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Short communication

An optimized method for the simultaneous determination of vitamins B_1, B_6, B_{12}, in multivitamin tablets by high performance liquid chromatography

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Abstract

A simple, precise, rapid and selective HPLC-RP method has been developed for the simultaneous determination of thiamine hydrochloride (B_1) 150 mg, pyridoxine hydrochloride (B_6) 150 mg, and hydroxocobalamine chloride (B_{12}) 0.150 mg in multivitamin tablets. The method uses a Hypersil-BDS C_{18} reversed phase column and gradient elution. The aqueous mobile phase contained 0.015% triethylamine adjusted to pH 2.7 with 1 N sulfuric acid and acetonitrile. Separation and quantitation was achieved by changing the proportion of the system linearly with a time-schedule programme. Detection was carried out using a dual-beam UV detector set at 280, 350 nm. Good linearity was observed between the concentration of the analytes and peak area ($r = 0.9999$, 0.9998). Sample preparation was relatively simple whereas excipients present in the dosage forms did not interfere with the peaks of interest. Recovery of the compound from the B_1, B_6, B_{12} was quantitative.

Keywords: Vitamins B_1, B_6, B_{12}; Determination; Multivitamin tablets; High performance liquid chromatography

1. Introduction

Vitamins B_1, B_6, B_{12} could be important in the human endogenous metabolism and they are found in small quantities in natural food. The B abitaminosis are usually attributed to nutritional deficiency, to competition between bacteria, to disturbances of the intestinal flora and to the excessive exposure to sunrays. Multivitamin tablets are regularly used in therapeutic management, in improper dietary feeding and for strengthening purpose [1–3].

The determination of water-soluble vitamins has always been a peculiar problem largely because of the instability of these compounds and the complexity of the matrices in which they usually exist. As their chemical structure is not related, a considerable number of publications have appeared using different physical, chemical and

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biological methods. For the determination of the compounds in single-and multi-component mixtures the number of papers is more restricted. They include, among others, the determination of vitamin B_{12} as cobalt by electrothermal atomic absorption spectrometric methods [4], higher order derivative spectrophotometry [5], and capillary electrophoresis [6]. Also, the determination of the B-complex mainly in tablets have been described extensively using HPLC methods [6–18] or HPLC after solid-phase extraction [19,20]. Most methods are successful in single component preparations. Other chromatographic systems separate B_1, B_6 and B_{12} when they exist in the same range [11] but hampered when the amount of B_1 and B_6 exceeds by a hundred or even a thousand times the amount of B_{12} present in the complex.

The described gradient elution procedure allows the simultaneous determination of the B-complex in the multivitamin tablets by setting two wavelengths, 280 and 350 nm through the computer program. Also, the chromatographic system developed clearly separates the three analytes from their degradation products and this makes the presented procedure advantageous with respect to the existing literature. The method is simple, rapid and validated according to USP 24 guidelines [27]. Moreover it is employed successfully for the quality control during the production process.

2. Experimental

2.1. Chemicals

The solvents, acetonitrile and triethylamine 99% were 'gradient grade for liquid chromatography', Merck and redistilled water from a 'Millipore-Q plus 185' was used. All the chemicals and reagents used were of HPLC, USP-NF grades and were used without further purification. The thiamine hydrochloride (B_1), pyridoxine hydrochloride (B_6) and hydroxocobalamine chloride (B_{12}) reference standard were purchase from Merck Chemical Company. The analyzed samples were placebo tablets containing 150, 150 and 0.150 mg B_1, B_6, B_{12} per tablet, respectively.

2.2. Chromatographic conditions

A Waters Alliance series 2690 HPLC equipped with two reciprocating pumps, a Waters series auto-sampler with an effective volume 100 μl and a Waters series 2487 spectrophotometric detector with a dual-beam, variable wavelength, system was used. The chromatographic peaks were recorded and elaborated automatically by employing a computerized Waters program 'Millenium 32'. The analytical column was a C_{18} Hypersil-BDS®, 100 × 4.6mm i.d., 3 μm particle size, end capped to minimize unreacted silanol effects. During the analysis the column was equilibrated at 30 °C. The samples were kept in amber vials at stable temperature, 20 °C. The wavelength was set at 280 nm for B_1, B_6 and 350 nm for B_{12}, respectively. The flow rate was 1.5 ml/min whereas the mobile phase was degassed by filtering through a Millipore HV 0.45 μm pore membrane filter. The gradient elution system consisting of aqueous 0.015% triethylamine, was adjusted to pH 2.7 with 1 N sulfuric acid (Solvent A), for 3 min. Then it was changed linearly in another 3 min to a binary mobile phase of Solvent A:Solvent B/Acetonitrile, 75:25 v/v. Afterwards the initial system in 4 min was returned linearly and finally the column was flushed for 5 min with Solvent A. The gradient elution programme for the proposed procedure is illustrated in Table 1.

