Journal of Chromatography, 541 (1991) 383–392
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 931

Determination of vitamin B_{12} in multivitamin–multimineral tablets by high-performance liquid chromatography after solid-phase extraction

JOHAN DALBACKE* and IRENE DAHLQUIST
Analytical and Quality Development Department, Gacell Laboratories AB, P.O. Box 839, S-201 80 Malmö (Sweden)

(First received May 2nd, 1990; revised manuscript received September 28th, 1990)

ABSTRACT

A method for the determination of vitamin B_{12} (cyanocobalamin) in multivitamin–multimineral tablets, containing 3 μg of vitamin B_{12} per tablet, is described. Powdered tablets are extracted with a mixture of ammonium pyrrolidinedithiocarbamate and citric acid in dimethyl sulphoxide and water. The extract is centrifuged and the supernatant is diluted with water before concentration and clean-up by solid-phase extraction using a quaternary amine and a phenyl column in series. The eluate from the solid-phase extraction is analysed by reversed-phase liquid chromatography using a methanol–water gradient with detection at 550 nm. A comparison with the frequently used microbiological method is presented. The validation of the procedure is described in terms of selectivity, linearity, accuracy and precision.

INTRODUCTION

Vitamin B_{12} is a water-soluble vitamin that belongs to the corrinoids. The core of vitamin B_{12} consists of a corrin ring with a central cobalt atom. The corrin ring has four pyrrole units. Vitamin B_{12} was isolated in crystalline form from liver in 1948 and its three-dimensional structure was elucidated by Hodgkin in 1956. The human need for vitamin B_{12} is about 1–2 μg per day. Vitamin B_{12} appears to be necessary for red cell formation because of its ability to make folic acid available to the bone marrow. A deficiency of vitamin B_{12} leads to a degeneration of both the sensory and motor columns in the spinal cord with loss of sensation and paralysis.

The determination of water-soluble vitamins has been a persistent problem largely because of the instability of these vitamins and the complexity of the matrices in which they are usually found. Vitamin B_{12} is usually determined by a time-consuming microbiological method using *Lactobacillus leichmannii* [1]. High-performance liquid chromatography (HPLC) has been used to determine vitamin B_{12} in pharmaceutical formulations [2–4]. Hudson *et al.* [5] described a method for the determination of vitamin B_{12} in multivitamin–multimineral tablets by HPLC. However, a combined preconcentration–sample clean-up step is required owing to the low level of vitamin B_{12} present in a large excess of potentially interfering compounds in multivitamin–

0021-9673/91/$03.50

multimineral tablets. A procedure for solid-phase extraction of vitamin B_{12} from multivitamin–multimineral tablets is described in a Baker publication [6].

This paper describes a method for the determination of vitamin B_{12} in multivitamin–multimineral tablets containing ten vitamins and eight minerals. Solid-phase extraction with a quaternary amine and a phenyl column in series is used to concentrate vitamin B_{12} and clean up the sample. Most compounds are retained on the quaternary amine column and vitamin B_{12} is retained on the phenyl column. The eluates are subjected to reversed-phase HPLC with binary gradient elution and detection at 550 nm.

EXPERIMENTAL

Chemicals and reagents

HPLC-grade methanol was obtained from Labscan (Dublin, Ireland). Ammonium pyrrolidinedithiocarbamate (APDC), citric acid and dimethyl sulphoxide of analytical-reagent grade were purchased from Merck (Darmstadt, Germany) and vitamin B_{12} from Sigma (St. Louis, MO, U.S.A.). Water purified with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) was used in all procedures. Multivitamin–multimineral tablets, Vitaplex mineral and placebo tablets (Vitaplex mineral without vitamin B_{12}) were kindly supplied by Ferrosan (Malmö, Sweden).

