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Kinetic determination of vitamin B_{12} in pharmaceuticals by the continuous addition of reagent technique

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Abstract: An automatic kinetic method for the determination of micro amounts of vitamin B_{12} in pharmaceutical samples based on the fast formation of a coloured complex between the cobalt contained by this vitamin and PAR [4-(2-pyridilazo)resorcinol] in a weakly alkaline medium and on the use of the continuous addition of reagent technique for the mixing of sample and reagent is reported. The reaction is monitored by measuring the changes in the absorbance at 510 nm characteristic of the complex formed. The linear range of the determination is 1.1–34.5 μg ml^{-1} and the relative standard deviation is 1.2%. The sample throughput is 75 h^{-1} (triplicate runs). The results obtained in analyses of pharmaceutical samples showed excellent correlation with nominal contents and the results of atomic absorption spectrophotometric analyses.

Keywords: *Vitamin B_{12}; automatic kinetic method; continuous addition of reagent technique; assay of pharmaceuticals.*

Introduction

The determination of B_{12} vitamins is of relevance to various fields such as clinical analysis, food processing and fermentation processes [1]. These vitamins, also called cobalamins because of the presence of cobalt in their chemical structures, are essential to the normal function of bone marrow, the nervous system and gastrointestinal tract. Vitamin B_{12} is usually determined either by direct absorbance measurements on aqueous solutions or, indirectly, and more frequently, by measuring the cobalt it contains by atomic absorption spectrometric [2–4], spectrophotometric [5–8], chromatographic [9, 10], radiochemmetric [11, 12] or catalytic kinetic [13–16] methods. The sensitivity thus achieved varies over a wide range (from a few ng ml^{-1} to a few μg ml^{-1}).

This paper reports a method for the kinetic determination of vitamin B_{12} based on the determination of the cobalt present in this molecule by the continuous addition of reagent (CAR) technique for automatic mixing of sample and reagents. The method is based on the complex formation reaction between cobalt and 4-(2-pyridilazo)resorcinol (PAR) in a weakly alkaline medium. As shown below, the CAR technique, principles and applications to routine analyses which are described in earlier papers [17, 18], improves the features of the determination of cobalt with regard to the conventional photometric methods. Although kinetic methods based on cobalt-catalysed reactions are more sensitive than the proposed method, they are more time-consuming. The sensitivity achieved in the direct determination of cobalt with PAR by the CAR technique is more than adequate for application to pharmaceutical samples containing vitamin B_{12}, with the additional advantage of its rapidity on account of the high sample throughput.

Experimental

Reagents

All experiments were performed with analytical reagent grade chemicals which were used as supplied without further purification. A standard cobalt solution (1000 μg ml^{-1}) was prepared by dissolving 1.000 g of cobalt metal in a minimum volume of (1:1) hydrochloric acid and diluting to 1.0 l with 1% (v/v) hydrochloric acid. More dilute solutions were prepared as needed. A stock 4-(2-pyridilazo) resorcinol solution was prepared by dissolving 100 mg of the chemical in 100 ml of distilled water. A buffer solution of pH 8.8 was made by adding the appropriate volume of 2 M hydrochloric acid to 38.1 g of sodium tetra-

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borate dissolved in 500 ml of water in order to ensure the above-mentioned pH in a final volume of 1.0 l.

Apparatus

All measurements were made by means of a CAR reagent system described elsewhere [17], using a microcomputer for data collection and processing. A Perkin–Elmer 380 atomic absorption spectrometer, equipped with a hollow-cathode lamp for cobalt, and a radiometer PHM62 pH-meter were also used.

