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Validation of a reversed-phase LC method for quantitative analysis of intravenous admixtures of ciprofloxacin and metronidazole

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Abstract

The admixture ciprofloxacin–metronidazole is nowadays well known as a clinical tool when anaerobic organisms are involved. The stability of ciprofloxacin with metronidazole depends on several variables and this has been scarcely studied. Thus, the aim of this study was the search for a specific, precise and accurate method that would allow the quantification of ciprofloxacin in the presence of metronidazole. The reversed-phase liquid chromatography method is the analytical technique that has proved to be the most adequate for stability and compatibility studies of this mixture. This method was shown to be linear ($r > 0.999$) in the range of concentrations of this study chosen, between 80 and 120% of the concentration of the commercial formulation. It showed precision with a repeatability (within-day) of 0.62% and reproducibility (between-day) of 1.14%, accuracy expressed as recovery percentage.

Keywords: Ciprofloxacin; Metronidazole; Reversed-phase chromatography; Validation

1. Introduction

Ciprofloxacin is a carboxyquinolone antibiotic with activity against gram-positive and gram-negative bacteria, belonging especially to the *Enterobacteriaceae*. Certain clinical situations require a combined therapy of ciprofloxacin and another antibacterial agent [1]. The mixture with metronidazole [2-(2-methyl-5-nitroimidazole-1-yl) ethanol] [2] is nowadays well known as a valuable tool for antibiotic therapy when anaerobic organisms are involved [3].

The stability and compatibility of ciprofloxacin with metronidazole has been scarcely studied and findings have failed to define its compatibility [4–6]. In searching for an adequate quantification method, a direct spectrophotometric ultraviolet–visible method was used initially. However, preci-

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sion and accuracy in working conditions to measure the mixture compounds were not calculated.

This study was undertaken to develop and validate a quantification method that could be specific, precise and accurate, and that would facilitate to determine the compatibility of the admixture ciprofloxacin–metronidazole. The study was carried out at ambient temperature, under room light as well as light-protected conditions, according to hospital use and the conventional definition of compatibility and incompatibility.

2. Experimental

2.1. Apparatus

The melting points of both drugs were determined by the capillary method in an Electrothermal 9100. The infrared absorption spectra were carried out with a Nicolet 5 SXC Fourier transform infrared detector in 1% BrK disks. The pH measurements were made with an Orion SA 520. The pHmetre was calibrated with buffer solutions pH 7.00 ± 0.02 and 4.00 ± 0.02 (Titrisol, Merck).

The spectrophotometric determinations were carried out with a Shimadzu UV 260. The high pressure liquid chromatography (HPLC) was performed in a KNK-500 G (Konik instruments) equipped with a microprocessor KNK 029-375 and a manual injector (Rheodyne model 7125 with loop 20 μl). An ultraviolet–visible spectrophotometric detector UVIS 200 (Linear Instruments) and an integrator Data Jet CH2 (Konik Instruments) were used.

2.2. Materials and reagents

Ciprofloxacin was obtained by neutralization and later precipitation of a hydrochloride ciprofloxacin solution, analytic grade (Airfarma, Spain), and was identified by its melting point (m.p.) and IR spectra. It was assayed by the technique described in the European Pharmacopoeia [7]. The potentiometric titrations were carried out in glacial acetic acid, analytic grade (Cicarelli), with perchloric acid 0.095 N, validated according to the technique USP 23 [8]. The m.p. for ciprofloxacin was 257–259°C with decomposition [9]. The IR spectra was compared with a national reference standard.

Metronidazole was kindly supplied by Laboratorio Rivero & Co. S.A. (lot No. 10781). It was identified by IR spectra and its melting point was determined. The IR spectra was compared with one in the literature and the m.p. obtained was 163–163.7°C [10].

The commercial formulations used in this study were:

Ocefax: ciprofloxacin lactate in 2 mg/ml (lot 5711005), kindly supplied by Laboratorio Roux Ocefa;

Flagyl: metronidazole in 5 mg/ml (Laboratory Rhône-Poulenc Rorer; lot 6Y024).

