

Accurate assessment and identification of naturally occurring cellular cobalamins

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Abstract

Background: Accurate assessment of cobalamin profiles in human serum, cells, and tissues may have clinical diagnostic value. However, non-alkyl forms of cobalamin undergo β -axial ligand exchange reactions during extraction, which leads to inaccurate profiles having little or no diagnostic value.

Methods: Experiments were designed to: 1) assess β -axial ligand exchange chemistry during the extraction and isolation of cobalamins from cultured bovine aortic endothelial cells, human foreskin fibroblasts, and human hepatoma HepG2 cells, and 2) to establish extraction conditions that would provide a more accurate assessment of endogenous forms containing both exchangeable and non-exchangeable β -axial ligands.

Results: The cobalamin profile of cells grown in the presence of [⁵⁷Co]-cyanocobalamin as a source of vitamin B₁₂ shows that the following derivatives are present: [⁵⁷Co]-aquacobalamin, [⁵⁷Co]-glutathionylcobalamin, [⁵⁷Co]-sulfitocobalamin, [⁵⁷Co]-cyanocobalamin, [⁵⁷Co]-adenosylcobalamin, [⁵⁷Co]-methylcobalamin, as well as other yet unidentified corrinoids. When the extraction is performed in the presence of excess cold aquacobalamin acting as a scavenger cobalamin (i.e., “cold trapping”), the recovery of both [⁵⁷Co]-glutathionylcobalamin and [⁵⁷Co]-sulfitocobalamin decreases to low but consistent levels. In contrast, the [⁵⁷Co]-nitrocobalamin observed in extracts prepared without excess aquacobalamin is undetectable in extracts prepared with cold trapping.

Conclusions: This demonstrates that β -ligand exchange occurs with non-covalently bound β -ligands.

The exception to this observation is cyanocobalamin with a non-covalent but non-exchangeable CN[−] group. It is now possible to obtain accurate profiles of cellular cobalamins.

Clin Chem Lab Med 2008;46:1739–46.

Keywords: β -axial ligand exchange; cobalamin; cold trapping; glutathionylcobalamin; vitamin B₁₂.

Introduction

Cobalamins (Cbls, B₁₂) are dietary micronutrients that serve as coenzymes for methionine synthase (EC 2.1.1.13) (1) and methylmalonyl-CoA mutase (EC 5.4.99.2) (2). The clinical consequences of Cbl deficiency include hyperhomocysteinemia, methylmalonic acidemia, megaloblastic anemia, and neurological disease (3). Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (4), and it is also associated with complications of pregnancy (5), cognitive dysfunction including Alzheimer's disease (6), and osteoporosis (7, 8).

Our laboratories have had a long-standing interest in intracellular Cbl processing and coenzyme biosynthesis in mammalian cells and proposed that glutathionylcobalamin (GSCbl) was an intermediate generated during processing (9–12). The standard protocols for extraction and purification of Cbls from biological samples involve heated ethanol extraction, treatment with very alkaline or acidic solutions, and a final organic-phase extraction step (13–17). The major Cbls extracted and identified in these early studies included methylcobalamin (MeCbl), adenosylcobalamin (Ado-Cbl) and aqua(hydroxo)cobalamin (H₂OCbl⁺/HOCbl) [reviewed by Gimsing and Nexø (18)]. Other studies have reported that sulfitecobalamin (SO₃Cbl), nitrocobalamin (NO₂Cbl), and the extremely air-sensitive derivative nitroxycobalamin (NOCbl) are present in biological samples (19–21).

The intracellular milieu contains a variety of ligands that rapidly and essentially irreversibly exchange with the β -axial ligand of H₂OCbl⁺ (22, 23). Exchangeable ligands include sulfite, glutathione and other thiols, nitrite and nitrate. We hypothesized that SO₃Cbl, GSCbl, and NO₂Cbl could be formed artifactually by β -axial ligand exchange reactions between H₂OCbl⁺ and the corresponding free anions (SO₃^{2−}, GS[−], NO₂[−]) during cell lysis and extraction. This work confirms that β -axial ligand exchange occurs during lysis and extraction of Cbls from cultured bovine aortic endothelial cells (BAECs), cultured human foreskin fibroblasts (HFFs), and cultured human hepatoma HepG2 cells. Understanding the dynamics of β -axial ligand exchange reactions during Cbl extraction is an impor-

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Received July 1, 2008; accepted September 11, 2008;
previously published online October 31, 2008

tant first step in the development of a clinical diagnostic assay based on the profiling of intracellular Cbls.

