Jagannath University Journal of Science
Volume 08, Number I, December 2021, pp. 53–58
https://jnu.ac.bd/journal/portal/archives/science.jsp
ISSN 2224-1698

JnUJSci

Analytical Assay Method Validation of Levofloxacin 250 mg Tablet by HPLC Using C8 Reversed-Phase Column

Research Article

Nazifa Anjum, Sabarni Sarker, Sukumar Bepary, Bishyajit Kumar Biswas*

Department of Pharmacy, Jagannath University, Dhaka-1100, Bangladesh.

Received: 04. July 2021
Accepted: 30 November 2021

Abstract : The assay testing is a very important tool to characterize the performance and activity of a pharmaceutical product. In this study, an attempt was taken to validate an analytical assay method for levofloxacin 250 mg tablet by HPLC using C8 reversed-phase column. The analytical method validation for assay was performed on Adept CECIL system equipped with UV Visible detector and Hyperclone 5 µm BDS C8 column (150 × 4.6 mm). The gradient chromatography was made and the run time was 15 minutes. The detection was performed at 295 nm and the flow rate was set to 0.5 ml/min. The system precision, method precision, linearity, range, accuracy, LOD, LOQ, specificity, robustness were performed as per International Conference on Harmonization (ICH) guidelines to validate the analytical assay method. The retention time of levofloxacin hemihydrate was found at 6.54 minute. According to ICH guidelines, the validation parameters were obtained within the acceptance limits. The % assay of test sample was 99.06% and % RSD value was 1.28%. The analytical validation method was simple, accurate and effective for routine assay analysis of Levofloxacin 250 mg tablet.

Keywords: *Levofloxacin • HPLC • Assay • Method validation*

1. Introduction

Levofloxacin[(-)-(*S*)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid hemihydrate] is a levorotatory isomer of ofloxacin (Croom *et al*.,2003). It is a synthetic fluoroquinolone antibiotic (Brunton *et al.*, 2017) having molecular formula $C_{18}H_{20}FN_3O_4.½H_2O$ & molecular weight 370.38 (Figure 1). It exerts broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria such as *Streptococcus pneumoniae, Streptococcus hemolyticus, Streptococcus pyogene, Escherichia coli, Salmonella, Klebsiella, Serratia* etc. (Katzung, 2017; Zhanel *et al.*, 2006). It has also antibacterial activity against *Chlamydia trachomatis* (Smelov *et al*., 2005).

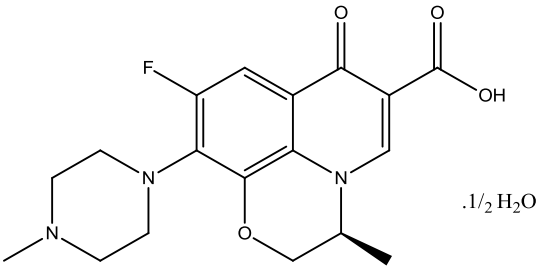

Figure 1. Structure of levofloxacin hemihydrate

Quantitative analysis of levofloxacin in single as well as combination drugs can be estimated by HPLC (Chepurwar *et al*., 2007; Lalitha Devi *et al*., 2009; Szerkus *et al*., 2017) and UV-VIS (Maleque *et al*., 2012) spectroscopy. Depending on the indication, the dosage

*Corresponding Author: Bishyajit Kumar Biswas
Email: biswas121@yahoo.com

regimen can be 250 mg, 500 mg or 750 mg daily. In our study, levofloxacin 250 mg tablet, single batch B# EXP002 was selected to perform the validation throughout the whole analytical process by HPLC. The main target was to ensure an accurate, reproducible, economical and easy method of assay validation that can be ultimately intended for routine analysis, stability testing and other related substances (impurities) testing of levofloxacin in 250 mg tablet dosage form by HPLC using C8 reversed-phase column.

