Journal of Chromatography, 590 (1992) 359–363
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 804

Short Communication

Ultramicrodetermination of cyanocobalamin in elemental diet by solid-phase extraction and high-performance liquid chromatography with visible detection

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(First received May 31st, 1991; revised manuscript received October 15th, 1991)

ABSTRACT

The ultramicrodetermination of cyanocobalamin (9 ng/g) in elemental diet containing 46 kinds of compounds, with concentrations at least 50–10^6 times higher than that of cyanocobalamin, was performed by Sep-Pak C_{18} purification and concentration of cyanocobalamin followed by HPLC with detection at 550 nm. The method is simple, rapid, sensitive and reproducible. The calibration graph was linear in the range of 0–0.2 μg. The recovery of cyanocobalamin was over 95% by the standard addition method. There was good agreement between the cyanocobalamin concentrations indicated and found.

INTRODUCTION

Cyanocobalamin (vitamin B_{12}) is widely distributed and plays an important role in animal and plant metabolisms. Ultramicro amouns of cyanocobalamin are contained in foods, foodstuffs and drugs. It is necessary to monitor the intermediates and finished products of drugs for process control and quality control purposes for good manufacturing practice, hence simple, rapid, sensitive and reproducible analytical methods are required.

Cyanocobalamin has been determined by spectrophotometry [1,2], microbiological method [3] and high-performance liquid chromatography (HPLC) [4–10]. Spectrophotometry is not suitable for complex sample matrices. Microbiological methods have generally been used for the routine determination of vitamins. Ujiue *et al.* [3] reported the analysis of samples of foods, foodstuffs and drugs for the content of cyanocobalamin by a microbiological method. However, this method requires culture of the tissue and preservation of its strain. Further, the procedure of sample preparation is tedious and the incubation is time consuming.

Analyses for many kinds of vitamins have been investigated by HPLC [4–15]. The separation of cyanocobalamin and its analogues has been reported by many workers [4–10]. However, HPLC could not be used for the routine ultramicrodetermination of cyanocobalamin, because experimental conditions for the sample preparation such as the concentration of ultramicro amounts of cyanocobalamin and removal of interferences caused by the complex sample matrix were not investigated in detail.

This paper deals with the routine ultramicrodetermination of cyanocobalamin (9 ng/g) in elemental diet (commercial name Elental, Ajinomoto, Kawasaki, Japan) by HPLC. Elental drug is in powder form to be dissolved prior to use, and it contains 46

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kinds of compounds (*e.g.*, amino acids, vitamins, organic acids, soybean oil, dextrin, minerals), the concentration of which is at least 50–10^6 times higher than that of cyanocobalamin [16]. A simple sample preparation was carried out, using a Sep-Pak C_{18} cartridge to concentrate the ultramicro amount of cyanocobalamin. Routine ultramicrodetermination of cyanocobalamin was performed by using a Sep-Pak C_{18} cartridge followed by HPLC with visible detection at 550 nm, which is specific to cyanocobalamin. This paper also describes the ultramicrodetermination of cyanocobalamin of further two elemental diets, Elental P for paediatrics and Hepan ED for hepatic failure.

EXPERIMENTAL

Reagents and materials

Cyanocobalamin was of Japanese Pharmacopoeia Standard. Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Milford, MA, USA). Other reagents were all of analytical-reagent or HPLC grade.

Apparatus and conditions

A Model 655 A-11 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Uvidec 100-IV detector (JASCO, Tokyo, Japan) set at 550 nm was used. The samples were applied with a Rheodyne Model 7125 sample loop injector with an effective volume of 2 ml. A 25 × 0.46 cm I.D. Column of Capcellpak C_{18} (5 μm) (Shiseido, Tokyo, Japan) was used under ambient conditions. Water–acetonitrile (87:13) was used as the mobile phase at a flow-rate was 0.6 ml/min. A Model UV-2100 variable-wavelength UV recording spectrophotometer (Shimadzu, Kyoto, Japan) was used for the absorption spectra.

Sample preparation

The Sep-Pak C_{18} cartridge was washed with 5 ml of acetonitrile and then with 10 ml of deionized water prior to use.

To a solution of Elental (20 g) dissolved completely in deionized water (60 ml) on a water-bath at 50°C was added sodium chloride (10 g). After this solution had been allowed to stand at room temperature for 30 min, it was diluted accurately to 100 ml with deionized water and then this solution was extracted with hexane (10 ml) for 3 min to remove oils. This aqueous layer was passed through a Sep-Pak C_{18} cartridge and then the cartridge was washed with 50% acetonitrile (8 ml). The eluate was concentrated to dryness in a water-bath at 50°C *in vacuo*. Concentrates were dissolved accurately in deionized water (4 ml) and an aliquot (2 ml) was injected into the chromatograph.

