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TLC-Densitometric Determination of Vitamins B_1, B_6 and B_{12} in Pure and Pharmaceutical Formulations Using Treated Aleppo Bentonite

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Determination of vitamins B_1, B_6 and B_{12} in pure and in pharmaceutical formulations by thin layer chromatography (0.30 mm thickness) using acid treated Aleppo bentonite and mobile phase consisted of iso-butyl alcohol:methanol:chloroform:acetic acid:ammonia (7.5:1:3.5: 0.4:0.5, v/v/v/v/v)has been applied. The specific surface area of bentonite was 168.25 m^2/g and mean pore size was 52.3 Å determined using nitrogen adsorption (BET). Quantification was carried out densitometerically at λ = 275 nm for vitamins B_1, B_6 and B_{12} (if their concentrations are in close proximity) and at λ = 525 nm for vitamin B_{12} (when $C_{B_6} \approx C_{B_1} >> C_{B_{12}}$ as in pharmaceutical products). This method is successful for vitamin B_1, B_6 and B_{12} formulation even in the ratios between vitamin B_{12} and others 1:100. The retardation factors (R_f) of B_1, B_6 and B_{12} were 0.37, 0.68 and 0.26, respectively. Calibration curves were obtained in the range of 5.0-40.0 µg/spot for vitamins B_1, B_6 and B_{12} (at λ = 275 nm) for standard solutions, while for pharmaceutical products, vitamins B_1, B_6 were determined at λ = 275 nm and vitamin B_{12} was determined at λ = 525 nm in the range of 0.5- 4.0 µg/spot.

Key Words: Aleppo bentonite, BET, Thin layer chromatography, Vitamins B_1, B_6 and B_{12}.

INTRODUCTION

Aleppo bentonite is rocky clay which originates from volcanic ash and consists of 47 % SiO_2, 14.4 % Al_2O_3 and some other oxides as Fe_2O_3, MgO, CaO and Na_2O[1,2]. Bentonite has large pore volumes and high specific surface area[3]. The thermal treatment causes decreasing of its specific surface area with increasing in the temperature of thermal treatment[4]. Bentonite clays are used in many industrial products and processes, drilling fluids, a certain lubricating grease[5,6] and it can be used as chromatographic supports in gas chromatography to separate many mixtures after grafting with different methods[7,8]. Bentonite is used as stationary phase in thin layer chromatography to separate some metal ions[9].

Vitamins are organic compounds present mostly in food and are needed in very small amounts for various metabolic processes and other functions[10]. The two types of vitamins are named as fat-soluble and water soluble. The water-soluble vitamins are thiamine (vitamin B_1), riboflavin (vitamin B_2), niacin, pyridoxine (vitamin B_6),

folate, vitamin B_{12}, biotin and pantothenic acid. These vitamins are widely distributed in foods. They function as coenzymes that help the body obtain energy from food. They are also important for normal appetite, good vision, healthy skin, healthy nervous system and red blood cell formation[11].

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 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[12], higher order derivative spectrophotometry[13] and capillary electrophoresis[14]. Also, the determination of the B-complex mainly in tablets have been described extensively using HPLC methods[15,16] or HPLC after solid-phase extraction[17]. 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 but are 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[18,19].

EXPERIMENTAL

Surface area and pore size measurement (BET) were recorded using a Micromeritics Gemini III 2375 under nitrogen atmosphere (USA). Scanner-densitometer CD60 (Desega, Germany) equipped with mercury, tungsten and deuterium lamps, CAMAG Hand Operated TLC Coater for preparation of TLC plates (Switzerland), CAMAG UV Cabinet for assessing and marking thin layer chromatograms under UV light (Switzerland) and different size of syringe (Hamilton, Switzerland) were used.

Thiamine hydrochloride (vitamin B_1) (99.0 %, Jiangsu, China), pyridoxine hydrochloride (vitamin B_6) (99.6 %, Sinochem, China) and cyanocobalamine (vitamin B_{12}) (99.1 %, Hebei, China) were used. Methanol, chloroform, acetic acid were of analytical grade, Merck, Germany. Ammonia solution and iso-butyl alcohol were of analytical grade, BDH, England.

Preparation of stationary phase: Bentonite was crushed to obtain small pieces, which have diameter less than 45 μm, followed by washing with concentrated hydrochloric acid at boiling point for 30 h to remove soluble oxides especially iron oxide. Then it was washed several times with distilled water and dried at 120 °C for 3 h.