Materials and methods

Materials

Dulbecco's phosphate buffered saline (DPBS), AdoCbl, MeCbl, HOCbl, cyanocobalamin (CNCbl), glutathione (GSH), sodium sulfite, and sodium nitrite were purchased from Sigma (Saint Louis, MO, USA). [^{57}Co]-CNCbl (specific activity 379 $\mu\text{Ci}/\mu\text{g}$ CNCbl) was purchased from MP Biomedical (Solon, OH, USA). Glacial acetic acid, acetonitrile (both HPLC grade), and ethanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). BAECs and fetal bovine serum (FBS) were purchased from Cambrex (East Rutherford, NJ, USA). SO_2Cbl , GSCbl, and NO_2Cbl were synthesized by reacting H_2OCbl^+ with sodium sulfite, GSH, and sodium nitrite, respectively, according to our recently published procedure (24). Synthesis of [^{57}Co]- H_2OCbl^+ was performed according to published procedures using [^{57}Co]-CNCbl as the starting material (25).

Cell culture and [^{57}Co]-cobalamin metabolic labeling

The following cultured cells were used in this study: 1) BAECs were cultured in 162 cm^3 flasks (Corning Inc., Corning, NY, USA) and grown in vitamin B_{12} -free, folic acid-free Ham's F12/DME (1:1) medium supplemented with 5% FBS (~ 33 pM Cbl), 2.0 mM L-glutamine, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and 50 nM (6S)- N^6 -methyltetrahydrofolic acid (Eprova AG, Schaffhausen, Switzerland); 2) HFFs were obtained from the Lerner Research Institute tissue culture core. HFFs were grown in advanced Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (~ 67 pM Cbl), 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (0.1 mg/mL); 3) HepG2 cells were obtained from ATCC (HB-8065TM). HepG2 cells were grown in American Type Culture Collection (ATCC) complete growth medium (#30-2003) supplemented with 10% FBS (~ 67 pM Cbl), penicillin (100 U/mL), and streptomycin (0.1 mg/mL). Total Cbl concentration in commercial FBS was determined using a commercial assay (MP Biomedicals, Solon, OH, USA). The amount of Cbl present in the culture medium via supplementation with FBS was shown to be sufficient to support normal growth in all three cell types. For [^{57}Co] metabolic-labeling experiments, cells were passaged at a ratio of 1:2. [^{57}Co]-CNCbl was added to achieve a final concentration of 0.2 nM (0.1 $\mu\text{Ci}/\text{mL}$ of culture medium), and cells were grown to 100% confluency (~ 48 h).

Extraction of cellular cobalamins

Confluent cells were harvested by trypsinization and washed three times with DPBS. Cell lysis was performed by treatment with 0.5 mL 50 mM Tris, pH 7.4, containing 0.15 M NaCl and 1% Triton X-100 for 20 min at room temperature. (Note: extraction and handling of the samples was performed under dim-red light to prevent decomposition of the photo-sensitive Cbls.) An aliquot of the cell lysate was taken for protein and total GSH determinations. Extractions were performed by addition of 0.75 mL of absolute ethanol to the cell lysate and incubated for 20 min at room temperature. Protein precipitates were removed by centrifugation at $9302 \times g$ for 3 min at room temperature. The resulting supernatant was taken to dryness in a Speed Vac at 45°C . The Cbl-

enriched residue was washed three times with acetone, re-suspended with 0.3 mL DPBS and passed through an Amicon microfuge filter (0.22 μm). Cbl extracts were stable for at least 2 months at -20°C .

Identification of intracellular cobalamins by HPLC

Extracted Cbls were separated by gradient reverse-phase HPLC using an Agilent 1100 System equipped with a Zorbax SB C-18 column (4.6 \times 250 mm, 5 μm particle size; Agilent, Santa Clara, CA, USA). Solvent A contained 0.1% acetic acid/acetate buffer titrated to pH 3.5 with NH_4OH , and solvent B was acetonitrile containing 0.1% acetic acid. Prior to injection, Cbl extracts were spiked with a mixture of pure non-radioactive Cbl standards to assist in the identification of the intracellular Cbls. Gradient conditions were as follows: 0–2 min, 5% B; 2–14 min, 15% B; 14–19 min, 18% B; 19–32 min, 35% B; 32–33 min, 60% B; 33–35 min, 5% B at a flow rate of 1 mL/min. Elution was monitored with UV detection at 254 nm. Peak areas were obtained using HP Chemstation LC 3D software (Agilent, Santa Clara, CA, USA) provided with the instrument. In typical runs, 70 fractions (~ 0.23 mL) were collected between 12 and 28 min. When required, a HPLC method consisting of a shallow linear gradient was used to improve separation of H_2OCbl^+ from GSCbl (see "cold-trapping" experiments below). Gradient conditions for such runs were as follows: 0–2 min, 5% B; 2–14 min, 15% B; 14–39 min, 18% B; 39–42 min, 35% B; 42–43 min, 60% B; 43–45 min, 5% B and 70 fractions (~ 0.23 mL) were collected between 15 and 30 min. The radioactivity associated with each fraction was counted using a gamma-counter (Gamma 4000; Beckman-Coulter, Fullerton, CA, USA).

