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Short communication

Validation of an HPLC method for the determination of ciprofloxacin in human plasma

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Abstract

A simple HPLC method with fluorescence detection of ciprofloxacin in human plasma was developed and validated. After protein precipitation, chromatographic separation of ciprofloxacin in plasma was achieved at 35 °C with a C18 column and acetonitrile–phosphate mixture, pH 3, as mobile phase. Quantitative determination was performed by fluorimetry after excitation at 278 nm. The method was specific and validated with a limit of quantification of 41 ng/ml. The intra- and inter-day coefficients of variation were between 0.5 and 6.6% and accuracy between −2.02 and 7.04%. Ciprofloxacin was stable in plasma for 40 days at −20 °C and after three freezing-thawing cycles. The method has been applied in a bioequivalence study of two formulation of 500 mg ciprofloxacin.

Keywords: Ciprofloxacin; Reversed-phase HPLC; Pharmacokinetics

1. Introduction

Ciprofloxacin is an antimicrobial agent, member of the quinolone class, with activity against both Gram-positive and Gram-negative bacteria and other microorganisms [1], with a large applicability in clinical practice. There are many works published in the last two decades regarding HPLC methods for determination of ciprofloxacin in biological fluids of different species. For a laboratory, to develop a method is sometimes a compromise between cost, time consuming and purpose of study. Some of the reported methods about ciprofloxacin quantification in human plasma supposed expensive sample extractions by using switching devices [2], time and materials consuming extraction methods such as liquid–liquid extraction [3–6] or derivatization methods [7].

The number of articles about determination of ciprofloxacin in human plasma after protein precipitation is great due to the relative simplicity of the sample treatment. In many works acetonitrile or methanol was used as precipitating agent [8–13]. The methods had good sensitivity, accuracy and precision, but internal standards were used, special devices or further sample treatment was applied to improve the results. Perchloric or

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trichloroacetic acid causes protein precipitation without significant dilution of the sample. Three of studied works [14–16] applied this kind of precipitation for quantification of ciprofloxacin in human plasma and all used an internal standard. There are also a few articles in which ciprofloxacin metabolites are chromatographic determined in plasma, applying a more or less complicate extraction or chromatographic procedure or using special stationary phase [4,11,12,16].

The aim of this study was to propose a simple method for quantification of ciprofloxacin in human plasma after protein precipitation with perchloric acid, without internal standard. The method was validated to provide enough selectivity, sensitivity and stability in pharmacokinetic and bioequivalence studies [17–19]. The clinical application of method allowed identification of some fluorescent peaks, well resolved from ciprofloxacin, which are probable ciprofloxacin metabolites.

2. Experimental

2.1. Reagents

Ciprofloxacin hydrochloride was an USP standard. Tetramethylammonium hydroxide (10% in water), HPLC-grade acetonitrile, potassium dihydrogen phosphate, ortho-phosphoric acid 85%, perchloric acid 70–72% were purchased from Merck (Merck KgaA, Darmstadt, Germany), sodium chloride from Chimopar (Bucuresti, Romania). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. The human blank plasma was supplied by the Local Bleeding Centre Tg. Mureş, Romania.

2.2. Standard solutions

A 128.8 μg/ml ciprofloxacin base stock solution (S_0) was prepared by dissolving 7.5 mg ciprofloxacin hydrochloride (weighed on a Mettler Toledo AB 54-S balance from Mettler Toledo, Greifensee, Switzerland) monohydrate in 50 ml of water. The solution was sonicated for 5 min in an Elma Transsonic 700/H (Singen, Germany) ultrasonic bath. Eight working solutions (S) were prepared by diluting with water appropriate volumes of stock solutions at 25 ml. All stock and working solutions were prepared freshly in every day analysis. Preparation of the plasma standards: 0.1 ml of fresh working solution S was used to spike 0.9 ml of blank plasma. The concentrations of the plasma standards (STD) ranged between 0.0412 and 3.1 μg/ml ciprofloxacin base. Quality control (QC) samples in plasma had the following concentrations: 0.0824 (QCA), 1.03 (QCB) and 2.576 (QCC) μg/ml ciprofloxacin.

2.3. Chromatographic system and conditions

The HPLC system was a LaChrom (Merck KgaA, Darmstadt, Germany and Hitachi, Tokyo, Japan) model consisted of a L7000 pump (with on line degaser), autosampler L7200 with a Peltier Sample Cooler L7200, fluorescence detector L7480, DAD detector L7400, column heater L7360. Data were achieved and computed by the HSM D-7000 (ver. 4). The detector was operated at 278 nm, wavelength of excitation, and 455 nm, wavelength of emission. Chromatographic separation was achieved in 15 min at 35 °C with a C18 column, LiChrospher RP18, 250 × 4 mm, 5 μm (Tracer, TR-015246, C23375, Teknokroma, Spain), protected by a C18 pre-column. Wash-solvent of the syringe was NaCl 0.85% (w/V).