Preparation of TLC plates: For preparation of thin layer chromatography, 8.8 g treated bentonite was mixed with 0.7 g fluorescence substance (F_{254}), then the mixture was added to 20 mL hot water containing 0.5 g corn starch as binder to obtain homogeneous slurry. The slurry was spread over glass plates by an applicator to form uniform thin layer 0.30 mm thick. The plates were dried at 105 °C.

Mobile phase: Iso-butyl alcohol:methanol:chloroform:acetic acid:ammonia (7.5:1:3.5:0.4:0.5, v/v/v/v/v) were used for the development method as mobile phase.

Standard solutions: Stock solution ($\mathbf{A_i}$) was prepared by dissolving 2.5 g of each vitamins B_1, B_6 and B_{12} in 25 mL mixture of distilled water and methanol (1:1), then transferred into a 50 mL volumetric flask and the final volume was completed to 10 mL with the same mixture of solvents. Volumes 1, 2, 3, 4, 5, 6, 7 and 8 mL from the former solution were transferred into 50 mL volumetric flasks and completed to the mark with the same mixture of solvents (these solutions content: 5, 10, 15, 20, 25, 30, 35 and 40 mg mL^{-1}, respectively for each vitamin).

Stock solution ($\mathbf{B_i}$) was prepared by dissolving 12.5 g of each vitamins B_1 and B_6 and 0.1250 g of vitamin B_{12} in 40 mL mixture of distilled water and methanol (1:1), then transferred into a 50 mL volumetric flask and the final volume was completed to 50 mL with the same mixture of solvents. Volumes 1, 2, 3, 4, 5, 6, 7 and 8 mL from former solution were transferred into 10 mL volumetric flasks and completed to the mark with the same mixture of solvents (these solutions content: 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 mg mL^{-1} of vitamin B_{12}).

Sample preparation: Twenty tablets were weighed and the average tablet weight determined (each tablet contains: 100 mg B_1, 100 mg B_6 and 1 mg B_{12}). The tablets were finely powdered and a portion of powder equivalent to the weight of eight tablets was dissolved in 20 mL mixture of distilled water and methanol (1:1) and vigorously shaken for a 20 min on a mechanical shaker, then filtrated and transferred into a 25 mL volumetric flask and the final volume was completed to 25 mL with the same mixture of solvents. This solution ($\mathbf{T_i}$) contains: 32 mg B_1 mL^{-1}, 32 mg B_6 mL^{-1} and 0.32 mg B_{12} mL^{-1}).

The ampoules were directly used for analysis (each ampoule contains: 100 mg B_1, 100 mg B_6 and 1 mg B_{12} in 3 mL).

Procedure (chromatographic conditions): 1 µL of standard solutions $\mathbf{A_i}$ were spotted on TLC-glass plates 20 cm × 10 cm pre-coated treated bentonite (F_{254} with 0.30 mm thickness). Mobile phase were used for development method, then the plates were dried at room temperature and quantification was carried out densitometerically at λ = 275 nm for vitamins B_1, B_6 and B_{12}. This process was repeated five times for each concentrations and calibration curves were obtained in the range 5-40 µg/spot for B_1, B_6 and B_{12}.

2 µL of standard solutions $\mathbf{B_i}$ were spotted on TLC-glass plates 20 cm × 10 cm pre-coated treated bentonite (F_{254} with 0.30 mm thickness). Mobile phase were used for development method, then the plates were dried at room temperature and quantification was carried out densitometerically at λ = 525 nm for vitamin B_{12}. This process was repeated five times for each concentrations and calibration curves were obtained in the range 0.50-4.00 µg/spot for B_{12}.

Pharmaceutical formulations (tablets and ampoules): 1 µL of solution ($\mathbf{T_i}$), for tablets or 1 µL for ampoules were spotted on TLC-glass plats for determination of vitamins (B_1 and B_6) and quantification was carried out densitometerically at λ = 275 nm. The concentrations were calculated from standard curves in the range 5-40

μg/spot for B_1 and B_6. 2 μL of solution ($\mathbf{T_i}$), for tablets or 2 μL for ampoules were spotted on TLC-glass plates for determination of vitamin B_{12} and quantification was carried out densitometerically at $\lambda = 525$ nm. The concentrations were calculated from standard curves in the range 0.50-4.00 μg/spot for vitamin B_{12}.