2.3. Standard solutions

B_{12} stock standard: about 31 mg of hydroxocobalamine chloride (B_{12}) reference standard accurately weighed, transferred into a 500-ml volumetric flask, and diluted with distilled water. Afterwards 10.0 ml of this solution was transferred to a 100-ml volumetric flask and made up to volume with distilled water.

Mixed stock standard: about 31 mg of thiamine hydrochloride (B_1) and about the same amount of pyridoxine hydrochloride (B_6) reference standard accurately weighed and transferred into a 50-ml volumetric flask. A portion of water and 5.0 ml of hydroxocobalamine chloride (B_{12}) stock solution standard were added to dissolve the analytes by

Table 1
Gradient profile programme for the simultaneous determination of B_1, B_6, B_{12}

Time (min)	Solvent A% 0.015% Triethylamine adjusted to pH 2.7 with 1 N H_2SO_4	Solvent B% Acetonitrile HPLC	Gradient type
0	100	0	Linear
3	100	0	
6	75	25	
10	100	0	
Post time 5 min		Total run time 15 min	

shaking and then made up to volume with distilled water.

An intermediate stock mixed solution was thus obtained. Portions of the latter were transferred into 25-ml volumetric flasks to yield six series of mixed standard solutions.

2.4. Sample preparation

No less than 20 tablets were weighed and the average tablet weight determined. The tablets were finely powdered and a portion of powder equivalent to one average tablet weight was quantitatively transferred into a 1000-ml amber volumetric flask. About 200-ml of water was added and the dispersion was vigorously shaken for 40 min on a mechanical shaker. Ultrasonication followed for 3 min and the solution was diluted to volume with water and left to precipitate. Appropriate dilutions were made from the clear supernatant solution so that the concentration of each sample solution approached the concentration of that in the middle of the standard solution range. Filtration with acrodisc GHP was used to ultraclean the solutions of particles 0.45 μm or greater.

3. Results and discussion

It is generally agreed that the selectivity of a chromatographic separation is described by the combined affinities that the mobile and stationary phase exert on the sample components. A reliable chromatographic assay also, requires acceptable resolution, reasonable retention times and good peak symmetry. As a consequence in the preliminary studies the optimum chromatographic conditions were investigated in isocratic and gradient elution systems by varying the tiny amount of sulfuric acid in the aqueous mobile phase. It was observed that various aqueous acidic mobile phases containing triethylamine, resulted in shorter retention times for the analytes. Similarly different proportions of acetonitrile in the mobile phase exerted a striking change in the k' values of hydroxocobalamine chloride whereas the peak behaviour of pyridoxine HCl and thiamine HCl remained unaffected.

It must be borne in mind that the profile of a gradient elution system affects the retention of solutes in a similar way to the proportions of the solvent with greater elution strength in a binary mobile phase under isocratic conditions. Under isocratic conditions, the band widths regularly increase as the retention time increases for all sample compounds. In contrast an advantage of gradient elution is that the band widths can be approximately constant both for early and late-elution analytes. Therefore a binary gradient elution system was developed with a beneficial effect on the sensitivity of hydroxycobolamine chloride, which exists 1000-fold less in the B-complex, using alternatively two wavelengths for detection. A representative chromatogram together with other observed chromatographic parameters of the system are illustrated in Fig. 1 and Table 2. The retention time of the B-complex was reproducible over a period of a month, which makes the method most suitable for routine analysis. The peak asymmetry for thiamine HCl and hydroxocobalamine chloride was very close to an ideal value of 1.2 whereas pyridoxine HCl yields a value of 2 [26]. The resolution factors Rs, between the chromatographic peaks, were calcu-

