DEVELOPMENT AND VALIDATION OF A UV SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION OF THE SYNTHESIZED LENTINAN–CONGO RED COMPLEX**

S. Trivedi [1*], V. Belgamwar [1], K. Wadher [2], M. Umekar [2]

[1] Department of Pharmaceutical Sciences,
Rashtrasant Tukadoji Maharaj Nagpur University Campus, Nagpur,
Maharashtra, India; e-mail: sagartrivedi2000@gmail.com
[2] Department of Pharmaceutics, Smt. Kishoritai Bhoyar College of Pharmacy,
Kamptee Nagpur, Maharashtra, India

Lentinan, a therapeutic bioactive molecule obtained from Lentinus edodes (Shitake mushrooms), possesses various pharmacological activities. A literature survey reveals that no simple UV-visible technique for the estimation of lentinan has yet been established; hence, there is a pressing need for a simple, yet precise and robust analytical method. We have developed a UV-visible spectrophotometric method for the estimation of lentinan in complex with Congo red azo dye in different concentrations of alkaline solutions. Lentinan was found to be freely soluble in deionized water, and the absorption maximum (λ_{max}) was found to be 486 nm. The validation of the developed method gave satisfactory results, the lentinan–Congo red solution complex gave acceptable linearity within the concentration range 2–10 μg/mL and the correlation coefficient (r^2) was found to be >0.99. The developed method was found to be accurate because the mean recovery value at various concentrations gave higher results than 90%. LOD and LOQ for lentinan were reported and found to be 0.014 and 0.0431 μg/mL, respectively. The developed method was found to be simple, specific, economic, reliable, accurate, precise, reproducible, and it could be used as a quality control tool for the analysis of pure lentinan and lentinan in formulations.

Keywords: *lentinan, Congo red, complex formation, validation, UV spectroscopy.*

РАЗРАБОТКА И ВАЛИДАЦИЯ УФ-СПЕКТРОФОТОМЕТРИЧЕСКОГО МЕТОДА ОЦЕНКИ СИНТЕЗИРОВАННОГО КОМПЛЕКСА ЛЕНТИНАН–КОНГО КРАСНЫЙ

S. Trivedi [1*], V. Belgamwar [1], K. Wadher [2], M. Umekar [2]

УДК 543.42.062

[1] Университет Раштрасанта Тукадоджи Махараджа Нагпура,
Нагпур, Махараштра, Индия; e-mail: sagartrivedi2000@gmail.com
[2] Фармацевтический колледж Кишоритай Бхояр,
Кампти Нагпур, Махараштра, Индия

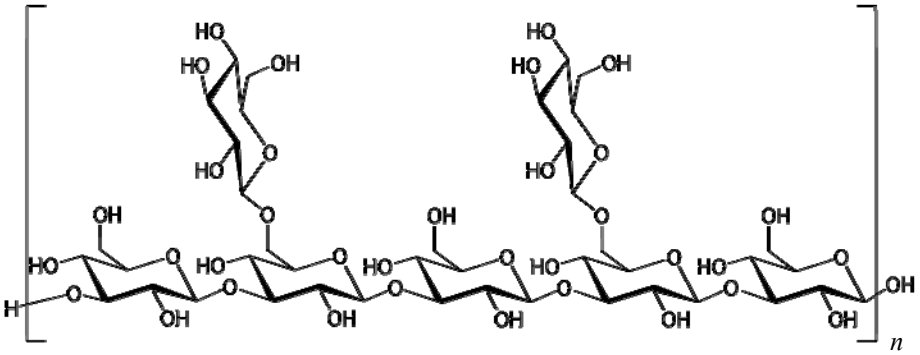
(Поступила 10 марта 2021)

Разработан простой УФ-спектрофотометрический метод определения содержания лентинана в комплексе с азокрасителем конго красным при различных концентрациях щелочных растворов. Обнаружено, что лентинан легко растворяется в деионизированной воде с максимумом поглощения при 486 нм. В результате проверки метода выявлено, что раствор комплекса лентинан–конго красный имеет приемлемую линейность в диапазоне концентраций 2–10 мкг/мл с коэффициентом корреляции $r^2 > 0.99$, среднее значение извлечения при различных концентрациях >90 %, для лентинана LOD = 0.014 мкг/мл и LOQ = 0.0431 мкг/мл. Разработанный метод может использоваться для контроля качества при анализе лентинана чистого и в лекарственных препаратах.