Extraction columns and vacuum apparatus

Bond-Elut quaternary amine (SAX 500 mg, 2.8 ml) and phenyl (PH 500 mg, 2.8 ml) columns were obtained from Analytichem International (Harbor City, CA, U.S.A.). The columns were used with a Vac-Elut 10 sample-processing station from Analytichem International.

Chromatographic equipment

The HPLC system consisted of a Model 600 multisolvent delivery system, a WISP Model 712 autosampler and a Model 490 programmable multiwavelength detector, all from Waters Assoc. (Milford, MA, U.S.A.). A Spectra-Physics (San Jose, CA, U.S.A.) Model SP4270 integrator was used to record chromatograms and calculate peak areas. A Spectra-Physics ChromStation-AT was used for data handling and storage. A Waters Assoc. Lambda-Max Model 481 LC spectrophotometer was also used during the method development. A Model HP 1040A diode-array detector was used together with an HP 79994A HPLC ChemStation from Hewlett-Packard (Waldbronn, Germany) when assessing the homogeneity of the vitamin B_{12} peak.

Extraction procedure

Approximately 25 mg of vitamin B_{12} was accurately weighed into a 50-ml volumetric flask and diluted to volume with water. This stock solution was diluted with water to a final concentration of 8 μg/ml. An extraction solution containing 0.25 g of APDC, 1.0 g of citric acid, 10.00 ml of dimethyl sulphoxide and 30.00 ml of water was added to 5.00 ml of the diluted stock solution. This mixture was shaken for 15 min at 40°C in a water-bath. The extract was centrifuged for 8 min at 2500 *g* and 15.00 ml of the supernatant were diluted with 100 ml of water.

Forty tablets were powdered in a mortar. An amount corresponding to ten

tablets was treated in the same way as the diluted stock solution, with the exception that 35.00 ml instead of 30.00 ml of water were added.

Solid-phase extraction

The solid-phase columns were conditioned immediately prior to use with one column volume of methanol followed by one column volume of water. The columns were held in a Vac-Elut 10 processing station operated at a pressure of 34 kPa. The columns were filled with water before the SAX columns were placed in adaptors on top of the phenyl columns, *i.e.*, the sample solution first passed through the SAX column and then through the phenyl column. Reservoirs with 20-μm frits were attached on top of the SAX columns.

The diluted extracts were aspirated through the columns by a vacuum of 85 kPa. After aspiration of the standard and sample solutions, the quaternary amine columns were removed and the phenyl columns were washed with one column volume of water followed by 1.0 ml of methanol–water (20:80). The columns were air-dried under vacuum (34 kPa) for 30 s. Vitamin B_{12} was eluted with two 0.50-ml aliquots of methanol–water (90:10) into 3-ml volumetric flasks. The eluates were diluted to volume with water.

Chromatography

The diluted eluates were analysed by reversed-phase HPLC on a 150 × 3.9 mm I.D. μBondapak C_{18} (10 μm) column (Waters Assoc.). A Waters Assoc. Guard-Pak precolumn module containing a Guard-Pak μBondapak C_{18} insert was used to protect the analytical column. Binary gradient elution was used (Table I). Reservoir A contained methanol–water (10:90) and reservoir B methanol–water (90:10). The reservoirs were continuously sparged with helium. Chromatography was carried out under ambient conditions at a flow-rate of 1.0 ml/min. The injection volume was 200 μl. Vitamin B_{12} was detected at 550 nm.

The HPLC system was considered to be acceptable when the relative standard deviation of the vitamin B_{12} peak area of six consecutive injections was less than 1.0%. A tailing factor (T) was calculated according to USP XXII [7] in order to check the asymmetry of the peak. The number of theoretical plates (n) was also calculated. Typical values of n were in the range 20 000–40 000 and of T in the range 1.1–1.2.

Quantification was achieved by comparing the vitamin B_{12} peak area of the sample preparation with that of the standard preparation.

