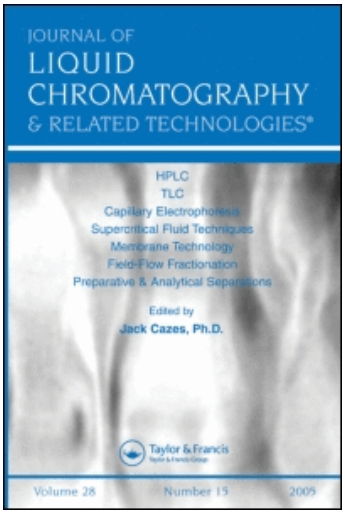

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DETERMINATION OF DIFFERENT SOLUBILITY VITAMINS IN PHARMACEUTICAL PREPARATIONS. II. METHODS VALIDATION

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ABSTRACT

Validation of methods for quantitative determination of vitamins in multivitamin preparations was performed. It was confirmed that tested methods are suitable for determination of both nonpolar vitamins (fat-soluble: A, D_3 and E) and polar vitamins (water-soluble: B_1, B_2, B_6, C, and PP). Determinations were quick and selective; they allowed a separation of peaks of vitamins to the baseline within 25 minutes. The ranges of concentration taken through the validation procedure were similar to daily doses of vitamins proposed by the Food and Drug Administration (FDA), and they were within the range of vitamin concentrations in common pharmaceutical preparations. In these ranges of concentration, the calibration graphs were linear (cor-

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relation coefficient r from 0.990 to 0.999). The repeatability of the methods (RSD) varied between 1.4 and 4.5% for fat-soluble vitamins and between 1.6 and 2.6% for water-soluble vitamins. The recoveries of added vitamins were between 98.1 and 100.4% for fat-soluble vitamins, and 97.2 and 99.0% for water-soluble vitamins. The sample solutions were stable for twenty-four hours after the preparation.

The obtained results show that these methods are usable for automatic quantitative determination of vitamins in pharmaceutical preparations for quality control purposes.

INTRODUCTION

Proposals of many parameters, which characterize analytical methods, can be found in the literature. There are such terms as: accuracy, precision (repeatability and reproducibility), specificity, linearity, detection limit, quantitation limit, robustness, ruggedness, range, sensitivity, bias, matrix effect, recovery, effort and cost.[1–5] The first three characteristics are mentioned in most publications (about 20%), the next two are in 10% of the publications, and the three following are in 3% of publications.[2]

The basic aim of the validation is to assure the appropriate reliability of the results of analysis and acceptability of the results derived from different laboratories. According to the present demands, validation takes into consideration the whole analytical method and not only one selected parameter. The problem of validation is increased vagueness and insinuations, in the case of many suggestive characteristics of analytical methods, and differences in the ways of their realization, as well as the lack of clear common criteria that would be accepted by different official organizations. Laboratories in the pharmaceutical industry proceed along guidelines given in USP,[6] as well as in a validation guide published by the commission of SFTP,[7] and ICH[8,9] documents. In the controlled methods that are applied in the pharmaceutical commodities, determination of accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity, range, and robustness are recommended. The choice of the mentioned characteristics depends on the aim and on the sample for which the method will be applied.[8]

Errors in the results of vitamins determination depend on: vitamin concentration, dosage form, matrix effects, method of sample preparation, and the conditions of the chromatographic analysis. For preparations that have a simple matrix, the errors are usually small and are contained between 1 to 5%, but they increase to 15% and more when the matrix is strongly complicated. Water-soluble vitamins are usually directly determined after dissolution or, in the case of

a more complex matrix, after preliminary purification of the samples by SPE. The accuracy and precision of the methods, in which the vitamins are determined directly after simple dissolution of the sample, are very good. Reported recoveries are between 97.4–105.4% when the precision (RSD) varied from 0.1 to 2.9%[10] or 99.7–100% when the RSD was below 0.6%.[11] According to another source, the precision of method (RSD) was contained between 1.3–3.0%.[12] Poorer parameters characterized methods in which SPE was applied. For example, recovery was between 98.3–116% when the precision (RSD) of the method was in the range of 1.7–6.2%.[13]