Ключевые слова: *лентинан, конго красный, комплексообразование, валидация, УФ-спектроскопия.*

**Full text is published in JAS V. 89, No. 2 (http://springer.com/journal/10812) and in electronic version of ZhPS V. 89, No. 2 (http://www.elibrary.ru/title_about.asp?id=7318; sales@elibrary.ru).

Introduction. Lentinan is a bioactive molecule mainly isolated from Lentinus edodes (Shitake mushrooms) [1] and chemically composed of β-(1→6) branched β-(1→3)-glucan



arranged in a repetitive sequence along with seven glucose molecules in a series, which gives the molecular formula $C_{42}H_{72}O_{36}$ and molecular mass 1152 Da [1]. Lentinan is reported to possess cytotoxic, anticancer, antiviral, anticoagulant, and immunological effects [2]. Lentinan is freely soluble in solvents such as alkaline solution and formic acid. It is slightly soluble in hot water and insoluble in cold water, acid solution, and organic solvents such as alcohol, ether, chloroform, pyridine, and hexamethylphosphoramide [3, 4]. A considerable number of high-performance liquid chromatography (HPLC) and liquid chromatography mass spectroscopic techniques are reported for the estimation of lentinan, but the literature survey has revealed that no simple ultra-visible spectroscopic method exists [5, 6]. Owing to the therapeutic importance of lentinan, the development of a simple and robust UV-Vis spectrophotometric analytical method for its estimation is of prime importance. The method for the estimation of lentinan was developed and optimized by complexing it with Congo red azo dye in different concentrations of alkaline solutions of NaOH.

Methods. Lentinan was received as a gift sample from Xi'an Pincredit Bio-tech Co., Ltd. (Xi'An, China), Congo red was purchased from Sigma-Aldrich Corporation (Mumbai, India). All other chemicals used were of analytical grade. A double beam UV-visible spectrophotometer (JASCO V-630) was used.

The standard lentinan (5 mg) was accurately weighed to make three standard stock solutions of 1000 μg/mL in a 5-mL volumetric flask containing 0.1 mL of Congo red azo dye of a concentration not exceeding more than 1.25×10^{-5} M in dilute alkaline solutions of NaOH (sodium hydroxide) of concentrations 0.1, 0.12, and 0.15 M. The lentinan and Congo red solutions of different concentrations in subsequent volumetric flasks were properly mixed and dissolved with a minimal quantity of deionized water and kept aside for 15 min, and the final volume was made up to mark with the same mobile phase, i.e., deionized water [7]. Further, a working solution of lentinan–Congo red dye (100 μg/mL) was prepared by diluting 1 mL of the above standard stock solution to 10 mL with deionized water for all concentrations. The prepared working stock solutions were further diluted with deionized water to get the 10 μg/mL concentration (1 to 10 mL). All three solutions were scanned in the wavelength regions from 200 to 500 nm against deionized water as a blank. The UV spectra of three concentrations are shown in Fig. 1 and Table 1. The alkaline solution of Congo red having a concentration of 0.15 M shows a characteristic absorption maximum at 486 nm and an ideal curve. The other concentrations have comparatively flat curves and higher absorption curves and absorbance. After acquiring the spectrum, λ_{max} at 486 was identified and this particular concentration was selected for the validation of the analytical method [8].

The calibration curve was prepared using a working stock solution with diverse calibration standards representing a series within the concentration range 2–10 μg/mL by further dilution of the stock solution with deionized water. The absorbance of every calibration standard was estimated at λ_{max} = 486 nm using the fixed wavelength measurement mode. The calibration curves representing the concentration versus absorbance values were plotted using Microsoft Excel 2016 [9].

The validation of the analytical method plays a vital role in authenticating the estimation method. Hence, the validation was done as per the ICH protocols. For this, different concentrations of the series of solutions ranging from 2 to 10 μg/mL were prepared and further subjected to linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) studies [3].