Recovery of total and individual cobalamins

Total recovery of Cbl was calculated as the radioactivity present in the final Cbl extract divided by the radioactivity present in the washed cell pellets. The individual recovery of the different Cbl derivatives during the extraction procedure was also assessed. Briefly, unlabeled cell pellets were spiked with a known amount of the [^{57}Co]-Cbl derivative to be tested immediately before cell lysis. The cells were then lysed, extracted, and the HPLC/radioactive assay performed as described before. Recovery of the individual Cbl derivatives was calculated as the ratio of the corresponding [^{57}Co]-Cbl radioactive peak present in the final Cbl extract versus the radioactivity initially added to the cell pellets.

Assessment of β -axial ligand exchange during cobalamin extraction

GSH is the primary soluble thiol in cells (26). Because GSH readily reacts with H_2OCbl^+ (12), formation of GSCbl is likely to be a product of β -axial ligand exchange during extraction. To test this hypothesis, cells were grown in the presence of non-radioactive CNCbl, harvested, and lysed in the presence of [^{57}Co]- H_2OCbl^+ (0.33 μCi , specific activity = 305 $\mu\text{Ci}/\mu\text{g}$; chemical level comparable to the amount of [^{57}Co]-CNCbl that is imported by cells in culture investigated herein). Intracellular GSH was determined by the Jacobsen et al. (27) method.

"Cold trapping" of exchangeable ligands with excess aquacobalamin

Cells were grown in the presence of [^{57}Co]-CNCbl, harvested, and lysed with buffer containing 7.5 mM H_2OCbl^+ . We

hypothesized that a high excess concentration of non-radioactive H_2OCbl^+ would trap ligands that might undergo β -axial ligand exchange reactions with non-alkylcobalamins during lysis and extraction (designated "cold trapping"). The ratio of excess H_2OCbl^+ to total intracellular GSH in these studies ranged from 150 to 2500 depending on cell type. Accurate identification and quantification of the radioactivity associated with GSCbl was conducted for Cbl profiles analyzed by the slow-gradient HPLC method as described herein. Finally, to further examine the nature of the radioactive Cbls extracted from cultured cells, an aliquot of the Cbl extract was treated with excess potassium cyanide (KCN) under alkaline conditions followed by exposure to light in the presence of acetic acid. This procedure converts true Cbl to CNCbl (28). The samples were then separated by HPLC and the resulting radioactive signal/s assigned according to pure Cbl standards as described.

Assessment of the exchange of endogenous $^{57}\text{[Co]}$ -Cbls with excess H_2OCbl^+

It was important to determine whether endogenously labeled $^{57}\text{[Co]}$ -Cbls undergo exchange with the trapping agent H_2OCbl^+ during the lysis and extraction procedures. Therefore, the following experiment was conducted. Briefly, each one of the following pure radioactive standards: $^{57}\text{[Co]}$ -GSCbl, $^{57}\text{[Co]}$ - SO_3Cbl , and $^{57}\text{[Co]}$ - NO_2Cbl (final concentration: 120 pM, i.e., comparable to the intracellular concentration of Cbls in vivo) was incubated with 7.5 mM H_2OCbl^+ (cold-trapping protocol), and the mixture subjected to the same conditions used for cell lysis and Cbl extraction. Samples were then separated by HPLC and individual recoveries were calculated as described. β -axial ligand exchange between $^{57}\text{[Co]}$ -Cbls and H_2OCbl^+ was <2%.

Statistical analysis

Statistical analysis was performed using Microcal Origin® v7.0 (OriginLab Corporation, Northampton, MA, USA). Unless otherwise indicated, all values are expressed as mean \pm standard deviation, with $n \geq 3$. The statistical significance for establishing presence and amount of each Cbl derivative was determined by a directional (one-tailed) Student *t*-test at the 99% confidence level. Briefly, an experimental HPLC baseline was obtained by injecting DPBS into the HPLC, collecting fractions, and counting them in the gamma-counter as described for the routine sample analysis. The limit of detection for a $^{57}\text{[Co]}$ signal to be considered different from the instrumental noise was set as the lowest positive value that differs from the mean of the baseline values at a 99% confidence level.

