Macedonian Journal of Chemistry and Chemical Engineering. Vol. **29**, No. 2, pp. 157–164 (2010)
MJCCA9 - 560 ISSN 1857–5552
Received: May 30, 2009 UDC: 543.422.3-76
Accepted: November 13, 2009

Original scientific paper

NEW, SIMPLE AND VALIDATED UV-SPECTROPHOTOMETRIC METHODS FOR THE ESTIMATION OF SODIUM USNATE IN PREPARATIONS

Ivana Savić[1]*, Goran Nikolić[1], Ivan Savić[1], Saša Zlatković[2], Dragiša Djokić[3]

[1]*Faculty of Technology, University of Niš, Bulevar oslobodjenja 124, Leskovac, Serbia*
[2] *Actavis Trading Ltd., Đorđa Stanojevića 12, Novi Beograd 11070, Serbia*
[3] *PCI Zdravlje-Actavis, Vlajkova 199, Leskovac, Serbia*
ici86@info-net.rs/ici_teh@yahoo.com

New, simple, cost effective, accurate and reproducible UV-spectrophotometric methods were developed and validated for the estimation of sodium usnate in pharmaceutical preparations. Sodium usnate was estimated at 290 nm in water and phosphate buffer (pH 3):methanol (11:20 *V/V*). Beer's law was obeyed in the concentration range of 0.1–5 $\mu g \cdot cm^{-3}$ ($r = 0.997$) in water and 1–12 $\mu g \cdot cm^{-3}$ ($r = 0.999$) in the phosphate buffer:methanol. The apparent molar absorptivity and Sandell's sensitivity coefficient were found to be 3.16×10^4 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ and 11.58 $ng \cdot cm^{-2}$/0.001 A in water and 3.72×10^4 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ and 9.83 $ng \cdot cm^{-2}$/0.001 A in phosphate buffer:methanol, respectively, indicating the high sensitivity of the proposed methods. These methods were tested and validated for various parameters according to ICH guidelines. The detection and quantitation limits were found to be 0.0721 and 0.2163 $\mu g \cdot cm^{-3}$ in water and 0.163, 0.489 $\mu g \cdot cm^{-3}$ in phosphate buffer:methanol, respectively. The proposed methods were successfully applied for the determination of sodium usnate in pharmaceutical preparations. The results demonstrated that the procedure is accurate, precise and reproducible (R.S.D. < 2 %).

Key words: sodium usnate; spectrophotometry; method validation; pharmaceutical preparations

НОВ, ЕДНОСТАВЕН И ВАЛИДИРАН UV-СПЕКТРОФОТОМЕТРИСКИ МЕТОД ЗА ОДРЕДУВАЊЕ НА НАТРИУМУСНАТ ВО ПРЕПАРАТИ

Развиен и валидиран е нов, едноставен, ефикасен, точен и повторлив UV-спектрофотометриска метод за определување на натриумуснат во фармацевтски препарати. Натриумуснат е одредуван на 290 nm во вода и фосфатен пуфер (pH = 3):метанол (11 : 20 *V/V*). Беровиот закон важи во опсег на концентрации од 0,1 до 5 $\mu g\ cm^{-3}$ ($r = 0,997$) за вода и од 1 до 12 $\mu g\ cm^{-3}$ ($r = 0,999$) за фосфатен пуфер:метанол. Вредноста на моларната апсорпција и Санделовиот коефициент на осетливост е $3,16 \times 10^4$ $dm^3 \cdot mol^{-1}\ cm^{-1}$ и 11,58 $ng \cdot cm^{-2}$/0,001 A за вода и $3,72 \times 10^4$ $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ и 9,83 $ng \cdot cm^{-2}$/0.001 A за фосфатен пуфер:метанол, соодветно, укажувајќи на висока осетливост на предложениот метод. Овие методи се тестирани и валидирани за различните параметри согласно со ICH регулативите. Добиените вредности на детекција и квантификација изнесуваат 0,0721 и 0,2163 $\mu g \cdot cm^{-3}$ за вода и 0,163, 0,489 $\mu g \cdot cm^{-3}$ за фосфатен пуфер:метанол, соодветно. Предложените методи успешно се применети за одредување на натриумуснат во фармацевтски препарати. Резултатите покажуваат дека методот е точен, прецизен и репродуцибилен (R.S.D. < 2 %).

