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ISOLATION OF 50-CARBOXYCYANOCOBALAMIN AND 32-CARBOXYCYANOCOBALAMIN IMPURITIES FROM CYANOCOBALAMIN DRUG SUBSTANCE BY PREPARATIVE HPLC TECHNIQUE AND THEIR CHARACTERIZATION USING DIFFERENT SPECTROSCOPIC TECHNIQUES

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Abstract:

Cyanocobalamin is the synthetic form of Vitamin B12. It helps to prevent deficiency of B12 in the body. Vitamin B12 plays an important role in the formation of red blood cells and also helps in absorption of iron in the body. A cost effective and high-throughput simple preparative LC method was developed with a runtime of 35 min for the isolation of the two process impurities 50-Carboxycyanocobalamin and 32-Carboxycyanocobalamin of cyanocobalamin drug substance by preparative LC. Since the impurity level was low in the sample, after trying several days, failed to enrich the impurity level by synthetic route. A thorough study has been undertaken to develop a preparative LC method to isolate and characterize these impurities by chromatographic and spectroscopic methods. The crude sample contains low level of the impurities, about 36% and 12% were isolated by preparative HPLC with a purity greater than 95%.

***Keywords**: Preparative LC (PREP), High performance liquid chromatography (HPLC), USP monograph spectroscopy; Isolation; Identification and Characterization*

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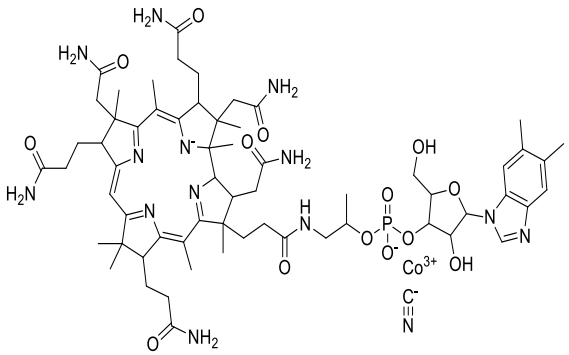
INTRODUCTION:

Cyanocobalamin is a form of vitamin B12. Vitamin B12 is important for growth, cell reproduction, blood formation, and protein and tissue synthesis. Cyanocobalamin is used to treat vitamin B12 deficiency in people with pernicious anemia or other conditions such as folic acid deficiency, pregnancy, thyroid problems, stomach and intestinal disorders, bleeding, liver or kidney disease, parasite infection, or cancer.

Cyanocobalamin drugs substance USP monograph is available for analysis. But two impurities (50-Carboxycyanocobalamin and 32-Carboxycyanocobalamin) mentioned in USP monograph are not available in market. So to prepare Reference standard of these impurities, it was decided to synthesize them in house, purify them and finally characterize by different spectroscopic techniques to prepare Reference standards.

As these impurities are photo degradation products of Cyanocobalamin API. So Cyanocobalamin API was degraded to enrich concentration of these impurities and this degraded solution of Cyanocobalamin API is termed as Crude sample. Retention time and percentage of these impurities are verified by using USP Monograph related substance method. **Refer figure 1 for crude sample chromatogram as per USP monograph.**

Chemical structure of Cyanocobalamin



B12 is the most chemically complex of all the vitamins. The structure of B12 is based on a corrin ring, which is similar to the porphyrin ring found in heme, chlorophyll, and cytochrome. The central metal ion is Co (cobalt). Four of the six coordination sites are provided by the corrin ring, and a fifth by a dimethylbenzimidazole group. The sixth coordination site, the center of reactivity, is variable, being a cyano group (-CN), a hydroxyl group (-OH), a methyl group (-CH3) or a 5'-deoxy adenosyl group (here the C5' atom of the deoxyribose forms the covalent bond with Co), respectively, to yield the four B12 forms mentioned above. The covalent C-Co bond is the only carbon-metal bond known in biology.

METHODOLOGY DEVELELOPED AND MATERIALS USED:

Chemicals and Reagents

Name of Material	Batch No.
Formic acid	Emparta ACS grade
Methanol	Merck HPLC grade
HPLC grade water	Milli-Q water

INSTRUMENTATION FOR METHOD DEVELOPMENT

HPLC, A Waters HPLC System e2695 separation module with 2489 UV/Visible detector with Empower3 software was utilized for this work.

