ORIGINAL ARTICLE

DERIVATIVE SPECTROPHOTOMETRY IN THE VISIBLE REGION USING ABSORBANCE VERSUS LOG WAVELENGTH OR WAVENUMBER DETERMINATION OF CYANOCOBALAMIN IN INJECTION SOLUTIONS

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ABSTRACT

Two approaches have been adopted to increase the steepness of the slopes and the sharpness of the curvature of an absorption curve in the visible region. These are recording (i) A (1cm) versus log wavelength or (ii) A (1cm) versus wavenumber. The computer program was tested by calculating the ratios of the first derivative optima for a Gaussian band, and proved that changing wavelength into log wavelength or wavenumber is effective to squeeze an absorption curve non-linearly. Spectrophotometric identification of cyanocobalamin in the visible region was carried out using the ratios ($\Delta D_1/D_2$) calculated for λ, log λ and ν scales. The ratios were highly reproducible and independent of concentration. First, second and fourth- order derivative determination of cyanocobalamin in injection solutions was performed in the visible region at 20040.08, 17452.01, (20040.08 - 17452.01) cm^{-1} for $D_{11}(\nu)$, $D_{12}(\nu)$ ΔD_1 (D_{11} -D_{12}) (ν), respectively, and at 18018.02 cm^{-1} for $D_2(\nu)$ and $D_4(\nu)$.

Keywords: Derivative spectrophotometry, cyanocobalamin, UV-visible spectroscopy.

INTRODUCTION

In the visible region, absorption spectra are sometimes of weak slopes and curvature, a situation that hinders recording acceptable derivative curves. This may inhibit promising applications of derivative spectrophotometry in the visible region for single and multi-component analysis.

Two approaches have been adopted to increase the steepness of the slopes and the sharpness of the curvature of an absorption curve. These are: recording (i) A(1cm) versus log wavelength, or (ii) A(1 cm) versus wavenumber curves.

Recording absorbance versus log wavelength

By changing the linear wavelength scale λ, into log λ, differences (Δ log λ) decrease non-linearly and converge as λ increases. The longer the wavelength, the greater the squeeze of the absorption curves. In the ultraviolet region, it is not advantageous to change λ (nm) into log λ. However, in the visible region, changing λ into log λ results into an increase in the steepness of the slopes and the sharpness of peaks of an absorption curve.

Recording absorbance versus wavenumber

Antonov (1997) and Petrov *et al* (2000) recorded absorption spectra in the visible region as absorbance versus wavenumber. Using a suitable computer program, they were able to obtain sharper derivative curves up to the fourth order derivative. The longer the wavelength, the greater the non-linear squeeze of the absorption curve.

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The computer program

A program has been used to obtain derivative curves from an absorption curve recorded against λ, log λ or ν cm^{-1}. A curve fitting BASIC program based upon using orthogonal polynomials (Wahbi *et al.*, 1986) has been modified to be in Visual BASIC and extended to be capable of recording derivative curves from D_1 to D_4 from an absorption curve.

The program can "Read from Excel file" or "Read from Text file". The derivative data can be saved as "Save to text file" or "Save to Excel file" that can be recorded as derivative curves. The Visual BASIC program is saved on a CD ROM with an installation program.

Program testing

Using first derivative curves, derived from a Gaussian band, ratios of the first derivative optima D_{11}/D_{12} where 1 and 2 stand for first optimum and second optimum are shown in table 1. In the wavelength scale, the ratio was found to be equal to 1 due to its symmetry. In the log wavelength and wave number scales the ratios were found to be equal to 1.18 and 1.40, respectively (greater than 1). This means that changing the wavelength scale, λ(nm), into wave number, ν(cm^{-1}), is more effective to squeeze an absorption curve non-linearly than changing wavelength, λ (nm), to log wavelength, log λ scale. Moreover, the longer

the wavelength (or the lower the wavenumber) the higher will be the crowdness.

Table 1: Ratio of D_{11}/D_{12} calculated for a Gaussian band Y against λ(nm), log λ and ν(cm^{-1}).

