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SHORT COMMUNICATION

A novel and rapid microbiological assay for ciprofloxacin hydrochloride

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Abstract The present work reports a simple, fast and sensitive microbiological assay applying the turbidimetric method for the determination of ciprofloxacin hydrochloride (CIPRO HCl) in ophthalmic solutions. The validation method yielded good results and included excellent linearity, precision, accuracy and specificity. The bioassay is based on the inhibitory effect of CIPRO HCl upon the strain of *Staphylococcus epidermidis* ATCC 12228 used as the test microorganism. The results were treated statistically by analysis of variance (ANOVA) and were found to be linear (r=0.9994, in the range of 14.0–56.0 μg/mL), precise (intraday RSD %=2.06; interday RSD%=2.30) and accurate (recovery=99.71%). The turbidimetric assay was compared to the UV spectrophotometric and HPLC methods for the same drug. The turbidimetric bioassay described on this paper for determination of ciprofloxacin hydrochloride in ophthalmic solution is an alternative to the physicochemical methods disclosed in the literature and can be used in quality control routine.

1. Introduction

Ciprofloxacin hydrochloride (CIPRO HCl), namely 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid hydrochloride (Fig. 1), is a second generation fluoroquinolone antimicrobial with a wide spectrum of activity against Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa* [1]. The mode of action of fluoroquinolones involves interactions with both DNA gyrase, the originally recognized drug target, and topoisomerase IV, a related type II topoisomerase [2].

The drug is official in British Pharmacopoeia [3] presenting an HPLC assay for CIPRO HCl tablets and ciprofloxacin lactate intravenous infusion. In Brazilian Pharmacopoeia [4] three methods are proposed to determine CIPRO injection, CIPRO HCl tablets and ophthalmic solution; an UV spectrophotometric, an HPLC and a microbiological diffusion agar methods. The United States Pharmacopoeia [5] describes an HPLC method for CIPRO and CIPRO HCl assay in bulk, CIPRO injection, ophthalmic ointment, ophthalmic solution and tablets.

Despite most methods presented in official compendia are physicochemical assays, these methods do not represent the potency of antimicrobials neither can predict the loss of activity.

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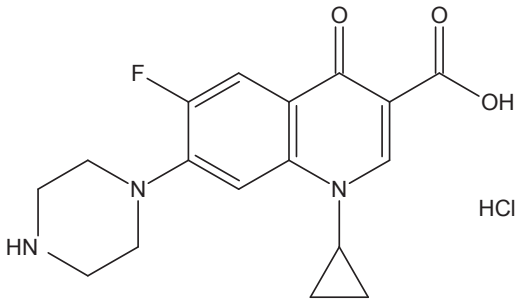

Fig. 1. Chemical structure of ciprofloxacin hydrochloride.

Furthermore, the low cost and simple procedures of bioassays have allowed them to become an alternative methodology for drug potency assessment in pharmaceutical formulations.

The literature has reported microbiological assays by agar diffusion method for determination of others fluoroquinolones in pharmaceutical formulations, such as norfloxacin [6], sparfloxacin [7], ofloxacin [8], enrofloxacin [9], lomefloxacin [10], gatifloxacin [11] and orbifloxacin [12]. However, no microbiological assay using turbidimetric method for the determination of quinolones has been reported yet. This assay is faster than agar diffusion method and presents easier management. The microbiological assay can reveal subtle changes not demonstrable by chemical methods and it gives the possibility to evaluate the potency of this substance, which is very important for the analysis of antibiotics. Bioassay is an ecological technique because it is not a residue or solvent producer. Moreover, microbiological assay requires no specialized equipment or toxic solvents [13].

In this paper, a novel, rapid, simple and sensitive turbidimetric bioassay method is described for determination of CIPRO HCl in ophthalmic solution as an alternative to the physicochemical methods described in the literature.