The reagents used to prepare the HPLC solvents were NaH_2PO_4 (Aldrich), H_3PO_4 analytic grade (Cicarelli) and acetonitrile grade HPLC (J.T. Baker). Distilled, deionized water with a Milli Rho Milli Q System was used for the preparation of all the solutions and mobile phase.

2.3. HPLC assay

The HPLC separation was performed on a LiChrosorb (Merck) column, 25 cm length, 4 mm i.d., and particle size 10 μm. An aqueous buffer was prepared with 0.1 M monobasic sodium phosphate solution and the pH was adjusted to 3.3 ± 0.1 with concentrated phosphoric acid. The mobile phase was a degassed and filtered (0.45 μm; Millipore) mixture of acetonitrile:buffer solution 20:80 (v/v). The HPLC pump was set at a flow rate of 1 ml/min, volumes of 20 μl were injected and the detector was set at 278 nm.

The ciprofloxacin solutions were prepared according to Ref. [8] with modifications in their concentrations. All solutions were prepared to yield concentrations of ciprofloxacin 0.04 mg/ml and metronidazole 0.10 mg/ml.

2.3.1. Validation of the chromatography method [8]

2.3.1.1. Specificity. To demonstrate the specificity of the method for determining ciprofloxacin and

metronidazole in the presence of their degradation products, ciprofloxacin, Ocefax, metronidazole and Flagyl underwent hydrolysis under extreme pH conditions and intense heating, without protection of light, in order to decompose them deliberately [11,12]. The working conditions to obtain the degradation products of each active principle and of the formulations are listed in Table 1. The degradated solutions were chromatographed under the conditions described in Section 2.3.

2.3.1.2. Linearity. Standards of ciprofloxacin were prepared for the calibration curves. The standards were prepared in a concentration range between 40 and 120% of the concentration used in this study.

2.3.1.3. Precision. To evaluate the precision of the analytical method, six repeated injections of a solution of 0.038 mg/ml were performed. The average of the areas was obtained and the relative standard deviation (R.S.D.) was calculated. This procedure was performed within-day and between-days.

Table 1
Working conditions to obtain the degradation products of each active principle and formulation

Name	Weight (mg)	Medium (10 ml)	Reflux time (h)
Ciprofloxacin	21.3	Sodium hydroxide, 1 N	6[a]
Ciprofloxacin	20	Hydrochloric acid, 1 N	6[a]
Ocefax	20	Sodium hydroxide, 1 N	6[a]
Ocefax	20	Hydrochloric acid, 1 N	6[a]
Metronidazole	21.1	Sodium hydroxide, 1 N	1[b]
Metronidazole	20.4	Hydrochloric acid, 1 N	1[b]
Flagyl	20	Sodium hydroxide, 1 N	1[b]
Flagyl	20	Hydrochloric acid, 1 N	1[b]

[a] From Ref. [4].
[b] From Ref. [1].

2.3.1.4. Accuracy. For the recovery assays of ciprofloxacin, an artificial matrix similar to that reported by the laboratory was prepared, containing: lactate acid, 95 mg; sodium chloride, 900 mg; and distilled water, to make 100 ml. Solutions containing 1.59, 1.87, 2.02 and 2.23 mg ciprofloxacin per ml was prepared and convenient dilutions were done in order to carry out the HPLC analysis.

2.3.1.5. The detection limit and the quantitation limit. These limits were determined from the slope of the calibration curve and the standard deviation of responses [13].

2.3.2. Assay of the commercial formulations

The commercial solution of ciprofloxacin (Ocefax) was evaluated with 1 ml (2 mg) taken up to a final volume of 50 ml with mobile phase. The final concentration of the injection was 0.040 mg/ml. These assays were done by duplicate.