2. Materials and Methods

Table 1. Chemicals and reagents**.**

Material	Purity	Manufacturer/ Supplier
Levofloxacin hemihydrate (Reference standard)	96.5% as it is; 99.1% on AB	Eskayef Pharmaceuticals Limited
Acetonitrile	HPLC grade	Daejung Chemicals Co., Korea
Water	HPLC grade	RCI Labscan, Thailand
Trifluroacetic acid	Reagent grade	Samchun Pure Chemical Co., Korea

Chromatographic condition: Chromatography was performed at ambient temperature. HPLC: CECIL; Column: HyperClone, BDS C8 (150 mm × 4.6mm, 5µm); Flow rate: 0.5 ml per minute; Runtime: 15 minutes; Detection: 295 nm; Injection volume: 10 µL; Mobile phase A: Acetonitrile 1L with 0.15 mL Trifluoroacetic acid (TFA); Mobile phase B: Water 1L with 0.15 mL Trifluoroacetic acid (TFA); Condition: Gradient condition. 0.0 -3 min, 15% -25% A; 3.0-5.0 min, 25% - 35% A, 5.0-7.0 min, 35% - 45% A; 7.0-10.0 min, 45% - 35% A; 10.0-12.0 min, 35% - 25% A; 12.0 – 15.0 min, 25% -15% A.

Standard solution preparation: Levofloxacin 100 mg reference standard was weighed accurately and placed in a clean 100 ml volumetric flask. The volume was made. From the above solution, 10 ml was diluted to 100 ml with mobile phase A to obtain the final conc. 100 µg/ml.

Preparation of sample solution: Twelve intact tablets were taken and finely powdered in which 284 mg was equivalent to 100 mg of levofloxacin and transferred into a clean 100 ml volumetric flask. The volume was made up to the mark with mobile phase A to get conc 1 mg/ml and sonicated for 60 minutes with intermediate shaking. Then**,** 10 ml was further diluted to 100 ml with mobile phase A to get the final conc. 100 µg/ml. Then it was filtered and injected.

Method validation parameters: The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) makes recommendations to achieve greater harmonization in the interpretation and application of technical guidelines and requirements for pharmaceuticals. According to ICH guidelines (FDA, *ICH-Q2 (R1)*, 1995; Sarah *et al*., 2005; Allport-Settle, 2010), validation parameters (Chan *et al*., 2008) such as system precision (repeatability), method precision (repeatability), linearity, range, accuracy, LOD, LOQ, specificity, and robustness were considered to be performed.

3. Results and Discussions

System precision and method precision: The precision of an analytical method is the degree of consistency among individual test results when the method is applied repetitively to multiple samplings (FDA, *ICH-Q2 (R1)*, 1995). Using the standard solution of levofloxacin (100 µg/ml), the system precision was determined from the results of 6 replicas consecutively and the method precision was calculated by 6 determinations from the sample solution (approximately 100 µg/ml) one by one. The areas of all the injections were calculated as followed (Table 2) with the statistical tools and approaches (Araujo, 2009).

Table 2. System precision and method precision

Parameters	System precision	Method precision
Number of replicas	6	6
Individual results (Areas)	10560.74, 10349.96, 10389.44, 10425.22, 10507.58, 10769.71	99.97, 100.26, 97.02, 98.50, 98.54, 100.04
Upper confidence limit	10400	100
Lower confidence limit	10600	98
Mean (Area)	10500.44	99.06
Standard deviation	152.85	1.26
%RSD	1.46	1.28
Acceptance criteria	Coefficient of variation not more than 2.0%	Coefficient of variation not more than 2.0%
Results and evaluation	Complies	Complies

Linearity: A dilution series were prepared from the standard solution in the range 20% to 140% (20, 40, 60, 80, 100, 120, 140 µg/ml concentration). The calibration curve was generated using regression analysis with Microsoft Excel (Figure 2).

Table 3. Linearity study at (20 µg/ml - 140µg/ml)

Conc. of levofloxacin (µg/ml)	Area of levofloxacin (mAs)
20	2063.31
40	4024.08
60	6332.36
80	8176.61
100	10603.36
120	12794.48
140	15824.36
Correlation coefficient, R^2	0.9959
Acceptance criteria	R^2 is ≥ 0.99
Result and evaluation:	R^2 is 0.9959 which complies the method is linear sufficiently.

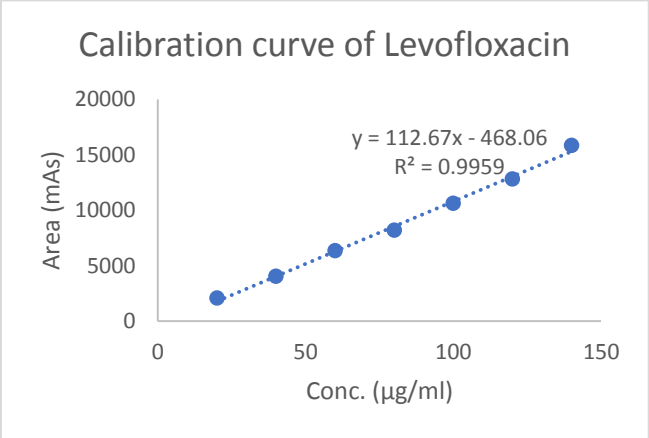

Figure 2. Calibration curve of standard solution (20 µg/ml - 140 µg/ml).

