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Microbiological assay for determination of ofloxacin injection

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Abstract

A simple, sensitive and specific agar diffusion bioassay for the antibacterial ofloxacin was developed using a strain of *Micrococcus luteus* ATCC 9341 as the test organism, ofloxacin at concentration ranging 12–27 μg ml^{-1} could be measured in injection. A prospective validation of the method showed that method was linear ($r = 0.9994$) and precise (CV = 1.14). UV spectrophotometric and high performance liquid chromatographic techniques were chosen as a comparison methods for the determination of ofloxacin. The results obtained by three methods were statistically analyzed by analysis of variance (ANOVA) and the results obtained indicate that there is no significant difference among these methods.

Keywords: Fluoroquinolone; Microbiological assay; Quality control; Ofloxacin

1. Introduction

Ofloxacin is a fluoroquinolone carboxylic acid antimicrobial with a good activity against Gram-negative and Gram-positive bacteria [1]. No official method is available for the assay of pure drug and its formulations. Some methods such as spectrophotometry based on the formation of yellow colored chromophore with Fe (III) ion in acid medium [2], spectrofluorimetry [3], high performance liquid chromatography and derivative UV spectrophotometry [4], potentiometry and conductometry [5] have been published. High performance liquid chromatography [6–11], thin layer chromatography [12], high performance capillary electrophoresis [13] were developed for determination of ofloxacin in biological fluids and for determination in growth media [14]. Bioassay for other fluoroquinolone has been described [15,16], but no bioassay of the ofloxacin drug and its formulations has been previously described for laboratory quality control.

In this paper, a simple, sensitive and specific agar diffusion bioassay method is described for determination of the ofloxacin injection, using UV spectrophotometry and HPLC methods as a comparison.

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2. Experimental

2.1. Chemicals and reagents

Ofloxacin drug was kindly supplied by CILAG (São Paulo, Brazil) and pharmaceuticals were available in the Brazilian market. Ofloxacin injection was claimed to contain 40 mg ml^{-1} in hydrochloric acid, sodium chloride and water.

All chemicals used were of analytical-reagent grade; acetonitrile was of HPLC grade. All solvents and solutions for HPLC analysis were filtered through a Millipore filter and degassed with helium gas.

2.2. Assay of antibacterial activity

Worked solutions in concentrations 12, 18 and 27 μg ml^{-1} of the phosphate buffer (pH 8.0) were prepared fresh for each assay by using a stock solution of 600 μg ml^{-1}. The indicator organism was *Micrococcus luteus* ATCC 9341 using the cylinder–plate method. Bacterium suspension (25% transmittance at 580 nm) was prepared in Grove–Randall's medium 3 and the inocula was prepared at the Grove–Randall's medium 11 at concentration of 0.5%. Grove–Randall's medium 11 (20 ml) was poured into microbiological plates (100×20 mm). The inocula (5.0 ml) was distributed to the surface of each plate. After 1 h, six cylinders were placed onto a plate and 200 μl of the standard or sample were added into each cylinder. The bioassay plates were incubated at 35 °C aerobically for 18 h. The zone sizes were carefully measured with calipers. The sample results were compared with the standard curve obtained in the same experiment. The percent activity of ofloxacin in commercially samples was calculated by Hewitt equation [17]. The precision was expressed as the percent coefficient of variation. The statistical analysis was calculated by analysis of variance (ANOVA).

2.3. UV spectrophotometry

A Shimadzu UV-visible spectrophotometer model UV-16A was used for analysis. The detector wavelength was set at 294 nm. The standard solutions were obtained by appropriate dilution in 5% acetic acid [5] for giving concentrations ranged from 2.0 to 10.0 μg ml^{-1}. The UV spectrophotometric calibration curve was constructed by plotting the absorbance values at 294 nm versus concentration of the solution. A recovery test was then performed.

2.4. HPLC

Liquid chromatography was performed using a Shimadzu apparatus consisting of two Model LC-10AD pumps, a Model SPD-10A variable wavelength UV spectrophotometric detector and a Model SCL-10A system controller with a Model C-R6A chromatopak date system. The analytical column was NovaPak C18 150×3.9 mm id., 4 μm particles (Waters Assoc.). The mobile phase consisted of 0.5% sodium acetate pH 2.5/acetonitrile (87:13, v/v). The flow-rate employed was 1 ml min^{-1} [9]. The HPLC system was operated at ambient temperature (20 ± 1 °C).

