × 50 mm, Inertsil ODS-3, 3 μm; LC-Packings, Amsterdam, The Netherlands) maintained at ambient temperature (25 ± 1 °C). The samples were eluted in gradient conditions with a mobile phase constituted of solution A (5% acetonitrile), solution B (70% acetonitrile) and solution C (5% acetonitrile), each solution was acidified with 0.1% TFA. When the switching valve was in the loading position, the samples were injected to the pre-column using the auxiliary system C (flow rate: 15 μl/min), while the binary pump system A/B eluted 20% B over the analytical column (flow rate: 5 μl/min). In this position the excess of OH–Cbl and some other unwanted polar contaminants, present in the injected sample, were passing the pre-column directly to the waste. Five minutes after the injection the valve was switched to the eluting position and the binary system A/B forward flushes 20% B over the pre-column to the analytical column where the DEB–Cbl and IS–Cbl were separated. The gradient was started immediately and 100% B was reached in 30 min. The mobile phases were filtered prior to use.

2.3. Mass spectrometry

The mass spectrometer was operated using an electrospray atmospheric pressure ionization source in positive ion mode (ESI^+) using multiple reaction monitoring (MRM) of the analytes.

The capillary voltage was set at 3.5 kV, with nitrogen used as nebulizing and drying gas at flow-rates of 20 and 300 L/h, respectively. Low-energy collision-activated dissociation (CAD) spectra were recorded using argon as the collision gas (gas cell pressure of 4×10^{-3} mbar). The collision energy used was 30 eV. The ion source temperature was 80 °C and the desolvation gas was 120 °C. The alkyl–Cbls gave the doubly protonated molecules $[M + 2H^+]^{2+}$ and fragments in accordance with previous studies [9,15]. The major fragment in product ion scan has been shown to be 665 which is a doubly protonated fragment with the loss of the alkyl moiety. The MRM precursor ion to product ion transitions used for DEB–Cbl and IS–Cbl were m/z 709 → 665, and m/z 695 → 665, respectively. For the data acquisition and integration MassLynx 3.5 (Micromass) was used.

2.4. Preparation of cob(I)alamin

Cob(I)alamin [Cbl(I)] was prepared according to the method of Haglund et al. [7]. Briefly, OH–Cbl dissolved in water (5 mM, 200 μl) was added, with a reduction catalyst (25 mM, 10 μl), to a septum-sealed amber vial (to protect from light). The solution was degassed with argon for a few minutes and the reducing