Results

HPLC separation of cobalamin derivatives

An efficient and reproducible HPLC method for the separation of 7 Cbl standards was developed. A representative HPLC chromatogram is shown in Figure 1. The run-time for this separation is 35 min with a resolution of at least 1 min between peaks. The retention times were shown to be highly reproducible with a within-run coefficient of variation between 2% to 4%. While AdoCbl, MeCbl, and CNCbl were stable under the HPLC conditions described herein (see materials and methods), the non-alkylcobalamin derivatives

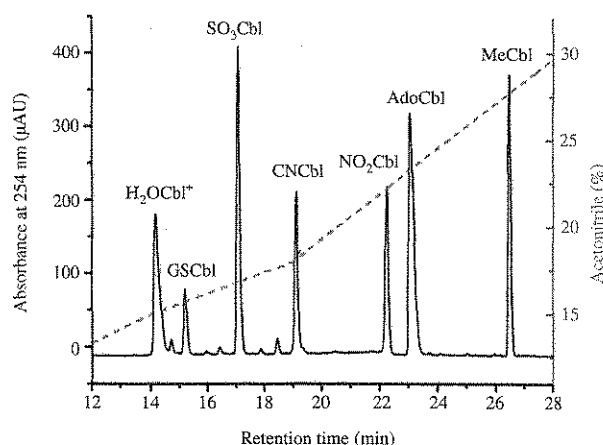


Figure 1 Reverse-phase HPLC of a mixture of seven cobalamin standards.

Gradient conditions: (A) 0.1% acetic acid, pH 3.5; (B) acetonitrile containing 0.1% acetic acid (dashed line, % change in B). Retention times were 14.1 min for H_2OCbl^+ , 15.2 min for GSCbl, 17.0 min for SO_3Cbl , 19.1 min for CNCbl, 22.2 min for NO_2Cbl , 23.1 min for AdoCbl, and 26.5 min for MeCbl.

SO_3Cbl , GSCbl, and NO_2Cbl decomposed to form H_2OCbl^+ (~5% decomposition).

Performance of the cobalamin extraction procedure

The overall recovery of Cbls ranged from 68% to 76% ($n=20$). Denatured proteins retain approximately 25% of cellular Cbls. Repeated extraction (5 times) of the protein-bound fraction led to an additional ~15% recovery of total Cbls. HPLC analysis showed that the relative amounts of each Cbl were similar to those found in the primary extraction step. Analysis of individual Cbl recoveries showed that $^{57}\text{[Co]}$ -MeCbl, $^{57}\text{[Co]}$ -AdoCbl, and $^{57}\text{[Co]}$ -CNCbl remained completely unchanged during the extraction procedure (recovery of each was ~80%), whereas the non-alkylcobalamins underwent different degrees of decomposition. Thus, 35% of $^{57}\text{[Co]}$ - SO_3Cbl , 30% of $^{57}\text{[Co]}$ - NO_2Cbl , and more remarkably 65% of $^{57}\text{[Co]}$ -GSCbl decomposed to form $^{57}\text{[Co]}$ - H_2OCbl^+ during the extraction procedure.

Profiling of cobalamins in cultured BAECs

Extracts from BAECs grown in the presence of $^{57}\text{[Co]}$ -CNCbl were analyzed by HPLC as shown in Figure 2A. Approximately 38% of the total Cbl was in the form of $^{57}\text{[Co]}$ -AdoCbl showing that these cells readily import, process, and synthesize AdoCbl from CNCbl (Figure 2A and Table 1). Lesser amounts of radioactive MeCbl (10%), HOCbl (8%), GSCbl (5%), and SO_3Cbl (3%) were also observed (Figure 2A and Table 1). When extracts from BAECs were treated with KCN under alkaline conditions and then run on HPLC, the major peaks shown in Figure 2A were converted to $^{57}\text{[Co]}$ -CNCbl, as shown in Figure 2B, demonstrating that they are authentic Cbls. Surprisingly, 21% of the total extracted radioactivity did not correspond to identifiable Cbls. These compounds may correspond to the so-called "analogs" (29, 30). Studies aimed at