Elental P and Hepan ED were also prepared in the same manner as described for Elental.

RESULTS AND DISCUSSION

Stability of cyanocobalamin in aqueous solution at 50°C

As a preliminary test, the stability of cyanocobalamin in aqueous solution was examined periodically, because the sample was heated at 50°C for complete dissolution. Cyanocobalamin was stable in aqueous solution at 50°C for 1 h. Thus it was found

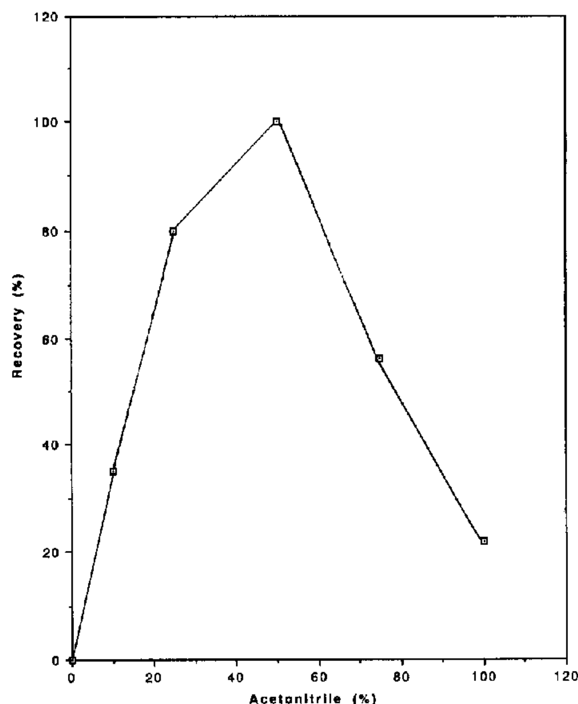

Fig. 1. Variation of recovery with acetonitrile content in acetonitrile–water eluent. A 100-ml volume of deionized water containing cyanocobalamin (1.8 ng/ml) was passed through a Sep-Pak C_{18} cartridge and cyanocobalamin was eluted with 8 ml of acetonitrile solution.

that cyanocobalamin was stable in this sample preparation.

Effect of acetonitrile concentration of elution of cyanocobalamin using a Sep-Pak C_{18} cartridge

Using a Sep-Pak C_{18} cartridge, optimum elution and retention characteristics of a cyanocobalamin standard were determined. An acetonitrile–water mobile phase was examined for the complete elution of cyanocobalamin on a Sep-Pak C_{18} cartridge. The optimum elution of cyanocobalamin achieved with acetonitrile–water (50:50) (Fig. 1). Cyanocobalamin in the aqueous fraction (unbound) was examined for the complete retention of cyanocobalamin on a Sep-Pak C_{18} cartridge. Cyanocobalamin was not detected in the aqueous fraction. From the above result, cyanocobalamin in aqueous solution was completely retentioned on one Sep-Pak C_{18} cartridge and eluted with acetonitrile–water (50:50).

Chromatography

Not only cyanocobalamin but also other vitamins, amino acids and organic acids absorb in the UV region. Cyanocobalamin shows UV absorbance at 260 and 360 nm and visible absorbance at 550 nm, that at 550 nm being specific to cyanocobalamin. Thiamine, riboflavin, ascorbic acid, nicotinamide, pyridoxine, folic acid, tyrosine, phenylalanine and tryptophan have absorbance at 260 nm and several compounds, such as folic acid and riboflavin, have absorbance at 360 nm.

Many overloading peaks were observed on the chromatograms at 260 and 360 nm, and cyanocobalamin could not be identified at 260 and 360 nm. Large amounts of co-existing compounds such as other vitamins and amino acids, with concentrations at least 50–10^6 times higher than that of cyanocobalamin, were observed after the retention time of cyanocobalamin, and it took about 50–60 min to obtain a stable baseline. Hence, monitoring at 260 or 360 nm was not suitable for the ultramicrodetermination of cyanocobalamin. On the other hand, a sharp and reproducible peak [relative standard deviation (R.S.D.) 1.5%] of cyanocobalamin in the sample treated with a Sep-Pak C_{18} cartridge and monitoring at 550 nm was observed with sufficient intensity (Fig. 2, *ca.* 9 ng/g), the baseline was stable and the retention time was about 20 min, irrespective of the large injection volume (2 ml). It was necessary to inject 2 ml of sample solution because cyanocobalamin was present in the sample in ultramicro amounts. As a possible mechanism of this method, sample enrichment at the top of column might be considered. A similar technique has been used in ion chromatography.