Chromatographic conditions for HPLC: Mobile phase-A: 0.1% Formic Acid, Mobile Phase-B: Acetonitrile, column; C18, 240x4.6 mm,5µm, column oven temperature was maintained at 35°C, flow rate 1.0ml/min, diluent: mixture of water and methanol in the ratio of 75:25, gradient program T/% of B: 0.0/15, 20/15, 21/60, 25/60, 25.5/15, 35/15 has been used for the analysis of the sample. The data was collected and processed using waters empower3 software.

Preparation sample solution:

The crude sample was synthesized in house by Mankind Research Centre. Crude sample contains low level of the impurities, about 36% and 12% (Refer figure xxxx) diluted to 1000ppm by diluent Methanol: Water (75:25) was used for development of HPLC method. **Refer figure 2 for chromatogram of method developed on HPLC for crude sample.** Desired two process impurities 50-Carboxycyanocobalamin and 32-Carboxycyanocobalamin retention time are observed at 8.8 and 6.0 respectively.

INSTRUMENTATION FOR IMPURITY SEPARATION AND COLLECTION

PREPARATIVE LC: A Shimadzu preparative system PC-20AP, photodiode array detector, auto liquid sampler handling system SIL-10AP fitted with 5ml loop and FRC-10A preparative fraction collector has been used. The data was collected and processed using shimadzu LC solution software.

Chromatographic conditions for Preparative LC: Mobile phase-A: 0.1% Formic Acid, Mobile Phase-B: Acetonitrile, column; C18, 240x21.2 mm,5µm,

column oven temperature was maintained at 35°C, flow rate 1.0ml/min, diluent: mixture of water and methanol in the ratio of 75:25, gradient program T/% of B: 0.0/15, 20/15, 21/60, 25/60, 25.5/15, 35/15 has been used for the analysis of the sample. The data was collected and processed using waters empower3 software.

The crude sample contains low level of the impurities, about 36% and 12% diluted to 40mg/ml by diluent Methanol: Water (75:25) was used for collection of impurities. **Refer figure 3 for chromatogram of Preparative LC for crude sample.**

Desired two process impurities 50-Carboxycyanocobalamin and 32-Carboxycyanocobalamin retention time are observed at 12.5 and 10.2 respectively.

The collected fractions were analyzed by the related substances HPLC method to check its purity. After checking the purity, the purified fractions were further lyophilized with the help of lyophilizer to get the solid impurity. Further the purity of the pure sample was confirmed by analyzing the sample in the related substances method by HPLC. Refer figure 4 and 5 respectively

Structure elucidation:

UV-VIS Spectroscopy: The UV spectrum of 50-Carboxycyanocobalamin and 32-Carboxycyanocobalamin has been recorded by PerkinElmer Lambda 365 UV-Vis spectrophotometer. The spectrum was recorded in a quartz cell of 1 cm path length.

Result: 50-Carboxycyanocobalamin

Solvent	Concentration (ppm)	λ_{max} (nm)	Absorbance (AU)
Water	~10.0	361.37	0.1725
		278.20	0.07802

Result: 32-Carboxycyanocobalamin

Solvent	Concentration (ppm)	λ_{max} (nm)	Absorbance (AU)
Water	~10.0	360.62	0.1771
		277.80	0.08673

IR Spectroscopy (FT-IR): The IR absorption spectrum of 50-carboxycyanocobalamin was recorded using a Perkin Elmer Spectrum Two FT-IR spectrophotometer. The major absorption bands and the corresponding assignments are listed below

Result: 50-Carboxycyanocobalamin

Type of vibration	**Theoretical value* (cm^{-1})**	**Observed absorption band (cm^{-1})**	**Intensity**
ν_{N-H} (amide)	3500-3100	3334.23	m
ν_{C-H} (aliphatic)	3000-2850	2977.26	m
$\nu_{C=O}$ (amide)	1680-1630	1662.23	s
$\nu_{C=C}$ (aromatic)	1600, 1475	1572.48, 1496.48	m
ν_{C-N} (amine)	1350-1240	1350.03,1212.64,	s
$\nu_{P=O}$ (phosphate ester)			
ν_{P-O} (phosphate ester)	845-725	850.57	s

