Multicomponent spectrometric assay of cyanocobalamin and its photoproduct hydroxocobalamin in the presence of ascorbic acid in photolyzed solutions

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Abstract: The simultaneous determination of cyanocobalamin (CC), hydroxocobalamin (HC) and ascorbic acid (AA) in aqueous solution has been achieved by a multicomponent spectrometric method. CC undergoes photolysis in acidic and alkaline media to form HC and the reaction is enhanced in the presence of AA. The method has been used to evaluate the kinetics of photodegradation reactions of the vitamin. CC, HC and AA present in the photolyzed solutions have been determined by absorbance measurement at 550, 525 and 265 nm at pH 4.0. These wavelengths correspond to the absorption maxima of the three substances and thus provide high specificity and sensitivity to the method. The method has been validated with respect to various parameters relating to the analytical performance characteristics. The recovery of the method for the three compounds ranges from 97.1-103.0% with a RSD value of ±3%. The accuracy of the method is shown by the linearity of the kinetic plots in the concentration range studied. The method is simple, rapid and convenient for the proposed work.

Keywords: Cyanocobalamin, hydroxocobalamin, ascorbic acid, multicomponent spectrometric assay, vitamin mixture.

INTRODUCTION

Ultraviolet and visible absorption spectrometry is one of the most useful techniques for the quantitative analysis of pharmaceutical compounds. Several examples of single-component spectrometric assay of vitamins such as cyanocobalamin, hydroxocobalamin, riboflavin, folic acid and vitamin A and other compounds are included in the British Pharmacopoeia (2013). In the case of mixtures of compounds with overlapping absorption spectra, the total absorbance of a solution at a given wavelength is the sum of the absorbances of the individual components in the solution. It is, therefore, possible to analyze the components of the mixture by absorbance measurements at two or three carefully selected wavelengths, preferably corresponding to the absorption maxima of the components to achieve high sensitivity, specificity, reproducibility and accuracy.

The method of a two-component spectrometric assay of mixtures of compounds is described by Christian (2005) and Skoog *et al*. (2004). It has been applied to the assay of cyanocobalamin (CC) and hydroxocobalamin (HC) (Ahmad and Hussain, 1993a, 1993b; Ahmad *et al*., 1992, 2003), formylmethylflavin and lumichrome (Ahmad and Fasihullah, 1991; Ahmad and Vaid, 2008), benzoic acid and salicylic acid (Ahmad and Vaid, 2009), salicylamide and paracetamol (Alkhami and Sarlak, 2005), acetylsalicylic acid and caffeine (Bharate and Bharate, 2010), paracetamol and caffeine (Vichare *et al*., 2010), paracetamol and meloxicam (Khan *et al*., 2010) and aspirin and paracetamol (Murtaza *et al*., 2011).

In several drug degradation studies, three-component spectrometric methods have been developed to evaluate the kinetics of the reaction. These methods include the assay of formylmethylflavin and its hydrolytic and photoproducts, lumichrome and lumiflavin (Ahmad *et al*., 1980, 2006a; Heelis *et al.*, 1980; Ahmad and Rapson, 1990), riboflavin and its thermal degradation products, β-keto acid and flavo-violet (Ahmad *et al*., 1973), riboflavin and its photoreduction products, formylmethylflavin, lumichrome and lumiflavin (Ahmad and Rapson, 1990; Ahmad *et al*., 2004a, 2008, 2009), and riboflavin and its photoreduction and photoaddition products, formylmethylflavin, lumichrome, lumiflavin and cyclodehydroriboflavin (2004b, 2005, 2006b, 2010), and sulphacetamide and its photoproducts, sulphanilamide and azobenzene-4,4'-disulphonamide (Ahmad and Ahmad, 1990).

There are only few other examples of three-component spectrometric assays including those of aspirin, paracetamol and caffeine (Sharma *et al*., 1990), B-vitamins (Ghasemi and Abbasi, 2005) and complex chemical mixtures (Monakhova *et al*., 2010). The object of the present work is the development and validation of a simple three-component spectrometric method to investigate the photodegradation of CC in the presence of its photoproduct, HC, and ascorbic acid (AA). AA is

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known to enhance the photolysis of CC (DeRitter, 1982) and the study requires the assay of all the three components to evaluate the kinetics of the reaction. This is necessary to account for the presence of HC and AA in the assay of CC in photolyzed solutions. The proposed method can be used for the stability analysis and shelf-life determination of CC in stored samples.