Клучни зборови: натриумуснат; спектрофотометрија; валидација; фармацевтски препарати

1. INTRODUCTION

Sodium usnate or 2,6-diacetyl-7,9-dihydroxy-8,9*b*-dimethyldibenzofuran-1,3(2*H*,9*bH*)-dione, sodium salt, (boiling point 594.8 ºC at 760 mmHg and flash point 219.1 ºC) coats mucous membrane like a protective film and as a surface antibiotic selectively inhibits causes of infection (Figure 1). The formed film covers lesions caused by the infection or mechanical injury and helps their healing, without damaging the healthy microflora of the mucous membrane. Sodium usnate is a compound isolated from lichen for its effective antibacterial properties. It is used in deodorants because it helps to reduce bacterial growth and its associated odour. Sodium usnate has been successfully used by Ark et al. (1960), for the control of various fungal diseases of plants in the green house [1]. The procedures of sodium usnate synthesis are described in detail in patent literature [2–7].

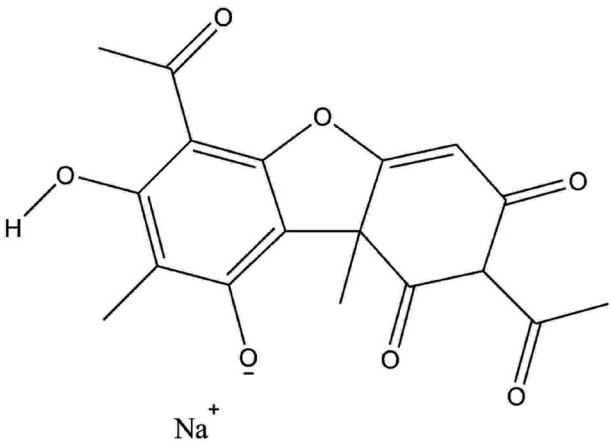

Fig. 1. Chemical structure of sodium usnate

For routine analysis a simple, rapid and cost effective analytical method is preferred. A survey of literature has not revealed any simple UV-spectrophotometric method for estimation of sodium usnate in bulk drug and preparations. Various methods have been reported in literature for monitoring sability sodium usnate in cosmetic preparations (shampoos, lotions and deodorants) including HPLC [8] and isothermal method [9]. Development of simple and accurate UV-spectrophotometric methods can provide a very useful alternative for routine analysis of bulk drug and formulations.

The objective of the present study was to develop simple, precise, accurate and validated, economic analytical methods for the estimation of sodium usnate in pharmaceutical formulations. Two analytical methods have been developed in different media for estimation of sodium usnate. Media used were water and phosphate buffer (pH 3):methanol. Sodium usnate showed absorption maxima at 290 nm in both media. The developed analytical methods were validated as per ICH guidelines and USP requirements [10, 11]. Statistical tests were performed on validation data [12, 13].

2. EXPERIMENTAL

Samples. Sodium usnate was obtained as gift samples from Zdravlje-Actavis, Leskovac. Formulations containing sodium usnate: Fitosept classic tablets, labelled to contain 0.1 mg of sodium usnate and 3 mg of menthol per tablet (Zdravlje-Actavis, Leskovac), Fitosept diet tablets, labelled to contain 0.1 mg of sodium usnate and 2 mg of menthol (Zdravlje-Actavis, Leskovac) and Fiotosept kids tablets, labelled to contain 0.05 mg of sodium usnate (Zdravlje-Actavis, Leskovac). Fitosept classic contained D-glucose as a sweetener, but Fitosept diet contained sorbitol. Fitosept kids did not contain menthol. All other chemicals and reagents used were of analytical grade.

Apparatus. A double-beam Varian Cary-100 Conc. UV-VIS spectrophotometer, connected to computer and loaded with Cary WinUV software was used. For intermediate precision study, a different Perkin Elmer Lambda-16 UV–VIS spectrophotometer connected to computer with UV-PC software was used. Both the instruments have an automatic wavelength accuracy of 0.1 nm and matched quartz cells of 10 mm (1.0 cm) cell path length.

Analytical method development. Different media were investigated to develop a suitable UV spectrophotometric method for the analysis

of sodium usnate in formulations. For selection of media the criteria employed were sensitivity of the method, ease of sample preparation, solubility of the drug, cost of solvents, applicability and robustness of the method to various purposes. Absorbance of sodium usnate in the selected medium at respective wavelength was determined and apparent molar absorptivity and Sandell's sensitivity coefficients were calculated according to the standard formulae (Table 1).