Result: 32-Carboxycyanocobalamin

Type of vibration	Theoretical value* (cm^{-1})	Observed absorption band (cm^{-1})	Intensity
ν_{N-H} (amide)	3500-3100	3346.44	m
ν_{C-H} (aliphatic)	3000-2850	2984.68	m
$\nu_{C=O}$ (amide)	1680-1630	1666.46	s
$\nu_{C=C}$ (aromatic)	1600, 1475	1572.78, 1497.11	m
ν_{C-N} (amine)	1350-1240	1350.58,1213.30, 1061.31	s
$\nu_{P=O}$ (phosphate ester)			
ν_{C-O} (ether)	1300-1000		s
ν_{P-O} (phosphate ester)	845-725	850.06	s

Mass Spectrometry: The mass spectral data of 50-carboxycyanocobalamin was recorded on Waters Xevo TQS LCMS spectrometer. The ESI +ve ionization mass spectrum displayed the molecular ion at m/z = 1357.66 which corresponds to the molecular formula $C_{63}H_{87}CoN_{13}O_{15}P$.

The mass spectral data of 32-carboxycyanocobalamin was also recorded on Waters Xevo TQS LCMS spectrometer. The ESI +ve ionization mass spectrum displayed the molecular ion at m/z = 1357.67 which corresponds to the molecular formula $C_{63}H_{87}CoN_{13}O_{15}P$.

NMR Spectroscopy:

(a) ^{1}H NMR: The ^{1}H NMR spectrum has been recorded using a Bruker Ascend 400 MHz spectrometer using $CDCl_3$ as solvent and 5 mm PABBO BB probe. All chemical shift values have been reported with respect to that of the TMS peak [δ_H(TMS)= 0.00 ppm].

Result of ^{1}H NMR: 50-Carboxycyanocobalamin

Chemical shift (ppm)	Multiplicity	Number of proton(s)
0.265	s	3
0.923-0.940	m	1
1.055-1.082	m	5
1.170	s	3
1.226	s	3
1.310	s	3
1.519-1.555	m	1
1.659-1.719	m	6
1.754-1.780	m	1
1.889-1.924	m	1
2.018-2.049	m	2
2.160-2.175	m	5
2.229-2.294	m	2
2.432-2.475	m	5
2.617-2.650	m	1
2.666-2.729	m	1
3.188-3.214	m	2

3.515-3.577	m	4
3.698-3.736	m	1
3.910-3.935	m	3
4.085-4.145	m	1
4.471-4.515	m	1
4.644-4.660	m	1
5.917	s	1
6.150	br s	1
6.248	br s	1
6.455	s	1
6.522	br s	1
6.693	br s	1
6.785	br s	1
7.042	br s	2
7.139-7.188	d(^{3}J=19.6)	2
7.312-7.343	d(^{3}J=12.4)	2
7.540-7.612	m	3
7.742	br s	1

Result of ^{1}H NMR: 32-Carboxycyanocobalamin

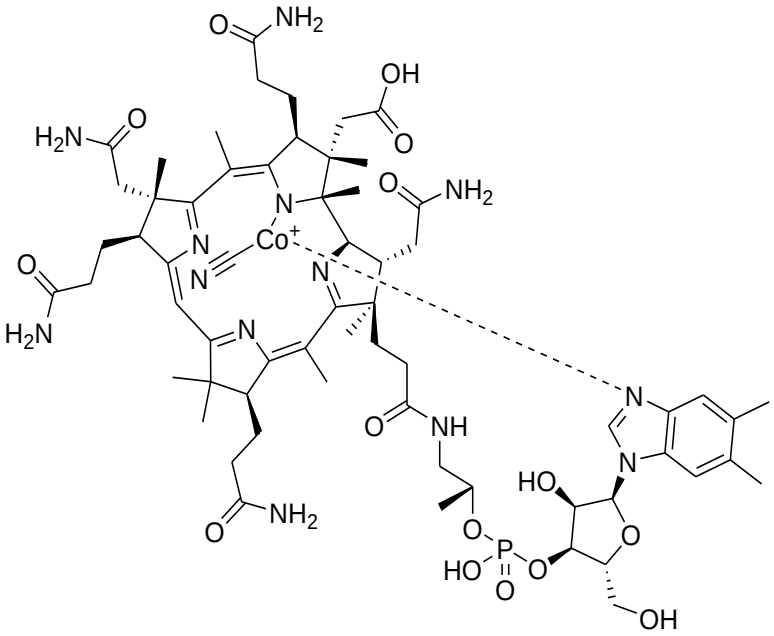
Chemical shift (ppm)	Multiplicity	Number of proton(s)
0.912-0.940	m	1
1.056-1.079	m	4
1.140-1.182	m	3
1.219-1.235	m	3
1.336	s	3
1.494-1.570	m	2
1.625-1.713	m	5
1.753-1.782	m	3
2.016-2.086	m	2
2.172-2.177	m	5
2.362-2.423	m	2
2.467-2.497	m	4+5
2.551-2.556	m	2
2.597-2.626	m	2
3.116-3.145	m	1
3.518-3.593	m	3
3.704-3.744	m	1
3.875-3.931	m	3
4.101-4.116	m	1

