Hydroxocobalamin association during cell culture results in pink therapeutic proteins

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Abbreviations: mAb, monoclonal antibody; OH-Cbl, Hydroxocobalamin; CN-Cbl, cyanocobalamin; Me-Cbl, methylcobalamin; Ado-Cbl, Adenosylcobalamin; H2O-Cbl, Aquocobalamin; CHO, Chinese hamster ovary; IgG, immunoglobulin; PBS, phosphate buffered saline; RP-UPLC, reversed phase ultra performance liquid chromatography; SEC, size exclusion chromatography; UV, ultraviolet; MS, mass spectrometer; ICP-MS, inductively coupled plasma mass spectrometry; LOQ, limit of quantification; DS, drug substance; HC, haptocorrin; IF, intrinsic factor; TC, transcobalamin II

Introduction

Successful control of biopharmaceutical manufacturing is critical for ensuring product quality and lot-to-lot consistency of therapeutic proteins. Although some amount of heterogeneity can be expected because of the complexity of large molecules, rigorous testing and characterization are used to define product consistency throughout process development. Product appearance, i.e., the state and color of a product, is a required specification for release and can be a simple test to identify product or process impurities.1 Although color may not affect safety or efficacy, even moderate color variation in a therapeutic product can indicate process inconsistency and possibly present complications for binding studies during clinical trials.

Although proteins commonly have some color due to light scattering at high concentrations, variability in color observed during manufacturing may be from process-related impurities.2,3 Cell culture media is a common source of colored components in biotherapeutic manufacturing, but these are normally cleared during downstream processing.4 Host cell proteins, however, have been observed to co-purify due to protein interactions.5 Similarly, vitamins in cell culture media have shown high affinity to monoclonal antibodies (mAbs) and may co-purify, resulting in a colored final product.6,7 Both vitamin B12 (riboflavin) and vitamin B9 (folic acid) are yellow, while vitamin B12 (cobalamin) is a vibrant pink and can dominate the color of the media.8 Vitamin B12, an essential vitamin for eukaryotes, has been described as the most complex of the B vitamins. It contains a corrin-ring surrounding a cobalt atom that is coordinated axially by a 5,6-dimethylbenzimidazole nucleotide tail in one plane and a variable R-group in the opposite position (Fig. 1). There are four naturally occurring forms of vitamin B12, which are distinguished by different R-groups: cyanocobalamin (CN-Cbl), hydroxocobalamin (OH-Cbl), methylcobalamin (Me-Cbl), and adenosylcobalamin (Ado-Cbl). CN-Cbl is the most stable of the vitamin B12 forms and is ubiquitous in commercially-available products such as vitamin supplements and cell culture media. The other three vitamin B12 forms are less stable, with ligand affinity in the order of OH- < Ado- < Me-Cbl. Me- and Ado-Cbl are biologically active forms of vitamin B12, and are used as cofactors for methionine synthase and methyl-malonyl CoA mutase.9,10

We investigated the cause of variable pink coloration of several purified mAbs and Fc-fusion proteins. Vitamin B12 used during
manufacturing was a likely source of pink color, and a combination of binding experiments and media photo stability studies identified OH-Cbl as the specific $B_{12}$ form responsible for pink color in product. Differential association between the $B_{12}$ forms was further leveraged to develop a rapid and simple sample pretreatment for accurate quantification. Analysis of multiple proteins and process conditions provides insight into the sporadic occurrence of pink coloration and suggests a path for eliminating future color variability.

Results

Proteins are well-known to have yellow color originating from a variety of sources; however, during process development, several mAbs and Fc-fusion proteins were observed to have pink color.\(^7\)\(^1\)\(^1\) The occurrences spanned multiple products, processes, sites and manufacturing scales over several years, and the color varied in intensities. Pink coloration usually did not occur with every production run, making investigation of the root cause problematic. In the most striking example, material purified from bioreactors run side-by-side under seemingly identical conditions produced pink and non-pink protein (Fig. 2). Only one of the two product lots was pink, which suggested the presence of a pink contaminant. Removal of the pink color using conventional protein purification techniques including affinity and size exclusion chromatography was unsuccessful, indicating co-purification of the pink contaminant.