TABLE I

GRADIENT USED

Time (min)	B (%)	Type of gradient
0	0	–
15	50	Linear
17	100	Linear
27	100	–
30	0	Linear
40	0	–

RESULTS AND DISCUSSION

Tablet extraction

It has been reported [8,9] that vitamin B_{12} is not stable in aqueous solutions containing vitamin C and copper(II) and/or iron(II) ions. Ascorbic acid (vitamin C) in aqueous solution is easily oxidized to dehydroascorbic acid. The oxidation is catalysed by copper(II) and iron(II) ions. The instability of vitamin B_{12} in aqueous solution is mainly due to the presence of dehydroascorbic acid [10]. As the multivitamin–multimineral tablets we were to analyse contained iron, copper and vitamin C, it was necessary to stabilize vitamin C in the tablet extracts so that vitamin B_{12} did not degrade. The active constituents of the tablets are given in Table II. Hudson *et al.* [5] have proposed an extraction solution containing APDC, citric acid and dimethyl sulphoxide when tablets containing iron and/or copper are to be analysed. These compounds have the ability to form stable coordination complexes with copper(II) and iron(II) ions. The extraction solution proposed by Hudson *et al.* was modified by decreasing the percentage of dimethyl sulphoxide. We investigated the stability of vitamin B_{12} in the extraction solution used in our method by analysing the supernatant 24 h after tablet extraction. A 10% degradation of vitamin B_{12} was observed in the tablet extract and no degradation was observed in the standard extract. It was not possible to increase the amount of tablets owing to greater problems with degradation of vitamin B_{12} in the extraction solution.

Solid-phase extraction

When tablets were extracted, readily oxidized and reduced constituents were dissolved and participated in several parallel reactions, which led to new potential interfering compounds. Hence a sample clean-up was needed. The mixture was too complex to be cleaned up by a single solid-phase extraction step.

The tablet extract was diluted prior to solid-phase extraction in order to decrease the solvent strength. Vitamin B_{12} was not retained on the phenyl column if this dilution step was excluded.

Most coloured substances in the extraction solution were retained on the SAX column and vitamin B_{12} was retained on the phenyl column. Vitamin B_{12} could be seen as a red band when it was eluted from the phenyl column. The stability of vitamin B_{12}

TABLE II

TABLET COMPOSITION

Active constituent	Amount per tablet	Active constituent	Amount per tablet
Vitamin A	0.9 mg	Nicotinamide	16 mg
Vitamin B_1	1.2 mg	Iron(II)	18 mg
Vitamin B_2	1.4 mg	Zinc(II)	15 mg
Vitamin B_6	2.1 mg	Copper(II)	2 mg
Vitamin B_{12}	3 μg	Iodine	0.15 mg
Folic acid	0.4 mg	Manganese	2.5 mg
Vitamin C	60 mg	Chromium	50 μg
Vitamin D_3	5 μg	Selenium(IV)	50 μg
Vitamin E	9 mg	Molybdenum	0.15 mg

in the eluates was investigated by analysing eluates 12 h after the elution. No degradation of vitamin B_{12} was observed.

Chromatography

The eluates were diluted three-fold with water in order to be able to inject as much as 200 μl without excessive peak broadening on the analytical column. The main reason for continuation of the gradient so long after vitamin B_{12} had been eluted was to ensure the elution of late-eluting compounds. If gradient elution was not used these compounds would be accumulated on the column and affect the stationary phase and thereby the chromatographic process. A chromatogram of the tablets registered at 360 nm (Fig. 1B) shows that there really were a large number of late-eluting compounds in spite of the clean-up procedure.