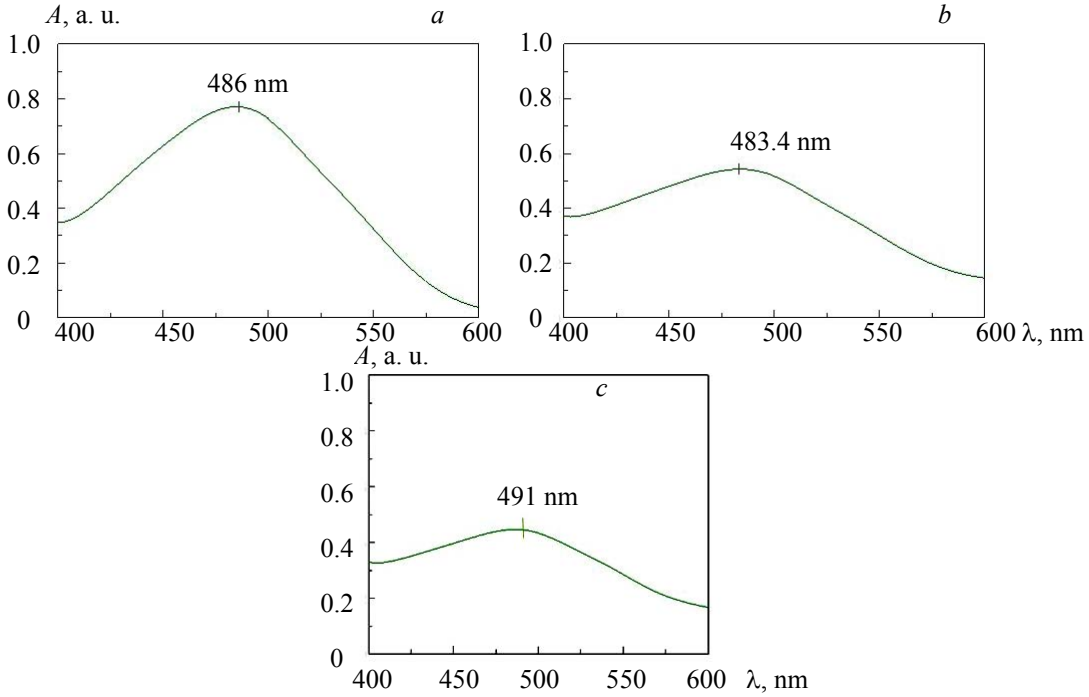


Fig. 1. UV-Vis spectra of dilute alkaline solutions of lentinan–Congo red complexes.

TABLE 1. Summary of the Estimation of the Maximum Absorbance

Alkaline Congo red solution, M	Lentinan concentration, μg/mL	λ_{max}, nm	Absorbance
0.1	10	491	0.621
0.12	10	483.4	0.548
0.15	10	486	0.771

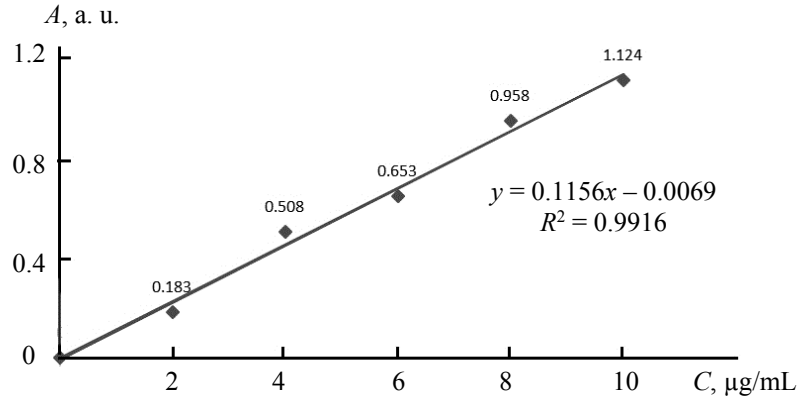


Fig. 2. Calibration curve of lentinan at 486 nm.

The linearity for the above developed method was justified by analyzing various concentrations of the prepared lentinan–Congo red complex between 2 and 10 μg/mL at 486 nm. The absorbance v/s concentration plot for lentinan was found to be linear in Fig. 2. Although considering linearity and range as an important parameter for the validation for the proposed method development, the *R* square value plays a vital role. To establish an acceptable linearity and range value, the interval between the upper and lower concentration of the test solution under analysis must be assessed [3].

