Research Paper

Photodegradation of Methylcobalamin and Its Determination in a Commercial Formulation

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Chamle *et al.*: Photodegradation of Methylcobalamin using RP-HPLC and MS/MS

Methylcobalamin is a highly photolabile and unstable molecule and hence, studies regarding photodegradation of methylcobalamin were carried out. In order to investigate the stability studies, the drug was subjected to photodegradation by exposing it to different light conditions in the validated photostability chamber as per ICH Q1B guideline. The drug was found to be less degraded in the blue light and was more prone to degradation under fluorescent light. Validated stability indicating liquid chromatography method was used for separating the methylcobalamin and its degradation products. The methylcobalamin peak with a retention time of 2.978 min was observed to decrease with a commensurate increase in a degradant peak at 4 min. The observed degradant peak was suspected to be hydroxocobalamin and was further confirmed by molecular weight determination. The fractions collected from high performance liquid chromatography were later injected into mass detector to determine the mass of the degradation products, which was found to be 665.78 amu.

Key words: Methylcobalamin, reversed-phase HPLC, MS/MS, hydroxocobalamin

The photostability of a drug substance might be distinct as the response of the drug or drug product to the exposure to solar, UV, and visible light leads to a physical or chemical change[1]. Methylcobalamin, Cobalt(3+); [(2R,3S,4R,5S)-5-(5,6-dimethylbenzimidazol-1-yl)-4-hydroxy-2-(hydroxymethyl)oxolan-3-yl]1-[3-[(1R,2R,3R,5Z,7S,10Z,12S,13S,15Z,17S,18S,19R)-2,13,18-tris(2-amino-2-oxoethyl)-7,12,17-tris(3-amino-3-oxopropyl)-3,5,8,8,13,15,18,19-octamethyl-2,7,12,17-tetrahydro-1H-corrin-24-id-3-yl]propanoylamino] propan-2-yl-phosphate, is a cobalamine form of vitamin B_{12}. It is used in the treatment of hyperhomocysteinaemia and peripheral neuropathy[2,3]. It was previously reported predicted that photolysis of methylcobalamin was initiated by a ligand to ligand charge transfer (LLCT) excited state, which included elevation of an electron of the cobalt-carbon adhesion to a π^* orbital of the corrin ligand. In possession by a determined proficiency of photolysis the absorption band at λ_{max}=317 nm was assigned to LLCT (CH_3 to corrin) transition of methylcobalamin. It was assumed that LLCT transition undergoes configuration interaction with the π-π^* corrin intraligand excitation at wavelength above 313 nm is then also associated through the proclamation of methyl radical however with abridged efficacy. This study mentioned about the photodegraded product of methylcobalamin as aquocobalamin based on the spectroscopic nature of the degradation product[4]. But this study never mentioned about the mass of the degraded product. Hence the present investigation involved the determination of the mass of the degradation product using mass spectroscopy.

From this study it is possible to predict the pathway based on the mass of the degradation product. The method is more reliable when compared to the UV/Vis spectroscopic methods[5]. The photodegradation process was carried out using photostability chamber, which was validated as per ICH Q1B guideline. The photodegradation products formed were separated by validated stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC)

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method[6] and the degradation products were further subjected to mass spectrometry (fig. 1).

MATERIALS AND METHODS

Biocon Limited, India provided an adequate amount of methylcobalamin as a gift sample. Commercial formulation A was used as the commercial formulation. HPLC grade acetonitrile (ACN) and methanol were procured from Finar Chemicals Limited (Ahemdabad, India). O-phosphoric acid (85 % pure) was purchased from Merck India Limited (Mumbai, India). MS grade methanol and MS grade ACN were obtained from Biosolve Chemicals, Bengaluru. Polytetrafluoroethylene (0.45 µ) membrane disc strainers were acquired from Pall Corporation (Mumbai, India). Millipore purification system (Siemens) provided the ultra clear water. Samples were examined on a Shimadzu HPLC (Kyoto, Japan) system equipped with LC Sol^{n} software and fortified by a LC-10 ADVP quaternary pump, a SIL-10 ADVP auto injector, a SPD M-10A VP photo diode array detector (PDA) and a SPD-10Avp UV detection system. Further samples were analysed by Thermo Scientific Dionex Ultimate 3000 series HPLC coupled to LTQMS Mass Spectrometry through HESI interface Chromeleon system equipped with LTQ Tune, Xcalibur software. C18 Hypersil BDS (5 µ, 150×4.6 mm) column was used for the separation.