Procedure

A volume of sample containing between 3.0–90 μg of cobalt or between 70–2075 μg of mineralized vitamin B_{12} as cyanocobalamin and 10 ml of borate buffer of pH 8.8 were mixed and diluted to 60 ml with distilled water in a reaction vessel. The complex-formation reaction between cobalt and PAR was developed by adding a 0.1% (w/v) solution of the chromogenic reagent from the burette at rate of 20 ml min^{-1} whilst stirring at 200 rpm. The fast changes in the absorbance were monitored at 510 nm (maximum absorption wavelength of the complex formed); data were collected and processed by the computer. The initial rate was determined in about 500 ms and the calibration graph was run by plotting the measured initial rate against the concentration of cobalt or vitamin B_{12}.

Determination of vitamin B_{12} in pharmaceutical preparations

The pharmaceuticals assayed were as follows:

- Imferon vials (Llorente), 500 μg of hydrocobalamin per vial;
- Nervobion 5000 vials (Merck Igoda), 5 mg of cyanocobalamin and 30 mg of lidocain hydrochloride per vial;
- Hidroxil B_{12}—B_6—B_1 vials (Almirall), 1 mg of hydroxycobalamin, 500 mg of pyridoxin hydrochloride and 250 mg of thiamine hydrochloride per vial;
- B_{12} Latino Depot vials (Syntex-Latino), 1 mg of cyanocobalamin per vial;
- Bester Complex capsules (Salvat), 1.5 mg of hydroxycobalamin, 60 mg of benzothiamine and 150 mg of pyridoxin hydrochloride per capsule;
- Sedionbel vials (Alter), 2 mg of hydrocobalamin, 10 mg of adenosin triphosphate, 100 mg of cocarboxylase, 25 mg of ornitine hydrochloride, 50 mg of pangamic acid, 100 mg of pyridoxal-5-phosphate, 20 mg of lidocain hydrochloride, 2 mg of desamethasone phosphate and 10 mg of uridin-triphosphate per vial.

These pharmaceutical samples were treated as follows: the contents of an adequate number of vials or capsules were placed in a porcelain capsule and ashed in an oven at 700°C for 30 min. Then the residue was dissolved in 5 ml of 1+9 (v/v) hydrochloric acid, neutralized with sodium hydroxide and diluted in a 25-ml volumetric flask with distilled water. Appropriate aliquots were then used for the CAR determination.

Results and Discussion

PAR is a classic photometric reagent for the determination of cobalt in a great variety of samples [19]. However, its use is rather tedious as the sample must be boiled twice for about 30 min before the absorbance can be measured in the organic layer after a 10 min extraction. In order to make this reagent useful in the routine kinetic determination of vitamin B_{12} via cobalt, with minimum handling at a high sampling rate, we applied the CAR technique for the mixing of sample and reagent.

Figure 1 shows a typical absorbance versus time curve provided by the CAR technique, which was processed by the data acquisition

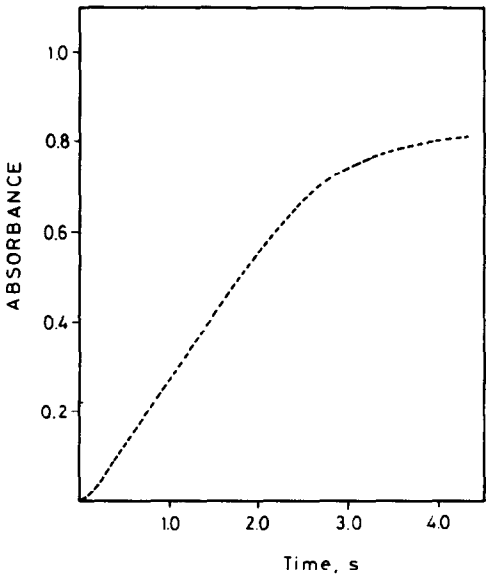

Figure 1
Absorbance versus time graph run at 510 nm. Cobalt concentration, 1.0 μg ml^{-1}. Other experimental conditions as described in the Procedure.

system. As can be seen, the initial rate was determined with good accuracy in about 500 ms, which makes the method particularly suitable for routine analyses compared with the classical photometric procedure.