The main pink-colored component in the manufacturing process is CN-Cbl, a component of cell culture media. The appearance of pink in multiple molecules and the persistence of this color through purification led to the hypothesis that CN-Cbl binds to product, and the binding should be ubiquitous, energetically favorable, and have a rapid association. Repeated attempts to turn proteins pink by incubation with CN-Cbl, however, were unsuccessful. This included titration of CN-Cbl with uncolored lots of products that had shown pink variability. When combined, these observations suggest CN-Cbl is not responsible for the pink coloration.

Identification of the pink colored contaminant. While CN-Cbl does not directly associate with protein, it was possible that a different form of vitamin $B_{12}$, such as OH-, Ado- or Me-Cbl, could bind. Like CN-Cbl, OH- and Me-Cbl are pink-colored and thus likely candidates.\(^1\)\(^0\) The lack of CN-Cbl-protein association was leveraged to determine if the pink-colored contaminant was a form of $B_{12}$ other than CN-Cbl. A dilute potassium cyanide (KCN) solution is known to convert all $B_{12}$ forms to CN-Cbl and is frequently used during $B_{12}$ analyses.\(^1\)\(^2\) Therefore, a pink Fc-fusion protein was incubated in the presence of KCN to convert all vitamin $B_{12}$ to CN-Cbl, then buffer-exchanged to remove disassociated CN-Cbl. After repeated washing, the protein-containing retentate and the filtrate were examined visually for pink color. In the presence of KCN, the retentate was colorless and the filtrate was pink, indicating that, by conversion to CN-Cbl, the pink contaminant had been released from the protein (Fig. 3). These results indicate a pink form of vitamin $B_{12}$, other than CN-Cbl, is associated with the Fc-fusion protein.

To identify which form of vitamin $B_{12}$ binds protein, samples of an uncolored IgG2 mAb were incubated with CN-, Me-, OH- or Ado-Cbl standards. Post-incubation, the IgG2/vitamin $B_{12}$ solution was buffer-exchanged and the retentate and filtrate visually inspected. Incubation without $B_{12}$ or with CN-Cbl resulted in colorless protein, while incubation with Ado-Cbl, Me-Cbl or OH-Cbl resulted in protein with some degree of pink coloration (Fig. 4A and B). The association of Me-Cbl and Ado-Cbl was unexpected because these forms have been shown to specifically bind the Asp-X-His-X-X-Gly motif, which is not present in the proteins used here.\(^1\)\(^3\) To gain further insight, the $B_{12}$ standards used in the experiment were analyzed.
excess vitamin, all were visibly pink (Fig. 4C). Taken together, these data strongly suggest that OH-Cbl is the pink-colored contaminant.

Since localization of OH-Cbl binding on mAb protein may provide insight into the mechanism of association, an IgG2 was incubated with OH-Cbl and subjected to limited proteolysis. Fc and F(ab')₂ fragments generated by IdeS digestion were separated by non-denaturing size exclusion chromatography (SEC) to maintain any OH-Cbl/protein associations. UV detection at both 280 nm and 360 nm was used to identify OH-Cbl association. While the UV 280 nm signal was unchanged by the presence of OH-Cbl, Fc and F(ab')₂ fragments showed an increase in UV 360 nm relative to the untreated control, demonstrating OH-Cbl association occurs in both regions (Fig. 6; Table 1).