The standard solutions were obtained by appropriate dilution in 5% acetic acid for giving concentrations ranged from 20.0 to 100.0 μg ml^{-1}. The high concentration of ofloxacin in HPLC method was used to quantify of ofloxacin in future photodegradation studies.

The calibration curve was constructed by plotting the peak area versus concentration of the solutions.

2.5. Reproducibility and validation

Accuracy and precision of microbiological method were determined intra-day and inter-day on three different days. Precision was expressed as the percent coefficient of variation of each curve. The statistical analysis employed mean, S.D., coefficient of variation and ANOVA [18].

3. Results and discussion

3.1. Assay of antibacterial activity

In the development phase of this microbiological assay, the parameters were evaluated as test

microorganism (*Staphylococcus epidermidis* ATCC 1228 or *Micrococcus luteus* ATCC 9341), inocula concentration (0.5, 1.0 and 2.0%) and solution concentration (4.0–8.0–16.0 μg ml^{-1}; 12.0–24.0–48.0 μg ml^{-1}; 12.0–18.0–27.0 μg ml^{-1} and 18.0–27.0–40.5 μg ml^{-1}). The conditions described in this paper showed the best results.

The results of growth inhibition zone diameter of ofloxacin reference substance and ofloxacin injection were presented in Tables 1 and 2.

There was a linear relationship between the $\log_{10}$ of the drug concentrations and growth inhibition zone diameter for concentrations 12, 18 and 27 μg ml^{-1}. The representative linear equation for ofloxacin was $13.885x - 4.5527$ and the correlation coefficient obtained was 0.9994, indicating good linearity. The coefficient of variation for individual standards ranged from 0.84 to 1.23% and for commercially samples ranged from 1.09 to 1.37% (Tables 1 and 2). The inter-day precision was evaluated by comparing the linear regressions of the three standards plots on the three different days over a 2 month period, the average coefficient of correlation was 0.9994 and the coefficient of variation of the slope of the three lines was 1.12%. The repeatability of the method was studied; the results in the determination of commercially sample of ofloxacin injection indicated the precision the method with coefficient of variation of 1.55 (Table 3). To assess the accuracy of the bioassay, three samples with concentration of 20.0, 30.0 and 40.0 μg ml^{-1} in three replicate were analyzed in the same day and on three different days, the results attained indicated the accuracy of the method proposed (Table 4). Photodegradation studies were carried out in our laboratory, the dates indicated the proposed procedure can be used as a stability-indicating method (in press).

In previous work it was possible to consider ofloxacin in water and injection stable at 90 °C for 60 days.

3.2. UV spectrophotometry

The UV spectrophotometry calibration curve was found to be linear with the correlation coeffi-

Table 1
Diameters of growth inhibition zones to obtain standard curve

Concentration (μg ml^{-1})	Diameters of growth inhibition zones (mm)[a]	Mean diameters of growth inhibition zones ± S.E.M.	CV (%)
12	10.60	10.48 ± 0.036	0.84
	10.41		
	10.36		
	10.44		
	10.53		
	10.52		
18	12.83	12.78 ± 0.060	1.14
	12.73		
	12.81		
	13.00		
	12.79		
	12.55		
27	15.01	15.37 ± 0.077	1.23
	15.42		
	15.50		
	15.44		
	15.30		
	15.52		

CV, Coefficient of variation; S.E.M., Standard Error Mean.
[a] Average of 10 plates.

Table 2
Zone diameters of growth inhibition for *Micrococus luteus* by ofloxacin injection

Concentration (μg ml^{-1})	Diameters of growth inhibition zones (mm)[a]	Mean diameters of growth inhibition zones ± S.E.M.	CV (%)
12	10.28	10.42 ± 0.058	1.37
	10.38		
	10.50		
	10.67		
	10.34		
	10.35		
18	12.74	12.71 ± 0.057	1.09
	12.66		
	12.48		
	12.89		
	12.72		
	12.80		
27	15.33	15.17 ± 0.071	1.14
	15.10		
	14.90		
	15.17		
	15.12		
	15.38		

CV, coefficient of variation; S.E.M., standard error mean.
[a] Average of 10 dish plates.

