Ultra-Performance Liquid Chromatographic Separation and Mass Spectrometric Quantitation of Physiologic Cobalamins

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Abstract

The current analytical high-performance liquid chromatography (HPLC) methods by which the various forms of cobalamin can be separated and quantified are limited to tedious chromatographic gradients with run times of 20–30 min and limits of detection (LOD) of 2 nM (2.7 ng/mL). This LOD is far above the physiological range of 148–443 pM (200–600 pg/mL) that is the normal total cobalamin level in human plasma. In this manuscript, benefits of ultra-performance liquid chromatography (UPLC) in which the stationary phase particle size may be reduced from 3.5 µm with a mobile-phase backpressure of 6000 psi in traditional analytical HPLC to a stationary phase particle size of 1.7 µm and a mobile phase backpressure of 15,000 psi in UPLC are reported. UPLC can more than double the chromatographic resolution and reduce each chromatographic run time by 10-fold, such that a complete analysis takes only 3 min per sample.

Introduction

Cobalamin is a micronutrient that animals and humans obtain through a diet of meat, symbiotic bacteria, or vitamin supplements. Two forms found in nature, 5'-deoxyadenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), are essential cofactors for methylmalonyl-CoA mutase and methionine synthase in humans. Minor forms of cobalamin are vitamin forms that can be converted to either of the two enzymatic cofactors through replacement of the β-axial ligand of cobalamin (Figure 1). The two most common vitamin forms of cobalamin are hydroxycobalamin (HOCbl) and cyanocobalamin (CNCbl).

An insufficient body burden of cobalamin in humans results in a complex spectrum of pathologies, including pernicious anemia, megaloblastic anemia, peripheral neuropathy, dementia, depression, impaired cognition, and autoimmune dysfunction (1–6). Cobalamin deficiency is often the result of poor intestinal absorption of cobalamin, but in some cases the cofactor form of cobalamin is specific to the underlying biological pathology of the disease. In cases of pernicious anemia, supplements of CNCbl, HOCbl, and MeCbl are given orally (PO) or by intramuscular (IM) injection (4,7). The form, dose, and route of administration are selected according to the therapeutic regimen that will most effectively achieve the goal of cobalamin replacement or supplementation, on the fundamental biochemistry of vitamin B_{12} deficiency, and on the medical traditions of the country where therapy is administered (4).

A unique role for HOCbl has been identified recently. HOCbl can be administered by bolus intravenous (IV) injection to scavenge cyanide in cases of cyanide poisoning (8–14). This lifesaving treatment has been used for years in Europe, and has only recently been approved for use in the United States (Cyanokit, Dey, L.P., Napa, CA). In cases of methylmalonic aciduria, AdoCbl is administered PO, IV, or IM to compensate for the inability of cells to convert other forms of cobalamin to AdoCbl (15). Similarly, certain forms of peripheral neuropathy that result from an inadequate body burden of cobalamin are being treated with IV or IM injections of methylcobalamin (1,16). Although cyanocobalamin (CNCbl) is not one of the bioactive enzymatic cofactors for either methylmalonyl-CoA mutase or methionine synthase, it gives the most stable pharmaceutical preparation as an aqueous suspension or solid powder. CNCbl can be crystallized most readily as the penultimate step to the preparation of

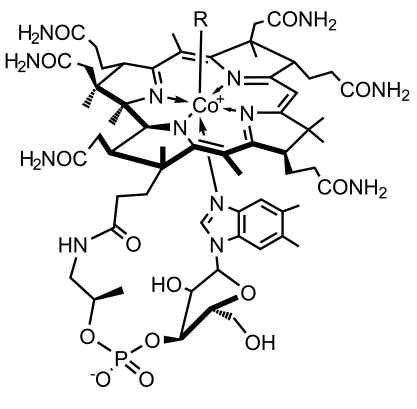

Figure 1. Structure of cobalamin. "R" represents the β-axial position. The major physiological forms of cobalamin include methylcobalamin, hydroxycobalamin, 5'-deoxyadenosylcobalamin, and cyanocobalamin.

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USP-grade cobalamin, and as such, this form is most commonly used for general replacement and supplementation therapies.