Band	Optima	D_{11}/D_{12}
Y vs. λ	652.5 & 547.5 nm	1
Y vs. log λ	2.815 & 2.742	1.18
Y vs. ν	15.326 & 18.0995(x 10^3)cm^{-1}	1.40

Derivative spectrophotometric identification and determination of cyanocobalamin in the visible region

Cyanocobalamin and its injection formulations are determined spectrophotometrically by measuring the absorbance of a solution of cyanocobalamin in water at 361 nm (BP 2003; USP 2004).

EXPERIMENTAL

Instrument

A Perkin Elmer Lambda EZ 201 UV/VIS spectrophotometer with 1-cm quartz cuvettes. The device was connected to a Panasonic impact dot matrix printer 24 pin KX-P 3626.

Materials and reagents

All experiments were performed with analytical grade chemicals and solvents. Authentic sample of cyanocobalamin was obtained from Pharco Pharmaceuticals, Alex., Egypt. Rubivamine® ampoules (Misr Co. for Pharm. Industries, Egypt) containing 1000µg/1ml ampoule, Tri-B® ampoules (The Nile Co. for Pharm. and Chem. Industries, Cairo, Egypt) containing 1000µg/1ml ampoule and Neurobion® ampoules (Glaxo Wellcome, Egypt), each 3ml ampoule contains 100mg thiamine hydrochloride, 100mg pyridoxine hydrochloride and 1000 µg cyanocobalamin were obtained from the local market.

Standard solution

Cyanocobalamin standard solution (0.2 mg/ml) was prepared in distilled water. Aliquot volumes of 2 to 6 ml (in 1 -ml steps) of standard cyanocobalamin solution were transferred into 10 -ml calibrated flasks and diluted to volume with the same solvent to contain final concentrations of 40 to 120 µg/ml.

Procedure

The contents of ten cyanocobalamin ampoules of each product were accurately emptied in a beaker and mixed well. 5-ml aliquots of Rubivamine and Tri- B ampoules

Table 2: Ratios of difference first derivative signals to second derivative signals ($\Delta D_1/D_2$) calculated for cyanocobalamin solution in water using *f*(λ), *f*(log λ) and *f*(ν).

Concentration µg/ml	$(\Delta D_1/D_2)\ \lambda^*$	$(\Delta D_1/D_2)$ log λ^+	$(\Delta D_1/D_2)\ \nu^{++}$
40	45.540	1.429	0.035
60	45.835	1.433	0.035
80	45.543	1.427	0.035
100	45.747	1.432	0.035
120	45.757	1.433	0.035
Mean	45.685	1.431	0.035
C.V.%	0.30	0.19	0.17

D_2 log λ at 2.7443 $^+$ ΔD_1 log λ at 2.6981 & 2.7582

D_2 ν at 18018.02 cm^{-1} $^{++}$ $\Delta D_1\nu$ at 20040.08 & 17452.01 cm^{-1}

Table 3: Ratios** of D_{12}/D_{11} calculated for cyanocobalamin injection solutions using *f*(λ), *f*(log λ) and *f*(ν).

Product	$D_{12}(\lambda)/D_{11}(\lambda)^*$	$D_{12}(\log\lambda)/D_{11}(\log\lambda)^+$	$D_{12}(\nu)/D_{11}(\nu)^{++}$
Rubivamine	2.61 (+1.56)	3.01 (+1.55)	3.48 (+1.69)
Tri-B	2.62 (+1.95)	3.03 (+2.23)	3.49 (+1.99)
Neurobion	2.60 (+1.17)	3.01 (+1.55)	3.47 (+1.40)
Authentic	2.570	2.964	3.422

** Results are the mean of three determinations.

* λ_1 and λ_2 = 499 & 573 nm, $^+$ log λ_1 and log λ_2 = 2.6981 & 2.7582, $^{++}\nu_1$ & ν_2 = 20040.08 & 17452.01 cm^{-1}

Figures in parentheses are the deviation from the authentic percent.