Conversion of CN-Cbl to OH-Cbl in cell culture media. Although OH-Cbl has been identified as the vitamin B₁₂ form that binds to product, CN-Cbl is the form added to the cell culture media. CN-Cbl is the most stable B₁₂ form, but can convert to OH-Cbl in the presence of light. Analysis of light-exposed and protected CN-Cbl confirms a small but distinct increase in OH-Cbl by RP-UPLC after only 30 min (Fig. 7A). Further investigation of CN-Cbl conversion to OH-Cbl was performed in Chinese hamster ovary (CHO) cell culture medium to determine whether light-induced conversion would occur in the complex matrix. Cell culture medium was initially prepared without CN-Cbl to prevent conversion to OH-Cbl during media preparation. After spiking medium samples with CN-Cbl, the effect of light exposure was evaluated over the course of 10 d at 37 °C. Formation of OH-Cbl was obscured due to co-elution with unidentified media components; however, CN-Cbl was well-resolved from other media components and easily quantified. CN-Cbl levels were significantly reduced upon light-exposure within a single day, and 50% or greater loss was seen over 10 d (Fig. 7B). In contrast, light-protected samples showed no significant loss of CN-Cbl, confirming that conversion to OH-Cbl occurs with light exposure. Approximately 90% of CN-Cbl was recovered after treatment of light-exposed samples with KCN, indicating CN-Cbl is converted to OH-Cbl as opposed to degraded in the presence of redox-active compounds (Fig. 7C).

Combined, these data indicate that light-exposure of cell culture media can convert CN-Cbl to OH-Cbl on a timescale significantly shorter than a typical production cell culture.

Vitamin B₁₂ analysis of purified protein samples. A simple procedure to quantify OH-Cbl in relatively pure protein samples was developed by exploiting the differential association of OH-Cbl and CN-Cbl. Fc-fusion or mAb was incubated in the presence of KCN to release bound OH-Cbl into solution by conversion to CN-Cbl. CN-Cbl and protein were then separated using protein A chromatography, and the unbound fraction containing CN-Cbl was analyzed by RP-UPLC with UV and MS detection. Each B₁₂ form had a distinct retention time, with OH-Cbl eluting first, followed by CN-Cbl, Ado-Cbl, and Me-Cbl (Fig. 5A and B). The Ado-Cbl and the Me-Cbl chromatograms, however, also contained OH-Cbl, as confirmed by mass spectrometry. The B₁₂ standards were purchased as >99% pure, so the presence of OH-Cbl was likely caused by Ado- and Me-Cbl degradation to OH-Cbl. This is further supported by the observation of lighter pink color and lower quantified B₁₂ levels for protein incubated with Ado- and Me-Cbl compared with the OH-Cbl sample, even though all standards were prepared and used at the same concentration (Fig. 4B). OH-Cbl was also incubated in the presence of IgG1, IgG2, and Fc-fusion proteins; after removal of excess vitamin, all were visibly pink (Fig. 4C). Taken together, these data strongly suggest that OH-Cbl is the pink-colored contaminant.

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spectrometry (ICP-MS) (Table 2). CN-Cbl is the main source of cobalt in the manufacturing process, with other minor contributors totaling less than 1%. Very good agreement was observed between the orthogonal analyses, indicating conversion of OH-Cbl to CN-Cbl is complete and CN-Cbl fully disassociates from the protein. The simple conversion, good recovery, and high throughput sample handling allows for analysis OH-Cbl levels in mAbs andFc-fusion protein samples of varying degrees of color and purity.

The levels of OH-Cbl were protein normalized in three different lots of an IgG1 mAb that exhibited pink intensities from colorless to strongly pink to determine the affect of purification (Table 3). After protein A purification, Lot 1 was a strong pink color with 116 ng B₁₂/mg mAb, while Lot 2 was less pink and contained 67 ng B₁₂/mg protein. The amount of OH-Cbl in Lot 2 was reduced by almost 50% across the second chromatography step, suggesting that there may be conditions that dissociate OH-Cbl from the product, but no clearance was observed across the third chromatography step or in the drug substance (DS). Lot 3 was visually colorless during downstream processing, although a small amount of OH-Cbl was detected in each in-process pool. Overall, the OH-Cbl concentration in the samples correlated well with the intensity of pink color. The most intensely pink sample had an OH-Cbl concentration of 1.5 mg/kg while samples below 0.5 mg/kg appeared visually colorless. The data also suggest that OH-Cbl associates with the protein during the cell culture, harvest, or protein A chromatography because the OH-Cbl levels do not increase during further downstream processing.