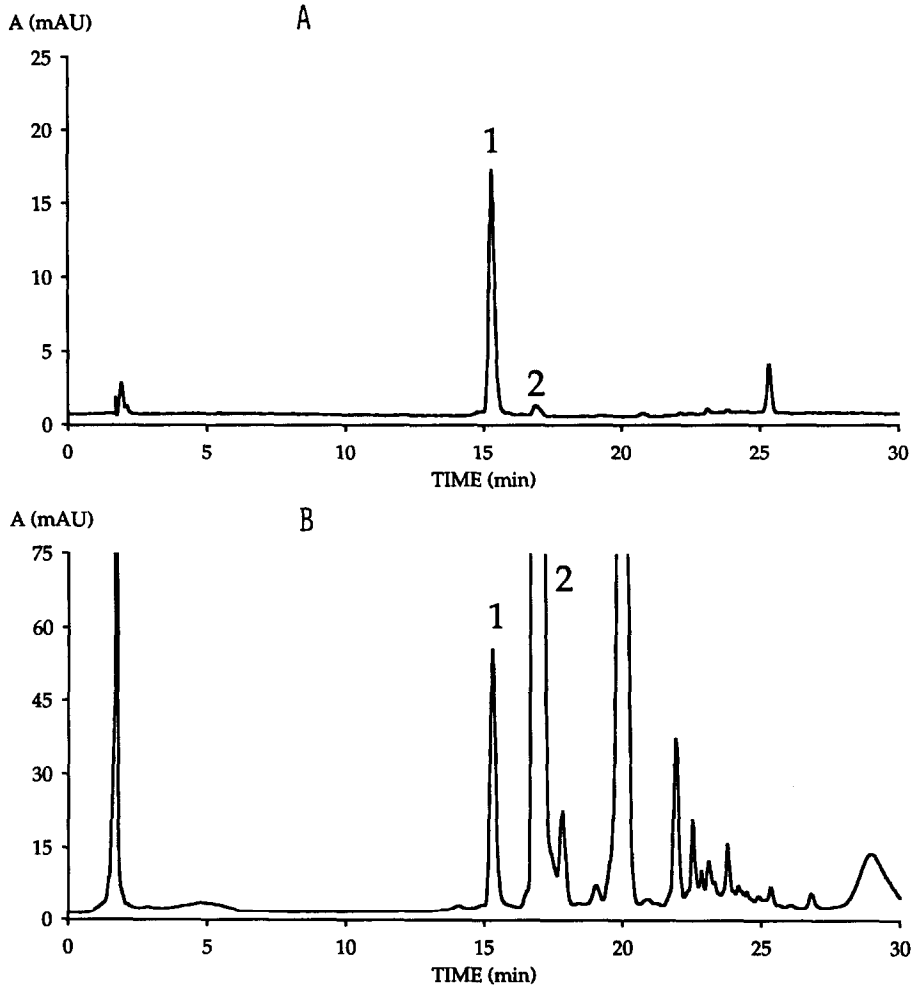

Fig. 1. Chromatogram of multivitamin–multimineral tablets at (A) 550 nm and (B) 360 nm after preconcentration and clean-up by solid-phase extraction. Peaks: 1 = vitamin B_{12}; 2 = vitamin B_2.

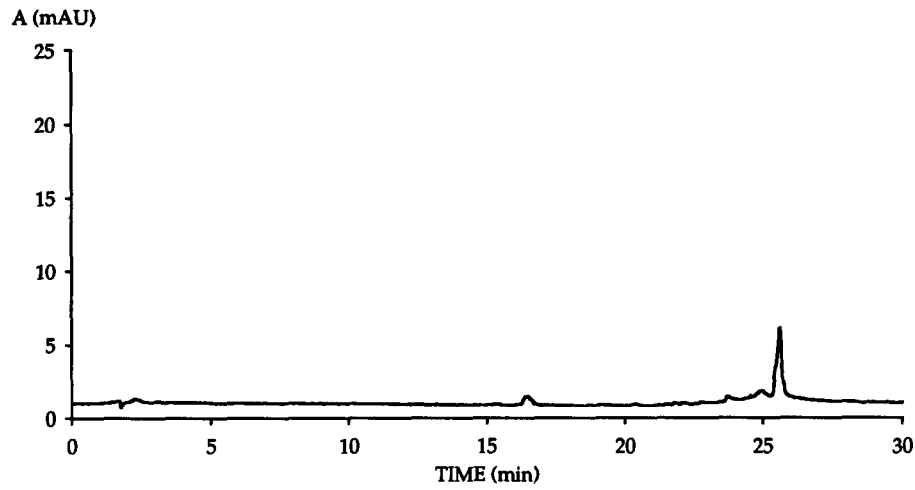

Fig. 2. Chromatogram at 550 nm of placebo tablets after solid-phase extraction.