4.457-4.512	m	1
4.648-4.663	m	1
5.914	s	1
6.091-6.101	m	1
6.272-6.278	m	1
6.390-6.421	m	1
6.460	s	1
6.523-6.544	m	1
6.676	br,s	1
6.937	br,s	1
7.005	s	1
7.039	br,s	1
7.157-7.203	m	2
7.315	s	1
7.521-7.590	m	1+2
7.689	br,s	1

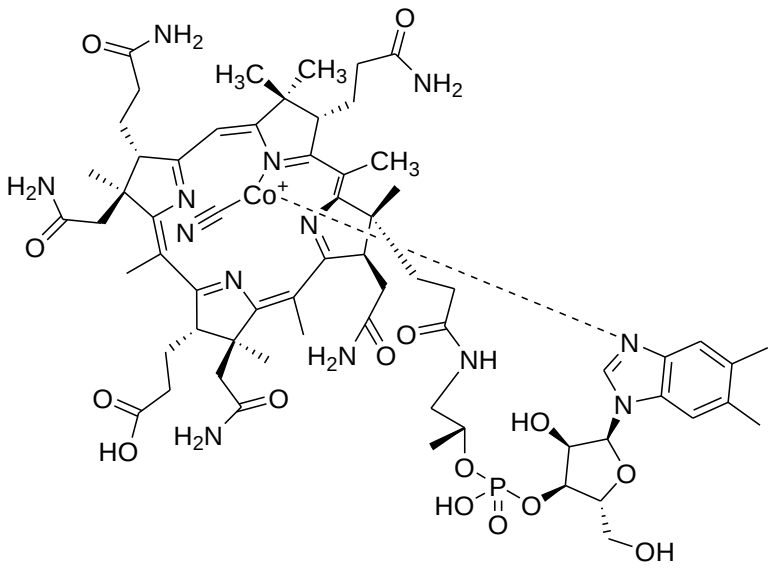
RESULTS AND DISCUSSION:

All the experimental data obtained from above analytical, spectral techniques and chromatographic retentive retention time as mentioned in the USP 40 monograph are in agreement with the proposed structure of 50-carboxycyanocobalamin and 32-carboxycyanocobalamin. The purity of both impurities as per USP monograph method is more than 95%.

50-carboxycyanocobalamin (*Co*-(cyno-kC)-dihydrogen phosphate (ester),inner salt,3'-ester with (5,6-dimethyl-1-α-D-ribofuranosyl-1*H*-benzimidazole-kN^3(9Cl) Cobinic acid-abcdg-pentamide)



32-carboxycyanocobalamin (*Co*-(cyno-kC)-dihydrogen phosphate (ester),inner salt,3'-ester with (5,6-dimethyl-1-α-D-ribofuranosyl-1*H*-benzimidazole-kN^3(9Cl) Cobinic acid-bcdeg-pentamide)



CONCLUSION:

This research paper describes novel method for the identification, isolation and structural elucidation of the process related impurity present in the Cyanocobalamin drug substance. These impurities were separated by reverse phase chromatographic technique, further purified by preparative LC. The isolated impurities were characterized by spectroscopic techniques. These impurities Reference standard were further used for analytical method validation studies. This work also supported the process development optimization Cyanocobalamin drug substance and facilitated to control the formation of this impurity during the process.