%Deviation=[(Ratio found- Ratio authentic)/ Ratio authentic] x 100

and 10-ml aliquot of neurobion ampoules were accurately transferred into three 25-ml calibrated flasks and completed to the mark with distilled water. Aliquot volumes of 4 to 6ml (in 1-ml step) of solutions of Rubivamine and Tri- B ampoules while 6 to 9 ml (in 1.5-ml step) of solution of neurobion ampoules were transferred into 10-ml calibrated flasks and diluted to volume with distilled water to contain final concentrations of 80 to 120 µg/ml. The absorbance (zero-order), first, second and fourth order derivative curves of the solutions

Table 4: Ratios**of difference first order derivative signals to second order derivative signals ($\Delta D_1/D_2$) calculated for cyanocobalamin injection solutions using *f*(λ), *f*(log λ) and *f*(ν).

Product[x]	($\Delta D_1/D_2$) λ*	(ΔD_1/ D_2) log λ[+]	($\Delta D_1/D_2$) ν[++]
Rubivamine	46.07 (+0.85)	0.0353 (+0.86)	1.445 (+0.98)
Tri-B	45.70 (+0.04)	0.0351 (+0.29)	1.433 (+0.14)
Neurobion	45.55 (-0.28)	0.0350 (0.00)	1.430 (-0.70)
Authentic	45.68	0.0350	1.431

**Results are the mean of three determinations.
D_2 λ at 555 nm *ΔD_1 λ at 499 & 573 nm
D_2 log λ at 2.7443 [+] ΔD_1 log λ at 2.6981 & 2.7582
D_2 ν at 18018.0 cm^{-1} [++] ΔD_1ν at 20040.08 & 17452.01 cm
Figures in parentheses are the deviation from the authentic percent.
%Deviation= [(Ratio found- Ratio authentic)/ Ratio authentic] x 100

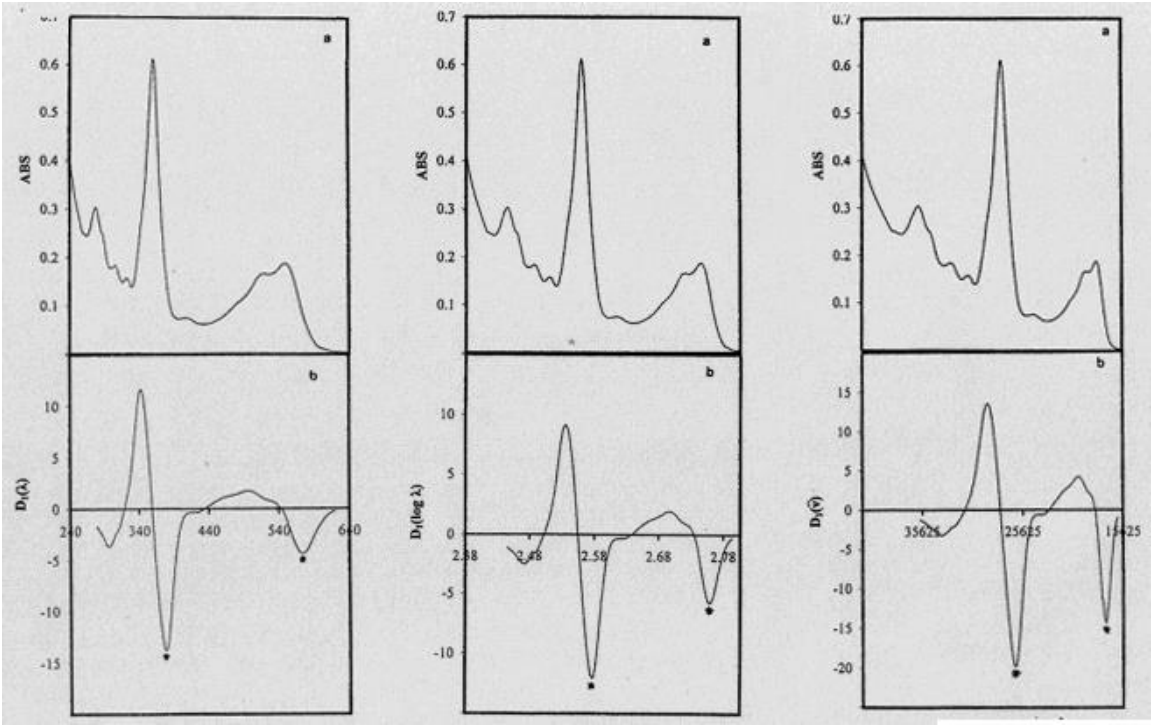
Table 5: Regression analysis of $D_{11}(\nu)$, $D_{12}(\nu)$, $\Delta D_1(\nu)$, $D_2(\nu)$ and $D_4(\nu)$ at 20040.08, 17452.01, (20040.08-17452.01) and 18018.02 cm^{-1}, respectively, to concentration of Cyanocobalamin solutions in water