Vitamin B_{12} was detected at 550 nm instead of 360 nm even though the response was three times higher at 360 nm. The reason for this was that the selectivity was much better at 550 nm than 360 nm. Typical chromatograms for multivitamin–multimineral tablets with detection at 550 and 360 nm are shown in Fig. 1. Vitamin B_{12} eluted as a symmetrical peak after about 15 min and vitamin B_2 2 min later.

Selectivity

A chromatogram at 550 nm of placebo tablets is shown in Fig. 2. No active or inactive ingredient interfered with the quantification of vitamin B_{12}, nor did hydroxycobalamin, which is a degradation product of vitamin B_{12}. The homogeneity of the vitamin B_{12} peak was assessed by analysing multivitamin–multimineral tablets and a standard solution according to the method and using a diode-array detector. Spectra acquired at the upslope, the apex and at the downslope of the vitamin B_{12} peak were normalized and overlayed (Fig. 3). The spectrum at the downslope differed in the

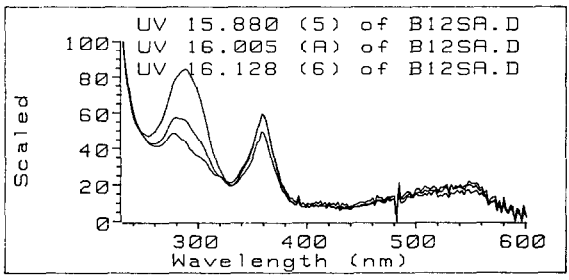

Fig. 3. Overlay of normalized spectra acquired at the upslope, apex and downslope of the vitamin B_{12} peak obtained after preconcentration and clean-up of multivitamin–multimineral tablets by solid-phase extraction.

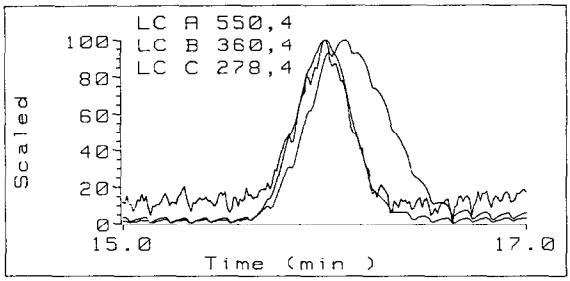

Fig. 4. Overlay of normalized signals of the vitamin B_{12} peak at 278, 360 and 550 nm obtained after preconcentration and clean-up of multivitamin–multimineral tablets by solid-phase extraction.

UV region from those at the apex and the upslope when multivitamin–multimineral tablets were analysed. This indicated that a UV-absorbing impurity eluted under the downslope of the peak. The spectra matched when a standard solution was analysed.

Signals acquired at 278, 360 and 550 nm were normalized and overlayed (Fig. 4). The retention time for the peak was not identical at these wavelengths when multivitamin–multimineral tablets were analysed. The peak at 278 nm had a longer retention time. The retention times were identical when a standard was analysed. A three-dimensional plot in which absorbance was plotted as a function of wavelength