MATERIAL AND METHODS

Cyanocobalamin (CC), hydroxocobalamin (HC) and ascorbic acid (AA) (Eur. Phr.) were obtained from Fluka (Switzerland) and the purity of these vitamins was confirmed by TLC. All solvents and reagents were analytical grade or of the purest form available from BDH/Merck. The buffer systems employed were: KCl-HCl, pH 2.0; citric acid-Na_2HPO_4, pH 2.5-8.0; citric acid-Na_2HPO_4-H_3BO_3, pH 8.5-12.0 (ionic strength 0.05 M).

Photolysis of cyanocobalamin solutions

A series of $5x10^{-5}$ M aqueous solutions of CC (100 ml) was prepared at pH 2.0-12.0 in Pyrex flasks and different amounts of A were added to each solution to obtain concentrations in the range of 1.0-5.0 $x10^{-4}$ M. The flasks were irradiated with a Philips HPLN 125W high-pressure mercury-vapor fluorescent lamp (emission in the visible region) fixed at a distance of 30 cm from the centre of the flasks. Samples were withdrawn at various intervals for chromatographic examination and spectrometric determination. Control solutions were placed in the dark and determined for CC and AA content on the completion of the reaction.

TLC of photolyzed solutions

a) Detection of CC and its photoproduct, HC (Ahmad *et al*., 1990).

TLC of the photolyzed solutions of CC was performed on 250-µm silica gel GF254 plates (Merck) using the solvent systems: A, 1-butanol-acetic acid-0.006 M potassium dihydrogen phosphate-methanol (36:18:36:10, v/v/v/v) (Cima and Mantovan, 1962); and B, methanol-water (95:5, v/v) (Covello and Schettino, 1964). The detection of spots was carried out visually (red color) or under UV light.

b) Detection of AA and its photoproducts; dehydroascorbic acid and 2, 3-diketogulonic acid (Ahmad *et al*., 2011, 2012).

The photolyzed solutions were subjected to TLC using 250-µm silica gel GF254 plates (Merck) and the solvent systems: C, acetic acid-acetone-methanol-benzene (5:5:20:70, v/v/v/v) (Ganshirt and Malzacher, 1960); D, ethanol-10% acetic acid (90:10, v/v); and E, acetonitrile-butyl nitrile-water (65:33:2,v/v) (Saari *et al*., 1967). The spots were located under UV light (254 nm) for dehydroascorbic acid or by spraying with a 3% aqueous phenylhydrazine hydrochloride solution for dehydroascorbic acid and 2,3-diketogulonic acid.

Assay of cyanocobalamin, hydroxocobalamin and ascorbic acid

5 ml of the degraded solution was placed in a 10 ml volumetric flask and made up to volume with acetate buffer of pH 4.0. The absorbance of the solution was measured at 550, 525 and 265 nm and the concentrations of CC, HC and AA were determined by solving the simultaneous equation by matrix method.

A three-component spectrometric assay (additive absorbances) requires the solution of three simultaneous equations which may be conveniently carried out by matrix method using a computer program. Thus absorbance measurements A_1, A_2, A_3 at carefully selected wavelengths λ_1, λ_2, λ_3 on the mixture of components 1, 2, 3, with molar absorptivities ϵ_1, ϵ_2, ϵ_3 at concentrations ${}_1C$, ${}_2C$, ${}_3C$ may be represented as:

Wavelength	Absorbance	Absorbance Sum
λ_1	A_1	${}_1\epsilon_1\,{}_1C + {}_2\epsilon_1\,{}_2C + {}_3\epsilon_1\,{}_3C$
λ_2	A_2	${}_1\epsilon_2\,{}_1C + {}_2\epsilon_2\,{}_2C + {}_3\epsilon_2\,{}_3C$
λ_3	A_3	${}_1\epsilon_3\,{}_1C + {}_2\epsilon_3\,{}_2C + {}_3\epsilon_3\,{}_3C$ (1a)

The matrix equation can be formulated as follows:

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \end{bmatrix} = \begin{bmatrix} {}_1\epsilon_1\,{}_1C + {}_2\epsilon_1\,{}_2C + {}_3\epsilon_1 \\ {}_1\epsilon_2\,{}_1C + {}_2\epsilon_2\,{}_2C + {}_3\epsilon_2 \\ {}_1\epsilon_3\,{}_1C + {}_2\epsilon_3\,{}_2C + {}_3\epsilon_3 \end{bmatrix} \begin{bmatrix} {}_1C \\ {}_2C \\ {}_3C \end{bmatrix}$$

(AM) (ASM) (CM) (1b)

Where,
(AM) = Absorbance matrix
(ASM) = Absorbance sum matrix
(CM) = Concentration matrix

The solution of (1b) for each concentration is carried out by replacing the appropriate column in the absorbance sum matrix in its determinant form and dividing the resultant by the ASM again in its determinant form.

The matrices are then expanded by any convenient method, e.g., for ${}_1C$ using the top row and Laplace's method.

$${}_1C = \begin{vmatrix} A_1 & {}_2\epsilon_1 & {}_3\epsilon_1 \\ A_2 & {}_2\epsilon_2 & {}_3\epsilon_2 \\ A_3 & {}_2\epsilon_3 & {}_3\epsilon_3 \end{vmatrix} \Big/ \begin{vmatrix} {}_1\epsilon_1 & {}_2\epsilon_1 & {}_3\epsilon_1 \\ {}_1\epsilon_2 & {}_2\epsilon_2 & {}_3\epsilon_2 \\ {}_1\epsilon_3 & {}_2\epsilon_3 & {}_3\epsilon_3 \end{vmatrix} \quad (2a)$$

$${}_2C = \begin{vmatrix} {}_1\epsilon_1 & A_1 & {}_3\epsilon_1 \\ {}_1\epsilon_2 & A_2 & {}_3\epsilon_2 \\ {}_1\epsilon_3 & A_3 & {}_3\epsilon_3 \end{vmatrix} \Big/ \left| \text{(ASM)} \right| \quad (2b)$$

$${}_3C = \begin{vmatrix} {}_1\epsilon_1 & {}_2\epsilon_1 & A_1 \\ {}_1\epsilon_2 & {}_2\epsilon_2 & A_2 \\ {}_1\epsilon_3 & {}_2\epsilon_3 & A_3 \end{vmatrix} \Big/ \left| \text{(ASM)} \right| \quad (2c)$$

$$ {}_1C = \frac{A_1 \begin{vmatrix} {}_2\epsilon_2 & {}_3\epsilon_2 \\ {}_2\epsilon_2 & {}_3\epsilon_3 \end{vmatrix} - {}_2\epsilon_1 \begin{vmatrix} A_2 & {}_3\epsilon_2 \\ A_3 & {}_3\epsilon_3 \end{vmatrix} + {}_3\epsilon_1 \begin{vmatrix} A_2 & {}_2\epsilon_2 \\ A_3 & {}_2\epsilon_3 \end{vmatrix}}{\text{ASM expanded}} \quad (3a) $$

$$ {}_1C = \frac{A_1 ({}_2\epsilon_2 \,.\, {}_3\epsilon_3 - {}_3\epsilon_2 \,.\, {}_2\epsilon_3) - {}_2\epsilon_1 (A_2 \,.\, {}_3\epsilon_3 - {}_3\epsilon_2 \,.\, A_3) + {}_3\epsilon_1 (A_2 \,.\, {}_2\epsilon_3 - {}_2\epsilon_2 \,.\, A_3)}{\text{ASM expanded}} \quad (3b) $$

Similarly, the matrices are expanded for ${}_2C$ and ${}_3C$. For each determinant of a different set of ${}_1C$, ${}_2C$ and ${}_3C$, the top line of equation (3b) is computed again since A_1, A_2 and A_3 vary with a change in concentration whilst ASM is always the same. The solution of the simultaneous equations may be carried out using a suitable software.

The assay procedure for the photolyzed solutions was performed in a dark room.

Spectral Measurements

The measurements of the absorption spectra and absorbances of photolyzed solutions were carried out on a Shimadzu UV-1601 recording spectrophotometer using quartz cells of 10 mm path length.

pH Measurements

The measurement of the pH of CC solutions was performed with a digital pH meter (model-CP 501; sensitivity ±0.01 units; Elmetron, Poland) using a combination electrode. The pH of the photolyzed solutions was adjusted to 4.0 (0.2 M acetate buffer) before assay.