	$D_{11}(\nu)$ cm^{-1}	$D_{12}(\nu)$ cm^{-1}	$\Delta D_1(\nu)$ cm^{-1}	$D_2(\nu)$ cm^{-1}	$D_4(\nu)$ cm^{-1}
Linearity range (µg/ml)	40-120	40-120	40-120	40-120	40-120
R	0.99997	0.99999	0.999999	0.99999	0.9997
A	8.25E-07	-1.30E-08	8.12E-07	0.78E-10	3.17E-15
B	1.03E-06	3.58E-06	4.62E-06	3.22E-09	4.36E-15
S_a	3.15E-07	4.78E-07	2.67E-07	0.58E-10	4.28E-15
S_b	4.05E-09	6.14E-09	3.42E-09	7.50E-12	5.49E-17
LOD (µg/ml)	10.09	4.41	1.91	0.60	3.24
LOQ (µg/ml)	3.058	1.34	5.78	1.81	9.84

Table 6: Determination of cyanocobalamin injection formulations by different methods using *f*(ν) and paired comparison method[(6)]

% Found per ampoule						
Method						
Product	$D_{11}(\nu)$	$D_{12}(\nu)$	$\Delta D_1(\nu)$	$D_2(\nu)$	$D_4(\nu)$	B.P. 2003
Rubi-vamine	108.3	109.5	109.2	109.0	108.1	110.3
Tri-B	103.3	104.8	104.5	105.0	104.5	106.0
Neurobion	122.1	123.5	123.2	123.0	122.0	123.6
Mean of difference*	-2.1	-0.7	-1.0	-1.0	-1.8	
S.E. = S.D/ $\sqrt{3}$	0.35	0.32	0.32	0.20	0.22	
Confidence limits[+]	±1.5	±1.4	±1.4	±0.9	±0.9	

*****Difference between percent found of each method and the spectrophotometric reference method for each product.
[+] Confidence limit = S.E. x $t_{0..95}$



Wavelength (nm) (A) | Log wavelength (B) | Wavenumber (cm^{-1}) (C)

Fig. 1: For a 30µg/ml solution of cyanocobalamin in water, (A) absorption (a) and first derivative (b) curves as a function of wavelength, (B) absorption (a) and first derivative (b) curves as a function of log wavelength , (C) absorption (a) and first derivative (b) curves as a function of wavenumber.

were recorded for products solutions and standard solutions at 2-nm intervals over the wavelength range 450 to 600nm against distilled water as blank at scan speed 100 nm/min.

RESULTS AND DISCUSSION

The sample of cyanocobalamin used was conforming to specifications of the B.P. 2004 with regard to position of wavelength maxima and absorbance ratios. The ratio of $A_{361\ nm}/A_{550\ nm}$ was found to be 3.275 (B.P. range 3.15-3.45) for six independent concentrations with a coefficient of variation of 0.71%.

The instrumentally recorded D_1, D_2 and D_4 curves at sensitivity =6, and the computed curves obtained using the present program were found to be superimposable with regard to position of optima (± 1 nm difference) and general shape over the wavelength range 240-640 nm. This proves that the present program gave derivative curves superimposable to instrumentally derived curves.

Absorption characteristics of cyanocobalamin as a function of wavelength, log wavelength and wavenumber and the computed derivative curves

The absorption curve of 30 µg/ml cyanocobalamin in water was plotted against wavelength λ(nm), log wavelength (log λ) and wavenumber, ν (cm^{-1}) over the ultraviolet-visible region The maxima were found to occur at 267, 361 and 550 nm and at the corresponding log λ and ν cm^{-1} scales (Figs. (1-3) Aa, Ba & Ca). The D_1, D_2 and D_4 curves were computed from the respective absorption curves as a function of λ(nm), log λ and ν cm^{-1} (figs. 1-3; Ab, 1Bb & 1Cb).