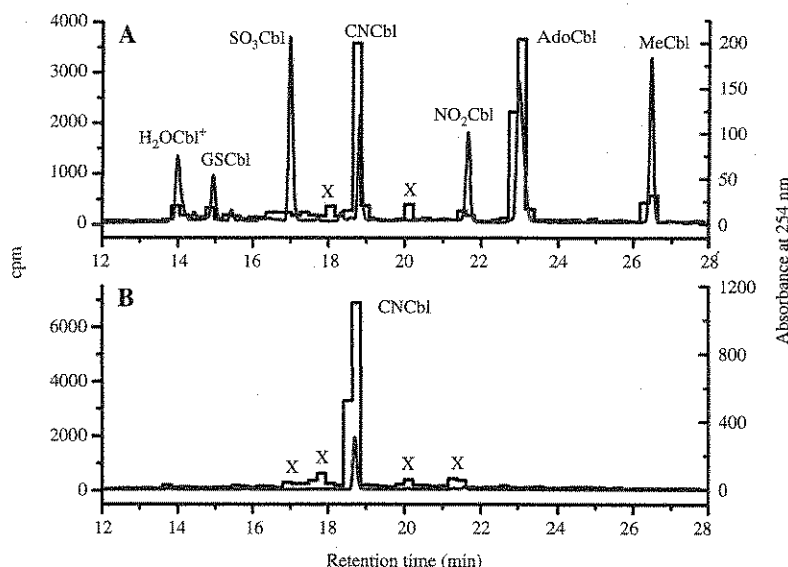


Figure 2 Profile of [^{57}Co]-cobalamins in cultured BAECs extracted in the absence of excess H_2OCbl^+ . (A) [^{57}Co]-AdoCbl, [^{57}Co]-MeCbl, and [^{57}Co]-CNCbl (the source of B_{12} given to the cells) were the most abundant forms present in BAECs. However, smaller amounts of [^{57}Co]- H_2OCbl^+ , [^{57}Co]-GSCbl, [^{57}Co]- SO_3Cbl , and [^{57}Co]- NO_2Cbl were also observed. X denotes as yet unidentified corrinoids. (B) Conversion of intracellular [^{57}Co]-Cbls to [^{57}Co]-CNCbl. X denotes unknown corrinoids that were not able to convert to [^{57}Co]-CNCbl.

their identification and characterization are currently in progress.

β -axial ligand exchange during extraction of cobalamins

We hypothesized that β -axial ligand exchange would occur with H_2OCbl^+ during the extraction of Cbls from BAECs. Evidence for β -axial exchange was obtained by adding [^{57}Co]- H_2OCbl^+ to a BAEC pellet immediately before extraction. During the extraction process, [^{57}Co]- H_2OCbl^+ was converted to [^{57}Co]-GSCbl (17%), [^{57}Co]- SO_3Cbl (15%), and [^{57}Co]- NO_2Cbl (4%), as shown in a typical experiment in Figure 3. Approximately 38% of the total extractable radioactivity was recovered as [^{57}Co]- H_2OCbl^+ . This experiment shows that intracellular GSH, SO_3^{2-} , and NO_2^- participate in β -axial ligand exchange reactions with [^{57}Co]- H_2OCbl^+ during cell lysis. Radioactivity was not detected under the AdoCbl and MeCbl standard peaks (Figure 3), demonstrating that these alkylcobalamins are not formed as artifacts during the extraction procedure. Interestingly, unidentified corrinoids (identified as "X" in Figure 3) accounted for approximately 27% of the total extractable radioactivity, suggesting that other, as yet unidentified, ligands react with [^{57}Co]- H_2OCbl^+ .

Identification of naturally occurring cellular cobalamins using cold trapping

Cbls were extracted from BAEC pellets in the presence of 7.5 mM H_2OCbl^+ , which was added to the cells just before lysis and extraction. We reasoned that excess H_2OCbl^+ would serve as a scavenger for intracellular ligands that might exchange with [^{57}Co]- H_2OCbl^+ and other labile [^{57}Co]-Cbls. The HPLC Cbl profile is shown in Figure 4A. As expected a broad

region of UV absorbance at 254 nm corresponding to excess H_2OCbl^+ in the extraction buffer was observed. The recovery of [^{57}Co]-AdoCbl, [^{57}Co]-MeCbl, and [^{57}Co]-CNCbl remained unchanged and comparable to the analyses shown in Figure 2A. However, the amount of the non-alkylcobalamins [^{57}Co]-GSCbl and [^{57}Co]- SO_3Cbl decreased substantially. [^{57}Co]- NO_2Cbl was undetectable under these conditions. A chromatogram of [^{57}Co]-Cbls separated using the slow-gradient HPLC method (see materials and methods section) is shown in Figure 4B.

Distribution of cobalamins in BAECs and human cell lines

The distribution of [^{57}Co]-Cbls and unknown corrinoids in cultured BAECs and human cell lines is summarized in Table 1. Values were taken from the cold-trapping experiments, which reflect relative amounts of naturally occurring Cbls only. The high amount of CNCbl present does not reflect natural intracellular levels, since it was the source with which cells were fed for the experiments. Cbl-like analogs ("unknowns" in Table 1) made up the second largest fraction in endothelial cells ($20.8 \pm 4.4\%$). Smaller amounts of SO_3Cbl ($2.8 \pm 1.0\%$) and GSCbl ($4.5 \pm 0.9\%$) were found in cultured BAECs. Also shown in the table are the total uptake of [^{57}Co]-CNCbl and the intracellular concentration of total GSH at the time of cell harvest (48 h).