2.4. Determination of ciprofloxacin in mixtures

2.4.1. Compatibility study

Equal volumes of the commercial products [14] were mixed in an erlenmeyer flask and kept closed in a bath at controlled temperature (25°C) under room light, simulating clinical conditions of administration. The mixture was immediately stirred, two aliquots of 2 ml each were taken, placed in vials of polypropilene (Nalgene Cryoware) and frozen with air liquid. These first aliquots were considered as zero time, other aliquots were taken at 30 min and at 1, 3, 6, 12, 18 and 24 h. The samples were stored at −20°C until analysis. The mixture and the aliquots were performed in a laminar airflow hood Type A (Labconco Model 36208). The pH of the commercial formulations was measured separately and the pH of the mixture was assessed at each time. During analysis, the samples were left until they reached room temperature and were diluted conveniently in mobile phase to HPLC analysis. The same working procedure was repeated with the ciprofloxacin–metronidazole mixture, which was protected from light.

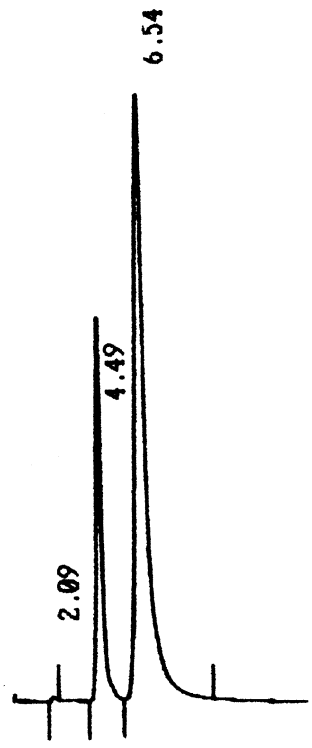

Fig. 1. Chromatogram of the mixture Flagyl–Ocefax. Peak (a) corresponds to metronidazole that elutes at 4.49 min and peak (b) corresponds to ciprofloxacin that elutes after 6.54 min. Resolution $R > 1.5$ (2.03).

Table 2
Linear regression data for the HPLC method ($n = 8$)

Parameters	Results
Range of concentrations	0.017 mg/ml at 0.051 mg/ml
Slope[a]	$(2.45 \pm 031) \times 10^8$
Intercept[b]	$-(0.56. \pm 1.08) \times 10^5$
(Correlation coefficient)^2 $(r)^2$	0.9998
Limit of detection	0.0007 mg/ml
Limit of quantitation	0.0024 mg/ml

[a] $\pm$ Confidence interval of the slope ($P = 0.05$).
[b] $\pm$ Confidence interval of the intercept ($P = 0.05$).

The concentrations were calculated on the basis of the calibration curve.

3. Results and discussion

Although direct spectrophotometric determination is widely used in pharmaceutical analysis and is cheaper than HPLC, it lacks specificity under the condition tested in this study [15]. Therefore, this study was undertaken using the HPLC technique. This is the only analytical technique that proved to be the most suitable for the stability and compatibility studies of the study mixture. Under the conditions described, the stability for the ciprofloxacin was determined by HPLC.

The HPLC system was developed by the authors based on the USP 23 monograph of ciprofloxacin. However the pH adjustment and other parameters were modified according the column and the equipment available.

The specificity in the working conditions of the mobile phase and flow rate were defined to separate adequately the ciprofloxacin from metronidazole, as well as the degradation products. Solutions of the degradation products coming from extreme acidic and basic conditions of both ciprofloxacin and metronidazole, were injected and their retention times were determined. The retention times of the degradation products of ciprofloxacin as well as of metronidazole were shorter than those of ciprofloxacin when injected under the same HPLC conditions, i.e. all eluted before 5 min. However, these products overlapped with the peak of metronidazole under these working conditions. For this reason, the concentration of ciprofloxacin was quantified along with the possible variations that it may present. The resolution achieved for ciprofloxacin and metronidazole is shown in Fig. 1.

The linear response of the method in the range of the concentrations used was evaluated. A good determination coefficient ($r^2 > 0.999$) was obtained with the calibration curve, where the area was plotted versus concentration of ciprofloxacin (mg/ml). It was performed with eight solutions of ciprofloxacin between 40 and 120% of the expected concentrations. Each area was the average of two determinations with a R.S.D. lower than 1%. The parameters are shown in Table 2.

The precision of the system was evaluated from the dispersion of at least six injections of a stan-

dard solution [8]. The acceptance criteria can vary. Thus, the USP 23 indicates a R.S.D. not higher than 2%, injecting five times the standard solution. The results in this study were within-days 0.62% and between-days 1.14%.