However, cyanocobalamin in an untreated sample was not detected (Fig. 3), owing to the presence of unknown peaks and a lack of sensitivity for cyanocobalamin. Hence, for the ultramicrodetermination of cyanocobalamin in complex mixtures, the procedure used here, involving Sep-Pak C_{18} purification and concentration of cyanocobalamin in Elental followed by HPLC with detection at 550 nm, seems appropriate.

From the above results, it seems that ultramicro amounts of cyanocobalamin were completely re-

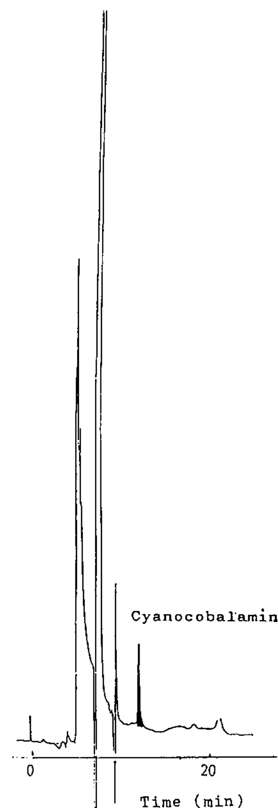

Fig. 2. Chromatogram of cyanocobalamin (treated wth a Sep-Pak C_{18} cartridge) with detection at 550 nm. Amount of cyanocobalamin injected, 0.09 μg in 2 ml.

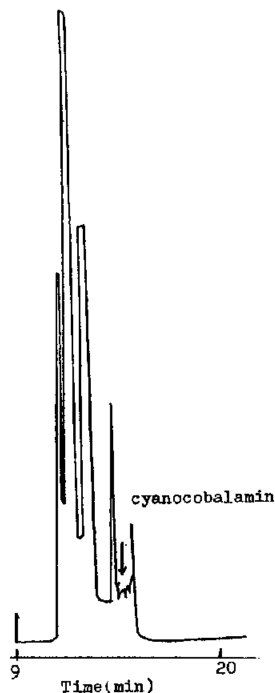

Fig. 3. Chromatogram of cyanocobalamin (not treated with Sep-Pak C_{18}) with detection at 550 nm. Amount of cyanocobalamin injected, 3.6 ng in 2 ml.

tained and concentrated on the Sep-Pak C_{18} cartridge, despite the large injection volume (100 ml) without interference from other compounds. Despite the large injection volume (2 ml), cyanocobalamin was concentrated on a Capcellpak C_{18} column, separated and detected at 550 nm without interference from any other compounds.

TABLE I

RECOVERIES OF CYANOCOBALAMIN ADDED ELENTAL

According to the label Elental contains 0.9 μg of cyanocob min per 100 g.

Cyanocobalamin (μg per 100 g)		Recovery (%)
Added	Found	
0	0.86[a]	–
0.22	1.07	95.5
0.45	1.29	95.6
0.90	1.75	98.9
1.80	2.63	97.8

[a] R.S.D. = 1.5% (n = 7) with no addition of cyanocobalan

Determination of cyanocobalamin

The calibration graph was linear ($y = 7.7x$) in t range 0–0.2 μg. The results in Table I show tl recovery of cyanocobalamin was over 95% by t standard addition method.

The results in Table II show that the analyti data for cyanocobalamin in Elental, Elental P a Hepan ED were excellent without an internal sta dard. Jansen and De Kleijn [10] reported the ass of cyanocobalamin in pharmaceutical preparatic using an internal standard, Co-α-(5-hydroxyben midazoyl)-Co-β-cyanocobamide, which was r commercialy available. If such an an internal sta dard were available, it would be better to use it routine analyses.

TABLE II

ANALYTICAL DATA FOR CYANOCOBALAMIN IN THREE ELEMENTAL DIETS

Diet	Concentration indicated (μg per 100 g)	Analytical data result (μg per 100 g)	Recovery (%)
Elental	0.9	0.86	95.6
		0.86	95.6
		0.87	96.7
Elental P	1.5	1.45	96.7
		1.44	96.0
		1.43	95.3
Hepan ED	2.7	2.61	96.7
		2.59	95.9
		2.57	95.2

There was good agreement between the cyanocobalamin concentrations indicated and found. This method was suitable for the routine ultramicrodetermination of cyanocobalamin in Elental, Elental P and Hepan ED because it is simple, rapid (retention time 20 min), sensitive, reproducible (R.S.D. 1.5%) and the recovery was over 95%. This method may be suitable for the ultramicrodetermination of cyanocobalamin in body fluids such as human plasma or serum, and this is currently under investigation.

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