Effect of reaction variables

The performance of proposed kinetic procedure for the determination of vitamin B_{12} is influenced by three major factors, namely pH, PAR concentration and rate of addition of the chromogenic reagent. These must be optimized to obtain as accurate and reproducible results as possible. To simplify matters, standard cobalt solutions were used whilst each variable changed in turn with others being kept constant. The optimum value of each variable was taken as that yielding the minimum relative standard deviation (RSD) in the initial rate measurements under conditions where the reaction order with respect to the variable concerned was zero or close to zero.

The influence of the pH on the reaction rate was studied over a wide range, between 2.0–11.1, as shown in Fig. 2. The plot of initial rate versus pH shows maximum dependence at about pH 8.8 at which the anion of PAR (HR^-, with one hydroxyl dissociated) is the ligand form reacting with cobalt [20]. Taking into account this kinetic dependence, a pH of 8.8 was selected for use in the kinetic determination of vitamin B_{12}; thus 10 ml of buffer of pH 8.8 were added to the reaction vessel as part of the procedure.

The effect of the PAR concentration and its rate of addition (u) from the autoburette are

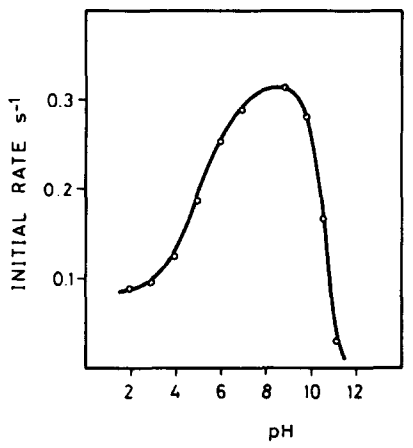

Figure 2
Effect of pH on the reaction rate. 1.0 μg ml^{-1} cobalt. (For details, see text.)

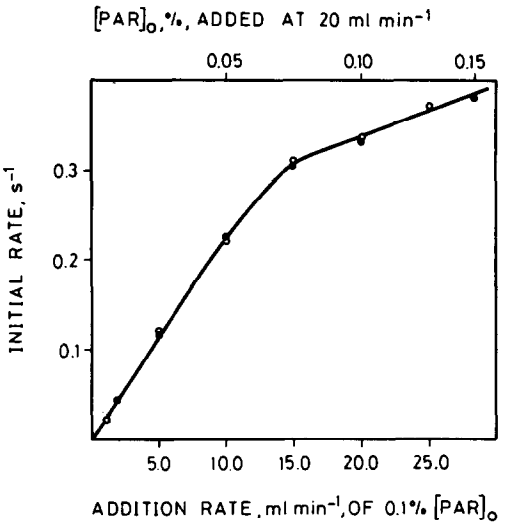

Figure 3
Influence of (○) addition rate and (●) concentration of PAR on the complex formation reaction of 1.0 μg ml^{-1} of cobalt. Experimental conditions as described in the Procedure.

closely related as is shown in Fig. 3. In fact, both exhibit the same dependence since the actual concentration of PAR reagent [PAR] in the reaction vessel at any time (t) can be expressed as follows:

$$[PAR] = u\,t\,[PAR]_0/V_0, \qquad (1)$$

where $[PAR]_0$ is the concentration of the chromogenic reagent in the autoburette and V_0 is the initial sample volume; the result is the same whether u or $[PAR]_0$ is varied. The two straight lines in Fig. 3 show a change in the slope at u = 15 ml min^{-1}, $[PAR]_0$ = 0.1%, or at $[PAR]_0$ = 0.075%, u = 20 ml min^{-1}. Taking into account that the kinetic curve shows an initial linear portion of about 2.5 s (see Fig. 1) and by substituting this time into equation (1), the actual concentration of PAR associated with the above-mentioned change is roughly twice that of the cobalt concentration, corresponding to the stoichiometric ratio of the cobalt–PAR complex. A PAR concentration of 0.1% (w/v) and an addition rate of 20 ml min^{-1} were chosen for further experiments.