Table 3
Recovery percentage of ciprofloxacin as average of two determinations[a]

Solution	Recovery percentage (%)
0.047 mg/ml (117%)	98.15 ± 1.01[b]
0.040 mg/ml (100%)	100.18 ± 0.50[b]
0.037 mg/ml (92%)	99.02 ± 0.30[b]
0.032 mg/ml (80%)	103.21 ± 0.34[b]

[a] The SD of the average of the recovery determination was lower than the RSD informed in Table.

[b] RSD of the response of one standard solution (0.038 mg/ml) with respect to the response obtained on the day the calibration curve was performed.

Table 4
Results from the determination of ciprofloxacin concentration in the mixture ciprofloxacin–metronidazole under room lighting

Time	Concentration (%) $\pm$ S.D.	pH
0	100.00 ± 0.22	4.35
30 min	91.98 ± 3.20	4.33
1 h	92.87 ± 0.57	4.33
3 h	92.24 ± 2.34	4.35
6 h	89.90 ± 7.03	4.31
12 h	92.08 ± 5.98	4.38
18 h	88.22 ± 8.25	4.41
24 h	90.31 ± 1.58	4.41

Table 5
Determination of ciprofloxacin concentration obtained in the admixture ciprofloxacin–metronidazole protected from light

Time	Concentration (%) $\pm$ S.D.	pH
0	100.00 ± 0.76	4.33
30 min	96.84 ± 4.72	4.33
1 h	97.59 ± 1.38	4.33
3 h	102.37 ± 8.01	4.34
6 h	97.95 ± 1.30	4.31
12 h	99.09 ± 2.88	4.37
18 h	97.82 ± 0.57	4.39
24 h	97.02 ± 0.00	4.38

The accuracy of the method is defined by applying the analytical method to a solution of the sample matrix components, to which known amounts of the analyte are added above and below the expected levels in the samples [16]. The accuracy is expressed as a percentage of recovery. The recovery assays of the method are listed in Table 3.

The evaluation of the commercial solutions of ciprofloxacin (Ocefax) was performed by HPLC and the concentration was calculated by interpolating the area obtained in the working curve. The result obtained was Ocefax, 1.962 ± 0.003 mg/ml, representing $98.09 \pm 0.16\%$ of the concentration reported by the manufacturer.

The results obtained from the determination of the concentration of ciprofloxacin in the compatibility studies for the mixture under room light are shown in Table 4, and those obtained for the mixture protected from light are shown in Table 5. The first aliquot, taken at the moment of the mixture, was determined at time zero and was defined as 100%. The values measured of ciprofloxacin are expressed as a percentage of the concentration at the initial concentration. Figs. 2 and 3 show the evolution of remaining amount of ciprofloxacin in both mixtures (exposed and unexposed) versus time (h).

The pH of the commercial formulations was determined separately to observe changes once the mixture was prepared: for example, Flagyl, pH 4.10 and Ocefax, pH 4.91. According to some authors, a variation of the pH in one unit is indicative of incompatibility [6]. However, in the present study, such changes were not observed in the pH value. The pH measured at each time for both samples is shown in Tables 4 and 5.

Measurements by HPLC usually involve injecting a standard solution to check the response of the system with respect to other days of the analysis. In the present work, the response of the system was controlled by analyzing one of the solutions (0.038 mg/ml) employed to draw the calibration curve (called ''standard solution'') before the problem solutions were measured. The error in the recovery assays, in the Ocefax evaluation, corresponds to the R.S.D. of the response of this standard solution with respect to the response