2.4. Mobile phase

The mobile phase consisted in an acetonitrile–phosphate (pH 3; 20 mM) mixture. A volume of 16 ml of tetramethylammonium hydroxide solution was added to 1000 ml phosphate to improve the ciprofloxacin peak shape. The pH 3 of the phosphate phase was adjusted with phosphoric acid 85% by a Mettler Toledo pH-meter MP225 (Mettler Toledo, Zchwerzenbach, Switzerland). The pump delivered the mobile phase at 1.5 ml/min with a gradient profile: for 7.5 min was an isocratic elution with 83% phosphate, after that the phosphate was decreased at 20% in 2 min and maintained at this value 1.5 min; the composition

changed at the initial values in 2 min; after 3 min a stable baseline was obtained.

2.5. Sample preparation

At 1 ml of the standard in plasma STD (or volunteer plasma sample) 20 μl phosphoric acid 85% were added [11,16] and mixed for 3 s via a vortex mixer (Falc, model Mix10, Falc Instruments SRL, Lurano, Belgium). After the perchloric acid adding (150 μl), the mixture was mixed for 30 s to achieve deproteinization. The mixture was centrifuged at 15 000 rot/min, 10 min at 20 °C, in a Sigma 2K15 centrifuge (Sigma, Osterode am Harz, Germany). A 30 μl aliquot of the supernatant was injected into the HPLC system.

2.6. Validation

The specificity of the method was verified using six different plasma blanks obtained from healthy human volunteers who did not take before ciprofloxacin. The anticoagulant (K_3EDTA) interference was also verified during this stage. In the lack of ciprofloxacin metabolites standards, the specificity of the proposed chromatographic conditions was also verified by monitoring the chromatographic behavior of human plasma of one healthy volunteer after oral administration of 500 mg ciprofloxacin tablet.

The linearity of the peak height against standard concentration was verified using least-squares linear regression in 5 different days. The calibration curves parameters were computed by the HSM D7000 software. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted, if the residuals were within $\pm 20\%$ at the lower limit of quantification and within $\pm 15\%$ at all other calibration levels and at least 2/3 of the standards meat this criterion.

To establish the lower limit of quantification in a single validation batch five replicates of QC sample with 0.0412 μg/ml ciprofloxacin were analyzed. On each of 5 different days, a single QC sample (0.0412 μg/ml) was analyzed against daily calibration (inter-day assay).

The intra- and inter-day precision (CV%) and accuracy (bias%) of the assay procedure were determined by the analysis of five samples at each lower, medium and higher QC concentration in the same day and one sample at each QC concentration in 5 different days, respectively.

The absolute recoveries at each concentration were measured by comparing the response of the pre-treated plasma standards (QC) with the response of standards diluted with water in the same proportion as the pre-treated standards.

On-instrument stability of ciprofloxacin in extract was verified at one level of concentration (0.5152 μg/ml) by performing the experiment five times during 10 h of storage at room temperature, looking for the change of signal height. The long-term stability of ciprofloxacin in human plasma was verified at three levels of concentration (0.0429, 1.288, 2.576 μg/ml ciprofloxacin in plasma) by performing the experiment after 7, 15, 26 and 40 days of storage at −20 °C. The freeze-thaw stability was also verified at two levels of concentrations, lower and higher, after three freeze-thaw cycles.

2.7. Clinical application and in-study validation

The validated method was successfully applied in a bioequivalence study of two dosage forms—tablets containing 500 mg ciprofloxacin. The collecting times were 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 h after oral administration. The accuracy and precision of the validated method was monitored to ensure that it continued to perform satisfactorily during analysis of volunteer samples. To achieve this objective, a number of QC samples prepared in duplicate at three levels of concentration were analyzed in each assay run and reported at the calibration curve of the day.