2. Materials and methods

2.1. Chemicals and instruments

CIPRO HCl reference standard (assigned purity 100%) was kindly supplied by EMS Sigma Pharma Group (São Paulo, Brazil). Pharmaceutical dosage form (ophthalmic solution) containing CIPRO HCl was obtained commercially and claimed to contain 3.5 mg/mL of drug and boric acid, sodium citrate, dissodium edetate (EDTA), benzalconium chloride and purity water as excipients.

All chemicals and reagents used were of analytical grade. High purity water was prepared using Millipore Milli-Q purification system (Millipore, Bedford, MA, USA). The absorbances were carried out in spectrophotometer Beckman model DU® 530 (California, USA).

2.2. Microorganism and inoculum

The cultures of *Staphylococcus epidermidis* ATCC 12228 were cultivated on Casoy agar and maintained in the freezer as stock. The cultures were pealed to brain heart infusion (BHI) broth (24 h before the assay) and kept at 36 ± 1 °C. A culture broth of $25 \pm 2\%$ turbidity (transmittance) was obtained at 530 nm, using a suitable spectrophotometer and a 10 mm diameter test tube as absorption cells against BHI broth as blank.

2.3. Preparation of the standard solutions

Accurately weighed 100 mg of CIPRO HCl reference standard was transferred to a 100 mL volumetric flask and dissolved in water (final concentration of 1000 μg/mL). Aliquots of this solution were diluted in water at concentrations of 14.0, 28.0 and 56.0 μg/mL, which were used in the assay.

2.4. Preparation of the sample solutions

Aliquots (40, 80 and 160 μL) of CIPRO HCl ophthalmic solution (3500 μg/mL) were transferred volumetrically into 10 mL volumetric flasks and added water to give a final concentrations of 14.0, 28.0 and 56.0 μg/mL.

2.5. Turbidimetric assay

1.0 mL of the inoculated BHI broth was added in tubes containing 10.0 mL of sterile BHI broth. Aliquots of 200 μL of CIPRO HCl reference standard and sample solutions were added in the respective tubes. Twenty tubes were used to carried out parallel lines 3×3 design, three tubes for each concentration of standard and sample, one tube for positive control (broth and inoculum), without addition CIPRO HCl and one for negative control (only broth).

After incubation at 35 ± 2 °C for 4 h in shaker incubator, the bacteria growing was discontinuous through adding 0.5 mL of 12% formaldehyde aqueous solution. The absorbance was determined in each tube using a spectrophotometer at 530 nm employing the negative control as blank.

2.6. Calculation

The potency of CIPRO HCl in ophthalmic solution was calculated by Hewitt equation [14]. The assay was statistically treated by the linear parallel model and by linear regression analysis. Analysis of variance (ANOVA) was also used to verify the validity of the method.

2.7. Method validation

The method was appropriately validated by determination of the following parameters: linearity, precision, accuracy, specificity and robustness.

2.7.1. Linearity

The calibration curve was obtained with three doses of the reference standard. The linearity was evaluated by linear regression analysis, which was calculated by the least squares regression method.

2.7.2. Precision

The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay). Repeatability was evaluated by assaying the samples in the same concentration and same day. The intermediate precision was studied by comparing the assays on three different days. The results were expressed in relative standard deviation (RSD%).

2.7.3. Accuracy

The accuracy was determined by % recovery of known amounts of CIPRO HCl reference standard added (4.5, 24.5 and 44.5 μg/mL) to the samples at the beginning of the process. Aliquots of 30 μL

of CIPRO HCl ophthalmic solution (3500 μg/mL) were transferred into 10 mL volumetric flasks containing 45, 245 and 445 μL of CIPRO HCl standard solution (1000 μg/mL). Then, distilled water was added to make up to volume and give the final concentrations of 15.0, 35.0 and 55.0 μg/mL. These solutions were assayed and the percentage recovery of added CIPRO HCl was calculated.

2.7.4. *Specificity*

The ability of the proposed method to determine CIPRO HCl in the presence of the excipients was assessed by comparing the results obtained in the bioassay with the whole sample against the results from the standard solution. The Student's *t*-test and *F*-test were performed to compare the ciprofloxacin standard and sample absorption values.