Procedure for calibration curve. Two different stock solutions of 100 μg·cm^{-3} of sodium usnate were prepared in water and phosphate buffer:methanol by dissolving 5 mg of sodium usnate in 50 cm^3 of each media. For preparation of different concentrations, aliquots of stock solutions were transferred into a series of 100 cm^3 standard volumetric flasks and volumes were made with the respective media. Five different concentrations were prepared in the range of 0.1–5 μg·cm^{-3} of sodium usnate in water. In a similar way, five different concentrations were prepared in the range of 1–12 μg·cm^{-3} of sodium usnate in the phosphate buffer:methanol for standard curve. Sodium usnate was estimated at 290 nm.

Sample preparation. Fitosept classic and Fitosept diet tablets were powdered and extracted with phosphate buffer:methanol. The solutions were then filtered and suitably diluted to get final concentration of 5 μg·cm^{-3}. Fitosept kids tablets were powdered and extracted with water and then suitably diluted to get final concentration of 5 μg·cm^{-3}.

2. 1. *Analytical method validation*

Specificity and selectivity. Sodium usnate solutions (5 μg·cm^{-3}) were prepared in both the selected media along with and without common excipients (PVP K-25, D-glucose, magnesium stearate, menthol) separately. All the solutions were scanned from 450 to 200 nm at a speed of 400 nm·min^{-1} and checked for change in the absorbance at respective wavelengths. In a separate study, drug concentration of 5 μg·cm^{-3} was prepared independently from pure drug stock solution in selected media and analyzed (n = 10). Paired t-test at 95 % level of significance was performed to compare the means of absorbance (Table 1).

T a b l e 1

Optical characteristics, statistical data of the regression equations and validation parameters for sodium usninate (n = 10)

Parameter	Water	Phosphate buffer (pH=3) : methanol
Optical characteristics		
Apparent molar absorptivity (dm^3·mol^{-1}·cm^{-1})	3.16×10^4	3.72×10^4
Sandell's sensitivity (ng·cm^{-2}/0.001 A)	11.58	9.83
Regression analysis		
Slope	64.75	55.74
Intercept	0.048	0.041
Regression coefficient (r)	0.997	0.999
Validation parameters		
Specificity and selectivity - t_{cal} (t_{crit})[a]	1.29 (2.225)	1.10 (2.225)
Linearity (μg·cm^{-3})	0.1–5	1–12
Limit of detection (LOD) (μg·cm^{-3})	0.0721	0.2163
Limit of quantification (LOQ) (μg·cm^{-3})	0.163	0.489
Robustness (mean % recovery ± S.D.)	101.03 ± 1.29	100.51 ± 0.97

[a] t_{cal} is calculated value and t_{crit} is theoretical value (at eight degrees of freedom) based on paired t-test at P = 0.05 level of significance.

Linearity. To establish linearity of the proposed methods, nine separate series of solutions of sodium usnate (0.1–5 μg·cm^{-3} in water and 1–12 μg·cm^{-3} in phosphate buffer:methanol) were prepared from the stock solutions and analyzed. Least square regression analysis was done for the obtained data (Table 1).

Accuracy. To determine the accuracy of the proposed methods, different levels of drug concentrations: lower concentration (LC), intermediate concentration (IC) and higher concentration (HC) (in both media) were prepared from independent stock solutions and analyzed (n = 10). Accuracy was assessed as the percentage relative error and mean %recovery (Table 2). To provide an additional support to the accuracy of the developed assay method, standard addition method was employed, which involved the addition of different concentrations of pure drug (1, 2, and 3 μg·cm^{-3} in water and the phosphate buffer:methanol to a known pre-analyzed formulation sample and the total concentration was determined using the proposed methods (n = 10) [11]. The % recovery of the added pure drug was calculated as, % recovery = $[(C_t - C_s)/C_a]\times 100$, where C_t is the total drug concentration measured after standard addition; C_s drug concentration in the formulation sample; C_a drug concentration added to formulation (Table 3).