3. Results and discussions

The endogenous compounds or the used anticoagulant did not interfere at the retention time of ciprofloxacin ($t_R = 4.7 \pm 0.2$ min) (Fig. 1a). In the studied published works regarding ciprofloxacin metabolites, not more than four metabolites were

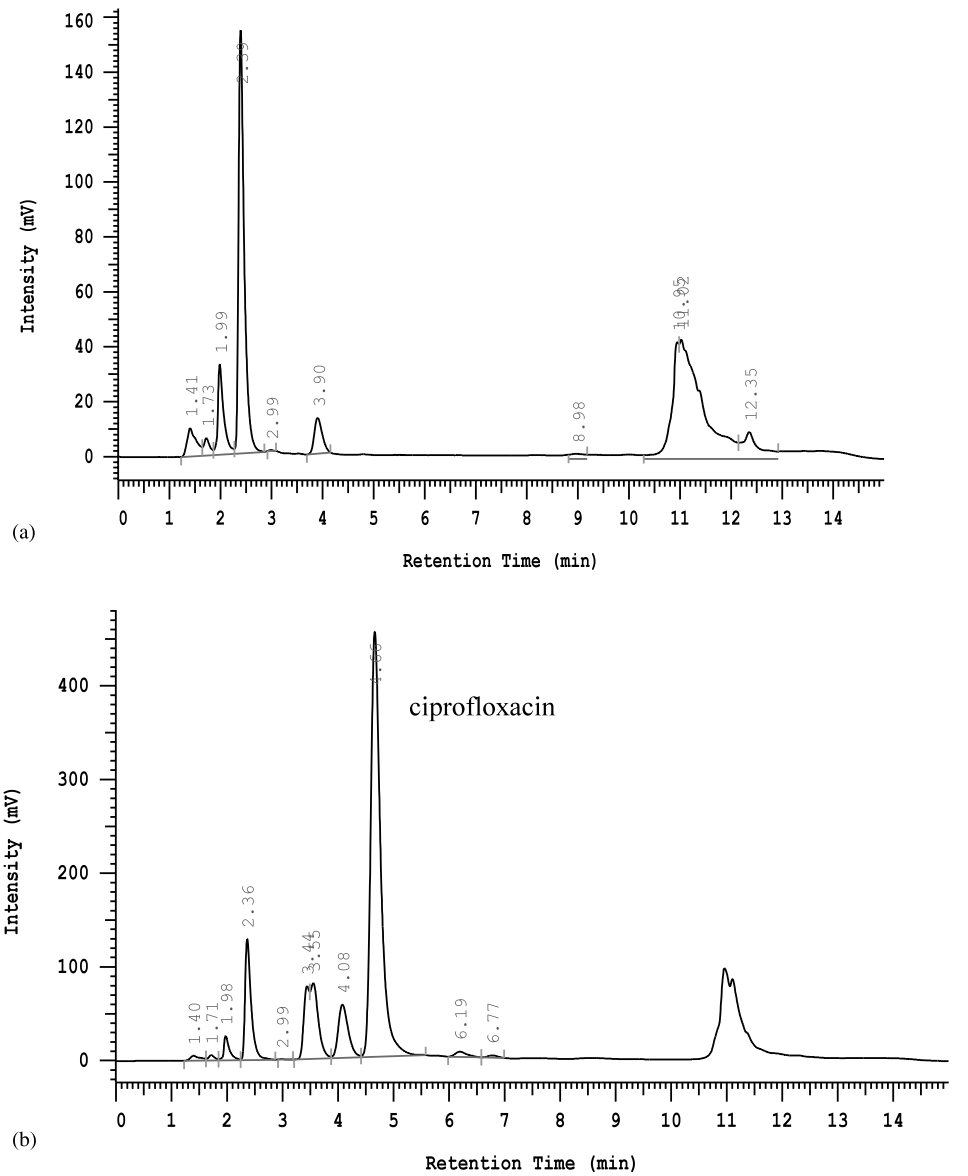

Fig. 1. Chromatograms of (a) blank plasma; (b) human plasma 2.5 h after oral administration of one tablet with 500 mg ciprofloxacin (1.007 μg/ml).

investigated [4,11,12,16]. Metabolites purchasing is sometimes an economical problem. In the lack of ciprofloxacin metabolites and taking account to the fact that only one metabolite has an intense fluorescence, the chromatographic conditions previously described were settled as ciprofloxacin was well separated from new four fluorescent peaks (two unresolved peaks at $t_R = 3.4$ and 3.5 min, respectively, and other two peaks at $t_R = 6.2$ and 6.8 min, respectively) observed in chromatograms of plasma samples, collected at different times after oral administration of 500 mg ciprofloxacin (Fig. 1b). Its area changed with the collection time and the compounds were probably ciprofloxacin metabolites. Because the aim of the study was only to establish the suitable chromatographic condi-

tions to separate ciprofloxacin from endogenous compounds and its metabolites, no further improvement of the resolution was performed. We accepted this separation satisfactorily in terms of specificity compared to the metabolites, taking account to the fact that ciprofloxacin metabolites have a relatively weak fluorescence and four new peaks observed in all 24 plasma volunteers were well separated from the main analyte. The profile of chromatograms (Fig. 1b), similar with those published by Krol et al. using a polystyrene divinylbenzene stationary phase, was obtained with an usual RP18 column and allowed to separate ciprofloxacin from four fluorescent peaks. The only maintenance required during the analysis of more than 500 samples were cleaning column at the end of each day of measurements and changing one time the guard column when pressures increased, during bioequivalence sample analysis. The on-line second detection (diode array) cannot get further qualitative information, due to the low concentration of the detected compounds. The introduction of a short gradient profile was caused by two late eluting compounds which were identified in all plasma blanks when the study was performed in isocratic conditions.