Figure 1: Crude sample HPLC chromatogram as per USP monograph

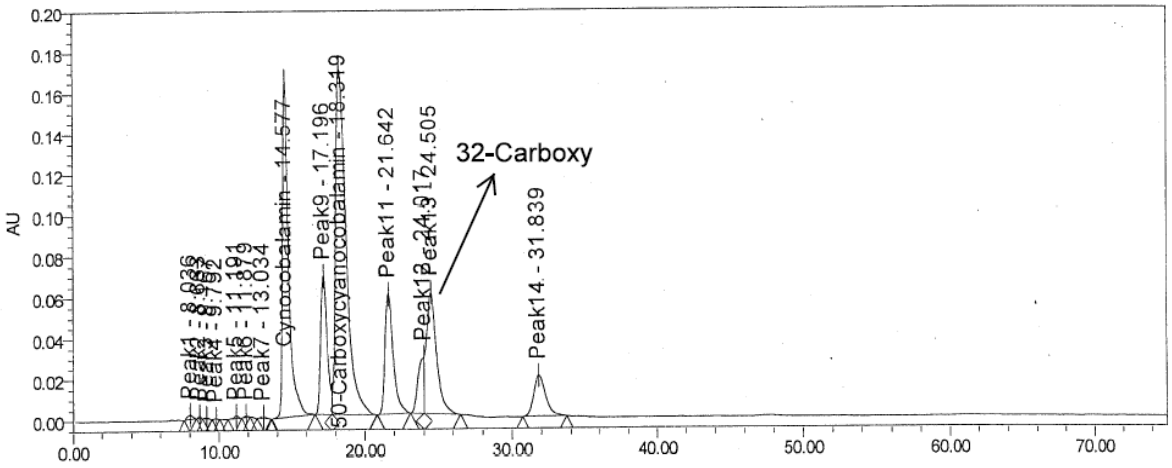


Figure 2: Chromatogram of method developed on HPLC for crude sample

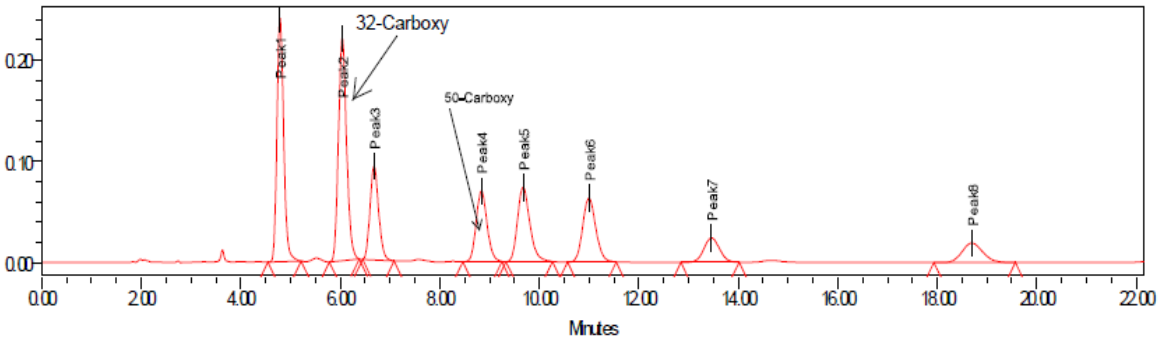


Figure 3: Chromatogram of Preparative LC for crude sample

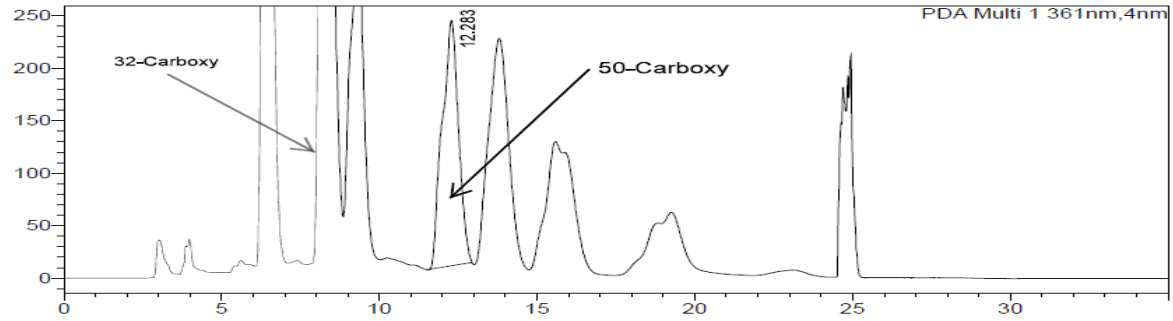
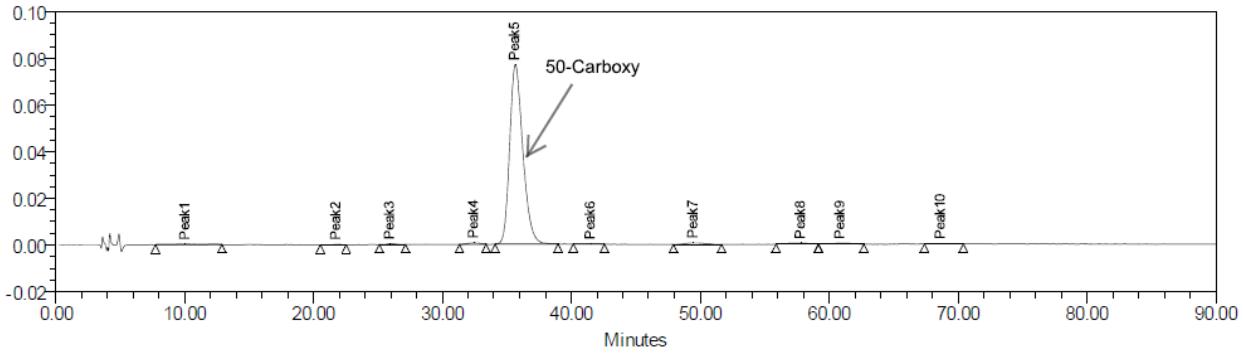


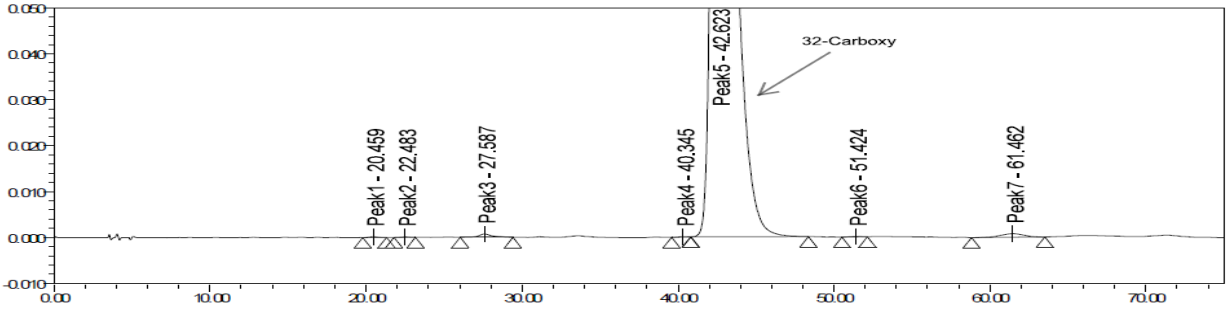
Figure 4: Purified 50-carboxycyanocobalamin HPLC chromatogram on USP monograph method



Peak Results

	Name	RT	Area	% Area	RT Ratio
1	Peak1	10.07	34173	0.59	0.28
2	Peak2	21.78	5006	0.09	0.61
3	Peak3	25.98	15939	0.28	0.73
4	Peak4	32.46	36706	0.64	0.91
5	Peak5	35.66	5476786	95.33	1.00
6	Peak6	41.52	11059	0.19	1.16
7	Peak7	49.50	59036	1.03	1.39
8	Peak8	57.83	47386	0.82	1.62
9	Peak9	60.89	40951	0.71	1.71
10	Peak10	68.70	18317	0.32	1.93

Figure 5: Purified 32-carboxycyanocobalamin HPLC chromatogram on USP monograph method



Peak Results

	Name	RT	Area	%Area	RT Ratio
1	Peak1	20.459	5048	0.03	0.48
2	Peak2	22.483	3656	0.02	0.53
3	Peak3	27.587	44057	0.24	0.65
4	Peak4	40.345	2800	0.02	0.95
5	Peak5	42.623	18036162	99.23	
6	Peak6	51.424	3769	0.02	1.21
7	Peak7	61.462	80474	0.44	1.44

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