During determination of the fat-soluble vitamins, the samples are usually first dissolved in a water–alcohol mixture, and then they are extracted for the purpose of separation of the vitamins from the matrix. Generally, the liquid–liquid extraction or SPE is most often applied, whereas SFE was rarely used. The accuracy of the method, which applies the liquid–liquid extraction, is in the range 94.7–103%[14] or 96.2–117% when precision (RSD) is between 1.1–5.1%.[15] In the case of the methods that apply SPE, the referred accuracy was between 78–100%, and the precision (RSD) was between 1.64–2.31%.[13] Methods, which apply SFE, showed similar results. One of these published methods had recovery between 97.8–110.1%, and the precision (RSD) varied from 1.7–3.9%.[16]

The aim of the present work was the validation of the methods of vitamin determination presented in the first part of this work[17] in compliance with international standards.

EXPERIMENTAL

Materials

Standard substances: vitamin A (retinol palmitate 250,000 I.U./g), vitamin D_3 (cholecalciferol 100,000 I.U./g), vitamin E (d, l-alpha-tocopheryl acetate 50%), vitamin B_1 (thiamine mononitrate), vitamin B_2 (riboflavin), vitamin B_6 (pyridoxine hydrochloride), vitamin C (ascorbic acid) (BASF, Ludwigschafen, Germany) and vitamin PP (nicotinamide) (Hoffmann LaRoche, Basel, Switzerland).

Solvents of purity for isocratic HPLC were: acetonitrile, acetic acid (J. T. Baker, Deventeer, Netherlands), ethyl acetate (E. Merck, Darmstadt, Germany), and n-hexane (Fisons, Loughborough, England). Water for HPLC purposes was prepared in the laboratory using the Elix 5 system (Millipore, Bedford, USA).

Other reagents: phosphoric acid 85% (POCh, Gliwice, Poland), hexane-1-sulfonic acid sodium salt and triethylamine (Merck), and ethanol 96% were of analytical grade.

Equipment

For sample preparation, the shaker model WU-4 (Premed, Warsaw, Poland), rotary evaporator model RE-111 (Büchi, Flawil, Switzerland), and water bath shaker Model 357 (Conbest, Warsaw, Poland) were used.

Chromatographic analysis was performed using a liquid chromatograph (Waters, Milford, USA) consisting of a quaternary gradient pump 60F with controller 600, autosampler 717 plus, and diode array detector PDA 996. The chromatograph was equipped with a two-position motorized valve TPMV 7750 (Rheodyne, Berkeley, USA). Data were recorded by means of a computer with software Millennium 2.10 (Waters).

Two different types of chromatographic columns were used: for fat-soluble vitamins, LiChrosorb RP-18 5 μm, 250 × 4 mm (Merck) and for water-soluble vitamins, Bakerbond BDC C_{18} 250 × 4.6 mm (J. T. Baker).

Standard Solutions

About 20 mg of vitamin A (250,000 I.U./g), 10 mg of vitamin D_3 (100,000 I.U./g), and 35 mg of vitamin E (50%) weighed accurately, were placed into a 300 mL Erlenmeyer flask. Then 25 mL 0.1 M solution of phosphoric acid was added, and next 25 mL of ethanol, and exactly 50.0 mL of n-hexane. The mixture was shaken for 30 minutes and left for 5 minutes to separate both layers. 25 mL portion of the clear hexane fraction was taken and evaporated to dryness on the rotary evaporator. The residue was dissolved in 3 mL of acetonitrile–ethyl acetate mixture (80 : 20, v/v).

For standard stock solutions of water-soluble vitamins 30 mg of vitamin B_1, 15 mg of vitamin B_2, 30 mg vitamin B_6, and 250 mg of vitamin PP weighed accurately, were carried into 100 mL calibrated flasks. About 60 mL of the mixture: water–acetonitrile–acetic acid (94 : 5 : 1, v/v/v) was added, and the vessels were shaken for 15 minutes, except vitamin B_2, which was shaken in a water bath with a temperature of 70°C. After cooling to room temperature, the volumes were adjusted to 100 mL with the same mixture. For working standard solution preparation, 1 mL of vitamins B_1, B_6, PP, 2 mL of vitamin B_2 standard stock solutions, and 10 mg of vitamin C were placed in a 10 mL calibrated flask, filled to volume with mixture of water–acetonitrile–acetic acid (94 : 5 : 1, v/v/v), and mixed. The concentration of vitamins in standard solution were: B_1 0.03 mg/mL, B_2 0.03 mg/mL, B_6 0.03 mg/mL, PP 0.25 mg/mL, and C 1 mg/mL.