Other experimental variables such as the initial sample volume, the stirring speed and the hydrogen peroxide concentration were also studied. As reported in the literature [19], the photometric determination of cobalt with PAR requires a prior step in which the sample is boiled with hydrogen peroxide to ensure the higher oxidation state of cobalt, i.e.

cobalt(III). The experiments involved in the proposed kinetic determination of cobalt gave the same results in the absence and presence of hydrogen peroxide, so this was not added to the reaction vessel. The very nature of the CAR technique, in which the continuous stirring of the sample solution facilitates aeration, could be responsible for this observation. The influence of the initial sample volume (between 50–80 ml) and the stirring speed (50–300 rpm) on the initial rate was also examined. The values advocated in the Procedure, i.e. V_0 = 60 ml and a stirring speed of 200 rpm lie in the zero-order dependence region.

Kinetic determination of vitamin B_{12}

The absorbance–time curves recorded by the CAR technique for different amounts of cobalt were examined by the initial rate method. The figures of merit of this determination and that of vitamin B_{12} expressed as cyanocobalamin obtained under the recommended procedural conditions are summarized in Table 1. Vitamin B_{12} was determined by assuming its cobalt content to be 4.34%.

The slope of the linear calibration graph given in the table was taken as the analytical sensitivity; the detection limit was calculated on the basis of the variation of the analyte response at low concentrations [21]; the RSD was calculated from 11 samples containing 0.5 μg cobalt ml^{-1} each; finally, the sample throughput was calculated from the time taken to run three replicates analyses on the same sample, including the time needed to change the reaction vessel in the CAR system.

The CAR technique considerably improves the results obtained in the determination of cobalt with PAR as chromogenic reagent, and hence the determination of vitamin B_{12} as well compared with the classic photometric procedure. The CAR technique enables the automatic determination of cobalt over a wider dynamic linear range at a higher sampling rate which makes the proposed method particularly suitable for the routine determination of this vitamin in real samples.

Analysis of pharmaceutical samples

The procedure for determination of vitamin B_{12} described above was applied to various pharmaceutical formulations manufactured by Spanish laboratories. The results found are summarized in Table 2, which also lists atomic absorption spectrometric (AAS) results. As can be seen, the results obtained by the CAR technique are consistent with the AAS results and with the nominal vitamin B_{12} contents quoted by the manufacturers.

Table 1
Analytical figures of merit for the determination of vitamin B_{12} and cobalt by the CAR technique

Feature	Cobalt	Vitamin B_{12}
Dynamic linear range	0.05–1.50 μg ml^{-1}	1.1–34.5 μg ml^{-1}
Sensitivity	0.328 ml μg^{-1} s^{-1}	0.014 ml μg^{-1} s^{-1}
Detection limit	0.020 μg ml^{-1}	0.365 μg ml^{-1}
Precision (RSD)	1.2%	
Sampling throughput	75 h^{-1}	

Table 2
Determination of vitamin B_{12} (as cobalt) in pharmaceutical preparations by the CAR technique

	Milligrams of vitamin B_{12} per vial or capsule		
		Found*	
Pharmaceutical	Claimed	CAR method	AAS
Imferon	0.5	0.51 ± 0.01	0.51 ± 0.01
Nervobion 5000	5.0	4.98 ± 0.04	4.81 ± 0.05
Hidroxil B_{12}—B_6—B_1	1.0	1.01 ± 0.01	0.99 ± 0.02
B_{12} Latino Depot	1.0	1.01 ± 0.02	1.01 ± 0.02
Bester Complex	1.5	1.47 ± 0.02	1.46 ± 0.02
Sendionbel	2.0	2.01 ± 0.02	2.00 ± 0.03

*Average of three determinations.

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