Range: Range of the method was determined by injecting 6 replicates of 20% conc. and 6 replicates of 140% conc. of standard solution one after another.

Table 4. Study of range

Parameters	20% concentration	140% concentration
Individual results (Areas)	2063.31, 2067.23, 2006.04, 2113.18, 2079.14, 2061.23	15824.36, 15661.28, 15378.04, 15599.18, 15688.43, 15269.77
Mean (Area)	2065.02	15570.18
Standard deviation	34.70	207.31
%RSD	1.68	1.33
Acceptance criteria	%RSD not more than 2.0%	%RSD not more than 2.0%
Results and Evaluation	Complies	Complies

Limit of detection (LOD): The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value (FDA, *ICH-Q2 (R1)*, 1995).

Limit of quantification (LOQ): The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy (FDA, *ICH-Q2 (R1)*, 1995).

Based on the Standard Deviation of the Response and the Slope: LOD= (3.3σ/s) and LOQ=(10σ/s) where σ is the standard deviation of the response and s is the slope of the regression line (Figure 2). From regression data, limit of detection (LOD) and limit of quantification (LOQ) were calculated (from EXCEL) as 0.5 µg/ml and 1.5 µg/ml respectively. From the test procedure, LOD was obtained 4 µg/mL Figure 3 and LOQ was obtained 8 µg/ml Figure 4.

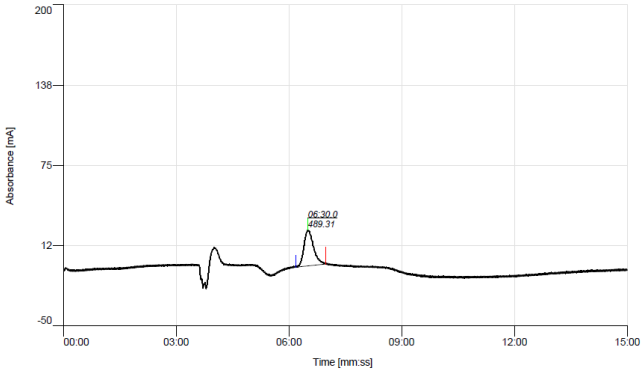

Figure 3. LOD at 4 µg/ml concentration level

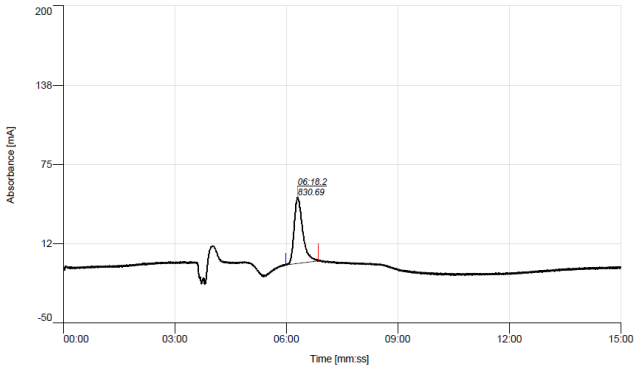

Figure 4. LOQ at 8 µg/ml concentration level.

Accuracy (Recovery): The accuracy of an analytical method indicates the closeness of test results obtained by that method to the true value (FDA, *ICH-Q2 (R1)*, 1995). For recovery testing, the quantity of excipients equivalent to 10 tablets were taken. Then approximately 80%, 100% and 120% of the declared amount of ingredient was mixed with excipients and analysed. The test procedure was followed thrice at each level.

Table 5. Recovery (Spiked placebo method)

Range relative to theoretical concentration	% Recovery			
	1st value	2nd value	3rd value	Mean Assay
80%	100.96	101.55	97.37	99.96
100%	98.72	97.49	102.69	99.63
120%	101.12	97.46	98.62	99.07

Mean Assay	99.71%
Standard deviation	1.71
%RSD	1.71
Number of determinations	9
Minimum	97.37
Maximum	102.69%

The results satisfied FDA criteria and demonstrated recovery (accuracy) of the developed method.

Specificity: By injecting blank, standard solution and the sample solution, the specificity of the method was checked to observe any interference to the peaks. Acetonitrile was used as blank and standard and sample were produced by 100 µg/ml concentration level. Placebo was injected also. For specificity, the chromatograms Figures 5-8 were observed.

