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EVALUATION OF THE CRITICAL POINTS IN THE VALIDATION OF 500 MG LEVOFLOXACIN TABLETS FOR DISSOLUTION PROFILE

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ABSTRACT

The method was validated by ultraviolet spectrophotometry for the determination of levofloxacin 500 mg tablets using dissolution profiles to establish therapeutic equivalence. The parameters were satisfactory according to accuracy and precision in a linear range between 2,0 µg/mL and 8.0µg/mL. Both, the relative error and relative standard deviation were less than 2%, the recovery was on average of 98,5%, the quantification limit (QL) obtained was 0,47 µg/mL. The effect of filters Varian ® Full FlowTM of 70 microns used in the dissolution equipment autosampler Varian ® 7010 and the effect of light were evaluated. For the effect of the filter, three concentrations levels were evaluated (3, 5, 7 µg/mL); no change in the recovery were observed applying a T-test (both sides, 95% limit of confidence). The effect of light was explored exposing levofloxacin standard's to natural and artificial light for 0, 6, 24, 48, 62 and 86 hours. We find that after 24 hours the response have significance's changes. With a both-sides, 95 % limit confidence ANOVA, the homocedasticity (Leven's test) and normality test (Shapiro-Wilk) were checked. In conclude the developed method can be used to determine levofloxacine in tablets

for profile dissolution, and the filter used does not affect, but light effect is important, and samples must not be handle for more than 24 hours.

KEYWORDS: levofloxacin, validation, filter effect, photosensitivity.

INTRODUCTION

Levofloxacin is an antibacterial agent belonging to the fluoroquinolone class and is the levorotatory isomer of ofloxacin.[1,2] Its IUPAC name is: (S)-(-)-9-fluor-2,3-dihidro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pirydo[1,2,3-de]-1,4-benzoxacin-6- carboxylic acid hemihydrate and its empirical formula $C_{18}H_{20}FN_3O_4 \cdot ½\ H_2O$ with a molecular weight of 370,38 µg/mol.[3]

It is important in the treatment for Gram-positive and some Gram negative bacteria pathologies as *Escherichia coli* and *Pseudomonas aeruginosa* among others.[4] For treatment of susceptible pathogens, levofloxacin can be administered orally or intravenously in doses of 250 or 500 mg once or twice a day; but in the case of complicated skin infections and in patients with hospital-acquired pneumonia, it is recommended to use a dose of 750 mg once a day. [5]

The chemical structure is shown below.

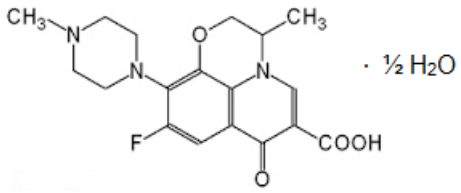

Figure 1. Structure of levofloxacin hemihydrate.

Its solubility keeps constant in a pH range of 0,6 to 5,8 (approximately 100 mg/mL), levofloxacin solutions are unstable when exposed to light so it is recommended to use amber vials for its storage. [3]

In the literature, various analytical techniques for quantification of levofloxacin appear such as spectrophotometry or high performance liquid chromatography (HPLC). The spectrophotometric method is preferred, because the results can be obtained more quickly, easily and with less control factors compared with HPLC technique. HPLC methods are used

when there is significant interference from the excipients in the formulation, to improve the analytical sensitivity and when the scan can be automated.[6]

El-Brashy and colaborators in 2004 published two procedures for the spectrophotometric determination of levofloxacin as raw material or in tablets by using chemical reactions for the formation of colored complexes.[6] Thakkar et al.[7], and Gupta et al.[8] also used spectrophotometric techniques to quantify levofloxacin in a dissolution study of tablets, in which absorbance measurements were performed at a wavelengths of 293,0 nm and 293,7nm respectively. Actually in the official monograph of the Pharmacopoeia of the United States American (USP) [5] for levofloxacin tablets assays, dissolution and dose uniformity, spectrophotometry is used as well as Desai et al describes. [9]

In order to verify the quality of levofloxacin tablets by a spectrophotometric method is intended to validate the method by standard addition not only for pharmacopoeia tests but also for "in vitro" therapeutic equivalence (dissolution profiles). For this reason, is that as validation parameters were considered: linearity, accuracy, precision (repeatability and intermediate precision), specificity for the system and for the method[10,11]; also evaluation of the critical points for this type of test were performed, such as the use of filters in the preparation of the samples and its stability to light exposure, even more that instability to light for these preparations is reported.[5]

MATERIALS AND METHODS

The reference standard used was levofloxacin hemihydrate EUROPHARMA, lot 0706161, 98,83% of purity, and as reference tablets were used ELEQUINE® 500 mg, batch LCE 120, made in Mexico by Janssen-Cilag.