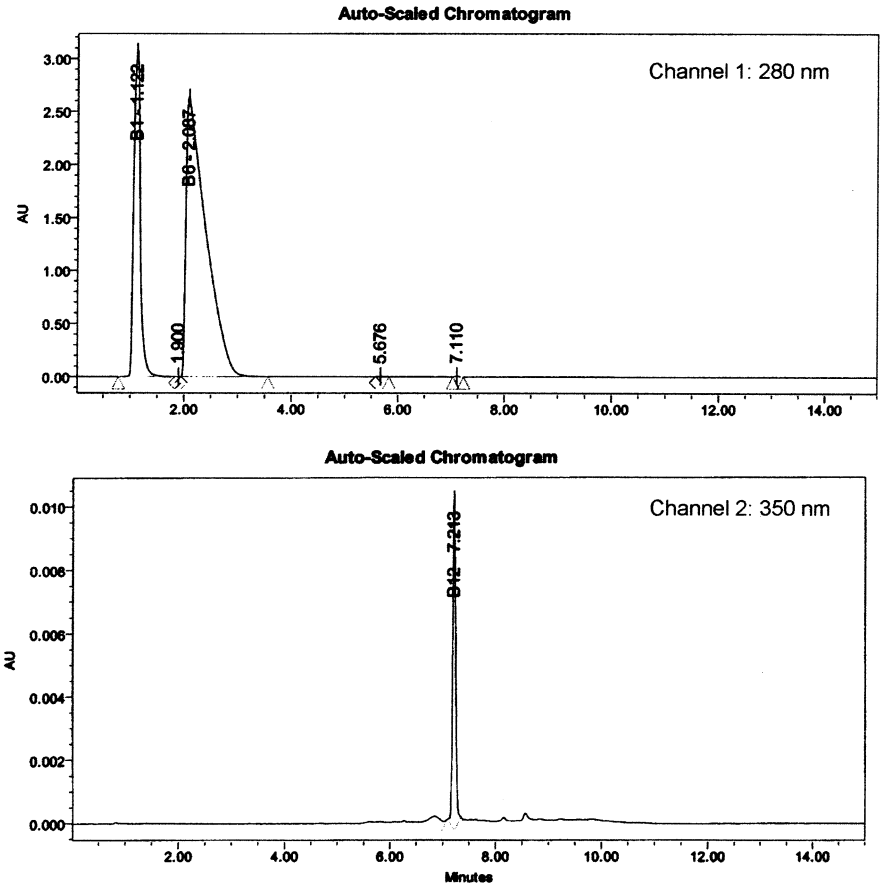

Fig. 1. Typical chromatograms showing resolution of thiamine hydrochloride (200 μg/ml), pyridoxine hydrochloride (200 μg/ml) and hydroxocobalamine chloride (0.2 μg/ml), respectively.

Table 2
High performance liquid chromatographic parameters of the drug separation, using the gradient elution system, $t_0 = 0.84$ min

Active ingredients	Chromatographic parameters			
	t_R (min)	k'	T	R_f
Thiamine–HCl	1.12	0.33	1.4	2.05 (B_1–B_6)
Pyridoxine–HCl	2.09	1.25	2	11.5 (B_6–B_{12})
Hydroxocobalamine–Cl	7.21	7.58	1.3	36.2 (B_1–B_{12})

lated from the equation $Rs = 2(t_2 - t_1)/W_1 + W_2$, where t_2, t_1 are the retention times of the two component and W_1, W_2 are the peak widths at the base of the two respective peaks, obtained by extrapolation of the relatively straight sides to the baseline. Other characteristic numerical values of the chromatographic parameters Rs, t_R and k' are shown in Table 2. It was also noticed that the mixed stock solutions yielded decomposition products within 4 h. The developed assay proved to be specific since these were well-separated as clearly shown in Fig. 2.