The calibration curves showed linear response over the range of concentrations used in the assay procedure. The mean calibration curve was $Y = 4.379(\pm 0.099)X - 2208(\pm 2514)$, $N = 8$, $n = 5$ days, $r^2 > 0.997$. No weight factor was applied. The residuals were within $\pm 8\%$.

The inter- and intra-day precision and accuracy results are showed in the Tables 1 and 2. The lower limit of quantification (0.0412 μg/ml) was accepted taking account to the fact that this level is the lowest on the calibration curve and its concentration can be still determined with acceptable precision (CV% < 20) and accuracy (bias < 20%) for the purpose of this particular application [17–19].

Table 1
Intra-day precision and accuracy ($n = 5$)

$c_{nominal}$, μg/ml	0.0515	1.03	2.576
Mean c_{found}, μg/ml	0.0544	1.01	2.683
CV%	1.3	0.6	0.5
Bias, %	5.63	−1.94	4.15

Table 2
Inter-day precision and accuracy ($n = 5$)

$c_{nominal}$, μg/ml	0.0515	1.03[a]	2.576
Mean c_{found}, μg/ml	0.0526	1.027	2.605
CV%	6.6	3.4	4.7
Bias, %	2.14	−0.29	1.13

[a] $n = 4$, one sample lost in process.

The limit of quantification (CV% = 3.3, bias = 7.1%) had a value better than obtained by Maya et al., Krol et al. (0.050 μg/ml) and Nix et al. (0.080 μg/ml) by applying a similar sample treatment.

The absolute recovery of ciprofloxacin ranged between 71.4 and 83.7%, an acceptable range for the purpose of study, with a CV% between 0.5 and 1.6.

No tendency of degradation of ciprofloxacin after 10 h of maintaining in autosampler was observed, the bias against zero time concentration being less than 2% for each collected data. Plasma samples spiked with ciprofloxacin showed no loss of analyte when reassayed four times, during 40 days. The slope of the linear regression $c_{measured} = f(c_{nominal})$ had a tendency to 1 value and the $r^2 > 0.999$. In the freeze-thaw stability study, no tendency of degradation after three freeze-thaw cycles was observed (bias < 2%).

The concentration profile of one healthy human volunteer after receiving one tablet with 500 mg ciprofloxacin is given in Fig. 2.

The results of the QC samples (Table 3) provided the basis of accepting or rejecting the run during clinical study. All six QC samples were

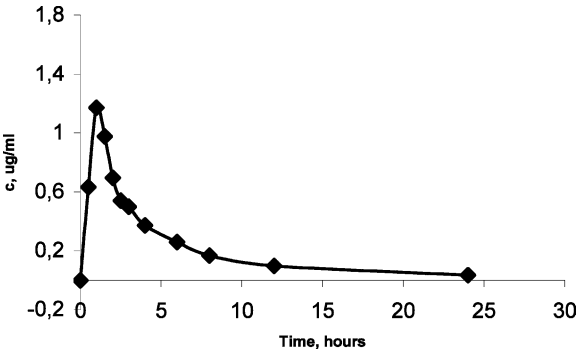

Fig. 2. Concentration profile in plasma in a healthy human volunteer receiving 500 mg of ciprofloxacin.

Table 3
QC samples results ($n = 24$)

	QCA1	QCA2	QCB1	QCB2	QCC1	QCC2
$c_{nominal}$, μg/ml	0.0824	0.0824	1.03	1.03	2.576	2.576
c_{mean}, μg/ml	0.0857	0.0862	1.084	1.077	2.69	2.68
CV%	6.1	7.1	4.9	5.3	4.1	3.7
Bias, %	4	4.6	5.2	4.6	4.4	4.0

within ±15% of their respective nominal value in each day of sample analysis, except 1 day when one QC sample was outside the ±15% of its nominal value. All the runs were accepted.

4. Conclusions

The proposed method has proven to be simple, specific, accurate and precise without need of internal standard. The limit of quantification (1 ng injected) allowed it to be successfully applied in a bioequivalence study of two formulas of ciprofloxacin. The results and statistical comparison between tested drugs are not published yet. The chromatographic conditions were suitable to separate ciprofloxacin from possible four fluorescent metabolites.

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