Being the levofloxacin a photosensitive active ingredient, samples and standards were covered with sheets of aluminium foil in a light restricted environment. An UV-VIS spectrophotometer UNICAM Helios β was used for system validation of the analytical method. Linearity was assessed by three standard calibration curves with the following concentrations: 2, 3, 4, 5, 6, 7 and 8 µg/mL, 25,0 mL of each standard was prepared with the appropriate dilution of a stock solution of the reference standard. The limit of quantitation (LOQ) and the limit of detection (LOD) were also determined referred to the levofloxacin base. The medium used as solvent was HCl 0,1 M.

The intermediate precision of three concentration levels (3, 5 and 7 μg/mL) were carried out for five days and repeatability of the system at a concentration of 3 ug / mL which was performed in triplicate.

The following parameters for the validation of the method were evaluated: linearity, intermediate precision, repeatability and recovery; for validation of the method for the determination of levofloxacin by standard addition[10.11]. Linearity was performed by standard addition, by grinding 10 tablets of the drug reference and measuring an equivalent powder tablets by triplicate, to prepare an equivalent stock solution of 1000 μg/ml levofloxacin base, which 50 mL placed stock solution in two 25,0 mL volumetric flasks and aliquots of the levofloxacin reference solution were added to each of the flasks respectively to have equivalent concentration solutions 3, 4, 5, 6, 7 and 8 μg/mL. The measurements were performed at a wavelength of 294,0 nm. All preparations were protected from light using an aluminum foil cover. The homocedasticity system and the method was evaluated by the Hartley test.[12] Validation`s parameters were calculated using Excel® Microsoft Office 2007.

Evaluation of the sensitivity to light was performed at three concentration levels (3, 5 and 7 μg/mL) in triplicate at 0, 6, 24, 48, 62 and 86 hours at each level, through a ANOVA, the Levene test and Shapiro Wilk test using the Statistic Software SPP 20. SPSS, IBM program. "IBM SPSS statistics based 20." Chicago, IL: SPSS Inc (2011). The effect of the filter Varian® Full FlowTM 70 microns, used in the dissolution equipment Dissolutor Varian® model with auto sampler unit VK7010 VK7010 Varian ® was assessed by triplicate at three concentration levels (3, 5 and 7 μg/mL) of levofloxacin basis reference standard, comparing the responses of the preparations before and after filtering.

Determination of the content and the test for uniformity of content for levofloxacin tablets was performed according to the guidelines of the USP.[5]

RESULTS AND DISCUSSION

Validation of the system and method, as well as assessments filter effect and light sensitivity of the preparations of levofloxacin was performed at 294,0 nm.[8]

Table 1 and 2. Shows the results for linearity for the validation of the system

Table. 1. Study of the linearity of the system to levofloxacin

	C (ug/ml)	Absorbance (UA)	FR
1	2,006	0,178	0,08875
2	2,006	0,183	0,09124
3	2,013	0,180	0,08940
4	3,009	0,265	0,08808
5	3,009	0,267	0,08875
6	3,020	0,261	0,08642
7	4,011	0,363	0,09049
8	4,011	0,359	0,08949
9	4,027	0,355	0,08816
10	5,014	0,440	0,08775
11	5,014	0,447	0,08915
12	5,034	0,441	0,08761
13	6,017	0,533	0,08858
14	6,017	0,541	0,08991
15	6,040	0,539	0,08923
16	7,020	0,622	0,08860
17	7,020	0,622	0,08860
18	7,047	0,627	0,08897
19	8,023	0,716	0,08925
20	8,023	0,716	0,08925
21	8,054	0,709	0.08803

Table 2. Results of analysis of variance of the regression line system

Parameter	Value obtained
Slope (m)	0,08882
Intercept (b)	-0,00003
Correlation coefficient (r)	0,9998
Coefficient of determination (r^2)	0,9995
SD slope l	0,00045
SD for the intercept	0,00245
Standard error for y ($S_{y/x}$)	0,00417
Average response factor (RF)	0.08884358
CV_{RF}	1.16 %
SD of the RF	0.00103
N (points number)	21
Confidence limits for the term b	(-0,00268;0,00758)

Linearity is between 2,0 and 8,0 ug / mL, which meets the criteria for homoscedasticity found by Hartley test[12] and the determination coefficient is between 0,98 and 1,00.[10]

For precision and accuracy of the system we found that both, repeatability (Table 3) and intermediate precision, (Table 4) meets the criteria as the variation coefficient percentage