T a b l e 2

Accuracy and method precision data for the developed method (n = 10)

Level	Predicted concentration[a] (μg·cm^{-3})		Mean recovery, %
	Mean (±S.D)	% R.S.D.	
Water			
LC (0.4 μg·cm^{-3})	0.423 ± 0.33	0.81	100.57
IC (0.5 μg·cm^{-3})	0.502 ± 0.34	0.67	100.42
HC (0.6 μg·cm^{-3})	0.599 ± 0.32	0.53	99.87
Phosphate buffer:methanol			
LC (8 μg·cm^{-3})	7.85 ± 0.137	0.683	99.25
IC (10 μg·cm^{-3})	10.21 ± 0.337	0.672	100.42
HC (12 μg·cm^{-3})	11.15 ± 0.179	0.303	98.58

[a] Predicted concentration of sodium usninate was calculated by linear regression equation.

Precision. The reproducibility was determined by using different levels of drug concentrations (the same concentration levels taken in the accuracy study), prepared from independent stock solutions and analyzed (n = 10) (Table 4). Inter-day, intra-day and inter-instrument variation were studied to determine the intermediate precision of the proposed analytical methods. Different levels of drug concentrations in triplicates were prepared three different times in a day and studied for intra-day variation. The same procedure was followed for three different days in order to study the inter-day variation (n = 10). One set of different levels of the concentrations was reanalyzed using another UV-VIS spectrophotometer, by proposed methods to study inter-instrument variation (n =10). The percent relative standard deviation (% R.S.D.) of the predicted concentrations from the regression equation was taken as precision (Table 3). The precision studies were also carried out by using the real samples of sodium usnate in a similar way to a standard solution to prove the usefulness of method.

Limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ of sodium usnate by the proposed methods were determinated using calibration standards. LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept of regresion equation (n = 10) (Table 1).

Robustness. Robustness of the proposed method was determined by (a) changing pH of the phosphate buffer : menthol by ± 0.1 units and (b) stability of the sodium usnate in both the selected

Table 3

Standard addition method for accuracy (n = 10)

Method	Concentration ($\mu g \cdot cm^{-3}$)	Pure drug added ($\mu g \cdot cm^{-3}$)	Total drug found ($\mu g \cdot cm^{-3}$) (±S.D)	% recovery (±R.S.D)
Water	0.5	0	0.502 ± 0.34	100.42 ± 0.67
	0.5	1	1.499 ± 0.32	99.87 ± 0.58
	0.5	2	2.503 ± 0.45	100.50 ± 0.64
	0.5	3	3.498 ± 0.42	99.94 ± 0.53
Phosphate buffer (pH=3) :menthol	8	0	7.85 ± 0.137	99.25 ± 0.683
	8	1	9.01 ± 0.075	100.12 ± 0.719
	8	2	10.007 ± 0.037	100.07 ± 0.375
	8	3	11.05 ± 0.061	100.45 ± 0.539

Table 4

System precision study (n = 10)

Concentration ($\mu g \cdot cm^{-3}$)	Estimated concentration Intra-day reproducibility % R.S.D., *n* = 10			Intra-instrument reproducibility % R.S.D., n = 10
	Day 1	Day 2	Day 3	
Water				
0.4	0.423 (0.81)	0.399 (0.67)	0.404 (0.51)	0.389 (0.89)
0.5	0.502 (0.67)	0.503 (0.21)	0.498 (0.08)	0.503 (1.41)
0.6	0.599 (0.53)	0.605 (0.84)	0.601 (0.08)	0.603 (1.55)
Phosphate buffer : methanol				
8	7.851 (0.68)	8.070 (0.45)	7.92 (0.71)	7.56 (1.23)
10	10.21 (0.67)	9.87 (0.56)	9.92 (0.34)	10.34 (1.07)
12	11.15 (0.30)	11.86 (0.73)	12.08 (0.84)	11.56 (0.78)

media at room temperature for 24 h. Three different concentrations (LC, IC and HC) were prepared in both the media with different pH and mean % recovery was determined (Table 1).

Estimation from formulations (Tablets). Twenty tablets each preparations were weighed and pulverized. Amount of the powder equivalent to 10 mg of sodium usnate was taken and extracted with both media separately for 30 min. These solutions were diluted suitably to prepare a 100 $\mu g\ cm^{-3}$ concentration in respective media. Finally solutions were filtered through Whatman filter paper number 40 and the filtrate was suitably diluted to prepare a 5 $\mu g \cdot cm^{-3}$ concentration in both the media separately and the samples were analyzed using proposed analytical methods (Table 5). The calculated Student's t-values did not exceed the tabulated values indicating no significant difference between the methods, as far as accuracy and precision are concerned.