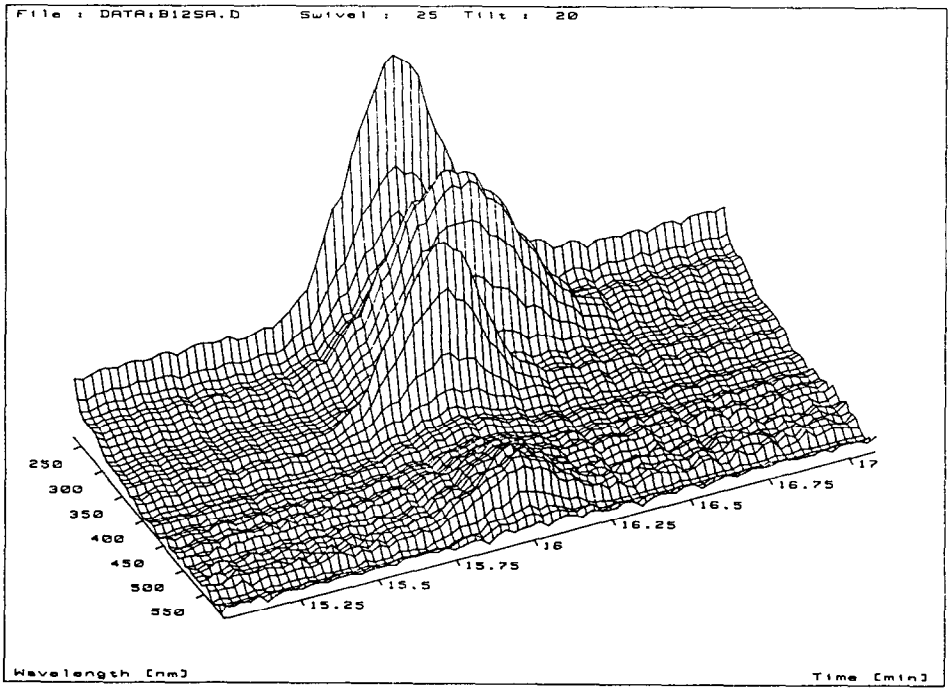

Fig. 5. Three-dimensional plot of the vitamin B_{12} peak obtained after preconcentration and clean-up of multivitamin–multimineral tablets by solid-phase extraction.

TABLE III

LINEAR REGRESSION ANALYSIS

Slope	144 773
Intercept	−2343
Correlation factor	0.9994

and time showed that the impurity did not interfere with the quantification of vitamin B_{12} at 550 nm (Fig. 5).

Two detectors from Waters Assoc., Models 481 and 490, were used during the method development. The chromatograms registered with the Model 490 at 550 nm did not have as many peaks as those registered with the Model 481 at the same wavelength, because the Model 490 automatically places an order filter which blocks all light below 300 nm when the operating wavelength is greater than 350 nm. When 550 nm is set as the detection wavelength the grating also gives a less intense band at 275 nm. As the sample contained many UV-absorbing compounds, the detector without the order filter (Model 481) gave chromatograms with a larger number of peaks.

Linearity

A series of six solutions of vitamin B_{12} in methanol–water (30:70) were prepared in the range 1.2–6.4 μg/ml, corresponding to 1.1–5.8 μg per tablet when tablets were analysed according to the method. Each solution was injected six times and the regression line was calculated by the method of least squares. The results from the linear regression analysis are given in Table III. The peak area was linear in the range 1.1–5.8 μg of vitamin B_{12} per tablet.

Accuracy

The accuracy of the described procedure was assessed by adding known amounts of vitamin B_{12} to powdered placebo tablets prior to analysis according to the method. The added amounts of vitamin B_{12} corresponded to 0.8, 3.3 and 5.4 μg of vitamin B_{12} per tablet. The recovery of vitamin B_{12} was determined by comparing the areas obtained with that obtained from a standard solution analysed according to the described method. The recovery of vitamin B_{12} ranged from 95 to 99% (Table IV). The areas obtained were also compared with those obtained from direct injections of

TABLE IV

ACCURACY

Added amount (μg per tablet)	Relative recovery (%)	Absolute recovery (%)
0.82	99	95
3.3	97	91
5.4	95	94

TABLE V

COMPARISON BETWEEN HPLC AND MICROBIOLOGICAL METHODS

Batch of tablets	Results (μg of vitamin B_{12} per tablet)	
	SPE + HPLC	Microbiological method
I	3.8	3.2
II	3.2	3.9
III	3.5	3.2

vitamin B_{12} in methanol–water (30:70). The absolute recovery of vitamin B_{12} ranged from 91 to 95% (Table IV).