Chromatographic conditions[6]:

Validated stability indicating liquid chromatography method was used for separating and quantifying the methylcobalamin and its degradation products[3]. The separation was attained using a C18 Hypersil BDS (5 micron, 150×4.6 mm) column using mobile phase comprising of (55:45 v/v) methanol:o-phosphoric acid 0.02 % v/v pH 2.3 with a flow rate of 1.0 ml/min and effluents were monitored at 223 nm. The retension time of methylcobalamin was observed to be 2.9 min.

Photodegradation studies:

To obtain O-phosphoric acid (0.02 % v/v) solution, 20 µl of o-phosphoric acid was taken and made up to 100 ml with water. In order to obtain the diluent (mobile phase), 55 ml of methanol and 45 ml of 0.02 % o-phosphoric acid were mixed for dilutions in ratio of 55:45. Methylcobalamin working standard 10 mg was weighed accurately and then transferred in 10 ml volumetric flask. The volume was made up to the mark with diluent. The concentration was 1000 µg/ml. The solution was further diluted to get 100 µg/ml concentration by using mobile phase. The sample was kept in photo stability chamber and different colour of light bulbs were used for the degradation study such as yellow light, sodium vapour lamp, orange light, fluorescent light (lab light), green light, and blue light (Table 1)[7,8]. The sample was taken initially and at different time intervals and loaded into HPLC system.

Commercial formulation A of 500 µg/ml strength was used as test formulation. One millilitre of commercial formulation A was diluted up to 5 ml with mobile phase to get 100 µg/ml concentration. The sample was kept in photo stability chamber and exposed to fluorescent light. The sample was taken initially and at different time intervals and loaded into HPLC system. Tuning parameters for the determination of mass of degradation product are as follows, Table 2[9,10].

RESULTS AND DISCUSSION

Methylcobalamine was subjected to photodegradation studies under different colour lights corresponding to various wave lengths (Table 3)[11], trend of photodegradation shows that under fluorescent light

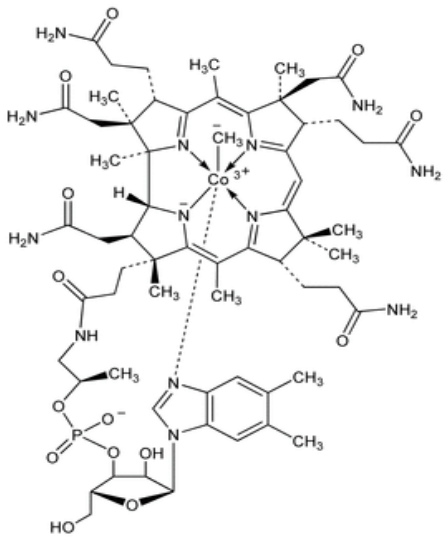

Fig 1: Structure of methylcobalamin

TABLE 1: DIFFERENT SOURCES OF LIGHT AND WAVE LENGTH

Light colour	Wavelength (nm)
Blue	492-455
Green	577-492
Orange	622-597
Yellow	597-577
Sodium vapor lamp	589
Fluorescent light	253.7 and 189

degradation of the methylcobalamine was maximum and under blue light drug degradation was minimum, which clearly indicated that blue light is best suitable for formulating methylcobalamine parenteral formulation. Chromatograms in figs. 2 and 3 can be used as an evidence for these studies.

Determination of mass of the degraded products and its determination in commercial formulations were carried out. Samples were introduced into the ion source by means of an axially mounted direct insertion probe, which was terminated with a stainless-steel sample support. Samples were deposited from ACN solution onto the sample support (figs. 4-7 and Table 4).

It was found that methylcobalamin decomposes under the ambient light of laboratory and other lights used for the photodegradation studies. The methylcobalamin was found to be very less degraded under blue light. The methylcobalamin peak decreased with a commensurate increase in a degradant peak at 4 min. The peak was suspected to be hydroxocobalamin as methylcobalamin is susceptible to photolysis. The fact was further confirmed by determination of molecular weight of the degradant. Therefore methylcobalamin is extremely light sensitive, enduring the homolytic cleavage of cobalt-carbon bond.

The peak corresponding with standard methylcobalamin was ionized. The ionized peak mass confirms the existence of methylcobalamin in the sample. The mass of methylcobalamin is *m/z* 1344. Similarly, spectra of methylcobalamin was achieved, which displays [M+H] ion at *m/z* 1344.73. The methylcobalamin provides an intense peak at *m/z* 1329 conforming to the loss of the axially involved ligand. The $[M+H]^+$ ion intensities qualified to this peak are in the 1344+ i.e. RCH3 where R' is the coenzyme of the axial group.