Table 5

Application of spectrophotometric method to the determination of sodium usninate from pharmaceutical preparations (n = 10)

Commercial formulations	Amount found[b]	% assay
Water		
Fitosept kids (5 $\mu g \cdot cm^{-3}$)		
Mean ± S.D. ($\mu g \cdot cm^{-3}$)	5.02 ± 0.02	100.37 ± 0.38
t[a]	1.75 (2.225)	
Phosphate buffer : methanol		
Fitosept classic (5 $\mu g \cdot cm^{-3}$)		
Mean ± S.D. ($\mu g \cdot cm^{-3}$)	4.985 ± 2.44	99.70 ± 0.61
t[a]	1.21 (2.225)	
Fitosept diet (5 $\mu g \cdot cm^{-3}$)		
Mean ± S.D. ($\mu g \cdot cm^{-3}$)	5.03 ± 1.69	100.55 ± 0.53
t[a]	1.74 (2.225)	

[a] The values in parenthesis are the tabulated values of *t* at $P = 0.05$.
[b] Amount found is represented as average ± S.D.

3. RESULTS AND DISCUSSION

For media optimization various aqueous media like acetate buffers (pH 3.6–5.8), phosphate buffers (pH 2.5–10.2) and 0.1 mol·dm^{-3} sodium hydroxide were investigated. Almost pH-independent UV absorption spectra of sodium usnate were observed for the determination of sodium usnate in the proposed analytical media (Figure 2). Addition of varying amounts of the methanol to various aqueous media did improve the sensitivity of the methods and the final decision of using water and phosphate buffer (pH 3) as a media was based on the criteria like: sensitivity of the method, cost of solvents, ease of preparation and applicability of the method to dissolution samples. The spectra of sodium usnate in water and the phosphate buffer:methanol are shown in Figure 2. The λ_{max} of sodium usnate in water and phosphate buffer : methanol were found to be 290 nm. Overlaied absorption spectra of sodium usnate at 0 and 24 h time intervals were recorded in both media (Figure 3). Apparent molar absorptivity of the drug was found to be 3.16×10^4 dm^3·mol^{-1}·cm^{-1} in water; and 3.72×10^4 dm^3·mol^{-1}·cm^{-1} in the mixture of phosphate buffer:methanol, respectively. Sandell's index represents the number of micrograms or nanograms of the determinant per millilitre of a solution having an absorbance of 0.001 for the cell path length of 1 cm and is a suitable parameter for expressing and comparing the sensitivities of developed UV-spectrophotometric methods [14]. Sandell's sensitivity coefficient of sodium usnate was found to be 11.58 and 9.83 ng·cm^{-2} /0.001 A in water and mixture of phosphate buffer and methanol, respectively (Table 1).

Calibration curve. In water, the linear regression equation obtained was: A_{290} nm = [64.75×concentration (μg·cm^{-3}) + 0.048]; with a regression coefficient of 0.997. In phosphate buffer:methanol, the linear regression equation obtained was: A_{290} nm = [55.74×concentration (μg·cm^{-3}) + 0.041]; with a regression coefficient of 0.999.

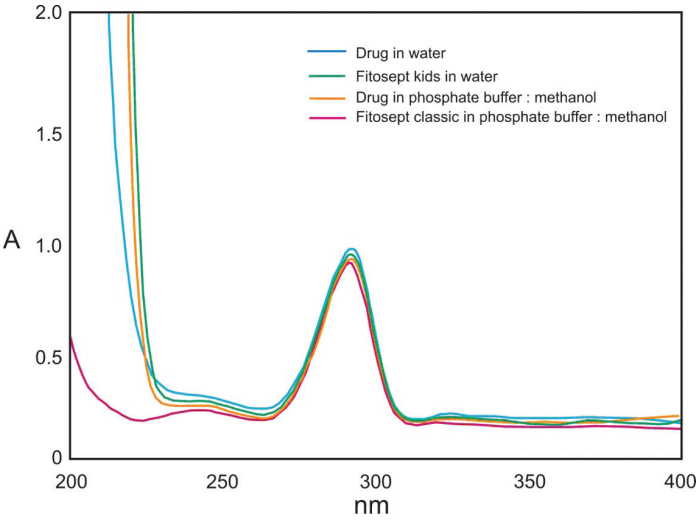

Fig. 2. UV-absorption spectra of 5 μg·cm^{-3} concentration of sodium usnate (bulk form and formulation) in water and phosphate buffer (pH 3) : methanol