3.1. Linearity and system suitability

Six working solutions for each analyte in the range of 50–300 μg/ml for B_1, B_6 and 0.05–0.3 μg/ml for B_{12} were prepared. Analysis was performed in triplicate to determine the linearity of the assay. The regression lines were calculated by the method of least squares of peak areas versus analyte

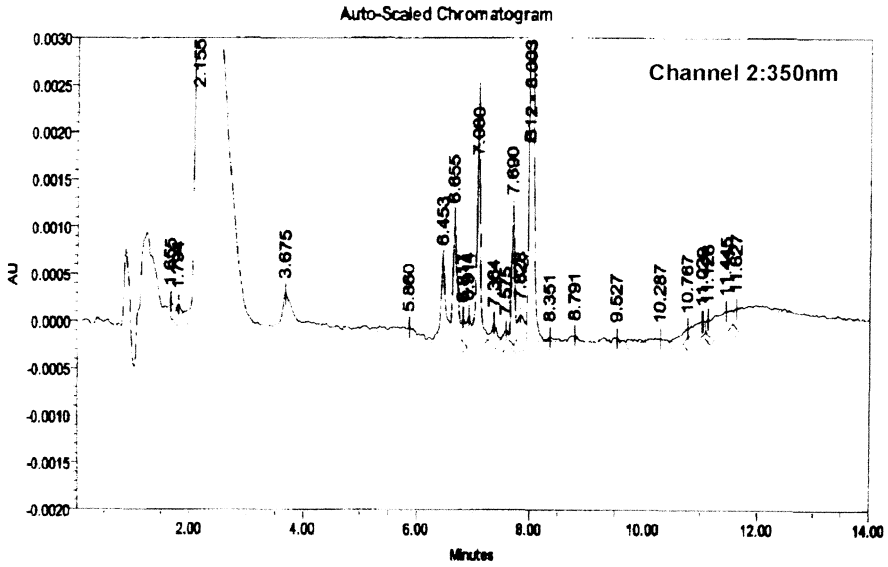

Fig. 2. The well-separated peaks of the degradation products derived within 4 h from the mixed stock standard solutions of the parent compounds using the developed chromatographic system.

concentrations. The equations corresponding to the three regression lines of the analytes were

$B_1, y = 35464330x + 273497;$
$B_6, y = 92229402x + 659983;$
$B_{12}, y = 60487x + 19.3.$

They were consistently linear in the already mentioned range for all compound. The determinations of the minimum detectable level LOD and minimum quantifiable level LOQ were defined as the signal-to-noise ratio 3:1 and 10:1 respectively based on the ICP guide line [21,22]. Other regression data for the compounds analyzed are presented in Table 3.

Also the system reproducibility—considering only the error contributed by the analytical method itself—was demonstrated: From an individual standard solution of the analytes, six replicates were injected onto the column, in five different days and the relative standard deviation (% RSD) was calculated. This for thiamin HCl (0.2 mg/ml) was in the range of 0.11–0.64%, for pyridoxine HCl (0.2 mg/ml) 0.06–0.39% and for hydroxocobalamine chloride (0.2 μg/ml) 0.25–1.17%.

3.2. Accuracy and precision

The accuracy of the described procedure was assessed by adding known amounts of vitamins, equal to one dosage form, to powdered placebo tablets prior to analysis in the presence of maize

Table 3
Concentration and peak area range, correlation data of the calibration curves and detection quantitation limits of the compounds determined

Compound	Concentration range, μg/ml	Range peak area	Correlation coefficient	Detection limits, μg/ml	Quantitation limits, μg/ml
Thiamine HCl	50–300	2 027 042–10 928 816	0.9999	0.014	0.046
Pyridoxine HCl	50–300	5 275 820–28 293 868	0.9998	0.005	0.017
Hydroxocobolamine–Cl	0.05–0.3	3044–18 161	0.9999	0.006	0.020

starch, PVP (kollidon N25), talc, magnesium stearate, eudragit E, aluminium hydroxide, sucrose. The recovery data was determined by comparing the areas obtained with that of standard solution analyzed according to the method. The results of the quantitation of thiamine hydrochloride (B_1), pyridoxine hydrochloride (B_6), and hydroxocobalamine chloride (B_{12}), 150, 150, 0.150 mg/tablet, respectively, in multivitamin placebo tablets are shown in Table 4. These were in good agreement with the added amount. Excipients present in the dosage forms did not interfere with the analysis (no noticeable interference from the excipients was observed in the chromatograms) whereas the recovery of the compounds from each dosage form was quantitative.