Considering the clinical utility of giving large doses of cobalamin (Cbl) to treat chronic and acute pathologies, an understanding of the systemic and cellular response to bolus doses of different forms of Cbl may help to elucidate new physiologic roles of cobalamin, including the possibility of physiological roles for free forms of Cbl. Current research maintains that binding to transcobalamin is requisite for cellular Cbl uptake, however, large IV and IM bolus doses may overwhelm the instantaneous, unsaturated B_{12} binding capacity of the cobalamin transport proteins, transcobalamin (TC), haptocorrin (HC), and intrinsic factor (IF).

The most common clinical assay for cobalamin levels in blood measures only the total concentration of cobalamin. In this clinical assay, venous blood is collected in a serum-separation tube that allows clotting to occur and separates serum from the packed red cell thrombus. The serum layer is decanted and incubated with potassium cyanide at elevated temperature to substitute the cyanide anion for all other ligands that may be coordinated to the Co atom at the β-axial ligand position. Quantitation of the resulting CNCbl reflects the total concentration of cobalamin in the blood and does not distinguish between the distribution of free cobalamin forms and the majority of circulating cobalamin that is protein-bound.

Since the pioneering reports of Jacobsen, Frenkel (17), and Binder (18), HPLC has remained the mainstay in chromatographic separation for the characterization and quantitation of the cobalamins in biological samples, vitamins, supplements, and food products (19–38). Recent advances in packed liquid chromatography have provided the opportunity for improved separation efficiency using ultra-performance liquid chromatography (UPLC) (39). UPLC improves separation through smaller particle size (1.7 µm) and increased pressure, which effectively decreases the theoretical plate height, and increases the particle diffusion pathway (Table I). The advancements in UPLC over traditional HPLC have become very clear in the investigation. The total run time for each chromatogram was decreased from 35 min to 3.5 min and achieved the baseline separation of the four common cobalamins. A consequence of shorter run times for each chromatogram was an accompanying decrease in mobile phase buffers. The sensitivity of detection was also increased 10-fold over traditional HPLC methods because sharper chromatographic elution peaks were observed due to a decrease in peak broadening (39).

Table I. Comparison of the Relative Improvements of UPLC over HPLC*

	UPLC	HPLC
Max Pressure	15000 psi	6000 psi
Minimum particle Size	1.7 µm	3.5 µm
Relative sensitivity	10	1
Relative resolution	2.1	1
Run time	1 min	10 min

* An increase in the maximum pressure of chromatographic instruments permits the use of smaller particle sizes of packed columns. A direct result is an increase in the sensitivity and resolution with decreased run times.

UPLC coupled to either quadruple or time-of-flight mass spectrometry was used to both identify and quantify cobalamins. The potential high throughput of this technique provides an ideal advancement in the investigation of cobalamins, analogues, and derivatives, which can assist in better understanding the pathological states that result from Cbl deficiency and help to define the optimal regimen for Cbl replacement and supplementation therapy. The main objective of this work was to establish a sensitive and selective UPLC method that incorporates both UV detection and mass spectrometric detection for the quantitation of four important forms of cobalamin.

Materials and Methods

Materials

Cyanocobalamin (CNCbl), methylcobalamin (MeCbl), 5'-deoxyadenosylcobalamin (AdoCbl), and hydroxocobalamin (HOCbl) were obtained from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC grade.

Sample preparation

Each cobalamin sample was dissolved in a minimal amount of CH_3OH (10 mg of cobalamin in 200 µL CH_3OH) and further diluted in a phosphate buffered saline (PBS) with fetal bovine serum (FBS) (1 × PBS, 5% FBS) to give final concentrations ranging from 100 µM to 100 pM.

Ultra-performance liquid chromatography

Separation was performed on a Waters Acquity UPLC system (Milford, MA) using a 1.0 × 50 mm BEH C18 column. The elution of Cbl was detected at 254 nm on a Waters Acquity photodiode array detector. The method used two mobile phases where mobile phase A was 0.1% TFA (v/v) in deionized H_2O, and buffer B was 0.1% trifluoroacetic acid (TFA) in acetonitrile. The sample injection volume was 5 µL using an automatic injector. A flow rate of 0.320 mL/min was maintained throughout the duration of each run (3.5 min). From 0–0.25 min 95% A and 5% B were combined in a linear gradient. From 0.25–2.50 min 95% A and 5% B were combined in a linear gradient to a final proportion of 60% A and 40% B. From 2.50–3.00 min 60% A and 40% B was maintained, and from 3.00–3.50 min. 60% A and 40% B were combined in a linear gradient to a final proportion of 95% A and 5% B. Five µL of DMSO were injected after every 3 sample runs to clean the column of proteinaceous material.