**Discussion**

This work demonstrates that pink coloration of purified mAb and Fc-fusion proteins is a result of OH-Cbl association. We found that incubation of colorless protein with OH-, Me-, or Ado-Cbl causes protein to become pink, although the appearance of pink coloration with both Me- and Ado-Cbl was determined to be OH-Cbl impurities generated during routine sample preparation.

CN-Cbl, the form of vitamin B₁₂ used in cell culture media, did not bind to mAbs or Fc-fusion proteins; however, pure CN-Cbl and CN-Cbl in cell culture media converted to OH-Cbl in the presence of light. This finding is consistent with previous studies on the conversion of CN-Cbl to OH-Cbl upon light exposure, and suggests a mechanism for OH-Cbl generation in media. It is likely that a combination of light exposure and other unknown factors facilitate conversion during media preparation, cell culture or harvest operations.¹⁵

In eukaryotes, vitamin B₁₂ is an essential vitamin that is used as a cofactor for the enzymes methionine synthase and methyl-malonyl CoA mutase. The different forms of B₁₂ interact with multiple proteins.⁹,¹⁰ To enter cells, vitamin B₁₂ is shuttled through a series of chaperone complexes: haptocorrin (HC), intrinsic factor (IF), and transcobalamin II (TC). HC, the first of the transport proteins, has the broadest specificity, binding vitamin B₁₂ and its analogs.¹⁷ IF, the second transport protein, has the narrowest specificity for vitamin B₁₂, and can be thought
Table 1. Localization of OH-Cbl association to an IgG2 monoclonal antibody

|          | Total area (mAU*sec) | Relative area (%) |
|----------|----------------------|-------------------|
|          | 280 nm | 360 nm | F(ab')2 | Fc | F(ab')2 | Fc |
| mAb control | 17182502 | 55548 | 63.4 | 36.6 | 57.2 | 42.8 |
| OH-Cbl + mAb | 17897236 | 119255 | 63.7 | 36.3 | 60.3 | 39.7 |

Percent change 4.2 114.7

Figure 7. (A) Analysis of freshly prepared CN-Cbl standard (solid) and CN-Cbl standard exposed to light at room temperature for 30 min (dashed). Inset is a zoom view of 7–9 min for visualization of OH-Cbl changes. (B) Stability plot of relative CN-Cbl levels in light exposed CN-Cbl standard (open diamond), and media at 37 °C either protected from light (filled squares) or light exposed (open squares). (C) RP-UPLC chromatogram of light protected media (top), light exposed media (middle), and light exposed then KCN incubated media (bottom) at 37 °C after 10 d.

of as a gatekeeper for the B₁₂ pathway because it binds only B₁₂. The third and final vitamin B₁₂ transport, TC, is the only protein in the pathway that is non-glycosylated and has an intermediate specificity to the vitamin. TC has been found to bind vitamin B₁₂ in a base-on configuration (nucleotide tail complexed to cobalt), and can change conformation to shield the weakly-bound OH-ligand in OH-Cbl. Once endocyctosed into the cell, vitamin B₁₂ is converted to the biologically relevant Me- or Ado-Cbl forms. Me-Cbl is a cofactor for methionine synthase in the cytoplasm, while Ado-Cbl is used by methyl-malonyl CoA mutase in the mitochondria. In both cases, the histidine in the Asp-X-His-XX-Gly motif of the coenzyme displaces the nucleotide tail in a base-off/His-on configuration.