RESULTS

Accuracy

Accuracy is normally measured as the percent recovery of the analyte by the proposed assay method. The accuracy has been determined by analyzing several synthetic mixtures of CC, HC and AA in the concentration range likely to occur in the photodegraded solutions of CC. The recoveries of CC, HC and AA range from 97.1-103.0 % (table 2) and this is reasonable for a method involving three-component spectrometric analysis.

Precision

The precision of the method has been evaluated by the analysis of several mixtures of CC, HC and AA in the concentration range of 1-5 x 10^{-5} M and determination of their relative standard deviation (RSD). The RSD of the method has been found to be within ± 3% (table 2).

Specificity

The selection of appropriate analytical wavelengths in multi-component spectrometric analysis is an important factor to achieve high specificity of the method. Wavelengths that show high sensitivity of measured absorbance have been found to be ideal for analytical work (Ahmad and Rapson, 1990). A careful examination of the absorption spectra of CC, HC (Ahmad *et al*., 1992) and CC and AA (fig. 1) indicated that these compounds possess distinct absorption maxima (pH 4.0) (550, 525 and 265nm, respectively) that can be conveniently used for the analysis of a three-component mixture of these compounds. The measurement of absorbance at the absorption maxima also imparts high sensitivity to the method. Therefore, the choice of analytical wavelengths at the absorption maxima would contribute maximum specificity and sensitivity to the method. This has already been shown in the analysis of CC and HC (Ahmad *et al*., 1992) and CC, HC and riboflavin mixtures (Ahmad and Hussain, 1992). The presence of HC as a photoproduct of CC was confirmed by TLC using solvent systems A and B (see Methods) before its determination. The degradation products of AA i.e., dehydroascorbic acid and 2,3-diketogulonic acid detected by TLC (solvent systems C, D and E) do not absorb in the region of the analytical wavelengths (Davies *et al.*, 1991), and, therefore, do not cause any interference in the assay of CC, HC and AA in photolyzed solutions. The values of molar absorptivities used in the calculation of the concentrations of CC, HC and AA (table 1) represent the means of five determinations (correlation coefficient 0.999).

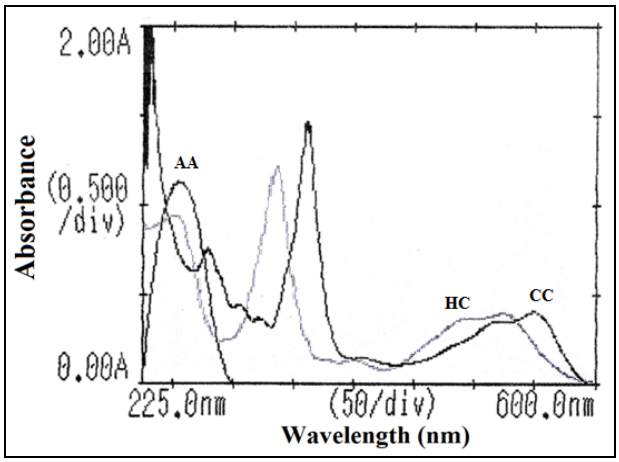

Fig. 1: Absorption spectra of CC (5×10^{-5} M) and AA (1×10^{-4} M) solutions at pH 4.0.

Limit of Detection (LOD)

LOD is the minimum amount of an analyte that can be detected in a sample. There are several approaches that can be used for the calculation of LOD. One of the methods in spectrometric analysis include the instrumental performance described by the signal-to-noise ratio (S/N), i.e., ratio of the average value of output signal to its standard deviation (Skooj *et al*., 2006). A S/N ratio of 3:1 has been considered adequate for the determination of LOD (Bharate and Bharate, 2012). Another approach estimates the LOD from the standard deviation of the

response and the slope of the calibration curve (Ahmed *et al*., 2013). It can be calculated by the following formula: LOD (M) = 3.3× (SD of intercept/slope).

The LOD values of $1.60x10^{-6}$, $1.64x10^{-6}$ and $3.10x10^{-7}$ M have been obtained for CC, HC and AA, respectively, in this method.