Sample Preparation

For the determination of fat-soluble vitamins, 5 g of the dry multivitamin preparation, weighed accurately, were placed in a 300 mL Erlenmeyer flask, then

25 mL 0.1 M of phosphoric acid, 25 mL of ethanol, and exactly 50 mL of hexane were added. The mixture was shaken within 30 minutes and then was left for 5 minutes to separate the phases. 25 mL portions of the hexane layer were taken and evaporated to dryness on a rotary evaporator. The residues were dissolved in 3 mL of the mixture acetonitrile–ethyl acetate (80 : 20, v/v).

For determination of water-soluble vitamins, 3 g portions of the same preparation were placed in a 50 mL flask, and then about 25 mL of the mixture water–acetonitrile–acetic acid (94 : 5 : 1, v/v/v) was added. The samples were shaken for 15 minutes in a water bath with a temperature of 70°C. The solutions were then cooled to room temperature and filled to volume with the same mixture.

Chromatographic Analysis

For determination of fat-soluble vitamins, a Lichrosorb RP-18 5 μm 250 × 4 mm column (Merck) was used. Mobile phase was the acetonitrile–ethyl acetate (85 : 15, v/v) mixture with the addition of 0.1 mL of triethylamine to 1 L. Flow rate was 1.5 mL/min, injection volume 50 μL, and detector UV-Vis λ 254 nm.

Analyses of water-soluble vitamins were performed using Bakerbond BDC C_{18} (Baker), 5 μm 250 × 4.6 mm analytical column. The mobile phase was water–methanol–acetic acid (730 : 270 : 10, v/v/v) mixture with the addition of 1.4 g hexane-1-sulfonic acid sodium salt and 0.3 mL triethylamine to 1 L. The flow rate was 1.0 mL/min, injection volume-20 μL, detector UV-Vis λ-280 nm.

Table 1. Comparison Between Vitamins Concentration in Tested Samples, Recommended Daily Allowances and Concentration in Preparations Available on the Polish Market

Vitamin	Unit	Concentration in Tested Samples (in 2 g)	Recommended Daily Allowances, FDA	Concentration in Commercially Available Prep.
A	IU	963.7–3867	1250–4 333	700–25 000
D_3	IU	271–752	200–400	50–1000
E	mg	2.89–13.71	3.3–13	0.5–50
B_1	mg	0.54–2.14	0.3–1.6	0.25–20
B_2	mg	0.55–2.30	0.4–1.8	0.3–15
B_6	mg	0.60–2.42	0.3–2.2	0.3–15
PP	mg	3.37–18.81	5–20	2.5–100
C	mg	27.2–116.38	30–90	10–600

Validation Samples

Five samples, containing vitamins in concentrations comprising the full range of application of the method, were prepared (Table 1). The analysis of each sample was performed thrice, and the repetitions were performed during three successive days. Fresh standard solutions were prepared every day. Moreover, the analysis of placebo, excipients, and raw materials of vitamins were performed. In order to estimate stability of the sample, chromatographic analysis of the sample solution was performed several times within 24 hours.

RESULTS AND DISCUSSION

Chromatographic Analysis

In conditions proposed for fat-soluble vitamin analysis, symmetrical peaks of the vitamins A and D_3 were obtained. Symmetry factor (f_{As}) (calculated as the ratio of the peak width from the peak end to the peak width, from peak start at 10% of the peak height) was 1.11 and 1.16 for the above mentioned vitamins. The worse symmetry, with the symmetry factor 2.11, was obtained for the vitamin E peak. Good peak area repeatability was obtained for the vitamins A and E, with relative standard deviation (RSD) not more than 1%. Somewhat worse repeatability for the vitamin D_3 was obtained with RSD 1.78%. Chromatographic system performance was about 10,000 theoretical plates (N/column), in comparison with 12,500 N per column (50,000 N/m) declared by the column manufacturer (Table 2).