First-order D_1 derivative curves

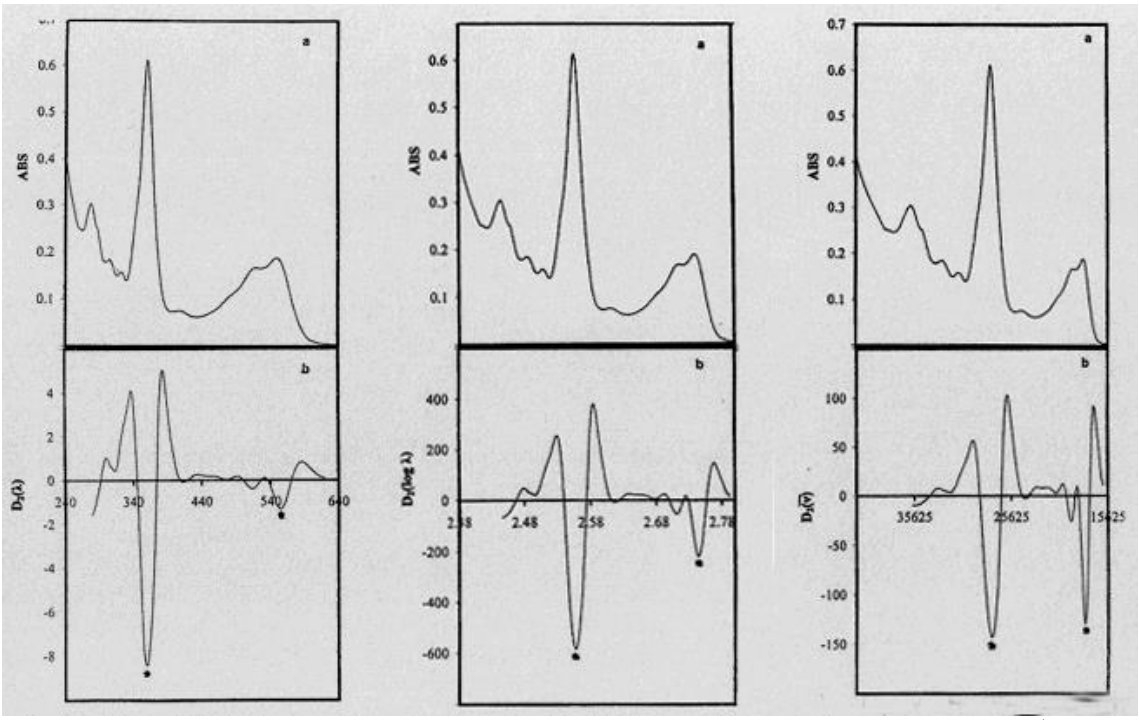
The prominent optima and sign of the D_1 curve were found to occur at 343 (+ve), 377 (-ve), 499 (+ve) and 573 (-ve) nm and at their corresponding log λ andν cm^{-1} curves (figs. 1Ab, Bb & Cb).

Second-order D_2 derivative curves

The prominent optima and sign of the D_2 curve were found to occur at 361 (-ve), 383 (+ve) and 555 (-ve) nm and at their corresponding log λ and ν cm^{-1} scales (figs. 2Ab, Bb & Cb).

Fourth-order D_4 derivative curves

The prominent optima and sign of the D_4 curves were found to occur at 361 (+ve), 375 (-ve), 535 (-ve) and 555 (+ve) nm and at their corresponding log λ and ν cm^{-1} scales (figs. 3Ab, Bb & Cb).