From the literature and our work, it has been observed that all the cobalamine molecules exhibit similar fragmentation patterns when subjected to MS fragmentation in the mass range of 1329-800 amu. From the fragmentation, we have observed loss of acetamide from corrin as well as its major fragmentation is focused on the chain, which is axially attached to cobalt. Direct injection of degradation sample was onto MS/MS and found out that its peak is seen at 665.0 amu, which corresponded to peak 1328.5 amu, first major fragmentation peak. Methylcobalamin on fragmentation lost dimethylbenzimidazol group at cobalt end and few sugar and phosphate groups, which gives peak at *m/z* 971.

A simple, efficient and sensitive method was developed using PDA detector by utilizing the λ_{max} of the drug methylcobalamin in order to determine its stability. The methylcobalamin peak decreased with a commensurate increase in degradant peak. The peak was found to be of hydroxycobalamin. Therefore, methylcobalamin

TABLE 2: TUNING PARAMETERS FOR MASS OF DEGRADATION PRODUCTS USING LC-MS/MS

Polarity	Positive
Capillary voltage	49.00 volts
Tube lens	250.00
Sheath gas flow rate	5 ml/min
Auxiliary gas flow rate	5 ml/min
Capillary temperature	180°
Multipole 00	-7.75
Lens 0	-8.00
Multipole 1	-11.00
Multipole 0	-8.50
Lens 1	-13.00
Gate lens	-54.00
Front lens	-11.00

TABLE 3: PHOTODEGRADATION STUDIES USING VARIOUS LIGHTS

ID	Sample	Time of exposure (min)	Retention time (min)	Area	% Degradation	Concentration (µg/ml)
Green light (577-492 nm)	Drug	60	3.073	2686981	35.31	100
	Degradant	60	4.049	388183	-	-
Orange light (622-597 nm)	Drug	60	3.078	2533636	33.24	100
	Degradant	60	4.041	680041	-	-
Yellow light (597-577 nm)	Drug	60	3.080	1198372	17.72	100
	Degradant	60	4.022	2660119	-	-
Blue light (492-455 nm)	Drug	60	3.088	2870092	37.32	100
	Degradant	60	4.051	328981	-	-
Sodium vapour lamp (589 nm)	Drug	60	2.852	1928701	12.23	100
	Degradant	60	3.696	931178	-	-
Fluorescent light (253.7 and 189 nm)	Drug	60	3.064	160281	44.46	100
	Degradant	60	4.054	637815	-	-