OH-Cbl has been suggested to associate with proteins other than B₁₂ transport proteins or enzymes. Additionally, OH-Cbl is used to treat B₁₂ deficient patients because it has a longer half-life due to its ability to bind serum proteins. We show OH-Cbl binds IgG1, IgG2 mAbs, and Fc-fusion proteins and is not localized to mAb F(ab')₂ or Fc regions, suggesting it does not bind a specific motif. The lack of CN-Cbl binding to product also suggests the mechanism of association is different than that used for normal B₁₂ uptake by trafficking proteins (HC, IF, and TC) since in that case both CN-Cbl and OH-Cbl are expected to bind. Furthermore, the Fc-fusion and mAbs lack the classic vitamin B₁₂ binding motif (Asp-X-His-XX-Gly), therefore it is unlikely that the binding mechanism is similar to base-off (released nucleotide tail) vitamin B₁₂ binding exhibited by Me- and Ado-Cbl as enzyme cofactors.

IgG-vitamin B₁₂ complexes have been found in humans, but the techniques used to quantify vitamin B₁₂ in these studies are not sensitive to a specific form. Considering the variety of Fc-containing molecules tested here and the documented IgG-B₁₂ complex in human subjects, this may suggest OH-Cbl association is universal to IgGs. The increased half-life of OH-Cbl utilized for vitamin B₁₂ deficiency treatment, the observation of IgG-vitamin B₁₂ complexes in serum and the association of OH-Cbl to proteins may all be linked to the unique binding properties of OH-Cbl. It is known that the H₂O of aquocobalamin (H₂O-Cbl), which is in equilibrium with OH-Cbl at neutral pH, is easily displaced by many ligands, including histidine. In the presence of electron-rich amino acids in proteins, OH may also be easily displaced from OH-Cbl, similar to base-on/His-on binding found in TC. The difficulty in removing OH-Cbl during routine downstream processing may then be due to a complex between the cobalt and protein. Additionally, the relative abundance of solvent-exposed, electron-rich amino acids across mAb sequences would explain the seemingly non-specific localization of OH-Cbl on IgG2. Transport proteins have been found to decyanate CN-Cbl during cellular trafficking; however, TC changes conformation when bound to OH-Cbl to protect the weakly bound OH ligand. The conformation change of TC upon binding OH-Cbl likely prevents non-specific binding with proteins similar to those seen in this study.

To quantify OH-Cbl in our purified proteins, we developed a rapid and specific technique using KCN to release OH-Cbl from recombinant therapeutic proteins by converting it to CN-Cbl. The released CN-Cbl is easily separated from bulk protein either by protein A chromatography or sized-based sieving. The separation of OH-Cbl from protein has allowed us to use a relatively simple UV-based RP-UPLC technique to measure and monitor OH-Cbl levels in purified protein during process development. Although we have solely focused on recombinant proteins in our study, the release procedure described could easily be adapted to an LC-MS/
MS assay to ensure that multi-vitamin measurements in complex mixtures (i.e., serum) accurately quantify total vitamin B$_{12}$ levels.\(^2\)

Analysis of OH-Cbl levels in samples from different stages of downstream processing indicates that OH-Cbl is present even in samples that lack pink color. This suggests that the variability of pink color in different development lots is due to varying OH-Cbl levels that occur during processing. Results from multiple recombinant mAbs and Fc-fusion proteins suggest a threshold between 0.3 and 0.5 mg/kg OH-Cbl for visible pink color, and highlights the inherent subjectivity in describing color. For example, mAb3 is alternately described as pink and peach at different stages (Table 2). Protein normalized results from analysis of in-process samples shows that there is never an increase in associated OH-Cbl after protein A purification, strongly suggesting that all OH-Cbl is generated during media compounding, cell culture, harvest, or protein A chromatography loading.