Mass spectrometry parameters

All mass spectrometry data were obtained on a Micromass Quattro II triple quadrupole mass spectrometer (Quattro II). The instrument uses electrospray (ES) to ionize the aqueous analyte, and samples can be introduced either by direct infusion or from HPLC–UPLC.

Analysis on the Quattro II mass spectrometer was carried out in positive-ion mode with a range of 400–1500 *m/z*. The following operating parameters were used: capillary 2.85, cone 55,

extractor 5, RF lens 0.2, source block temperature 100°C, desolvation temperature 200°C, ion energy ramp 0, lens 6, multiplier 650.

Data processing methodology

Cobalamin elution from UPLC separation was detected by monitoring the absorbance at 254 nm. Data processing was carried out using Empower 2 Chromatography Data software from Waters, Inc. Individual Cbl peaks were identified by mass spectrometry on the Quattro II, and by matching detected spectra to entries in a library of standards. Peak areas were integrated using the Empower 2 software ApexTrack function in order to standardize the integration of peak area. Calibration curve linear regression and graphing calculations were performed using Graphpad Prism software (La Jolla, CA).

Results

UPLC separation of cobalamins

Elution conditions were optimized to minimize run times and injection volumes, while still maintaining the baseline separation of the four cobalamins. The complete separation of cobalamin standards was accomplished in 3.5 min elution run time. Figure 2 shows a representative chromatogram using the method described and illustrates the separation of a mixture of four cobalamins (HOCbl, CNCbl, AdoCbl, and MeCbl). Each cobalamin was diluted to give final concentrations of 100 µM in the mixture, and 5 µL of the mixture was injected for each run.

Cobalamins were identified by matching the absorbance profile and mass spectrometric profile against entries in a library of standards. The retention time (t_R) of HOCbl in this method is 1.32 min; the t_R of CNCbl is 1.70 min; the t_R of AdoCbl is 2.15 min; and the t_R of MeCbl is 2.44 min. Minor peaks seen at 1.82 min and 2.37 min are components of buffer (1 × PBS, 5% FBS) used to dilute samples as detected from "blank" injections (data not shown). The detected masses of these components do not overlap with any of the cobalamins analyzed. The minor peak at 1.90 min is a contaminant of HOCbl that is present in all solutions made from the solid HOCbl standard.

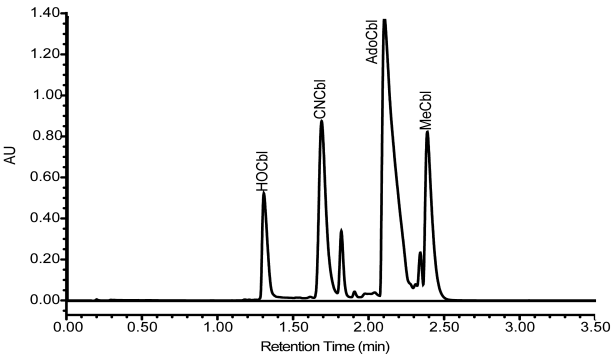

Figure 2. UPLC absorption trace of a mixture of cobalamins at 254 nm. The major peaks correspond to HOCbl (t_R = 1.32), CNCbl (t_R = 1.70), AdoCbl (t_R = 2.15), and MeCbl (t_R = 2.44). The minor peaks are buffer components.

Running buffers containing phosphate or acetate were also studied, however no improvement in separation was found. Addition of a small amount of trifluoracetic acid (0.1%, v/v) in elution buffers greatly increases the ionization of the cobalamins, and improves elution peak shape.

Standard curves were prepared for each cobalamin by plotting known concentrations of the cobalamin standard against the integrated peak area obtained from the spectrometric detector. For each cobalamin, a quantitative relationship was obtained between 50 µM and 500 pM. The limit of quantitation (LOQ) for all cobalamins was 500 pM with a signal-to-noise ratio of 5:1. The limit of detection (LOD) was 250 pM with a signal-to-noise ratio of 3:1.