RESULTS AND DISCUSSION

Surface properties of treated bentonite: Surface areas of treated bentonite was determined by the adsorption of nitrogen at 77 K (BET). For determination of textural properties, the adsorption was carried out until near saturation (P/Po ≈ 1.0), then the desorption was completed until closure of the hysteresis loop. Representative adsorption-desorption isotherms of nitrogen are shown in Fig. 1. The isotherms are IV type of Sing (1982) and BDDT (1940) classifications, which indicate the presence of mesoporous structure. Application of the linear BET equation to the nitrogen adsorption data obtained was within the range of relative pressures (0.02-0.25). The representation linear BET plots are shown in Fig. 2. From these plots, it is found that the BET surface areas (S_{BET}) was 168.25 m^2/g. The data also allows the determination of pore volume distribution (Fig. 3). The total pore volume v_p (0.440 mL/g) was determined from the adsorbed volume at P/Po = 0.95 in the liquid form. The mean pore radii r_a (52.3 Å), were determined from the equation: $r_a = 2 \times 10^4 \times v_p/S_{BET}$. Results of surface properties for treated bentonite can be showed in Table-1.

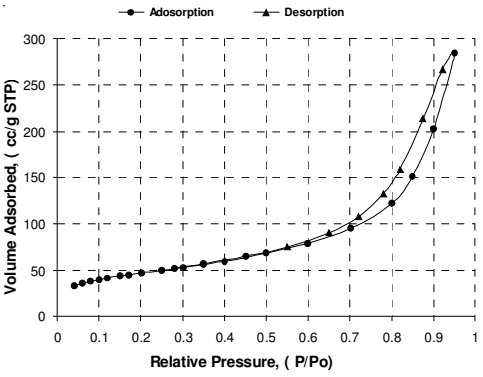

Fig. 1. Adsorption-desorptoin isotherm of nitrogen at 77 K on treated bentonite

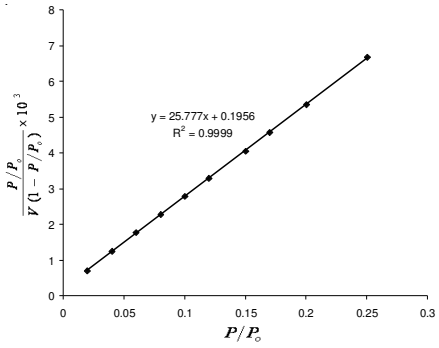

Fig. 2. BET plots for treated bentonite

TABLE-1
SURFACE PROPERTIES OF TREATED ALEPPO BENTONITE

S_{BET} (m^2/g)	v_p (mL/g)	r_a (Å)
168.25	0.440	52.3

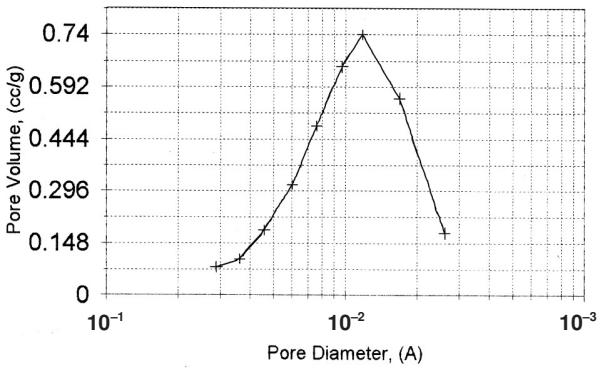

Fig. 3. Pore volume distribution of treated bentonite

Chromatograms processing: The position of the spots from the front on the chromatographic plate for different concentrations (5 to 40 μg/spot) of B_1, B_6 and B_{12} at $\lambda = 275$ nm was shown in Fig. 4.

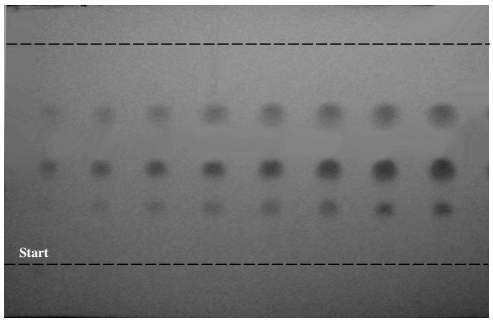

Fig. 4. TLC plate of standard vitamin B_1, B_6 and B_{12} for concentrations of each vitamin 5, 10, 15, 20, 25, 30, 35 and 40 μg/spot (mobile phase: iso-butyl alcohol:methanol:chloroform: acetic acid:ammonia 7.5:1:3.5:0.4:0.5 v/v/v/v/v)