Further analysis of in-process samples demonstrates that polishing chromatography steps may remove some associated OH-Cbl, although in either case there was insufficient clearance to remove the pink color (Table 3). It does suggest the potential for optimizing downstream processes to improve OH-Cbl clearance. If the OH ligand is displaced by electron-rich elements in the protein and the Cbl-protein complex is not further stabilized by both nucleotide tail interactions and OH-ligand protection as seen in TC-OH-Cbl, it is likely that process conditions can be evaluated. Ultimately, a combined effort of upstream and downstream process development will be necessary to completely eliminate the risk of pink color variability. As the source of OH-Cbl is introduced prior to downstream processing, and begins as CN-Cbl, the level of vitamin B$_{12}$ required in the media could be evaluated. Ultimately, a combined effort of upstream and downstream process development will be necessary to completely eliminate the risk of pink color variability.

### Materials and Methods

**Protein stocks.** The mAbs and Fc-fusion protein used in these studies were produced in CHO cells and captured using protein

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**Table 2.** Comparison of RP-UPLC quantification of purified protein with elemental analysis for cobalt

| Sample                      | Reported color | Conc. (g/L) | ICP-MS B$_{12}$ (mg/kg) | RP-UPLC B$_{12}$ (mg/kg) | ng B$_{12}$/mg protein$^1$ |
|-----------------------------|----------------|------------|-------------------------|-------------------------|---------------------------|
| Fc-Fusion: Protein A Pool   | Very pink      | 12.8       | N/T                     | 44.53                   | 3478.77                   |
| mAb1: Lot 1 DS             | Pink           | 70         | 4.4                     | 3.40                    | 48.54                     |
| mAb1: Lot 2 DS             | Non-pink       | 70         | 0.2                     | < LOQ$^2$               | < LOQ$^2$                 |
| mAb2: Protein A Pool       | Pink           | 12.6       | 0.7                     | 0.70                    | 55.82                     |
| mAb3: Protein A Pool       | Pink           | 16.5       | 0.7                     | 0.67                    | 40.42                     |
| mAb3: Column 2 Pool        | Non-pink       | 25         | 0.3                     | 0.29                    | 11.47                     |
| mAb3: DS                   | Peach          | 72.9       | 0.8                     | 0.68                    | 9.35                      |

$^1$For ease of comparison, elemental ICP-MS results were converted to B$_{12}$ by ratio of Co-CN-Cbl molar mass. $^2$Assuming a molecular weight of 150 kD for mAbs, a conversion factor of 150 kD for mAbs, a conversion factor of 11 070 may be used to obtain B$_{12}$/protein molar ratio. $^3$< LOQ = peak below limit of quantification.

**Table 3.** Quantification of purified lots of mAb2 by RP-UPLC

| Lot  | Process intermediate | Reported color | Conc. (g/L) | RP HPLC B$_{12}$ (mg/kg) | ng B$_{12}$/mg Protein$^1$ |
|------|----------------------|----------------|------------|-------------------------|---------------------------|
| Lot 1| Protein A Pool       | Strongly pink  | 12.8       | 1.49                    | 116.4                     |
| Lot 2| Protein A Pool       | Pink           | 12.2       | 0.81                    | 66.7                      |
|      | Column 2 Pool        | Colorless      | 6.5        | 0.25                    | 39.2                      |
|      | Column 3 Pool        | Pink           | 12.6       | 0.49                    | 39.2                      |
| Lot 3| Protein A Pool       | Colorless      | 21.9       | 0.88                    | 40.4                      |
|      | Column 2 Pool        | Colorless      | 21.6       | 0.18                    | 8.3                       |
|      | Column 3 Pool        | Colorless      | 11.9       | 0.06                    | 5.1                       |

$^1$Assuming a molecular weight of 150 kD for mAbs, a conversion factor of 11 070 may be used to obtain B$_{12}$/protein molar ratio.
A chromatography. Additional purification was performed using multiple polishing chromatography steps to a high purity. Protein types used in this study include Fc-fusion, IgG2 (mAb 1, mAb 3), and IgG1 (mAb 2) subclasses.