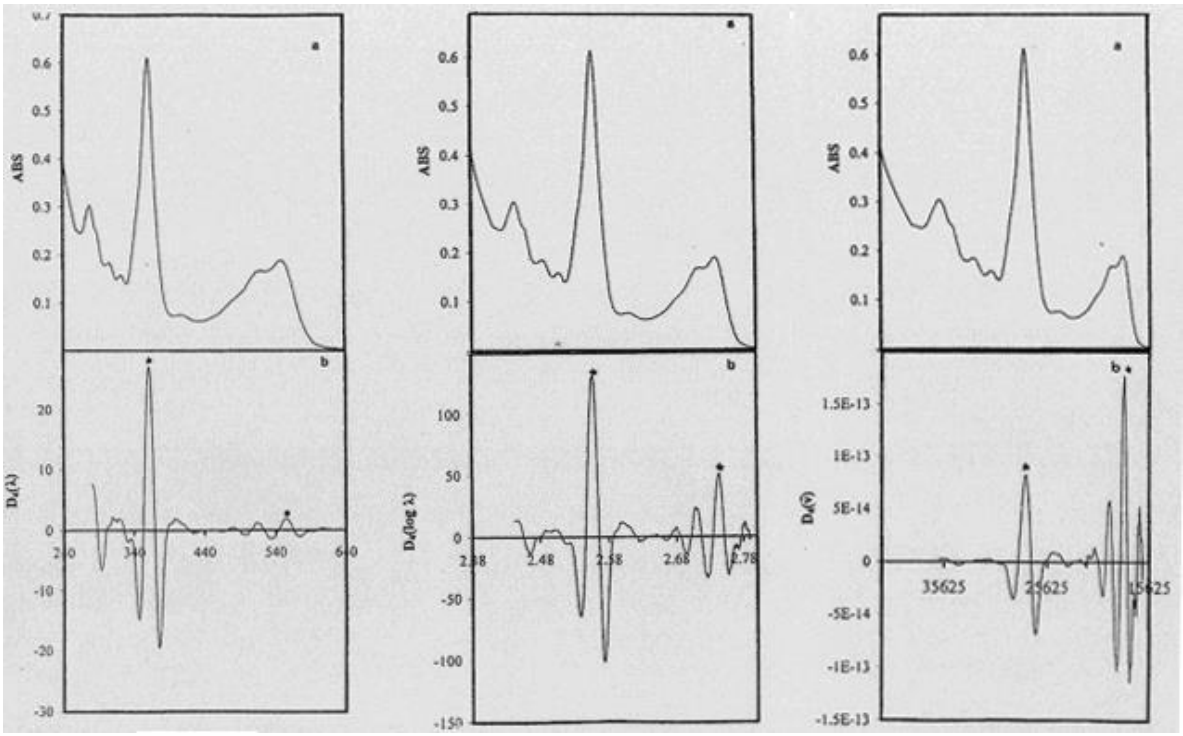
(A) (B) (C)

Fig. 2: For a 30µg/ml solution of cyanocobalamin in water, (A) absorption (a) and second derivative (b) curves as a function of wavelength, (B) absorption (a) and second derivative (b) curves as a function of log wavelength, (C) absorption (a) and second derivative (b) curves as a function of wavenumber.

Ratios of derivative signals

For each function, ratios of D_{12}/D_{11} (Wahbi *et al.*, 1986), have been calculated where subscripts 1 and 2 stand for 377 nm and 573 nm for λ, and 2.5763, 2.7582 for log λ and 26525.20 and 17452.01 cm^{-1} for ν, respectively. The mean ratios (n=5) were found to be 0.318, for wavelength scale, 0.481 for log wavelength scale, and 0.725 for wavenumber scale. The coefficients of variation were found to be 0.14, 0.17, and 0.20, respectively, indicating high reproducibility. These results means that by changing the wavelength scale (nm) into log λ the intensity of the peak in the visible region at 573 nm increased (fig. 1). Ratio of the two ratios (0.481/0.318& 0.725/0.318 for log λ and ν, respectively); which is a criterion of this phenomenon was found to be 1.5 and 2.3 (for log λ and ν, respectively). This indicates that the intensity of the peak, and accordingly sensitivity, increased 1.5 times in the log λ scale and 2.3 times in the ν (cm^{-1}) scale more than the corresponding peak in the wavelength scale curve. Ratios of D_{22}/D_{21} for the different functions have been calculated where subscripts 1 and 2 stand for 361nm and 555 nm for λ, 2.5575 and 2.7443 for log λ, and 27700.83 cm^{-1} and 18018.02 cm^{-1} for ν, respectively. The mean (n=5) of the calculated ratios were found to be 0.158, for wavelength scale, 0.378 for log wavelength scale, and 0.906 for wavenumber scale. The coefficients of variation were found to be 0.28 for the three ratios. Ratios were found to be highly reproducible and independent of concentration. These results means that by changing the wavelength scale into log λ the intensity of the peak in the visible region (at 555 nm) increased (fig. 2). Ratios of the two ratios (0.378/0.158 & 0.906/0.158 for log λ and ν scales, respectively) were found to be 2.4 and 5.7 (for log λ and ν scales, respectively). This indicates that the intensity of the peak, and hence the sensitivity, increased 2.4 times in the log λ scale and 5.7 times in the ν scale more than the corresponding peak in the wavelength scale curve.