2.7.5. *Robustness*

Robustness was determined by analyzing the same sample under a variety of conditions. The considered factors were incubation time and volume of the inoculated BHI broth. The variation of the mean absorbance between the different assays was statistically analyzed by ANOVA.

2.8. *Comparison of methods*

The results obtained in this study were compared with those by HPLC method described previously [15] and the UV spectrophometric method also developed by the authors.

2.8.1. *Chromatographic conditions*

The HPLC method was performed isocratically using a mobile phase consisting of 2.5% acetic acid solution, methanol and acetonitrile (70:15:15; v/v/v). The wavelength of the UV detector was set at 275 nm. A Symmetry Waters C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size) was used [15].

2.8.2. *UV spectrophotometric conditions*

The UV spectrophotometric method was performed on a UV–vis Shimadzu, model UVmini-1240. CIPRO HCl was detected at 275 nm using 0.5 M hydrochloride acid as solvent.

3. Results and discussion

The experimental conditions were adjusted to accurately determine the performance of the assay. Some parameters were tested earlier to establish the conditions described and it is shown in Table 1. To develop and validate this bioassay a strain of *Staphylococcus epidermidis* was found to be the appropriate microorganism test allowing quantitation of CIPRO HCl.

The microbiological assay described in this work was performed in 3 × 3 design (three doses of standard and three doses of sample), according to British [3], Brazilian [4], The United States [5] and European Pharmacopoeias [16]. The calculation procedure usually assumes a direct relationship between the observed absorbance and the logarithm of the applied dose. The corresponding mean absorbance for reference solutions was 0.480 ± 0.0012 (RSD = 0.43) for lower dose, 0.408 ± 0.0044 (RSD = 1.85) for medium dose and 0.344 ± 0.0021 (RSD = 1.05) for higher dose and that for ophthalmic solution was 0.476 ± 0.0069 (RSD = 2.51), 0.402 ± 0.0038 (RSD = 1.63) and 0.338 ± 0.0033 (RSD = 1.71) for concentrations of 14.0, 28.0 and 56.0 μg/mL, respectively (Table 2).

Table 1 Conditions tested to establish the parameters for microbiological assay of ciprofloxacin hydrochloride.

Parameters	Conditions
Microorganism	*Bacillus subtilis* ATCC 9372 *Micrococcus luteus* ATCC 9341 *Staphylococcus epidermidis* ATCC 12228 *Escherichia coli* ATCC 10536
Culture medium	BHI broth Casoy broth Müeller–Hinton broth
Concentration of inoculum (%)	4.0 8.0 10.0
Diluents	Water Phosphate buffer pH 8.0
Concentrations (μg/mL)	1.75, 3.5, 7.0 14.0, 28.0, 56.0

Table 2 Experimental values of absorbance for ciprofloxacin hydrochloride reference solutions obtained by microbiological assay—turbidimetric method.

Concentration (μg/mL)	Range of reference solutions absorbance	Mean absorbance[a] ± RSD (%)
14.0	0.478–0.482	0.480 ± 0.43
28.0	0.401–0.416	0.408 ± 1.85
56.0	0.340–0.347	0.344 ± 1.05

[a]Mean of three assays.

The calibration curves for CIPRO HCl were constructed by plotting log concentrations (μg/mL) versus absorbance and showed good linearity between 14.0 and 56.0 μg/mL concentration range. The representative linear equation for CIPRO HCl was $y = -0.0983\ \mathrm{Ln}(x) + 0.7385$, where x is log dose and y is absorbance. The correlation coefficient (r) was 0.9994. There was no deviation from parallelism and linearity in our results ($p < 0.05$). The precision and accuracy of the assay were also demonstrated. The results obtained on different days (intermediate precision/inter-assay) showed a relative standard deviation of 2.30% and those on the same day (repeatability/intra-assay) showed a mean RSD of 2.06%. The accuracy was 99.71% (Table 3). The applicability of this method was tested and the value obtained was 102.27% of drug in the ophthalmic solutions.