The chromatogram of mixture of B_1, B_6 and B_{12} (20 μg/spot for each vitamin) can be observed with three peaks at different wavelengths (λ) at 235 to 315 nm (Fig. 5). The first peak for B_{12} increase to $\lambda = 275$ nm then decrease, the second for B_1 decrease with increasing of λ and the third for B_6 increase to $\lambda = 275$ nm then sharply decrease (Fig. 6). It is infered from the Figs. 4 and 5 that, the best wavelength to determine the vitamins B_1, B_6 and B_{12} is 275 nm. The retardation factors (R_f) of B_1, B_6 and B_{12} were 0.37, 0.68 and 0.26, respectively.

It is seen from the previous study that the peak areas of the three vitamins at the wavelength 275 nm were convergent, while showing only peak of vitamin B_{12} at the wavelength 525 nm. When the concentration of vitamins B_1 and B_6 very larger than the concentration of vitamin B_{12} (as in pharmaceutical formulations: $C_{B_6} = C_{B_1} = 100\ C_{B_{12}}$) become identified vitamin B_{12} very difficult and interfere with the peak of vitamin B_1. Therefore be possible to select the wavelength at $\lambda = 525$ nm.

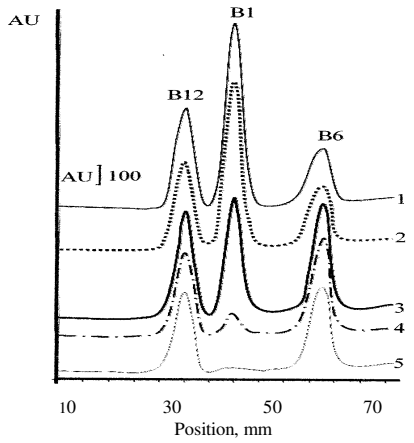

Fig. 5. Chromatograms of mixture of B_1, B_6 and B_{12} (20 µg/spot) disposed at different wavelengths (λ) at 235 to 315 nm: 1- λ = 235 , 2- λ = 255, 3- λ = 275, 4- λ = 295 and 5- λ = 315 nm

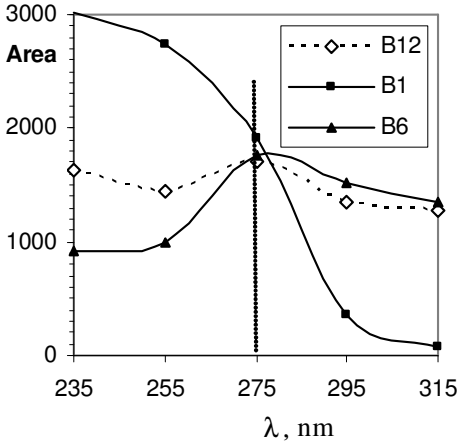

Fig. 6. Effect of wavelengths (λ) on peak areas of vitamins B_1, B_6 and B_{12} by TLC-densitometric method using acid treated Aleppo bentonite and mobile phase: iso-butyl alcohol:methanol:chloroform:acetic acid: ammonia (7.5:1:3.5:0.4:0.5, v/v/v/v/v) ($C_{B_6} = C_{B_1} = C_{B_{12}}$ = 20 µg/spot)

Quantitative evaluation: The method being validated through precision, linearity and accuracy for the determination of different standard mixtures of vitamins B_1, B_6 and B_{12} in the range of 5.0 to 40.0 µg/spot using λ = 275 nm (Fig. 7, Table-2). Determination of vitamin B_{12}, when $C_{B_1} >> C_{B_{12}}$; when it is not possible to use the wavelength 275 nm, the wavelength 525 nm was uses (only the peak of B_{12} appears). The calibration curve for vitamin B_{12} at λ = 525 nm was studied. It was found that the equation of this curve in the concentration range of 0.50 to 4.0 µg/spot was as the following: $y = 158.11x + 10.902$ and $R^2 = 0.9996$.