Table 6. Specificity study

Sample	Active ingredient	Expected retention time	Findings	Comments
Blank	Levofloxacin	7	No peak appeared	Method was specific for levofloxacin
Placebo	Levofloxacin	7	No peak appeared	
Standard	Levofloxacin	6.54	Respective peak appeared	
Test sample	Levofloxacin	6.58	Respective peak appeared	

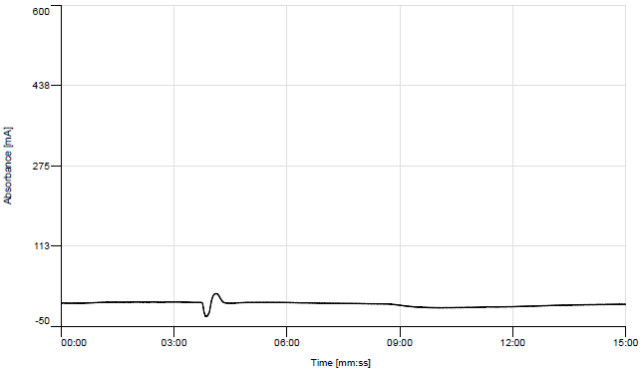

Figure 5. Chromatogram for blank.

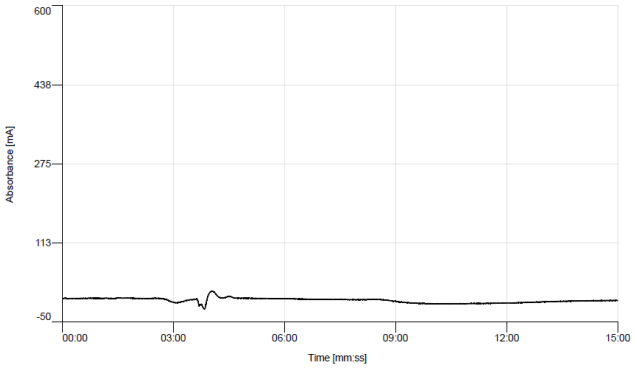

Figure 6. Chromatogram for placebo.

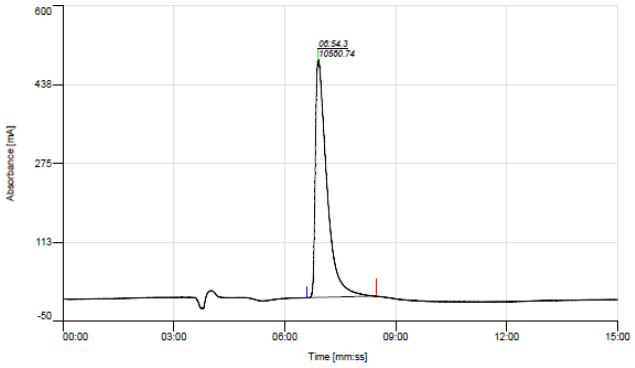

Figure 7. Chromatogram for standard solution.

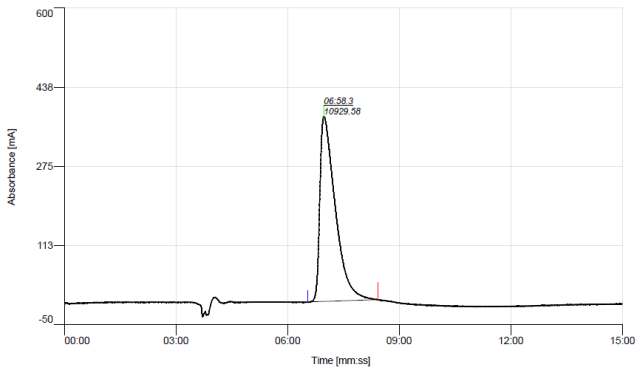

Figure 8. Chromatogram for sample solution

Robustness: For checking the robustness of the method, the flow rate of standard solution was changed. Retention times (RT), and asymmetry were obtained from chromatograms as shown in Figures 9-11 (Table 7).