The precision of the method was studied by assaying one batch of multivitamin tablets on three occasions during 1 week. On each occasion 40 tablets were powdered and four determinations of each vitamin were made. To estimate the components of variance, the concentrations % found were subjected to analysis of variance (ANOVA) and expected mean squares for the 'one way classification-balanced design', Table 5 [23–25]. A one-tailed F-test with a probability of 0.05 was carried out to test whether the mean squares differed significantly. The critical value of F for two and nine degrees of freedom for B_1, B_6, B_{12} was 4.26. As the calculated value of F was not greater than the F critical value, for all the cases, the null hypothesis that the population variances are equal, was adopted.

4. Conclusion

In the preceding method for determination of vitamin B_1–B_6–B_{12} levels in tablets, use of a BDS-C_{18} column and a gradient elution mode was applied. This method is successful for vitamin B_1–B_6–B_{12} formulations even in the ratios between B_{12} and the others 1:100 or 1:1000. Isocratic conditions could not be used mainly because the chromatographic peaks are not well resolved. The method is inexpensive, simple and rapid with a high degree of accuracy and precision and applied successfully to the routine analysis of B_1–B_2–B_6 in B-complex tablets. Also it is characterized by the required sensitivity and selectivity to measure the analytes and their possible degradation products in pharmaceutical formulations.

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Table 4
Recovery study of the determination of placebo multivitamin tablets by adding 150 mg/tablet for vitamins B_1, B_6, and 0.15 mg/tablet for B_{12}

No. tablets	Thiamine HCl		Pyridoxine HCl		Hydroxocobalamine–Cl	
	Found mg/tab	Recovery %	Found mg/tab	Recovery %	Found mg/tab	Recovery %
1	147.8	98.5	146.9	97.9	153.9	102.6
2	147.9	98.6	148.4	98.9	146.6	97.7
3	149.1	99.4	152.6	101.7	146.3	97.5
4	147.9	98.6	151.2	100.8	152.9	101.9
5	147.5	98.3	149.9	99.9	147.5	98.3
6	150.0	100.0	153.0	102.0	149.6	99.7
7	149.4	99.6	154.2	102.8	148.4	98.9
8	148.5	99.0	150.8	100.5	147.9	98.6
9	149.7	99.8	146.4	97.6	147.9	98.6
10	150.0	100.0	147.6	98.4	145.4	96.9
Mean		99.2		100.0		99.1
% RSD		0.67		1.8		1.9

Table 5
Precision data of four powdered tablet samples, based on the analysis at three different days, for thiamine hydrochloride (B_1), pyridoxine hydrochloride (B_6) and hydroxocobalamine chloride (B_{12})

Day	Concentration % found (B_1)	Mean $\pm tS/\sqrt{N}$	%RSD		
1	101.4,101.3, 98.0, 99.7	100.1 ± 2.5	Between-day	1.3	
2	100.1, 98.7, 97.9, 100.4	99.3 ± 1.9	Within-day	1.2	
3	98.5, 98.6, 99.4, 98.6	98.8 ± 0.64	Total	1.2	
	Variance Estimate				
	Source of variation	Degrees of freedom	SS	MS	F-ratio
	Between-day	2	3.58	1.79	
	Within-day	9	12.39	1.38	1.30
	Total	11	15.98	1.45	
Day	Concentration % found (B_6)	Mean $\pm tS/\sqrt{N}$	%RSD		
1	101.2, 100.1, 100.0, 99.8	100.3 ± 0.9	Between-day	1.2	
2	101.6, 98.5, 96.1, 100.2	99.1 ± 3.8	Within-day	1.7	
3	97.9, 98.9, 101.7, 100.8	99.8 ± 2.7	Total	1.7	
	Variance Estimate				
	Source of variation	Degrees of freedom	SS	MS	F-ratio
	Between-day	2	2.81	1.4	
	Within-day	9	27.04	3.0	0.47
	Total	11	29.85	2.7	
Day	Concentration % found (B_{12})	Mean $\pm tS/\sqrt{N}$	%RSD		
1	99.2, 103.0, 99.8, 98.2	100.0 ± 3.3	Between-day	1.0	
2	99.6, 97.0, 100.2, 102.9	98.7 ± 2.3	Within-day	1.5	
3	98.3, 98.9, 98.6, 98.6.	98.9 ± 0.9	Total	1.5	
	Variance Estimate				
	Source of variation	Degrees of freedom	SS	MS	F-ratio
	Between-day	2	4.31	2.16	
	Within-day	9	20.43	2.27	0.95
	Total	11	24.75		

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