(CV %) as the relative error percentage (RE %) it´s not greater than 2 % , in each of the three concentration levels.[10]

Table 3. Repeatability for the system at three different concentration levels

	3 µg/mL		5 µg/mL		7 µg/mL	
Replicates	**ABS (UA)**	**RE %**	**ABS (UA)**	**RE %**	**ABS (UA)**	**RE %**
1	0,262	0,13	0,438	0,15	0,618	0,22
2	0,262	0,13	0,439	-0,08	0,622	-0,43
3	0,263	-0,25	0,439	-0,08	0,618	0,22
Average	0,262		0,439		0,619	
DS	0,001		0,001		0,002	
RSD%	0,22		0,13		0,37	

Table 4. Intermediate precision for the system by five days at three concentration levels

	3 µg/mL		5 µg/mL		7 µg/mL	
Replicates	**ABS (UA)**	**RE %**	**ABS (UA)**	**RE %**	**ABS (UA)**	**RE %**
1	0,266	0,97	0,443	0,94	0,632	0,60
2	0,268	0,22	0,449	-0,40	0,641	-0,82
3	0,268	0,22	0,448	-0,18	0,637	-0,19
4	0,271	-0,89	0,449	-0,40	0,635	0,13
5	0,270	-0,52	0,447	0,04	0,634	0,28
Average	0,269		0,447		0,636	
SD	0,002		0,002		0,003	
RSD%	0,73		0,56		0,54	

The limit of detection (LOD) and the limit of quantitation (LOQ) were respectively 0,14 µg/mL and 0,47 µg/mL of levofloxacin base, so the range of linearity could be extended.

According to Table 5 (t exp <t critical) there's no significant difference in the effect of the filter, indicating that the filter material does not absorb the analyte.

Table 5. Results obtained to evaluate filtering preparations of levofloxacin base solutions with a Varian® Full FlowTM 70 µm filter.

Condition	**No filtration**		**Filtrated**		t_{exp}	$t_{critic(2,0.005)}$
Concentration (µg/mL)	ABS (UA)	DS	ABS (UA)	DS		
3,02	0,286	0,003	0,287	0,003	1,00	4,30
5,03	0,465	0,001	0,463	0,001	1,51	
7,05	0,649	0,006	0,647	0, 006	0,65	

Note: The results represent the average of three replicates in each of the conditions and to each concentration level.

Evaluation for light influence is shown in Table 6, these data were used to perform a two tails ANOVA at a confidence level of 95%; for this, a Levene test were performed previously, from which was observed that there is homoscedasticity in the data (p = 0.06, greater than or equal to α= 0.05 for a confidence level of 95%) and Shapiro-Wilk test, which indicates that the data meets the assumption for normality (p = 0.05, greater than or equal to α= 0.05 for a confidence level of 95%). The ANOVA analysis showed that there are significant differences in the data (p = 0.001, greater than or equal to α = 0.05 for a confidence level of 95%), while 0 and 24 hours have no significant differences (p = 0.071, greater than or equal to α= 0.05 for a confidence level of 95%). There is a difference between time of 24 hours and 48 hours. Turkey test found no significant differences between times 0 and 6 hours but since 24 hours there is a significant difference. Therefore, it requires working solutions in a shorter period, less than 24 hours.

Table 6. Evaluation of the stability of levofloxacin exposed to natural and artificial light

	Day 0			Day 1	Day 2	Day 3
Cn (µg/mL)	0 hours	6 hours	24 hours			
3,02	0,279	0,281	0,283	0,287	0,286	0,288
5,03	0,461	0,464	0,466	0,471	0,469	0,470
7,05	0,650	0,646	0,649	0,652	0,650	0,653

Note: The results represent the average of three replicates in each of the conditions and to each concentration level.

With USP method we determine the content and labeling percentage for reference levofloxacin tablets, table 7 and 8.[5]

Table 7. Determination of content for levofloxacin base tablets for the reference (Elequine ®)

Muestra	Mass (g)	ABS sample (UA)	Concentration (mg/mL)	Content (mg/mL)	% of labeling
I	0,1402	0,456	0,0051	511,65	102,33
II	0,1402	0,450	0,0050	504,92	100,98
III	0,1404	0,456	0,0051	510,92	102,18
		Promedio		509,2	101,8
		DS		3,7	0,7
		DSR%		0,7	0,7
		Límite de confianza 95 %		9,2	1,8

Table 8. Recovery obtained for levofloxacin tablets by standard addition at three concentration levels.