Limit of Quantitation (LOQ)

The LOQ value of a particular analyte gives the least determinable concentration with desirable precision. A S/N ratio of more than 10 could provides reasonable accuracy in the determination of LOQ (Bharate and Bharate, 2012). Like LOD it can also be calculated by using the following formula:
LOQ (M) = 10 × (SD of intercept/slope)

In the present work concentration levels of $4.84x10^{-6}$, $4.96x10^{-6}$ and $9.40x10^{-7}$ M have been determined as LOQs of CC, HC and AA, respectively.

Table 1: Calibration data for CC, HC and AA*

	CC**	HC**	AA
λ_{max} (nm)	550	525	265
Molar absorptivity (M^{-1} cm^{-1})	8660	8460	11600
Linearity range ($Mx10^5$)	1.0 - 5.0	1.0 - 5.0	1.0 - 5.0
Correlation coefficient (r^2)	0.9999	0.9999	0.9999
Slope	8.66×10^3	8.46×10^3	11.63×10^3
Intercept	-0.00040	-0.00039	-0.00130
SE(±)of intercept	0.00188	0.0019	0.0024
SD(±)of intercept	0.0042	0.0042	0.0011
SE(±)of slope	1.80×10^{-3}	1.79×10^{-3}	0.0023
Recovery range (%)	98.15-100.46	98.11-101.06	98.02-100.60
Accuracy (%) ± SD	99.68±0.974	99.68±1.094	99.44±1.004
%RSD	0.978	1.098	1.009
LOD (M/L)	1.60×10^{-6}	1.64×10^{-6}	3.10×10^{-7}
LOQ (M/L)	4.84×10^{-6}	4.96×10^{-6}	9.40×10^{-7}

*values represent five determinations of each compound. **values taken from previous work (Ahmad et al., 1992).

Table 2: Analysis of Synthetic Mixtures of CC, HC and AA*

CC				HC				AA			
Added ($Mx10^5$)	Found ($Mx10^5$)	Recovery (%)	RSD (%)	Added ($Mx10^5$)	Found ($Mx10^5$)	Recovery (%)	RSD (%)	Added ($Mx10^5$)	Found ($Mx10^5$)	Recovery (%)	RSD (%)
5.000	4.968	99.4	1.7	0.50	0.488	97.6	2.7	5.000	5.104	102.0	0.9
4.500	4.481	99.6	2.5	1.00	0.982	98.2	1.9	4.500	4.515	100.3	1.2
4.000	4.024	100.6	1.8	1.50	1.542	102.8	1.7	4.000	3.927	98.2	1.9
3.500	3.570	102.0	2.4	2.00	1.955	97.8	1.2	3.500	3.552	101.5	1.6
3.000	2.942	98.1	1.4	2.50	2.542	101.7	1.4	3.000	3.021	103.0	2.8
2.500	2.535	101.4	0.7	3.00	3.081	102.7	2.5	2.500	2.452	98.1	1.4
2.000	2.052	102.6	1.6	3.50	3.425	97.8	1.9	2.000	1.954	97.7	1.7
1.500	1.470	98.0	2.2	4.00	3.920	98.0	0.7	1.500	1.545	103.0	2.2
1.000	0.982	98.2	0.8	4.50	4.621	102.7	1.2	1.000	0.981	98.1	0.7
0.500	0.485	97.1	2.7	5.00	4.981	99.6	1.8	0.500	0.485	97.2	1.9

*values expressed as a mean of five determinations.

Table 3: Photolysis of 5×10^{-5} M CC solution in the presence of 5×10^{-4} M AA at pH 4.0

Time (min)	CC ($M\times10^5$)	HC ($M\times10^5$)	Total[a] ($M\times10^5$)	AA[b] ($M\times10^4$)
0	5.000	---	5.000	5.000
30	4.466	0.532	4.998	3.451
60	3.924	1.088	5.012	2.354
90	3.630	1.296	4.886	1.778
120	3.268	1.747	5.015	1.127

[a]Molar balance of CC and HC is equivalent to the initial concentration of CC. [b]Loss of AA during the reaction.

Linearity

The calibration curves for CC, HC and AA were prepared in the concentration range of 1.0-5.0x10^{-5} M (pH 4.0) at the appropriate wavelengths to confirm the validity of Beer's law. The linearity was evaluated by least square regression analysis and the calibration data are presented in table 1.