Release of pink color from protein. Samples were incubated in the presence of 0.01% (w/v) KCN (Ricca Chemical Co, 60-10-16) at 37 °C for 30 min. To easily visualize color changes, excess KCN and unbound material was removed by filtering the solution and washing three times with phosphate buffered saline (PBS) through a 50 kD filter (Millipore, UFC505096). The retentate was transferred to a 3 cc glass vial and visually examined for color.

Association of Vitamin B12 with protein. Binding of B12 forms (Sigma-Aldrich, OH-Cbl: H7126; CN-Cbl: C3607; Me-Cbl: M9756; Ado-Cbl: C0884) to recombinant protein was evaluated by incubating 5 mg/mL protein with 1 mg/mL of each form. Samples were protected from light, and held at room temperature overnight. After incubation, excess B12 was removed as above and examined for color.

RP-UPLC separation of B12 forms with MS/MS identification. Vitamin B12 standards were separated by RP-UPLC with UV detection at 360 nm (Waters Acuity UPLC, Waters) on a Waters HSS T3 C18 2.1 × 150 mm, 1.8 μm particle column (Waters Corp) An in-line Thermo LTQ XL (Thermo Scientific) ion-trap mass spectrometer was used to positively identify the B12 forms.

Quantification of OH-Cbl associated with protein. Protein-associated OH-Cbl was quantified, by incubating samples with 0.01% (w/v) KCN (37 °C, 30 min) to convert protein-bound OH-Cbl to free CN-Cbl. To simplify the solution for quantification, the now free CN-Cbl was separated from product by using small amounts of protein A chromatography resin in batch uptake mode automated on a Tecxan Freedom Evo robotic liquid handling system (Tecan US). Protein A flow through was quantified against a standard curve ranging from 0.03–10 mg/kg and recovery was confirmed through spiking. Vitamin B12 results are reported both in concentration (mg/kg) and protein normalized (ng B12/mg protein).

Elemental analysis of samples for cobalt. ICP-MS was utilized to analyze elemental cobalt concentrations in protein solutions. An Elan DRC II ICP-MS (Perkin Elmer) was set to measure cobalt (Co) at 59 m/z and internal standard cadmium (Cd) at 112 m/z. Co standard curve ranging between 0.2–200 ng/mL was run with Cd at 50 ng/mL. Each sample was diluted 30-fold with 0.5% nitric acid, spiked with 50 ppb Cd, and analyzed. The acquired response against the standard curve gave the observed Co concentration. Observed Co concentration was converted to CN-Cbl levels by ratio conversion of Co:CN-Cbl molar masses.

Cell culture media photo-stability study. A CHO cell culture medium containing CN-Cbl was freshly prepared and sterile filtered. Medium samples were held for 10 d at 37 °C, either exposed to or protected from light. Time points were analyzed on different days by RP-UPLC for CN-Cbl. Loss of CN-Cbl by conversion to OH-Cbl was confirmed by incubation of light exposed medium with 0.01% KCN (37 °C, 30 min) and RP-UPLC analysis.

Localization of hydroxocobalamin binding to a monoclonal antibody. A colorless IgG2 recombinant antibody (5 mg/mL in 10 mM acetate pH 5.2) was incubated (RT, 17 h) in the dark with 0.05 mg/mL OH-Cbl, then buffer exchanged to remove unassociated vitamin. The pink-colored antibody was digested using fabRICATOR™ enzyme (IdeS; Genovis AB, A0-FR1-020) into F(ab)2 and Fe fragments. The digest was separated by non-denaturing SEC using a Tosoh TSKgel G3000WxH 4.6 × 300 mm column (Tosoh Bioscience LLC, Japan). Detection of antibody fragments was performed at 280 nm for protein quantification and 360 nm for B12 quantification.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

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