Nivel	Sample	Real quantity (sample+standard added)	Quantity obtaines	% Recuperación
3 µg/mL	1	76,08	75,42	99,1
	2	76,08	74,31	97,7
	3	76,15	74,87	98,3
5 µg/mL	1	126,41	125,64	99,4
	2	126,41	124,25	98,3
	3	126,49	123,42	97,6
7 µg/mL	1	176,75	175,03	99,0
	2	176,75	173,92	98,4
	3	176,82	174,47	98,7
		Average		98,5
		SD		0,65
		RSD%		0,63
		% Recovery	Higher	99,4
			Lower	97,6

As found in Table 7, the tablets are within the specification for the product, knowing this information recovery determination was performed at three levels by adding standard with equivalent concentrations to 3, 5 and 7 ug / mL. The recovery is between 97,6 and 99,4 % which is within recommended specification for this type of methodology, 97,0 to 103,0 %, with a lower RSD of 2% (Table 8). [10]

For standard addition method for levofloxacin tablets it complies to homoscedasticity criteria (evaluated by test Hartley) and the determination coefficient is between 0,98 and 1, at table 9 and 10 linearity for standard addition data is presented.

Table 9. Correlation between the results found by adjustment and by added to the sample, and linearity for the added amount + sample quantity vs ABS

Stock solution	Quantity added+ Sample quantity (µg)	Adjust quantity (µg)	ABS (UA)
1	25,17	25,30	0,271
2	25,17	24,18	0,267
3	25,17	24,74	0,269
1	50,34	52,21	0,367
2	50,34	51,09	0,363
3	50,34	50,25	0,360
1	75,50	76,04	0,452
2	75,50	74,64	0,447
3	75,50	73,79	0,444

1	100,67	100,70	0,540
2	100,67	101,83	0,544
3	100,67	100,70	0,540
1	125,84	125,93	0,630
2	125,84	124,81	0,626
3	125,84	125,37	0,628
1	151,01	146,96	0,705
2	151,01	153,68	0,729
3	151,01	153,12	0,727

Table 10. Parameters for data of table 9.

Parámetro	**Valor obtenido**
Pendiente (m)	0,00357
Intercepto (b)	0,18074
Coeficiente de correlación (r)	0,9996
Coeficiente de determinación (r^2)	0,9992
DS de la pendiente	0,00002
DS del intercepto	0,00204
Error estándar de y ($S_{y/x}$)	0,00518

The repeatability of the method is shown in Table 11, where it is found that it meets the RSD % according to the criteria established for accuracy wich are less than 2 %, at each of the concentration levels. For intermediate precision is found that for each day, criterion is fulfilled of less than 2 %. In the case of intermediate precision, Table 12, F-test was applied to establish whether there was similarity or not for the variances of both groups and therefore there is no significant difference being the values obtained by the same analyst , on different days($F_{experimental} < F_{critic}$ for (Fn1-1 / Fn2-1 $\alpha = 0.05$). By applying the T-Student test using Microsoft Office Excel 2007 (n-2 and $\alpha = 0.05$) the experimental value turned out to be less than the table value, demonstrating no significant difference between the average achieved by the same analyst on different days.

Table 11. Repeatability by addition standard method at three concentration levels

3,02 µg/mL		**5,03 µg/mL**		**7,05 µg/mL**	
Réplica	**Adjusted concentration, µg/mL**	**Replicas**	**Adjusted concentration,, µg/mL**	**Replicas**	**Adjusted concentration, µg/mL**
1	3,08	1	5,07	1	7,05
2	3,04	2	5,03	2	7,03
3	3,02	3	5,09	3	7,05
Average	3,05	Average	5,06	Average	7,04
SD	0,03	SD	0,03	SD	0,01
RSD%	0,97	RSD%	0,58	RSD%	0,18

Table 12. Intermediate precision by standard addition method.

Same analyst at different days (µg/mL)	
5,06	5,07
5,06	5,03
5,07	5,09
5,03	5,04
5,10	5,07
Conc media= 5,06	Conc media= 5,06
S =0.02510	S= 0,02449
F_{exp}= 1,05	F_{tab}=6,39 (Fn1-1/Fn_2-1), α= 0,05)
T_{exp}=0,26	T_{tab}=1,86 n-2 α = 0,05

CONCLUSIONS

The analytical method complies with the specifications in a linear range of 2,0 to 8,0 µg/ mL, with respect to linearity, accuracy and precision. Full FlowTM Varian® filters of 70µm used for determining the dissolution profiles do not absorb levofloxacin; therefore does not affect its recovery. This implies that the methodology is appropriate for the analysis of dissolution profiles of tablets of levofloxacin by the automated system dissolutor Varian VK7010 ® model.

Because of its light stability, levofloxacin preparatiions should be protected from light and used within a period no longer than 24 hours.

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