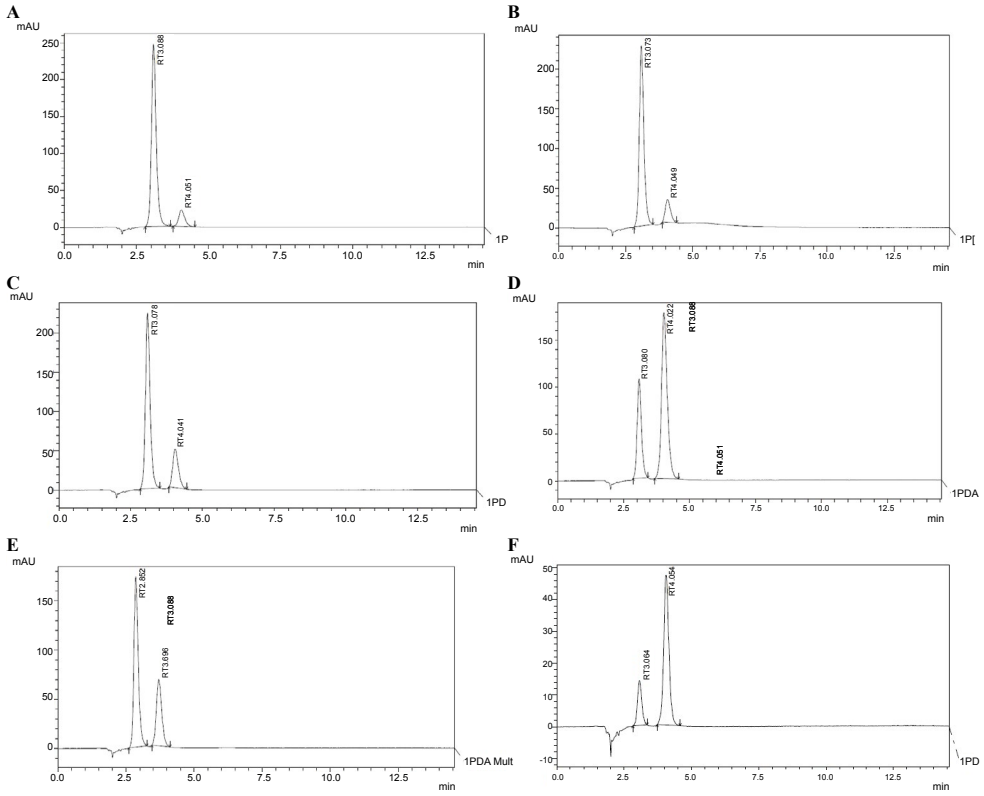

Fig. 2: Chromatograms of methylcobalamin
Chromatographs under (A) blue light (492-455 nm), (B) green light (577-492 nm), (C) orange light (622-597 nm), (D) yellow light (597-577 nm), (E) sodium vapor lamp (589 nm), (F) fluorescent light (253.7 and 189 nm) at 60 min exposure

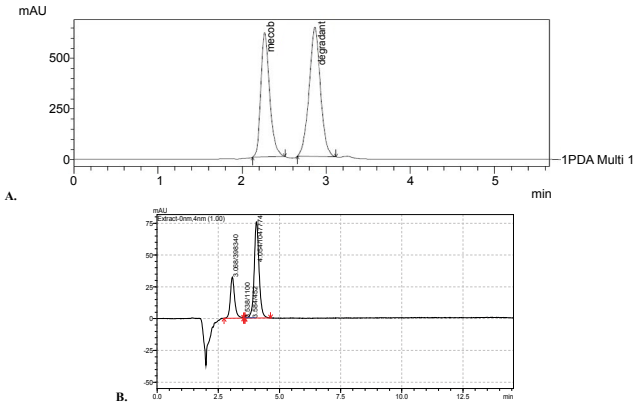

Fig. 3: Chromatograms of methylcobalamin after exposing the sample to lab light for 60 min
A: Methylcobalamin in commercial formulation A and B: methylcobalamin

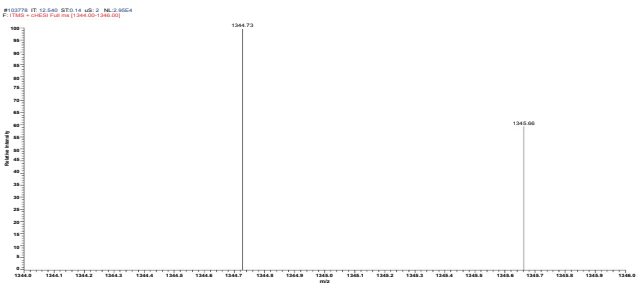

Fig. 4: The positive ion mass spectrum of methylcobalamin and vitamin B_{12}

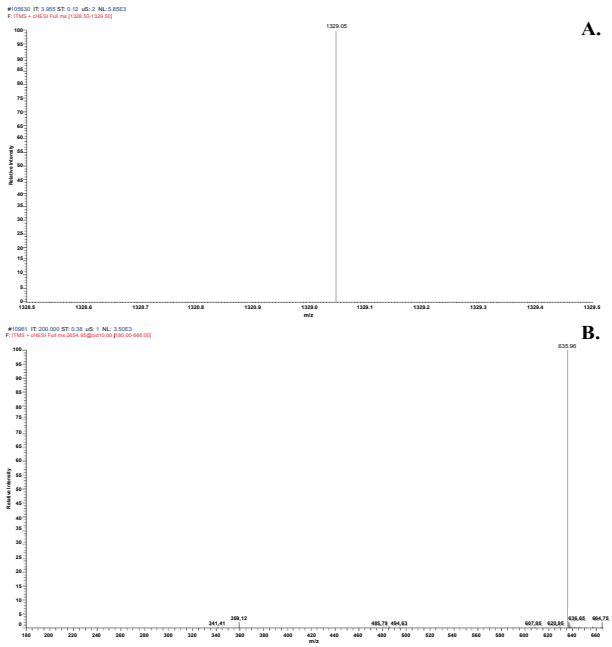

Fig. 5: Mass fragmentation pattern of the sample
A. Methylcobalamin m/z 1344; M/2, 1329; B. methylcobalamin m/z 665.78; M/2, 635.96

is very light sensitive and undergoes the homolytic cleavage of the cobalt-carbon bond. This method could be used for stability studies of methylcobalamin API and its commercial formulations.