In order to assess the robustness, some parameters were modified from the normal conditions: incubation time (from 4 h to 3 h 30 min and 4 h 30 min) and volume of the inoculated BHI broth (from 10 mL to 9.8 and 10.2 mL). The Student's *t*-values obtained showed that the method is robust to the parameter volume of the inoculated BHI broth, but not for the incubation time, thus it is established the need of exacts 4 h of incubation test.

Table 3 Experimental values obtained in the recovery test for ciprofloxacin hydrochloride in ophthalmic solutions by turbidimetric method.

Sample concentrations (μg/mL)	Concentration of added standard (μg/mL)	Concentration of found standard (μg/mL)	Percentage recovery[a]	Mean percentage recovery ± RSD
15.0	4.5	4.49	99.72	99.71 ± 1.52
35.0	24.5	24.06	98.19	
55.0	44.5	45.05	101.23	

[a]Each value is the mean of 3 determinations.

Table 4 Assay results of ciprofloxacin hydrochloride by three different methods.

Day	HPLC (%)	UV (%)	Microbiological (%)
1	107.09	101.34	99.96
2	101.01	99.35	106.22
3	101.64	99.67	100.63
Mean ± RSD%	103.25 ± 3.24	99.79 ± 1.39	102.27 ± 3.36

The data obtained in the analysis of CIPRO HCl in ophthalmic solution using the microbiological assay were compared with declared amounts and with those obtained by HPLC and UV spectrophotometry methods (Table 4). Analysis of variance indicated no significant differences between these methods ($p < 0.05$).

The development and validation of analytical methods for the determination of drugs has received considerable attention in recent years because of their importance in pharmaceutical analysis. A turbidimetric microbiological assay was proposed as a rapid, simple and suitable method for the determination of CIPRO HCl in ophthalmic solution.

The potency of an antibiotic may be demonstrated under suitable conditions by comparing the inhibition of growth of sensitive microorganisms produced by known concentrations of the antibiotic to be examined and a reference standard [5].

According to British [3], Brazilian [4], The United States [5] and European [16] Pharmacopoeias when a parallel-line model is chosen the two log dose–response lines of the preparations to be examined as well as the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $p = 0.05$. The assays were validated by means of the analysis of variance, as described in these official codes.

Precision is usually expressed as the variance, relative standard deviation (RSD%) of a series of measurements. The accuracy is shown by the percentage of recovery. The data obtained in this study confirm the precision and accuracy of the turbidimetric bioassay developed.

The results of analysis of the commercial colirium and the recovery study suggested that there is no interference from any excipients, which are present in pharmaceutical samples. Furthermore, the Student's t-values and F-tests values calculated for assay, 0.98 and 1.55, respectively, are below tabulated values ($n = 6$). The tabulated values of t and F at 95% confidence limit are $t = 2.18$ and $F = 4.28$. These results showed that the microbiological assay was specific and the impurities did not interfere in the capacity of the method to assess the analyte.

It was also considered necessary to evaluate small variations in the analytical conditions. Thus, the method was robust for all parameters, except by the incubation time, thereby it is established the need to observance this factor.

The quantification of antibiotic components by chemical methods such as HPLC and UV spectrophotometry, although precise, cannot provide a true indication of biological activity. Attempts to correlate antibiotic bioassay results with those from chemical methods have proved disappointing.

Although the biological assays have a high variability, the analysis of the obtained results demonstrated that the proposed method might be very useful for determination of this drug in pharmaceutical dosage forms, being an acceptable alternative method for the CIPRO HCl quality control routine.

4. Conclusions

The microbiological turbidimetric assay validated for determination of CIPRO HCl in ophthalmic solution demonstrated simplicity, linearity, precision, specificity and accuracy. Moreover, bioassay requires not specialized equipment and it is rapid execution, being an acceptable alternative method for the CIPRO HCl quality control routine.

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