Discussion

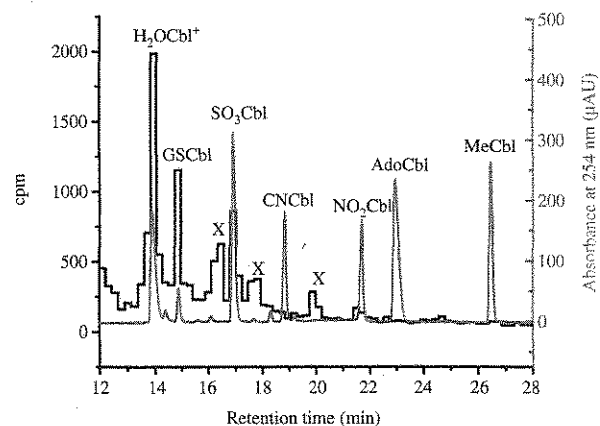
The objective of this study was to determine the extent of β -axial ligand exchange that occurs during the extraction of Cbls from mammalian cells. We

Table 1 Total Cbl uptake, intracellular distribution of [^{57}Co]-cobalamins and total GSH in BAECs, HFFs and HepG2 cells.

	Total [^{57}Co]-CNCbl uptake, pmol/mg protein ^a	[^{57}Co]-Cobalamin, % ^b					MeCbl	AdoCbl	Unknown corrinoids	Intracellular GSH, mM ^c
		HOCbl	GSCbl	SO ₃ Cbl	CNCbl	NO ₂ Cbl				
BAECs	0.54	7.8 ± 2.3 ^d	4.5 ± 0.9	2.8 ± 1.0	15.5 ± 2.5	ND	10.2 ± 3.5	37.6 ± 2.9	20.8 ± 4.4	2.39 ± 0.48
HFFs	0.36	7.2 ± 0.6	ND ^e	ND	29.4 ± 8.1	ND	51.8 ± 7.1	11.5 ± 1.9	5.1 ± 2.2	6.39 ± 0.27
HepG2 cells	5.1	11.9 ± 1.7	5.1 ± 0.8	ND	10.7 ± 0.5	ND	26.7 ± 4.8	42.5 ± 0.4	ND	0.30 ± 0.04

^aTotal [^{57}Co]-CNCbl uptake was determined by measuring the amount of radioactivity in the washed cell pellets divided by total mg of protein. [^{57}Co]-Cobalamin (%) values were expressed as mean ± standard deviation (n = 3-6). ^bIntracellular GSH was determined by the HPLC method of Jacobsen et al. (27). GSH concentrations are expressed as mean ± standard deviation (n = 3).

^cAll values are significant at a 99% confidence level as assessed by the Student t-test. ^dND, not detected.

**Figure 3** Formation of [^{57}Co]-cobalamins as a result of β -axial ligand exchange.

BAECs grown in the presence of non-radioactive CNCbl were harvested and pelleted. Immediately before lysis, the pellet was spiked with [^{57}Co]-H₂OCbl⁺, lysed, and extracted in the absence of excess H₂OCbl⁺. This figure shows that substantial amounts of [^{57}Co]-GSCbl, [^{57}Co]-SO₃Cbl, and [^{57}Co]-NO₂Cbl were formed during the extraction in contrast to the lack of formation of [^{57}Co]-AdoCbl and [^{57}Co]-MeCbl. Unknown corrinoids (X) were also observed.

hypothesized that non-covalent β -axial ligands would undergo exchange during the extraction procedure. We further hypothesized that non-covalent β -axial ligand exchange could be minimized by performing the extraction in the presence of a large excess of a ligand scavenger, in this case non-radioactive H₂OCbl⁺, which serves as a "cold-trapping" agent.

Accurate identification and quantification of Cbls in biological samples requires that the extraction procedure: a) efficiently releases Cbl from proteins [little or no Cbl exists in free form in mammalian cells (31)], b) maintains the chemical structures of the different cellular Cbl derivatives throughout the extraction procedure, and c) results in a near quantitative recovery of total cellular Cbl. Therefore, the recovery and stability of all the Cbls expected to be present in the cell lines studied was assessed and taken into consideration when interpreting the results. The proportion of each Cbl derivative recovered after successive extraction steps remained basically unchanged, suggesting that the observed relative amounts of AdoCbl and MeCbl indeed represent the natural abundance of each derivative in cultured cells.

Analysis of individual recoveries showed that the [^{57}Co]-non-alkylcobalamins underwent different degrees of decomposition during the extraction procedure, which leads to an underestimation of their actual biological abundance. [^{57}Co]-H₂OCbl⁺, the product of decomposition, is in turn concomitantly overestimated. [^{57}Co]-AdoCbl and [^{57}Co]-MeCbl remained essentially unchanged under our experimental conditions, supporting the notion that the alkylcobalamins can be considered as reliable indicators of Cbl metabolic activity in the cells.