3. 1. *Analytical validation*

Specificity and selectivity. The UV-spectrum of sodium usnate was not changed in the presence of common excipients used in the formulation of various Fitosept tablets, in both the selected media. Absorption spectrum of pure drug sample was matching with the formulation samples in both the selected media (Figure 2). The calculated *t*-values were found to be less than that of the tabulated *t*-values, indicating that statistically there was no significant difference between the mean absorbance of solutions prepared from pure drug sample and the formulation samples (Table 1). Therefore proposed analytical methods are specific and selective for the drug.

Linearity. The linearity range for sodium usnate estimation was found to be 0.1–5 μg·cm^{-3} (r = 0.997) and 1–12 μg·cm^{-3} (r = 0.999) in water and phosphate buffer medium, respectively (Table 1). Goodness of fit of the regression equations was supported by high regression coefficient values and lower calculated *F*-values (Table 1).

Accuracy. The accuracy of the method was checked by determining recovery values. Series of solution were made containing 80, 100 and 120 % of sodium usnate regarding the declared content. The accuracy ranged from 0.4 to

0.6 μg·cm^{-3} in water and from 8 to 12 μg·cm^{-3} in phosphate buffer:methanol (Table 3). The excellent mean % recovery values, close to 100%, and their low standard deviation values (SD < 1.0) represent high accuracy of the analytical methods. The validity and reliability of the proposed methods was further assessed by recovery studies via standard addition method. The mean % recoveries (% RSD) for concentration of 0.5 μg·cm^{-3} in water and 8 μg cm^{-3} in phosphate buffer:menthol showed in Table 3. These results revealed that any small change in the drug concentration in the solutions could be accurately determined by the proposed analytical methods.

Precision. Precision was determined by studying the repeatability and the intermediaprecision. The repeatability results indicated the precision under the same operating conditions over a short interval of time and the inter-assay precision. The intermediate precision expresses within-laboratory variations in different days and in different instruments. In the intermediate precision study, % RSD values were not more than 2.0 % in all the cases (Table 4). RSD values found for the proposed analytical method were well within the acceptable range indicating that the method have excellent repeatability and the intermediate precision.

LOQ and LOD. In water, LOD and LOQ were found to be 0.0721 and 0.2163 μg·cm^{-3} in water and 0.163, 0.489 μg cm^{-3} in phosphate buffer: methanol, respectively for sodium usnate.

Robustness. Variation of pH of the selected media by ±0.1 did not have any significant effect on the absorbance value of the sodium usnate. The mean %recovery (±S.D.) values were found to be 101.03 (±1.29) and 100.51 (±0.97) in water and phosphate buffer:methanol respectively (Table 1). The sodium usnate solution in selected media exhibited no spectrophotometric changes over a period of 24 h, when kept at room temperature (Figure 3).

Estimation of formulations. In water, the assay values of sodium usninate for Fitosept kids from 100.37 % with standard deviation from 0.38 %. In phosphate buffer medium the assay values of sodium usnate for different preparations (Fitosept classic and Fitosept diet) ranged from 99.70 % to 100.55 % with standard deviation not more than 0.61 %. Assay values of preparations were same as mentioned in the label claim indicating that the interference of excipient matrix is insignificant in estimation of sodium usnate by proposed analytical methods. The estimated drug content with low values of standard deviation established the precision of the proposed methods. The results obtained from the two analytical methods were compared statistically (Table 5). The calculated Student's *t*-values did not exceed the tabulated values indicating no significant difference between the methods, as far as accuracy and precision are concerned.