Analysis of hydroxycobalamin

Spectral data for hydroxycobalamin (HOCbl) is shown in Figure 3. The UV absorption spectrum of HOCbl was measured directly from the UPLC photodiode array, and shows major absorption peaks at wavelengths of 350.8, 273.5, and 232.5 nm. Direct infusion of a HOCbl standard at a concentration of 10 µM was analyzed to detect the fragmentation pattern. The parent peak from infusion is seen at 1346.1 amu $[M+H]^+$ and this peak was monitored for daughter fragments. The major daughter fragment at 1200.1 amu is consistent with the loss of the 5,6-dimethylbenzimidazole base that, with ribose 3'-phosphate, makes up the nucleotide portion of cobalamin. The next daughter peak at 1182.7 likely corresponds to the loss of the hydroxyl β-axial ligand, which is consistent with the fragmentation of other cobalamins.

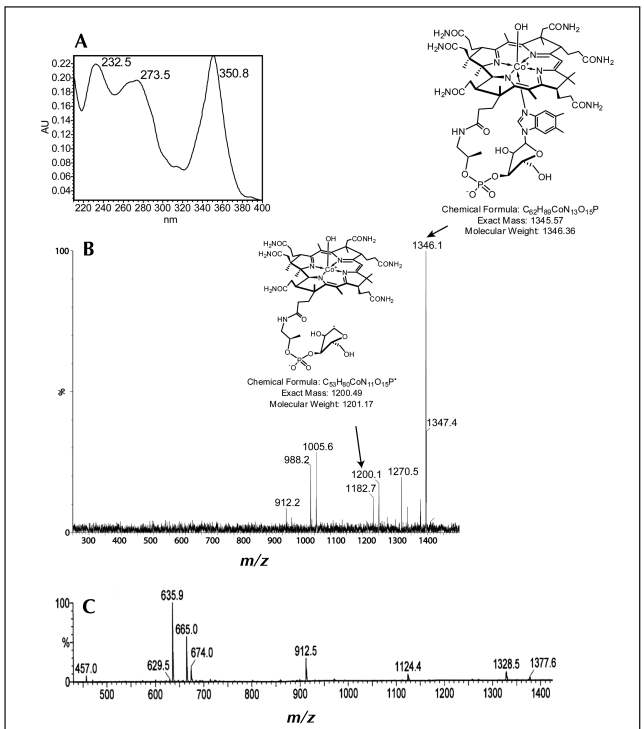

Figure 3. Spectral data obtained from UPLC–MS analysis of HOCbl. (A) UV absorption spectrum, (B) fragmentation pattern from direct infusion of HOCbl standard, the parent peak is seen at 1346.1 amu $[M+H]^+$ and the major daughter fragment at 1200.1 amu, (C) mass spectrum taken directly from UPLC–MS injection, the parent peak here is seen as 1328.5 amu $[M+Na]^+$.

The mass spectrum taken directly from a UPLC–MS injection was also analyzed, and the parent peak here is seen as 1328.5 amu, which is most likely the loss of the β-axial ligand. The composition of peak at 1377.6 is not clear, but may rise from an adduct of $[M+CH_3OH+H]^+$ Other peaks in the spectrum are consistent with fragmentation with the exception of the series of peaks around 600 amu. The peak at 674.0 is the result of the multiply charged ion of the parent peak $[M+2H^+] / 2$. Similarly, the peaks at 665.0 amu and 635.9 amu are multiply charged ions with mass-to-charge ratio (*m/z*) of 2. These peaks correspond to the single *m/z* ions 1328.5 and 1270.5 respectively.

Analysis of cyanocobalamin

Spectral data for cyanocobalamin (CNCbl) are shown in Figure 4. The UV absorption spectrum of CNCbl was measured directly from the UPLC photodiode array, and shows major absorption peaks at wavelengths of 361.4, and 278.4 nm. Direct infusion of a CNCbl standard at a concentration of 10 µM was analyzed to detect the fragmentation pattern. The parent peak from infusion is seen at 1355.4 amu $[M+H]^+$ and this peak was monitored for daughter fragments. The major daughter fragment at 1209.4 amu is consistent with the loss of the 5,6-dimethylbenzimidazole base, and mirrors the pattern seen in HOCbl.

The mass spectrum taken directly from a UPLC–MS injection was also analyzed, and the parent peak here is seen as 1355.6 amu $[M+H]^+$. The next peak detected is at 1209.5 and matches that seen in the fragmentation analysis. A similar pattern of multiply charged ion peaks is seen in the spectrum. The peak at 678.4 is the result of the multiply charged ion of the parent peak $[M+2H^+] / 2$. Similarly, the peak at 636.0 amu is multiply charged ion of the fragment at 1270.5 amu.