Robustness

The robustness of an analytical method indicates its capacity for not being affected by small variations in experimental parameters. It also provides assurance that the method is reliable for the desired analytical work. It has been found that minor alterations in the analytical wavelengths (1-2 nm), pH of the medium (±0.01-0.02 units) and composition of the mixture do not affect the limits of accuracy and precision of the method (table 2). However, it is suggested that a strict control of experimental parameters should be exercised to achieve reliable results on the application of the method to the analysis of vitamin mixtures and kinetic studies.

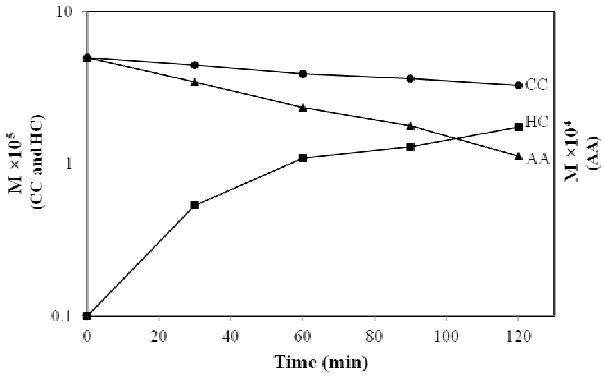

Fig. 2: First-order plot for the photolysis of CC (●); formation of HC (■) and the loss of AA (▲) at pH 4.0.

DISCUSSION

Pharmaceutical compounds may often be formulated as multi-ingredient dosage forms. This could create a problem in the analysis of these compounds and may require the development of a specific analytical method. It is advisable to apply those methods in the quality control of pharmaceuticals that are simple, rapid, selective, accurate and economical. In many cases spectrometric methods have been developed for the analysis of two- or three-component mixtures carrying out the calculations using a suitable programmed computer. The multicomponent spectrometric methods can not only be used for the analysis of mixtures but also to follow the kinetics of drug degradation in stability studies (see Introduction). These methods are rapid (need a few absorbance measurements), selective (in the presence of other components) and above all economical (do not require expensive solvents) compared with the chromatographic methods.

Method development

The present study involves the development of a three-component spectrometric method for the assay of CC and its photoproduct, HC (Ahmad *et al*., 1992) in the presence of AA in aqueous solutions. It involves the selection of appropriate analytical wavelengths for maximum specificity, choice of assay pH imparting maximum wavelength difference among the components, establishment of the validity of Beer's law and identification of the photoproducts of CC and AA. The method has been validated and successfully applied to the study of the kinetics of degradation of CC in the presence of AA. The various validation parameters of the method indicate that the method is reliable for kinetic studies.

Application of the assay method to kinetics studies

The kinetics of photodegradation of CC in the presence of AA has been studied by the application of the multicomponent assay method. AA is a well-known reducing agent and promotes the degradation of CC (DeRitter, 1982). A set of results for the assay of the three components (CC, HC and AA) in the photolyzed solution of CC at pH 4.0 is reported in table 3. The assay method on application to the photolysis of CC, gives increasing values of HC and a constant molar balance with respect to CC and HC. The gradual decrease in CC and AA concentrations, with time, indicates the loss of the two compounds during the photolysis. When the assay data for CC at pH 4.0 in a photodegradation reaction were subjected to kinetics analysis, the data followed first-order kinetics as observed by Ahmad *et al*., (2003) and fitted well around the first-order plot (fig. 2). The formation of HC and loss of AA during the reaction is also shown in fig. 2. This indicates the accuracy of the method in the assay of CC and the other two components involved. The method is specific, rapid and convenient and may be used for the photolysis studies of CC.

CONCLUSION

Cyanocobalamin, hydroxocobalamin and ascorbic acid have been determined by a multicomponent spectrometric method in aqueous solutions. The method involves absorbance measurements of the mixture of these compounds at their respective maxima at 550, 525 and 265 nm and calculation of the concentrations by matrix method. These wavelengths impart high specificity and sensitivity to the measured absorbance. The method has been validated with respect to the various assay parameters including accuracy, precision, specificity, detection limit, quantitation limit, linearity and robustness. The reproducibility of the method for the three components is within ± 3 %. It has been used to study the kinetics of cyanocobalamin photolysis in aqueous solution

and the accuracy of the analytical results is indicated by the linearity of the first-order plots for the reaction.

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