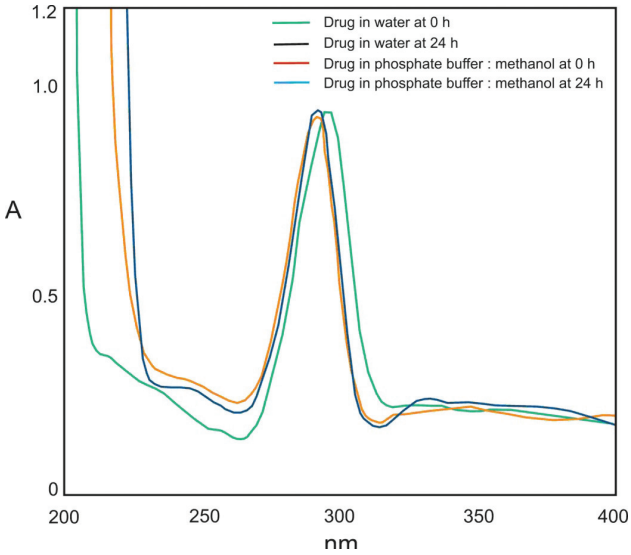

Fig. 3. UV-absorption spectra of 10 μg·cm^{-3} concentration of sodium usnate in water and phosphate buffer (pH 3) : methanol at 0 and 24 h time interval

4. CONCLUSION

The proposed analytical methods are simple, rapid, accurate, precise and inexpensive and hence can be used for the routine analysis of sodium usnate in bulk and pharmaceutical preparations. The sample recoveries from all preparations were in good agreement with their respective label claims, which suggested non-interference of formulations excipients in the estimation.

Acknowledgments. This work was supported by the the Ministry of Science and Technological Development of the Republic of Serbia, project TR-19035. The authors are grateful for the financial support provided by this Ministry.

REFERENCES

[1] P. A. Ark, A. T. Bottimi, J. P. Thompson, Sodium usnate as an antibiotic for plant diseases, *Plant disease Report*, **44**, 200-203 (1960).

[2] M. Cocchietto, N. Skert, P.L. Nimis, G. Sava, Review on usnic acid, an interesting natural compound, *Naturwissenschaften*, **89**, 137–146 (2002).

[3] S. Huneck, I. Yoshimura, *Identification of Lichen Substances*, Springer, 1996.

[4] K. Ingolfsdottir, G. Chung, V. Skulason, S. Gissurarson, M. Vilhelmsdottir, Antimycobacterial activity of lichen metabolites in vitro, *E. J. Pharm. Sci*., **6**, 141–144 (1998).

[5] M. Cakić, G. Nikolić, N. Nikolić, M. Stanković, FTIR spectra of usnic acid dihydrate natrium salts and its deuterated analogs, *III Simpozijum Savremene tehnologije i privredni razvoj*, Proceedings, Leskovac, OHT-3, 74 (1998).

[6] S. Shibata, Y. Miura, Antibacterial effects of lichen substances, *Jpn. Med. J.*, **1**, 518–521 (1948).

[7] M. Stanković, S. Stanković, Z. Đorđević, S. Đorđević, Process for obtaining natrium salt of (+)-usnic acid, Pat. Yu 43978 (1989).

[8] C. Coiffard, L.J.M. Coiffard, F. Peigne, and Y. De Roeck-Holtzhauer, Effect of pH on Sodium Usnate Thermostability in Dilute Aqueous Solutions, *Arch. Pharm. pharm. Med. Chem.* **331**, 128–132 (1998).

[9] C. Coiffard, L. J. M. Coiffard, Y. De Roeck-Holtzhauer, Photodegradation Study of Sodium Usnate Solution: Influence of pH, *Drug Development and Industrial Pharmacy*, **25** (12), 1295–1299 (1999).

[10] The European Agency for the Evaluation of Medicinal Products. ICH Topic Q2B Note for Guideline on Validation of Analytical Procedures: Methodology GPMP/ICH/281/95, 1996.

[11] *United States Pharmacopoeia, Validation of Compendial Methods*, 26th edition, Pharmacopoeial Convention Inc., Rockville, MD, 2003. pp. 2439–2442.

[12] S. Bolton, *Pharmaceutical Statistics: Practical and Clinical Application*, 3rd ed., Marcel Dekker, New York, 1997, pp. 216–264.

[13] J. C. Miller, J. N. Miller, *Statistics for Analytical Chemistry*, 2nd ed.,Wiley, New York, 1984, pp. 83–117.

[14] Y. J. Wei, K. A. Li, S. Y. Tong, A linear regression method for the study of the Coomassie brilliant blue protein assay, *Talanta,* **43**, 1–10 (1996).