Table 3
Determination of ofloxacin injection commercially available samples using microbiological assay

Sample	Declared amount (μg ml^{-1})	Found amount (μg ml^{-1})	Found amount (%)	CV (%)
1	40.00	40.25	100.62	1.55
2	40.00	38.98	97.45	
3	40.00	39.68	99.92	
4	40.00	39.29	98.23	
5	40.00	40.41	101.03	

CV, Coefficient of variation.

Table 4
Recovery test of ofloxacin injection

Know concentration (μg ml^{-1})	Found concentration (μg ml^{-1}) ± S.E.M.[a]		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
20	19.73 ± 0.67	20.36 ± 0.42	98.65	101.80
30	30.42 ± 1.38	29.81 ± 1.67	101.40	99.36
40	39.45 ± 0.91	39.04 ± 1.18	98.62	97.60

[a] S.E.M., Standard error mean.

Table 5
Determination of ofloxacin injection commercially available samples using UV spectrophotometric method at 294 nm

Sample	Declared amount (μg ml^{-1})	Found amount (μg ml^{-1})	Found amount (%)	CV (%)
1	40.00	40.50	101.25	
2	40.00	39.75	99.37	
3	40.00	39.30	98.25	1.49
4	40.00	40.80	10.00	
5	40.00	39.92	99.80	

CV, Coefficient of variation.

Table 6
Experimental values obtained in the recovery test for ofloxacin injection by UV spectrophotometric method at 294 nm

Sample	Added (μg ml^{-1})	Found (μg ml^{-1})[a]	Recovery (%)
1	2.00	1.99	99.50
2	4.00	4.05	101.17
3	6.00	6.15	102.50

[a] Average of two determinations.

cient (r) 0.9999, the regression equation was $y = 0.0631x - 0.0013$. The detailed precision is shown in Table 5 indicate good results for this method (coefficient of variation = 1.49). The recovery test indicated excellent accuracy of the ofloxacin from the spiked samples (Table 6).

3.3. HPLC

The calibration curve for HPLC analysis was obtained by plotting the peak area against drug concentration. The regression equation was $y = 2.4897^5x + 0.0228^5$ and the correlation coefficient (r) 0.9999. The results of the determination of ofloxacin injection by HPLC and recovery tests were presented in Tables 7 and 8, respectively. The method is precise with coefficient of variation 0.72%. Recovery tests confirmed the accuracy of the HPLC proposed method.

The data obtained by microbiological assay, UV spectrophotometric analysis and HPLC method (Table 9) were statistically comparable by ANOVA test, which indicated there is no significant difference among three methods at the $P < 0.05$.

It was also found that the temperature (90 °C) during 60 days did not interfere with ofloxacin stability.

4. Conclusions

The microbiological method developed for determination of ofloxacin injection in this study is linear, precise, reproducible and simple, which provides it valuable method for quality control of this drug in the formulation studied. All methods proposed showed coefficient of variation less than 1.5%. These results can indicate a good precision. The reported methods for the determination of ofloxacin injection gave accurate and precise re-

Table 7
Determination of ofloxacin injection commercially available samples using HPLC method

Sample	Declared amount (μg ml^{-1})	Found amount (μg ml^{-1})	Found amount (%)	CV (%)
1	40.00	40.18	100.45	
2	40.00	39.49	98.72	
3	40.00	39.93	99.82	0.72
4	40.00	40.15	100.37	
5	40.00	40.12	100.31	

CV, Coefficient of variation.

Table 8
Experimental values obtained in the recovery test for ofloxacin injection by HPLC

Sample	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)[a]	Recovery (%)
1	20.00	20.06	100.30
2	40.00	39.31	98.28
3	60.00	61.49	102.48

[a] Average of two determinations.

Table 9
Analysis of ofloxacin injection by three different methods

Sample	UV	HPLC	Microbiological
1	101.25	100.45	100.62
2	99.37	98.72	97.45
3	98.25	99.82	99.92
4	99.81	100.37	98.23
5	102.00	100.31	101.03

sults. Moreover bioassay requires not only no specialized equipment but also no toxic solvents.

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