Three batches of multivitamin–multimineral tablets were analysed according to the described method and by a microbiological method. The results obtained are given in Table V. The inter-method correspondence was satisfactory.

Precision

One batch of multivitamin–multimineral tablets was analysed on five occasions during 2 weeks. On each occasion 40 tablets were powdered and three determinations of vitamin B_{12} were made. One analyst performed the analyses using different batches of solid-phase columns and HPLC columns but the same pump, autosampler, detector and integrator. The results obtained are given in Table VI. A one-way analysis of variance (ANOVA) was used to evaluate the variance [11]. The results are given in Table VII. A one-tailed *F*-test was carried out to test whether the mean squares differed

TABLE VI

ASSAY OF TABLETS

Day	Individual results (μg of vitamin B_{12} per tablet)	Mean (μg of vitamin B_{12} per tablet)
1	3.8, 3.6, 3.9	3.8
2	3.4, 3.7, 3.6	3.6
3	3.2, 3.3, 3.3	3.3
4	3.4, 3.6, 3.5	3.5
5	3.5, 3.5, 3.5	3.5

TABLE VII

ANOVA TABLE

Source of variation	Degrees of freedom	Sum of squares	Mean squares	*F*-ratio
Between days	4	0.384	0.096	8.000
Within days	10	0.120	0.012	
Total	14	0.522		

TABLE VIII

PRECISION (RELATIVE STANDARD DEVIATION, R.S.D.)

Source of variation	R.S.D. (%)
Within days (repeatability)	3.1
Between days	4.8
Total (reproducibility)	5.7

significantly. The critical value of F for four and ten degrees of freedom was 3.48 ($P = 0.05$). As the calculated value of F exceeded the critical value, the variance between days, σ_1^2, differed significantly from zero. The within-day mean square gave an estimate of the variance within days, σ_0^2. Since the between-day sample mean square estimated $\sigma_0^2 + n\sigma_1^2$, where n is the number of replicate determinations on each day, the precision could be calculated. The results are given in Table VIII.

In conclusion, the method was found to be selective, linear, accurate and precise. The method is rapid compared with the frequently used microbiological method.

REFERENCES

1 *United States Pharmacopeia, XXII Revision*, U.S. Pharmacopeial Convention, Rockville, MD, 1989, pp. 1516–1518.
2 M. Amin and J. Reusch, *J. Chromatogr.*, 390 (1987) 448.
3 M. Amin and J. Reusch, *Analyst (London)*, 112 (1987) 989.
4 E. Wang and W. Hou, *J. Chromatogr.*, 447 (1988) 256.
5 T. S. Hudson, S. Subramanian and R. J. Allen, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 994.
6 *Baker-10 SPE Applications Guide*, Vol. 1, J. T. Baker, Philipsburg, NJ, 1982, p. 40.
7 *United States Pharmacopeia, XXII Revision*, U.S. Pharmacopeial Convention, Rockville, MD, 1989, p. 1566.
8 H. Hulchin, P. Cravioto and T. Macek, *J. Am. Pharm. Assoc.*, 45 (1956) 806.
9 A. J. Rosenberg, *J. Biol. Chem.*, 219 (1956) 951.
10 A. J. Bartilucci, R. Di Giralamo and H. Eisen, *J. Am. Pharm. Assoc.*, 47 (1958) 42.
11 J. C. Miller and J. N. Miller, *Statistics for Analytical Chemistry*, Ellis Horwood, Chichester, 2nd ed., 1988, pp. 83–84.