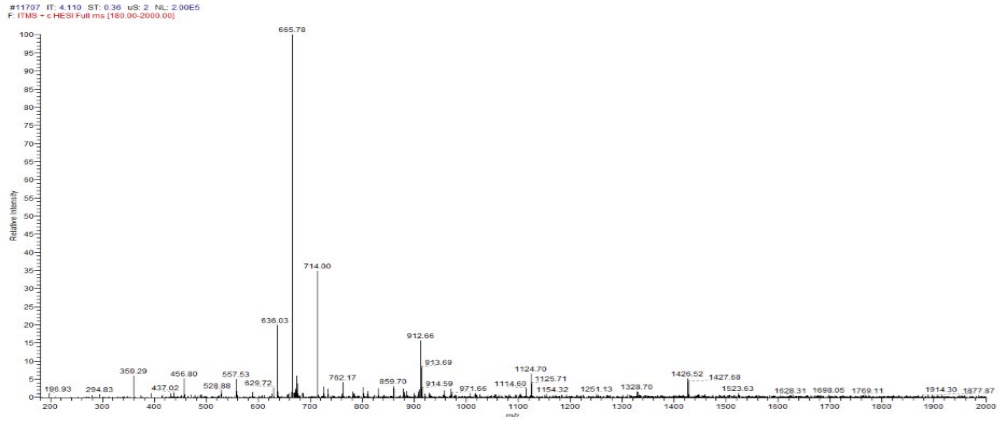

Fig. 6: Mass spectrum of the photo degraded sample exposed to light for 60 min

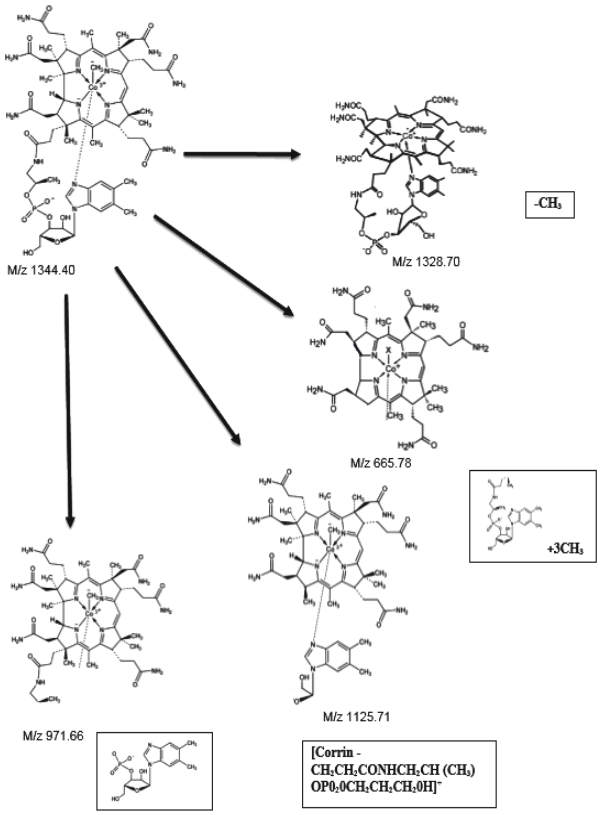

Fig. 7: Proposed fragmentation pathway of degradation products of methylcobalamin under photo degradation

TABLE 4: POSSIBLE COMPOSITIONS OF THE MAJOR COBALAMINE FRAGMENTS

m/z	Possible composition
1329	**[M + H -axial ligand]$^+$**
1286	[1329-CONH]$^+$
1270	[1329-CH_3CONH_2]$^+$ or [1329-CO]$^+$
1257	[Corrin-benz-sugar-$OPO_2OCH(CH_3)CH_2$]$^+$
1199	[Corrin-benz-sugar-OPO_2]$^+$
1183	[1329-benz]$^+$
1126	**[Corrin-$CH_2CH_2CONHCH_2CH(CH_3)$ $OPO_2OCH_2CH_2CH_2OH$]$^+$**
1069	[Corrin-CH_2CH_2 $CONHCH_2CH(CH_3)0PO_2OH$+ H]$^+$
1051	[1069-H_2O]$^+$
989	[Corrin - $CH_2CH_2CONHCH_2CH(CH_3)OH$]$^+$
971	**[989-H_2O]$^+$**
957	[Corrin-$CH_2CH_2CONHCHCH_2$]$^+$
914	[Corrin-CH_2HCO]$^+$
841	[Corrin-H_2O]$^+$
801	[Corrin+H-CH_3CONH_2]$^+$ or [Corrin+H-CO]$^+$

Benz = dimethylbenzimidazol

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Conflict of interest:

None.

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