Table 2. Parameters of the Chromatographic Systems

Analyte	Retention Time, T_r [min]	Number of Theoretical Plates, N	Peak Area Repeatability (RSD) n = 6	Resolution, R_s
A	13.25	12,904	0.99	6.3 (from D_3)
D_3	8.21	11,103	1.78	4.3 (from E)
E	6.43	8080	1.00	–
B_1	15.28	14,661	0.20	12.0 (from vanilin)
B_2	8.05	10,261	0.17	4.4 (from B_6)
Vaniline	9.11	13,942	–	2.2 (from B_2)
B_6	6.33	11,687	0.17	7.1 (from PP)
PP	4.05	8831	0.18	4.0 (from C)
C	2.99	2182	0.22	–

Water-soluble vitamins were analyzed in reverse phase systems using ion pair technique. For all of the tested vitamins, including basic substances such as vitamin B_1 and B_6, symmetrical peaks were achieved. The symmetry factor for all of the vitamins was not more then 1.20. All vitamins had excellent peak area repeatability with relative standard deviation not more then 0.25%. Chromatographic system performance was about 10,000 theoretical plates per column for all of the vitamins, except vitamin C for which 2000 theoretical plates were obtained. The total time of the one chromatographic analysis in both cases was not longer than 25 minutes (Table 2).

Validation

According to commonly accepted rules,[20] first, the imprecision of the methods were verified, and then the parameters of the methods' accuracy: linearity, recovery, selectivity, and ruggedness, measured as analyte stability, were tested.

Precision

The method was elaborated for internal control purposes and, therefore, only the measures of repeatability, i.e., within-day imprecision and between-day imprecision were determined (Table 3).

Table 3. Methods Precision: Repeatability (Within-Day Imprecision), Between-Day Imprecision and Uncertainty

	Repeatability		Between-Day Imprecision	
Vitamin	RSD, % (n = 15)	Uncertainty (*)	RSD, % (n = 15)	Uncertainty (*)
A	4.3	9.22	–	–
D_3	4.5	9.98	4.6	10.14
E	1.4	3.10	2.2	4.73
B_1	2.3	5.10	4.1	9.18
B_2	1.6	3.47	2.8	6.14
B_6	2.6	5.67	3.9	8.62
PP	2.2	5.15	2.6	5.99
C	1.8	3.92	2.8	6.08

(*) uncertainty as confidence interval for single analysis, % (P = 0.95).

The within-day imprecision measured as the relative standard deviation of the results of fat-soluble vitamins determination was 1.4% for vitamin E, 4.3 and 4.5% for vitamins A and D_3. For the water-soluble vitamins, RSD was between 1.6 and 2.6%. The between-day imprecision (RSD) was 2.2% for vitamin E and 4.6 for vitamin D_3. For the water-soluble vitamins, RSD was in the range 2.6–4.1% (Table 3).

Linearity

On the basis of the data from analysis-fortified samples prepared for the validation for each of the vitamins, the regression equation and the correlation factor of the chromatographic peak area vs. concentration were calculated. In the proposed range of concentrations, the methods were linear for all vitamins (Table 4). For none of vitamins, was the correlation coefficient lower than 0.99.

Range

The scope of the method was the determination of the fat-soluble vitamins first, and water-soluble vitamins second, in pharmaceutical multivitamin preparation in the form of the powders.

The range of the concentrations (Table 1) was chosen for the validation was selected in such a way that it was similar to daily doses of vitamins suggested by FDA.[18] Simultaneously, the range was included in the range of vitamin concentration in the multivitamin preparations available on the market.[19]

Table 4. Methods Linearity: Range and Correlation Coefficient (R)

Vitamin	Unit	Concentration in 1 mL	R
A	IU	402–1611	0.995
D_3	IU	113–313	0.991
E	mg	1.20–5.71	0.999
B_1	mg	0.016–0.064	0.990
B_2	mg	0.017–0.069	0.998
B_6	mg	0.018–0.073	0.999
PP	mg	0.10–0.56	0.999
C	mg	0.82–3.49	0.999

Table 5. Recovery of the Vitamins

Vitamin	Mean Recovery, % (n = 15)	RSD, %
A	100.4	4.1
D_3	98.3	4.7
E	98.1	2.0
B_1	97.9	5.0
B_2	97.2	2.6
B_6	97.6	3.7
PP	98.8	3.2
C	99.0	2.6

Recovery

The results of the analysis of 5 laboratory-prepared samples in the same known matrix and of different vitamin concentration were used for the recovery estimation. Average recovery and the relative standard deviation of the results were calculated for each vitamin. Recovery for fat-soluble vitamins was in the range of 98.1–100.4% and for the water-soluble vitamins in the range of 97.2–99.0% (Table 5 and Figure 1).