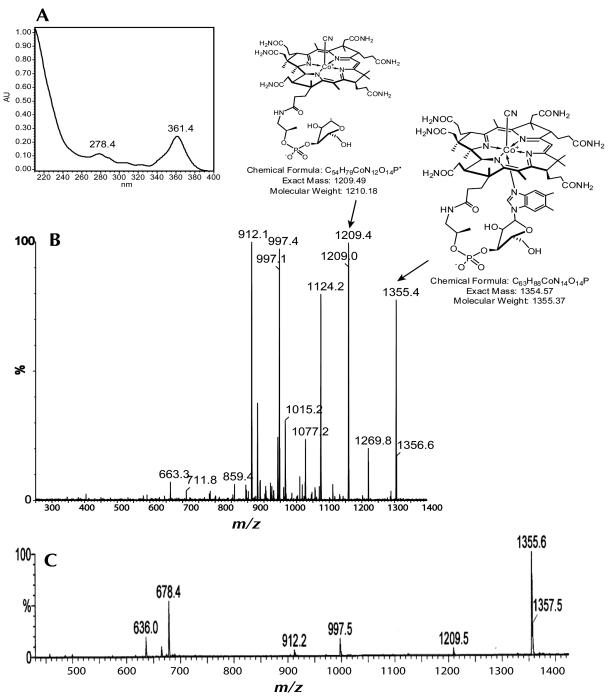

Figure 4. Spectral data obtained from UPLC–MS analysis of CNCbl. (A) UV absorption spectrum, (B) fragmentation pattern from direct infusion of CNCbl standard, the parent peak is seen at 1355.4 amu [M+H]+ and the major daughter fragment at 1209.4 amu, (C) mass spectrum taken directly from UPLC–MS injection, the parent peak here is seen as 1355.6 amu $[M+H]^+$.

Analysis of 5′-deoxyadenosylcobalamin

Spectral data for 5'-deoxyadenosylcobalamin (AdoCbl) is shown in Figure 5. The UV absorption spectrum of AdoCbl was measured directly from the UPLC photodiode array, and shows major absorption peaks at wavelengths of 303.1, and 263.7 nm. Direct infusion of an AdoCbl standard at a concentration of 10 µM was analyzed to detect the fragmentation pattern. The parent peak from infusion is seen at 1602.4 amu $[M+Na]^+$ and this peak was monitored for daughter fragments. The major daughter fragment at 1351.4 amu is consistent with the loss of the relatively large β-axial ligand of AdoCbl.

The mass spectrum taken directly from a UPLC–MS injection was also analyzed; however the parent peak is not seen here. Instead the first peak is seen as 1329.5 amu which corresponds to the protonated mass after the loss of the β-axial ligand.

A similar pattern of multiply charged ion peaks is also seen in the spectrum, and mirrors that of HOCbl.

Analysis of methylcobalamin

Spectral data for methylcobalamin (MeCbl) is shown in Figure 6. The UV absorption spectrum of MeCbl was measured directly from the UPLC photodiode array, and shows major absorption peaks at wavelengths of 346.5, and 264.9 nm. Like the other cobalamins, direct infusion of a MeCbl standard at a concentra-