Application: The results of the validation varify the fitness of the proposed analytical procedure for the identification and quantitative determination of the three vitamins in the mixture. Two different commercial products (that contained of the three studied vitamins were analyzed in order to assign the values of their contents using λ = 275 for B_1 and B_6 and λ = 525 nm for B_{12}, because $C_{B_6} \approx C_{B_1} >> C_{B_{12}}$. The pharmaceutical formulations were selected for the study as the following:

Tablets: Each tablet contains 100 mg vitamin B_1, 100 mg vitamin B_6 and 1 mg (1000 µg) vitamin B_{12} (+ 20 % over dose of all vitamins for stability) of brand Vitaneurin Fort (manufactured by Asia Pharmaceutical Industries, Aleppo-Syria).

Ampoules: Each ampoule contains 100 mg vitamin B_1, 100 mg vitamin B_6 and 1 mg (1000 µg) vitamin B_{12} (+ 20 % over dose of all vitamins) in 3 mL of brand Vitaneurin Fort (manufactured by Asia Pharmaceutical Industries, Aleppo-Syria).

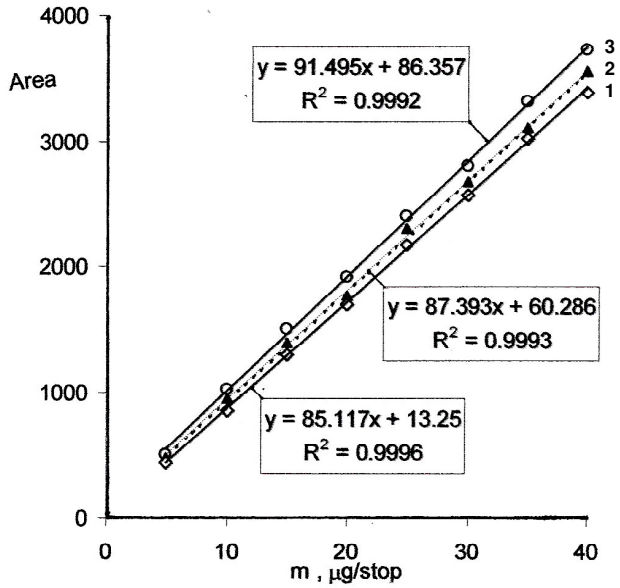

Fig. 7. Calibration curves for determination of vitamins B_1 (3), B_6 (2) and B_{12} (1) in pure forms by TLC-densitometric method using acid treated Aleppo bentonite and mobile phase: iso-butyl alcohol:methanol:chloroform:acetic acid:ammonia (7.5:1:3.5:0.4:0.5, v/v/v/v/v) at λ = 275 nm

The three vitamins in the mentioned pharmaceutical formulations were simultaneously evaluated, on the same plate, reducing the time and the amount of the materials required for the analysis. The obtained results and their confidence intervals are listed in Table-3.

The results are in good agreement with the results of HPLC[20]. It can be observed that the difference between the results by HPLC[20] and the found values by this method are less than 5 % in the worst case and the relative standard deviation is did not exceed ± 4.8 %. The proposed method has been successfully applied to determine vitamins B_1, B_6 and B_{12} formulation even in the ratios between vitamin B_{12} and others 1:100.

Conclusion

In the preceding method, determination of vitamins B_1, B_6 and B_{12} in pure and pharmaceutical formulations by TLC-densitometric method using acid treated Aleppo bentonite and mobile phase: iso-butyl alcohol:methanol:chloroform:acetic acid:ammonia (7.5:1:3.5:0.4:0.5, v/v/v/v/v) has been applied. Quantification was carried out densitometerically at λ = 275 nm for vitamins B_1, B_6 and B_{12} (if their concentrations were close) and at λ = 525 nm for vitamin B_{12} (when $C_{B_6} \approx C_{B_1} >> C_{B_{12}}$ as in pharmaceutical formulations). This method is successful for determination of vitamins B_1, B_6 and B_{12} formulation even in the ratios between vitamin B_{12} and others 1:100. The retardation factors (R_f) of B_1, B_6 and B_{12} were 0.37, 0.68 and 0.26,

TABLE-2
DETERMINATION OF VITAMINS B_1, B_6 AND B_{12} IN PURE FORMS BY TLC-DENSITOMETRIC METHOD USING ACID TREATED ALEPPO BENTONITE AT λ = 275 nm (MOBILE PHASE: ISO-BUTYL ALCOHOL:METHANOL: CHLOROFORM:ACETIC ACID:AMMONIA 7.5:1:3.5:0.4:0.5 v/v/v/v/v)