The repeatability (intra-day) and intermediate precision (inter-day) are the parameters that help in defining the precision of the developed method. The intra-day precision was done by evaluating the lentinan–Congo red complex solution having a concentration of 10 μg/mL at three different time points within a single day. The inter-day precision was performed in a similar manner with the same concentration of the sample but instead sampling was done at three different points for 3 days and the average %RSD was calculated [3].

The closeness of the obtained results to the true value is the main aim behind assessing the accuracy of the developed method. Generally, the validation of accuracy is performed by spiking recovery studies; the pre-analyzed sample solution of lentinan–Congo red was spiked at three different concentrations of 8, 10, and 12 μg/mL and the % recovery was computed [3].

Limit of detection is mostly determined to assess the nethermost concentration of the analyte in the sample that can be differentially measured from the background levels, whereas LOQ helps in defining the lowest concentration for calibration at which acceptable accuracy and precision can be obtained. For the validation of the developed method LOQ and LOD were resolute with the help of Eq. (1) and the standard deviation of the response and slope of the calibration curve:

$$\text{LOD} = 3.3\ S/M,\ \text{LOQ} = 10\ S/M, \quad (1)$$

where S is the standard deviation of the absorbance of the sample and M is the slope of the calibration curve [5].

Results and discussion. Lentinan was found to be freely soluble in deionized water, and the absorption maximum (λ_{max}) was found to be 486 nm, as shown in Fig. 1. The absence of the chromophore group in lentinan leads to no absorption in the UV spectrum, which ultimately demands conjugation of lentinan with a chromophore adding agent. Electrostatic interaction is the possible mechanism lying behind the complex formation between the azo dye Congo red and polysaccharide (lentinan) in dilute alkaline solution and leaving the side chain alpha and beta glucan in the charged state. The basic aqueous solutions of Congo red bring conformational transitions in the uncharged molecules; these structural changes occurring in polysaccharide lead to absorption spectra and a sharp peak in the visible UV spectrum region at 486 nm [10]. The formation of a complex between lentinan and Congo red in dilute alkaline solutions (0.1, 0.12, and 0.15 M sodium hydroxide) gave conclusive results that as the alkalinity of the formed complex decreases in the solution, it directly reduces the average number of glucose residues accommodating at the binding site of a single dye molecule. The absorption maximum (λ_{max}) curves were found to be ideal at the maximum concentration of Congo red alkaline solutions being tested. When lentinan was exposed to the dilute alkaline solution of sodium hydroxide comparatively higher than 0.15 M containing Congo red azo, it caused conformational transitions leading to a random coiled arrangement in the narrow range from 0.12 to 0.15 M. This conformational transition occurred at a high rate when exposed to higher concentrations of alkaline solution. The random coiled ordered structure of lentinan in complex with 0.1 M aqueous NaOH solution of Congo red offers a spectral red shift because of the presence of a glucan ring in the lentinan core structure [4]. Figure 1c gives suggestive results that the maximum absorption of the lentinan–Congo red complex solution (486 nm) shifted towards a longer wavelength up to 491 nm upon successive additions. The maximum absorption of the lentinan–Congo red complex solution (486 nm) was observed because of the equilibrium achieved by the sum of the free dye and the complexed dye. It was observed when a single moiety of the Congo red combined with a glucan segment of lentinan, which led to the occurrence of this reversible reaction in the solution. The validation of the developed method gave satisfactory results: the lentinan–Congo red solution complex gave acceptable linearity within the concentration range 2–10 μg/mL. The correlation coefficient (r^2) was found to be >0.99. At the maximum absorption of 486 nm the linearity curves gave the regression equation $y = 0.1156x - 0.0069$. Table 2 shows the precision data for intra-day and inter-day having good re-

TABLE 2. Results of Intra- and Inter-day Precision at λ_{max} = 486 nm

Lentinan-CR complex (0.1M), μg/mL	Intra-day		Inter-day	
	Absorbance ±SD	Relative standard deviation, %RSD, n = 6	Absorbance ±SD	Relative standard deviation, %RSD, n = 6
10	0.771±0.0002	0.0875	0.771±0.0002	0.1241
10	0.791±0.0005		0.831±0.0012	
10	0.811±0.0014		0.925±0.0004	

TABLE 3. Recovery Study of Lentinan at λ_{max} = 486 nm

Level of recovery, %	Amount spiked recovery, μg/mL	Amount recovered, μg/mL	Recovery, %	Mean recovery, %
80	8	7.32	91.5	93.8
100	10	9.21	92.1	
120	12	11.74	97.8	