Table 7. Robustness study

Conditions applied for standard (Flow rate)	Retention time in minutes	Asymmetry and peak shape	Remarks
0.4 ml/min	7.58	0.85	Complies
0.5 ml/min	6.50	0.80	Complies
0.6 ml/min	5.04	0.82	Complies

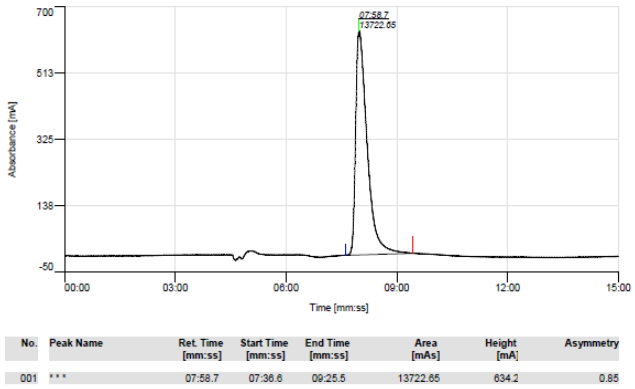

Figure 9. Chromatogram for standard solution at 0.4 ml/min flow rate level.

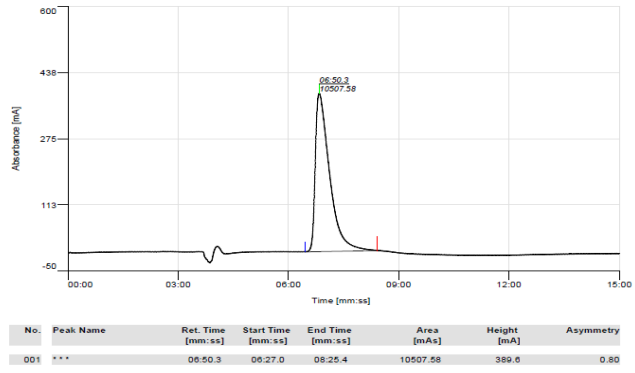

Figure 10. Chromatogram for standard solution at 0.5 ml/min flow rate level

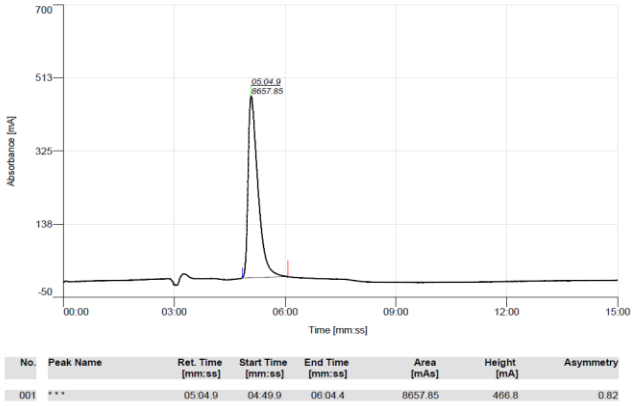

Figure 11. Chromatogram for standard solution at 0.5 ml/min flow rate level

Acceptance Criteria: In order to meet the acceptance criteria, the tailing factor should be T≤ 2. (CDER guidance document, FDA)

Result and Evaluation: From the findings, there were no major changes in the retention time and tailing factor. This robustness study reflects that the method is unaltered by small variations in the chromatographic conditions.

Assay calculation: The assay was calculated (Table 8) from the Levofloxacin 250 mg tablet.

$$\% \text{ Assay} = \frac{\text{Area of Sample x Conc. of Standard}}{\text{Area of Standard x Conc. of Sample}} \text{ x Potency or Assay of standard}$$

Table 8. Assay calculation

Sample Wt. taken (mg)	Sample Conc (µg/ml)	Sample Number		Area (mAs)	Average Area	% Assay
282.5	100.18	1	rep1	10929.58	10897.70	99.97
			rep2	10865.81		
292.3	102.92	2	rep1	11194.19	11228.82	100.26
			rep2	11263.45		
284.8	100.28	3	rep1	10546.81	10586.82	97.02
			rep2	10626.82		
288.0	101.41	4	rep1	10931.36	10868.50	98.50
			rep2	10805.64		
287.5	101.23	5	rep1	10883.55	10854.50	98.54
			rep2	10825.44		
294.5	103.70	6	rep1	11124.07	11288.31	100.04
			rep2	11452.54		
					Mean	99.06
					SD	1.26
					%RSD	1.28

Result and Evaluation: The percentage of levofloxacin 250 mg tablet was found to be 99.06 % and %RSD value was within limit of ≤ 2.