Ratios of D_{42}/D_{41}, have been calculated where subscripts 1 and 2 stand for 361 nm and 555 nm for λ, 2.5575 and 2.7443 for log λ, and 27700.83 cm^{-1} and 18018.02 cm^{-1} for ν, respectively. The mean (n=5) of the calculated ratios were found to be 0.072 for wavelength scale, 0.390 for log wavelength scale, and 2.121 for wavenumber scale. The coefficients of variation were found to be 1.35, 1.02, and 1.10, respectively. Ratios were found to be reasonably reproducible and independent of concentration. By changing the wavelength scale (nm) into log λ the peak in the visible region (at 555 nm) increased in signal and sharpness (fig. 3). Ratios of the two ratios (0.39/0.072 and 2.121/0.072 for log λ and ν scales, respectively), were found to be 5.42 and 29.46 (for



(A) (B) (C)

Fig. 3: For a 30µg/ml solution of cyanocobalamin in water, (A) absorption (a) and fourth derivative (b) curves as a function of wavelength, (B) absorption (a) and fourth derivative (b) curves as a function of log wavelength, (C) absorption (a) and fourth derivative (b) curves as a function of wavenumber.

log λ and ν scales, respectively). In other words the signal in the visible region, and hence the sensitivity, increased 5.42 times in the log λ scale and 29.46 times in the ν scale more than the corresponding peak in the wavelength scale.

Derivative spectrophotometric identification of cyanocobalamin in the visible region

The derivative ratios, D_{ji}/D_{ki} (where $j \neq k$) calculated at optima at the same λ_i, D_{j1}/D_{j2} (where 1 and 2 refer to different wavelengths i.e. λ_1 and λ_2 (optima); and D_{j1}-D_{j2}/D_{k3} (where $j \neq k$ and 3 stands for λ_3 wavelengths optimum for D_k are constants. Their values are highly characteristic for a particular compound and can be used for its identification. Position of the derivative optima and the derivative ratios can be used for the identification of cyanocobalamin in pharmaceutical preparations in the visible region. These ratios are applicable to absorption curves expressed as a function of λ, (log λ) and (ν).

Ratio of difference first order derivative signals to second order derivative signals

For each function, ratios of $\Delta D_1/D_2$ have been calculated where ΔD_1 stands for (D_1 at 499 nm –D_1 at 573 nm) for λ, (D_1 at 2.6981- D_1 at 2.7582) for log λ and (D_1 at 20040.08 - D_1 at 17452.01 cm^{-1}) for ν, respectively. While D_2 stands for D_2 at 555 nm (for λ), at 2.7443 (for log λ) and at 18018.02 cm^{-1} (for ν), respectively. The difference derivative ratios ($\Delta D_1/D_2$) for different functions (table 2) were found to be highly reproducible and independent of concentration. The ratios are highly characteristic for cyanocobalamin in water at the specified settings.

Derivative spectrophotometric identification of cyanocobalamin in injection formulations in the visible region applied to A(ν) cm^{-1} spectra

For the identification of cyanocobalamin in the visible region, the products have been suitably diluted for absorbance measurements. Ratios of D_{12}/D_{11} and $(\Delta D_1)/D_2$, where $\Delta D_1 = D_{11}$-D_{12}, have been calculated for the different solutions of the products. Results obtained are shown in tables 3 and 4. For comparison, the mean of the authentic ratios are enclosed in each case.