The coenzymes AdoCbl and MeCbl were the major Cbl forms present in BAECs, HFFs, and HepG2 cells (Table 1). Non-metabolized [^{57}Co]-CNCbl, the sub-

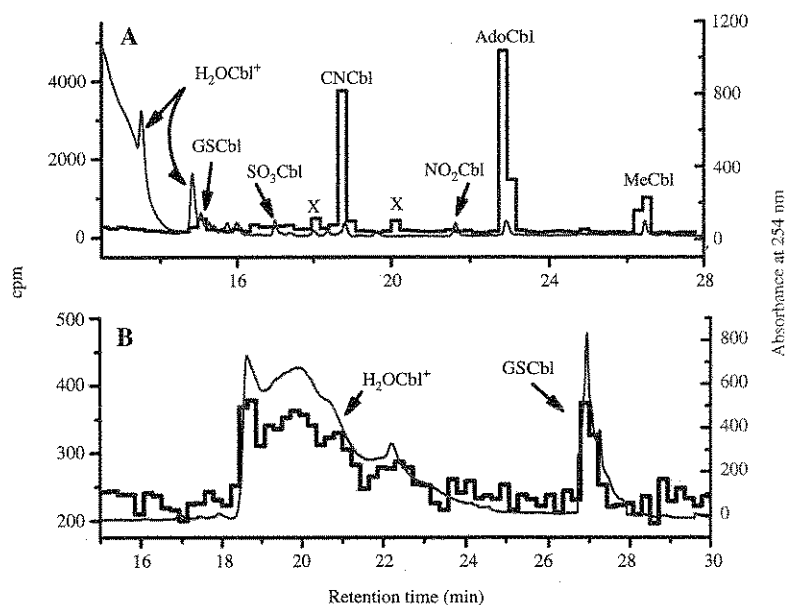


Figure 4 Profile of [^{57}Co]-cobalamins in cultured BAECs extracted in the presence of excess H_2OCbl^+ ("cold trapping"). (A) [^{57}Co]-AdoCbl, [^{57}Co]-MeCbl, and [^{57}Co]-CNCbl (the source of B_{12} given to the cells) were the most abundant forms present in BAECs and were similar to the levels shown in Figure 2A. However, the level of the non-alkylcobalamins [^{57}Co]-GSCbl and [^{57}Co]- SO_3Cbl decreased significantly, and [^{57}Co]- NO_2Cbl was undetectable when the extraction was carried out in the presence of excess H_2OCbl^+ . Unknown corrinoids (X) were also observed. (B) Improved separation of [^{57}Co]- H_2OCbl^+ and [^{57}Co]-GSCbl using the "shallow" HPLC method.

strate used in these studies, was also present in substantial amounts (10.7% for HepG2 cells to 29.4% for HFFs). Based on model studies (12, 22, 23), we hypothesized that upon cell lysis [^{57}Co]- H_2OCbl^+ would react with intracellular anions, such as nitrite (NO_2^-), sulfite (SO_3^{2-}), and glutathione thiolate anion (GS^-) to form the corresponding Cbl derivatives [^{57}Co]- NO_2Cbl , [^{57}Co]- SO_3Cbl , and [^{57}Co]-GSCbl, respectively (Figure 5). Since these derivatives are readily formed upon cell lysis, a strategy was used to distinguish natural versus artifactual Cbls based on the use of an efficient anion trap (non-radioactive H_2OCbl^+) to scavenge intracellular anions during the lysis and extraction procedure. To our knowledge, this is the first time that "cold trapping" of exchangeable anions has been utilized in the extraction of cellular Cbls.

Previous studies carried out in our laboratories suggested that GSCbl was an intermediate in the biosynthesis of AdoCbl and MeCbl (10, 32, 33). The Cbl profile of cells grown in the presence of [^{57}Co]-CNCbl as a source of vitamin B_{12} showed that the following derivatives are formed: [^{57}Co]- H_2OCbl^+ , [^{57}Co]-GSCbl, [^{57}Co]- SO_3Cbl , [^{57}Co]-CNCbl, [^{57}Co]-AdoCbl, [^{57}Co]-MeCbl, as well as other yet unidentified corrinoids. When the extraction was performed in the presence of excess cold H_2OCbl^+ , the recovery of both [^{57}Co]-GSCbl and [^{57}Co]- SO_3Cbl decreased significantly. Moreover, preparation of cell lysates in the presence of freshly added [^{57}Co]- H_2OCbl^+ resulted in the formation of [^{57}Co]-GSCbl and [^{57}Co]- SO_3Cbl but not [^{57}Co]-AdoCbl or [^{57}Co]-MeCbl, suggesting that β -ligand exchange occurs but only with non-covalently bound β -ligands. [^{57}Co]-GSCbl was present in BAECs and HepG2 cells but not in HFFs. The strikingly high

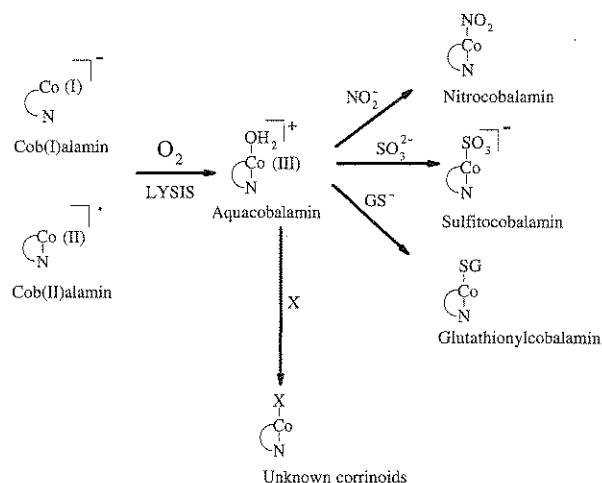


Figure 5 Reactions of aquacobalamin (H_2OCbl^+) with relevant biological ligands leading to the formation of artifactual derivatives during the cell lysis and cobalamin extraction procedures.