Taken standard m (µg/spot)	Vitamin	Found $\overline{m}$ * ± SD (µg/spot)	RSD (%)	$\frac{SD}{\sqrt{n}}$ (µg/spot)	$\overline{m} \pm \frac{SD}{\sqrt{n}} \times t$ (µg/spot)	Recovery (%)
5.00	B_1	4.83±0.15	3.1	0.067	4.83±0.19	96.7
	B_6	4.96±0.13	2.6	0.058	4.96±0.16	99.2
	B_{12}	4.88±0.17	3.5	0.076	4.88±0.21	97.6
10.00	B_1	10.14±0.30	3.0	0.134	10.14±0.37	101.4
	B_6	9.93±0.26	2.6	0.116	9.93± 0.32	99.3
	B_{12}	9.94±0.34	3.4	0.152	9.94±0.42	99.4
15.00	B_1	15.20±0.44	2.8	0.197	15.86±0.55	101.3
	B_6	15.02±0.36	2.3	0.161	15.02±0.45	100.1
	B_{12}	14.80±0.49	3.3	0.218	14.80±0.61	98.7
20.00	B_1	19.86±0.50	2.5	0.224	19.86±0.62	99.3
	B_6	20.10±0.40	2.0	0.180	20.10±0.50	100.5
	B_{12}	20.07±0.62	3.1	0.278	20.07 ±0.77	100.3
25.00	B_1	24.87±0.57	2.3	0.255	24.87±0.71	99.5
	B_6	25.23±0.48	1.9	0.215	25.23±0.60	100.9
	B_{12}	24.92±0.75	3.0	0.334	24.92±0.93	99.7
30.00	B_1	29.89±0.66	2.2	0.294	29.89±0.82	99.6
	B_6	30.17±0.58	1.9	0.259	30.17±0.72	100.6
	B_{12}	29.93±0.90	3.0	0.402	29.93±1.12	99.8
35.00	B_1	34.49±0.86	2.5	0.386	34.49±1.07	98.5
	B_6	34.52±0.72	2.1	0.322	34.52±0.89	98.6
	B_{12}	34.46±1.14	3.3	0.510	34.46±1.41	98.5
40.00	B_1	38.62±1.27	3.2	0.570	38.62±1.58	96.6
	B_6	39.01±0.94	2.4	0.421	39.01±1.17	97.5
	B_{12}	38.24±1.83	4.8	0.821	38.24±2.28	95.6

*n = 5, t = 2.776.

TABEL-3
DETERMINATION OF VITAMINS B_1, B_6 AND B_{12} IN PHARMACEUTICAL FORMULATIONS BY TLC-DENSITOMETRIC METHOD USING ACID TREATED ALEPPO BENTONITE AT λ = 275 nm FOR B_1 AND B_6 AND λ = 525 nm FOR B_{12} (MOBILE PHASE: ISO-BUTYL ALCOHOL:METHANOL:CHLOROFORM: ACETIC ACID:AMMONIA 7.5:1:3.5:0.4:0.5 v/v/v/v/v)

Product	Compound and dose	Found $\overline{m}$ * ± SD (µg/spot)	RSD (%)	$\frac{SD}{\sqrt{n}}$ (µg/spot)	$\overline{m} \pm \frac{SD}{\sqrt{n}} \times t$ (µg/spot)	Recovery (%)
Vitaneurin Fort (Tablets)	B_1 100 mg/tab.	113.0±3.630	3.2	1.620	113.0±4.500	113.0
	B_6 100 mg/tab.	114.1±3.420	3.0	1.530	114.1±4.250	114.1
	B_{12} 1 mg/tab.	1.197±0.051	4.3	0.023	1.197±0.064	119.7
Vitaneurin Fort (Ampoules)	B_1 100 mg/amp.	115.5±3.580	3.1	1.600	115.5±4.440	115.5
	B_6 100 mg/amp.	116.2±3.250	2.8	1.450	116.2±4.020	116.2
	B_{12} 1 mg/amp.	1.198±0.050	4.2	0.022	1.198±0.061	119.8

*n = 5, t = 2.776.

respectively. Calibration curves were obtained in the range of 5.0- 40.0 μg/spot for vitamins B_1, B_6 and B_{12} (at λ = 275 nm) for standard solutions, while for pharmaceutical formulations, vitamins B_1, B_6 were determined at λ = 275 nm and vitamin B_{12} was determined at λ = 525 nm in the range of 0.5-4.0 μg/spot.

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