4. Conclusion

A selective, accurate and precise method was developed for quantitative analysis of levofloxacin 250 mg tablet by C8 HPLC using reversed-phase column. The validation parameters were found within the recommended limits according to ICH guidelines. The tailing factor in changing flow rate was 0.80 to 0.85 which reflected that the method was robust in terms of little variation of chromatographic conditions. From the readings, the analytical assay method validation was found to be simple, effective, reliable and reproducible at ambient temperature. Thus, the C8 column reversed-phase HPLC can be used for routine analysis of Levofloxacin 250 mg tablet effectively and for regulatory approval.

Acknowledgement

The authors are grateful to the Department of Pharmacy,Jagannath University, Dhaka, Bangladesh for providing chemicals and instrumental supports and Eskayef Pharmaceuticals Limited, Tongi, Gazipur, Bangladesh for supplying reference standard.

References

Allport-Settle, M. J. (2010). *International Conference on Harmonisation (ICH) Quality Guidelines: Pharmaceutical, Biologics, and Medical Device Guidance Documents Concise Reference*. PharmaLogika

Araujo, P. (2009). Key aspects of analytical method validation and linearity evaluation. *Journal of Chromatography B*, 877(23): 2224–2234.

Brunton, L., Knollmann, B., & Hilal-Dandan, R. (2017). *Goodman and Gilman's The Pharmacological Basis of Therapeutics, 13th Edition* (13th ed.). McGraw-Hill Education / Medical.

Center for Drug Evaluation and Research, U.S. Food and Drug Administration. *Reviewer Guidance, Validation of Chromatographic Methods;* FDA, Rockville, MD; Nov 1994.

Chan, C. C., Lee, Y. C., Lam, H., & Zhang, X. (2008). *Analytical Method Validation and Instrument Performance Verification* (1st ed.). Wiley-Interscience.

Chepurwar, S. B., Shirkhedkar, A. A., Bari, S. B., Fursule, R. A., & Surana, S. J. 2007. Validated HPTLC Method for Simultaneous Estimation of Levofloxacin Hemihydrate and Ornidazole in Pharmaceutical Dosage Form. *Journal of Chromatographic Science*, 45(8): 531–536. https://doi.org/10.1093/chromsci/45.8.531

Croom, K.F., Goa, K.L. Levofloxacin. (2003). *Drugs* 63: 2769–2802. https://doi.org/10.2165/00003495-200363240-00008

FDA, *ICH-Q2 (R1).* (1995). *Validation of Analytical Procedures: Text and Methodology*, vol. 60, U S Food and Drug Administration, Washington, DC, USA

Katzung, B. (2017). *Basic and Clinical Pharmacology 14th Edition* (14th ed.). McGraw-Hill Education / Medical.

Lalitha Devi, M., & Chandrasekhar, K. (2009). A validated stability-indicating RP-HPLC method for levofloxacin in the presence of degradation products, its process related impurities and identification of oxidative degradant. *Journal of Pharmaceutical and Biomedical Analysis*, 50(5): 710–717. https://doi.org/10.1016/ j.jpba.2009.05. 038

Maleque, M., Hasan, M. R., Hossen, F., & Safi, S. (2012). Development and validation of a simple UV spectrophotometric method for the determination of levofloxacin both in bulk and marketed dosage formulations. *Journal of Pharmaceutical Analysis, 2*(6): 454–457. https://doi.org/10.1016/j.jpha. 2012.06.004

Sarah K. Branch. (2005). Guidelines from the International Conference on Harmonisation (ICH), (2005). *Journal of Pharmaceutical and Biomedical Analysis*, (38): 798-805, https://doi.org/10.1016/ j.jpba.2005.02.037.

Smelov, V., Perekalina, T., Artemenko, N., Smelova, N., Ukleeva, G., & Gorelov, A. (2005). Chlamydia trachomatis survival in the presence of two fluoroquinolones (lomefloxacin versus levofloxacin) in patients with chronic prostatitis syndrome. *Andrologia*, *37*(2–3): 61–64. https:// doi.org/10.1111/j.1439-0272.2005.00654.x

Szerkus, O., Jacyna, J., Gibas, A., Sieczkowski, M., Siluk, D., Matuszewski, M., Kaliszan, R., &Markuszewski, M. (2017). Robust HPLC–MS/MS method for levofloxacin and ciprofloxacin determination in human prostate tissue. *Journal of Pharmaceutical and Biomedical Analysis*, *132*: 173–183. https://doi.org/10.1016 /j.jpba.2016.10. 008

Zhanel, G.G., Fontaine, S., Adam, H. et al. (2006). A Review of New Fluoroquinolones. *Treat Respir Med* 5: 437–465. https://doi.org/10.2165/ 00151829-200605060-00009