TABLE 4. Validation Parameters

λ_{max}, nm	486
Beer's law range, μg/mL	2–10
Correlation coefficient (r^2)	0.9916
Slope, *m*	0.1156
Intercept, *c*	0.0069
Accuracy	93.8
Precision (%RSD)	
Intra-day	0.0875
Inter-day	0.1241
LOD, μg/mL	0.014
LOQ, μg/mL	0.0431

N o t e. RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification.

producibility with %RSD lower than 2.0%, which assures that the developed method is precise. The developed method was found to be accurate because the mean recovery value (Table 3) at various concentrations gave higher results than 90%. LOD and LOQ for lentinan were reported (Table 4) and found to be 0.014 and 0.0431 μg/mL [10–12].

Conclusions. The developed method was found to be simple, specific, economic, reliable, accurate, precise, and reproducible. It could be used as a quality control tool for the analysis of pure lentinan and lentinan in formulations.

Acknowledgments. The authors are thankful to the Government of India, Ministry of Science and Technology, Department of Science and Technology (DST), India, for their financial support through the DST-INSPIRE Fellowship (IF 190486) and express their gratitude to Rashtrasant Tukadoji Maharaj Nagpur University Nagpur, Maharashtra, India, for carrying out this project.

REFERENCES

1. C. K. Miyaji, A. Poersch, L. R. Ribeiro, A. F. Eira, I. M. S. Cólus, *Toxicol. Vitr.*, **20**, 1555–1559 (2006), https://doi.org/10.1016/j.tiv.2006.07.004.
2. X. Chen, Z. Sheng, S. Qiu, H. Yang, J. Jia, J. Wang, C. Jiang, *PLoS One*, **14** (2019), https://doi.org/10.1371/journal.pone.0208611.
3. D. Brauer, T. E. Kimmons, M. Phillips, D. E. Brauer, *Curr. Res., Technol. and Ed. Top. in Appl. Microbiol. Microbial Biotech.*, Microbiol., Book Ser., **2**, Formatex Res. Center, Badajor, Spain, 1136–1142 (2010).
4. Y. Wang, H. Wang, D. Yan, L. Wang, Z. Sun, H. Sun, *Aquac. Int.*, **21**, 1261–1277 (2013), https://doi.org/10.1007/s10499-013-9628-7.
5. A. Maruca, F. Moraca, R. Rocca, F. Molisani, F. Alcaro, M. C. Gidaro, S. Alcaro, G. Costa, F. Ortuso, *Molecules*, **22** (2017), https://doi.org/10.3390/molecules22091571.
6. M. L. Ng, A. T. Yap, *J. Altern. Complement. Med.*, **8**, 581–589 (2002), https://doi.org/10.1089/107555302320825093.
7. X. Ma, S. Yuan, L. Yang, L. Li, X. Zhang, C. Su, K. Wang, *Cryst. Eng. Commun.*, **15**, 8288–8299 (2013), https://doi.org/10.1039/c3ce41275j.

8. N. A. Mohd Jamil, N. Rahmad, N. Mohd Nor Rashid, M. H. Y. Mohd Yusoff, N. S. Shaharuddin, N. Mohd Saleh, *J. Mycol*., 1–8 (2013), https://doi.org/10.1155/2013/718963.
9. P. S. Jain, A. J. Chaudhari, S. A. Patel, Z. N. Patel, D. T. Patel, *Pharm. Methods*, **2**, 198–202 (2011), https://doi.org/10.4103/2229-4708.90364.
10. H. H. Lee, J. S. Lee, J. Y. Cho, Y. E. Kim, E. K. Hong, *J. Microbiol. Biotechnol.*, **19**, 455–461 (2009), https://doi.org/10.4014/jmb.0809.542.
11. K. Ogawa, T. Dohmaru, T. Yui, *Biosci. Biotechnol. Biochem*., **58**, 1870–1872 (1994), https://doi.org/10.1271/bbb.58.1870.
12. M. M. M. Tomassen, E. A. H. J. Hendrix, A. S. M. Sonnenberg, H. J. Wichers, J. J. Mes, *Proc. 7th Int. Conf. World Soc. Mushroom Biology and Mushroom Products*, Arcachon, France, 04.10.2011, INRA, 259–267 (2011).