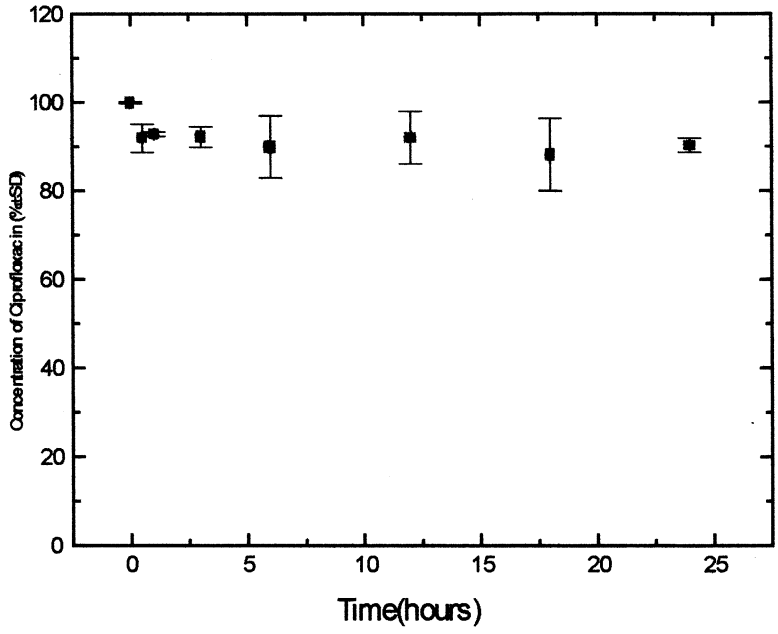

Fig. 2. Plot showing the evolution of the remaining amount of ciprofloxacin in mixtures under room light.

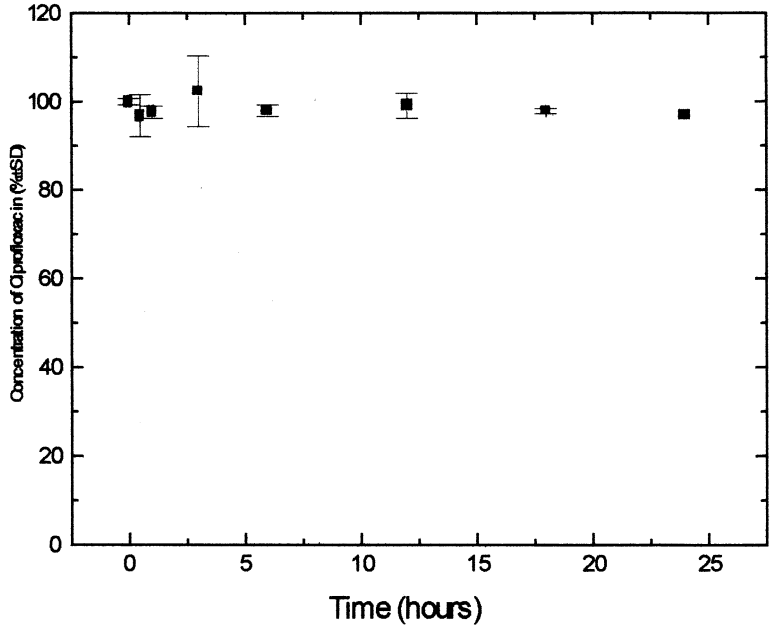

Fig. 3. Plot showing the evolution of the remaining amount of ciprofloxacin in mixtures protected from room light.

obtained on the day the calibration curve was performed.

The HPLC was useful for this type of study. It was also employed with a fluorescence detector in the search for an exact, accurate and specific method. It was observed that under these working conditions, the use of an ultraviolet spectrophotometry detector improved the precision of the method. It should be noted that the concentration of ciprofloxacin was quantified regardless of metronidazole.

Trissel refers to "compatibility" when a minimum 90% of the drug remains unchanged and available for administration [17]. From the results obtained here, the ciprofloxacin present in the mixture protected from light can be compatible with metronidazole during 24 h at 25°C. However, at room light conditions, the ciprofloxacin in the mixture seems to be degradated in 30 min. The ciprofloxacin percentages obtained from the sample under room light are very close to 90%. No compatibility can be confirmed with these results. Therefore, additional studies are necessary to assess the chemical stability of the metronidazole in the combinations tested.

4. Conclusions

The method developed in this study was validated by its specificity as a stability indicative method of ciprofloxacin, by linearity in the range of concentrations employed, and by accuracy and precision, in terms of repeatability and reproducibility.

The quality parameter determined supports the suitability of the method to quantify ciprofloxacin in the mixture with metronidazole.

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