Ratio of first derivative optima (table 3) for the different functions λ, log λ and ν cm^{-1} of the samples were found to be +1.17% to +2.23% higher than the authentic ratios which could be due to sample differences and instrumental reasons. Difference derivative ΔD_1 has been calculated which eliminates any constant interference. The D_2 signal, of course, corrects for constant and linear interferences. Thus, the ratio $\Delta D_1/D_2$ of the different functions calculated for the samples were found in good agreement to authentic values. Accordingly, $\Delta D_1/D_2$ of the different functions should be considered the ideal

identification test for cyanocobalamin in different pharmaceutical products in the visible region. Furthermore; position of first order derivative optima for injection solutions were found to coincide exactly with those of standard solution of cyanocobalamin in water. These findings added an important criterion for identification.

Derivative spectrophotometric determination of cyanocobalamin in injection formulations using derivative curves applied to A(ν) curves in the visible region

Derivative spectrophotometry as a function of wave-number, ν, in the visible region proved to be more sensitive. The signals obtained for first, second and fourth order derivative curves are much prominent in the ν scale, than the respective signals in the λ scale. Accordingly, spectrophotometric determination using derivative curves has been carried out in the ν scale.

VALIDATION

Specificity

Specificity has been confirmed by calculating the ratio $\Delta D_1/D_2$ (ν) (table 4).

Linearity

D_{11}(ν) at 20040.08 cm^{-1} , D_{12}(ν) at 17452.01 cm^{-1} and ΔD, (D_{11} -D_{12}) (ν) , D_2(ν) and D_4(ν) at 18018.02 cm^{-1} for cyanocobalamin solutions in water were found to be linearly related to concentration over the concentration range 40 to 120 µg/ml . The statistical analysis of the linearity proves that the derivative values are highly satisfactory to be applied for quantitative determination of cyanocobalamin in injection solutions (table 5). In all cases the regression coefficient, r, was found to be higher than 0.999.

Precision

Three different concentrations of cyanocobalamin 40, 60 and 120 µg/ml have been prepared. Each solution was measured three times to obtain nine D_{11}(1%, 1cm), D_{12}(1%, 1cm) and ΔD_1(1%, 1cm) D_2(1%, 1cm) and D_4(1%, 1cm). The coefficients of variation were found to be 1.333, 0.419 0.259, 0.82 and 3.27 for D_{11}, D_{12}, ΔD_1, D_2 and D_4, respectively.

The average D_{11} (1%, 1cm), D_{12} (1%, 1cm) and ΔD1 (1%, 1cm) were found to be 0.0105, 0.0358 and 0.0463, respectively. These results prove that D_1, D_2 methods are highly reproducible. On the other hand, D_4(1%, 1cm) proved to show relatively low reproducibility (C.V.% = 3.27%). It is noteworthy to mention that smoothness of the absorption curve before calculating the D_4(1%, 1cm) will certainly lead to improve the reproducible coefficient.

Limit of detection and limit of quantitation

Table 5 shows that the limits of detection were found to be 10.09, 4.41, 1.91, 0.60 and 3.24 µg/ml for D_{11}, D_{12}, ΔD_1, D_2 and D_4, respectively. Meanwhile, limits of quantitation were found to be 30.58, 13.35, 5.78, 1.81 and 9.84 µg/ml for D_{11}, D_{12}, ΔD_1, D_2 and D_4, respectively.

Assay of cyanocobalamin injection solutions

Table 6 shows the collective results for the determination of cyanocobalamin in the injection solutions. Paired comparison (Davies and Goldsmith, 1972) has been applied between the derivative methods and the B.P. method. It was found that D_{11}(ν), D_{12}(ν), ΔD_1(ν), D_2(ν) and D_4(ν) methods gave results within -2.1±1.5%, -0.7±1.4%, -1.0±1.4%, -1.0±0.9% and -1.8±0.9% from the results obtained using the B.P. 2003[5] method for each product. Derivative spectra in the visible region using A versus ν (cm^{-1}) proved to be successful in analyzing cyanocobalamin and are more sensitive than using A versus λ (nm).

CONCLUSION

Derivative spectrophotometry in the visible region would be more prominent when absorption curves are recorded against wavenumber rather than wavelength.

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