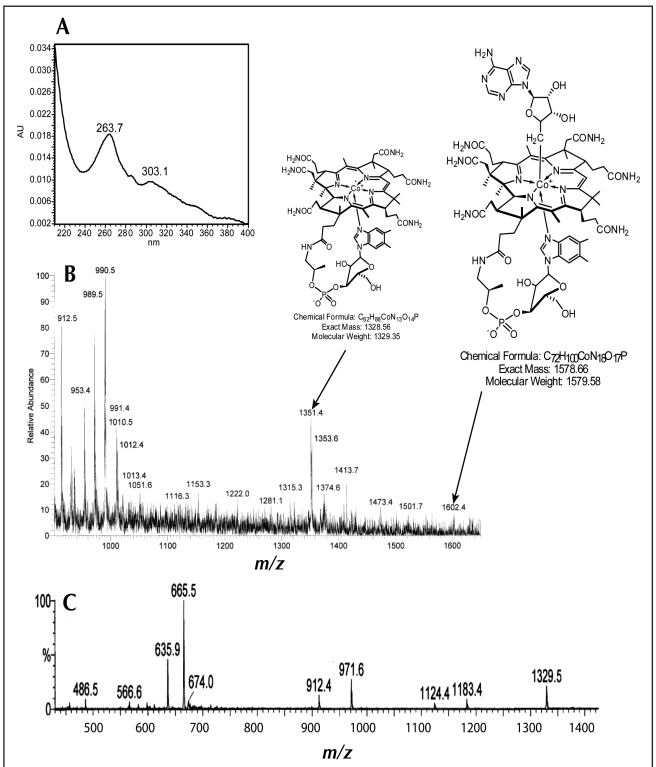

Figure 5. Spectral data obtained from UPLC–MS analysis of AdoCbl. (A) UV absorption spectrum, (B) fragmentation pattern from direct infusion of AdoCbl standard, the parent peak is seen at 1602.4 amu $[M+Na]^+$ and the major daughter fragment at 1351.4 amu, (C) mass spectrum taken directly from UPLC–MS injection, the main peak here is seen as the main daughter fragment at 1329.5 amu $[M+H]^+$.

tion of 10 µM was analyzed to detect the fragmentation pattern. The parent peak from infusion is seen at 1344.6 amu $[M+H]^+$ and this peak was monitored for daughter fragments. The major daughter fragment at 1329.2 amu is consistent with the loss of the β-axial ligand.

The mass spectrum taken directly from a UPLC–MS injection was also analyzed, and the parent peak is seen as 1344.6 $[M+H]^+$. The next major peak detected is at 1329.5 amu, and similar to AdoCbl, corresponds to the protonated mass after the loss of the β-axial ligand. A similar pattern of multiply charged ion peaks is also seen in the spectrum, and mirrors that of HOCbl and AdoCbl.

Monitoring of cobalamins in cell culture media

The method described here has been used to monitor cellular uptake and processing of these four cobalamins in order to determine the mean residence time of each cobalamin form in various cell lines. The extensive results of these experiments are the subject of a separate manuscript; however, it is important to include representative results here to demonstrate the utility of the UPLC method. A representative series of chromatographic traces for CNCbl is shown in Figure 7. Samples were taken from cell culture media (Dulbecco's Modified Eagle Medium, DMEM) that included 5% FBS after incubation with an immortalized fibroblast cell line (NIH3T3) for 10 min. Multiple samples were taken to test the repeatability of the method (n = 8). Figure 7 shows the stacked chromatograms of eight sequential injections of cell culture media. Each sample was filtered using a 0.22-µm filter to remove large proteinaceous material, and then injected directly onto the UPLC as described previously. The injections show CNCbl detected at a retention time of 1.70 min (indicated by the arrow). Based on calibration curves, the CNCbl in these injections is at a concentration of 25 ± 0.8 nM. Both the sharp peak at 1.45 min and the broad peak at 2.75 min are components found in the media.

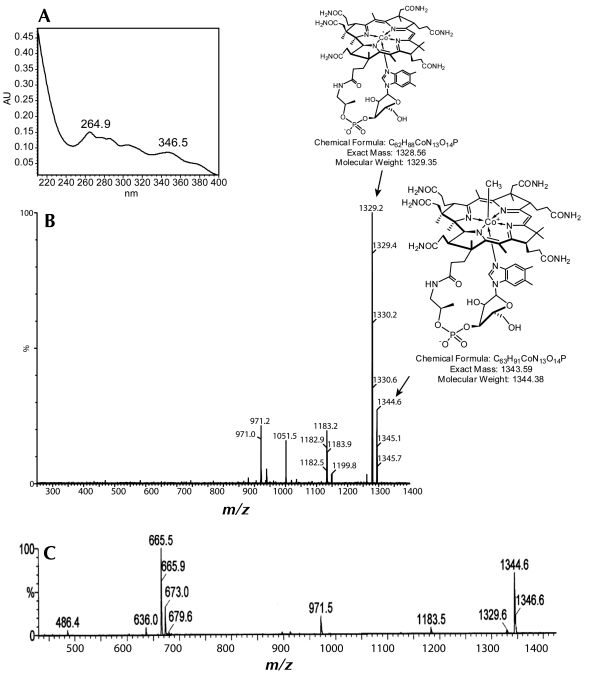

Figure 6. Spectral data obtained from UPLC–MS analysis of MeCbl. (A) UV absorption spectrum, (B) fragmentation pattern from direct infusion of MeCbl standard, the parent peak is seen at 1344.6 amu $[M+H]^+$ and the major daughter fragment at 1329.2 amu, (C) mass spectrum taken directly from UPLC–MS injection, the parent peak here is seen at 1344.6 amu $[M+H]^+$.