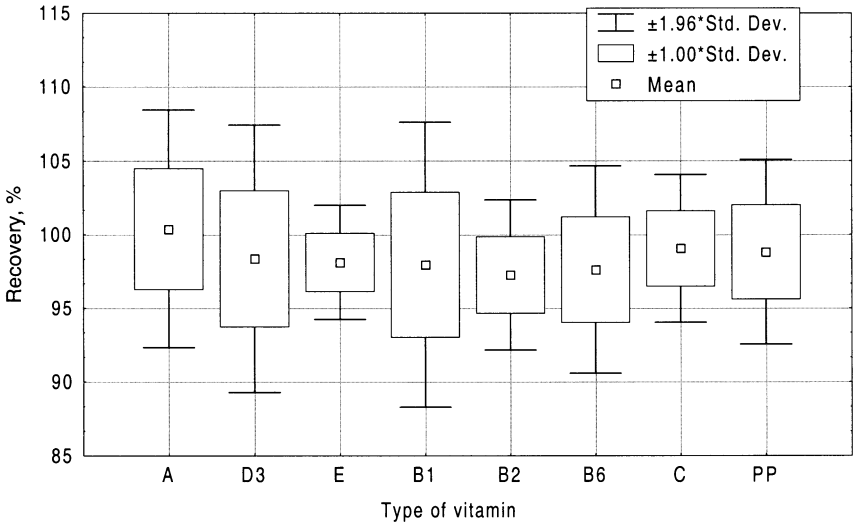

Figure 1. Methods recovery: mean recovery, standard deviation (SD) and 95% confidence interval (1.96 SD).

Selectivity

The assumption that the methods are selective was based on the stability of retention times and lack of coeluting peaks in fortified samples.[20]

The relative standard deviation of the retention times of the fat-soluble vitamins was in the range 0.35–0.5% and for the water-soluble vitamins 0.054–0.14% (Table 6).

On the chromatograms of all tested solutions, the peaks were separated to baseline. The resolution factor (R_s), for the worst separated pair of peaks for fat-soluble vitamins was 4.3 (vitamins E and D_3). For the water-soluble vitamins, the lowest resolution with $R_s = 2.2$ was obtained for vitamin B_2 and one of excipients, added to the tested preparation.

The asymmetry factor for peaks in single standard solutions and for the same peaks in fortified samples was similar. Both factors strongly suggest the lack of coeluting peaks (Table 6).

Stability of the Sample Solutions

In the solutions of the samples protected from the light and stored in room temperature, the differences in the measured vitamin concentrations did not exceed 1% within 24 hours. Thus, the methods are suitable for automated analyses. For samples which were exposed to the light, decrease in the concentration of vitamin B_2 was about 50%.

Table 6. Methods Selectivity

Analyte	Resolution, R_s	Variation of the Retention Time, (RSD) %	Symmetry Factor, f_{As}	
			Standards	Samples
A	6.3 (from D_3)	0.50	1.12	1.11
D_3	4.3 (from E)	0.38	1.26	1.16
E	–	0.35	2.09	2.11
B_1	12.0 (from vanilin)	0.10	1.23	1.10
B_2	4.4 (from B_6)	0.054	1.09	1.07
Vanilin	2.2 (from B_2)	0.051	–	1.19
B_6	7.1 (from PP)	0.078	1.08	1.09
PP	4.0 (from C)	0.14	1.02	1.03
C	–	0.14	0.96	0.94

CONCLUSIONS

The advantage of the chromatographic method in comparison with other methods (titration, UV-Vis or fluorimetry) is that it allows the determination of several various vitamins at the same time. This decreases the amount of time needed for performing analysis and decreases the expenses as well.

The tested methods were suitable for routine analysis of vitamins in multivitamin preparations with a range of concentrations, which is widespread for these preparations. Precision and accuracy of the methods are similar to the literature values for the HPLC methods of the vitamin determination.

The method, which was proposed for the water-soluble vitamin determination, allows determination not only of the B group vitamins but also the C vitamin, in spite of its considerable excess in comparison with the other vitamins.

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