In the presence of oxygen, intracellular cob(I)alamin and cob(II)alamin are converted to H_2OCbl^+ [cob(III)alamin]. H_2OCbl^+ reacts with free nitrite (NO_2^-), sulfite (SO_3^{2-}), and glutathione thiolate anion (GS^-) and other currently unidentified ligands (X) to produce the corresponding derivatives NO_2Cbl , SO_3Cbl , GSCbl, and XCbl, respectively.

level of [^{57}Co]-MeCbl (52%) seen in HFFs may suggest that GSCbl is being rapidly consumed as the proximal precursor in MeCbl coenzyme biosynthesis. Little or no [^{57}Co]- SO_3Cbl and [^{57}Co]- NO_2Cbl were present in any of the human cell lines, suggesting that previous findings by other investigators on the isolation of SO_3Cbl and NO_2Cbl may have been misleading due to the artifactual formation of these derivatives during

the extraction procedures. NOCbl, which immediately decomposes to NO₂Cbl in the presence of O₂ (34) is very unlikely to be an isolatable naturally occurring Cbl.

According to the literature, most tissues contain higher levels of AdoCbl than MeCbl (18), and this was the case for BAECs and HepG2 cells. In contrast, it has been reported that human fibroblasts show higher amounts of MeCbl than AdoCbl (35). In agreement, this study showed that HFFs contained 52% MeCbl and 12% AdoCbl (Table 1). Whether these results represent actual physiological patterns or are just a result of cell culture conditions adjusted by manufacturers for optimal cell growth remains to be investigated. It is important to note that the ratio AdoCbl/MeCbl could be affected by cell culture conditions, such as folic acid content, supplementation with different forms of Cbl, cell passage, and time of incubation. Indeed, previous studies published by Quadros and coworkers have shown that AdoCbl and MeCbl are synthesized at different rates in leukemia cells, and as a result the corresponding yields of each cofactor vary over time (36). Moreover, the metabolic need for AdoCbl and MeCbl may be cell specific, as these results suggest. Higher mutase activity could be responsible for and therefore correlate with a higher requirement of the corresponding cofactor, AdoCbl, as it was observed in BAECs and HepG2 cells. However, this possibility does not hold true for the case of human fibroblasts, in which higher levels of MeCbl were observed.

The total Cbl uptake was very similar for BAECs and HFFs, although considerably higher in the case of HepG2 cells (Table 1). This result was not surprising, since it is well established that tumor cells take up and accumulate higher amounts of Cbl than normal cells in order to sustain high rates of cell division (37).

In summary, we have designed a simple procedure for the characterization of naturally occurring Cbls in cultured cells. The distinction between natural versus artifactual formation of non-alkylcobalamins was made possible by means of "cold trapping" of exchangeable anions with excess non-radioactive H₂OCbl⁺. β -axial ligand exchange does occur during the extraction procedure, causing an increase in the relative recovery of the radiolabeled non-alkylcobalamins. Scavenging of GS⁻ and SO₃²⁻ did not result in the total loss of [⁵⁷Co]-GSCbl and [⁵⁷Co]-SO₃Cbl in BAECs, suggesting that these derivatives do occur naturally in endothelial cells. However, their abundance is probably underestimated given the instability of these compounds, especially of GSCbl, during the extraction procedure. The isolation and identification of [⁵⁷Co]-GSCbl as a naturally occurring Cbl in both BAECs and HepG2 cells strengthens the notion of its role as a key intermediate in the biosynthesis of AdoCbl and MeCbl (10). Although proposed by others as naturally occurring, [⁵⁷Co]-NO₂Cbl was not present in detectable amounts in any of the cell types investigated herein. Significant levels of the analogs have been previously observed in biological samples (29, 30), and their structure and function are still a matter of debate. Further research toward the isolation and

identification of the Cbl analogs in cultured BAECs is currently in progress. It is recommended that metabolic labeling studies on cultured cells and tissues and perfused organs use the cold-trapping approach to provide more reliable estimates of Cbls in these systems.

Acknowledgements

The authors would like to thank Dr. Ruma Banerjee, Department of Biochemistry, University of Michigan for helpful discussions.

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