Discussion

Despite shorter run times of 3.5 min for UPLC compared to common run times of 35 min for HPLC, baseline separation of the four cobalamins was achieved. Each sample injection volume was held to 5 µL while still providing a LOD of 250 pM. In contrast, updated HPLC methods require the injection of relatively large sample volumes (100 µL) in order to obtain a LOD of ~2000 pM (37,38). Additionally, the extraction protocols utilized by most cobalamin analysis converted all forms of cobalamin to CNCbl. Although the process is important for releasing bound forms of cobalamin to free forms, the actual identity of the original cobalamin form was lost.

All four of the cobalamins monitored in this study depict a multiply charged peak when analyzed at high concentrations (100 µM). The multiply charged peaks correspond to either the parent mass, or the first major fragment mass. In HOCbl, a multiply charged peak is seen at 665.0 amu, which corresponds to the first major fragmentation peak of 1328.5 amu with the addition of a proton. In CNCbl, a multiply charged peak is seen at 678.4 amu, which corresponds to the parent peak of 1355.6 amu with the addition of a proton. In AdoCbl, a multiply charged peak is seen at 665.5 amu, which corresponds to the first major fragment peak of 1329.5 amu with the addition of proton. Importantly, the spectrum of HOCbl and AdoCbl can also be distinguished by differences in both multiply charged peaks, and fragmentation patters. AdoCbl shows a strong peak at 971.5 amu (fragment), and a very weak peak at 674.0 amu (multiply charged peak). In contrast, HOCbl shows no significant peak at 971.5 amu, but a very strong multiply charged peak at 674.0 amu.

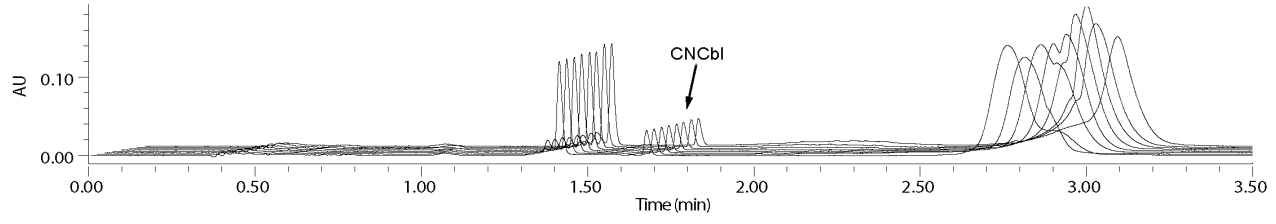

Figure 7. Stacked spectral data obtained from repeat injections (n = 8) of cell culture media containing CNCbl. Media was removed from culture after incubating with cells (NIH3T3) for 10 min and analyzed for the presence of Cobalamins. The injections show CNCbl detected at a retention time of 1.70 min (indicated by the arrow) at a concentration of 25 ± 0.8 nM. The sharp peak at 1.45 min and the broad peak at 2.75 min are components found in the media.

In MeCbl, a multiply charged peak is seen at 665.5 amu, which corresponds to the first major fragment peak of 1329.5 amu with the addition of proton. Although fragmentation and multiply charged peaks do not alter the absorption intensity detected on the chromatograph, these patterns do confound the MS quantitation of cobalamins at high concentrations. Therefore, samples of high concentrations should be sufficiently diluted to eliminate the formation of multiply charged peaks, and all samples should be protected from light to prevent the photolytic cleavage of the β-axial ligand of HOCbl, AdoCbl, and MeCbl.

In addition, the research detected a change in ion distribution depending on the source of samples (tissue, plasma, etc.). Most likely this phenomenon is the result of various salt concentrations in the samples. The addition of trifluoracetic acid greatly suppresses this problem; however, the change in ion distribution should be taken into account for each sample.

Conclusion

In this paper, a novel UPLC–MS method for the rapid and simultaneous analysis of four physiologic cobalamins has been demonstrated. This technology has already been applied to the investigation of several cobalamin derivatives in cell culture samples, and can readily be expanded to include additional cobalamin derivatives and analogues from different physiologic samples. Thus, the critical need for characterization of cobalamins from a variety of sources can be more efficiently met.

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