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NEW LIQUID CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY METHOD FOR CIPROFLOXACIN MONITORING IN HUMAN PLASMA

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ABSTRACT A new simple, sensitive and selective liquid chromatography coupled with mass spectrometry (LC-MS) method for quantification of ciprofloxacin in human plasma was validated. Ciprofloxacin and ofloxacin, as internal standard, were analysed on a SB-C18 column (Agilent Technologies, 100 mm x 3 mm I.D., 3.5 µm) under isocratic conditions using a mobile phase of a 70:30 (v/v) mixture of 0.1% (v/v) formic acid in water and acetonitrile. The flow rate was 0.5 mL/min at the column temperature of 25 ºC. The detection of the analyte was in SIM mode using a triple quadrupole mass spectrometer with electrospray positive ionisation. The monitored ions were m/z 332 for ciprofloxacin and m/z for 362 for ofloxacin. The sample preparation was very simple and consisted of protein precipitation from 0.2 mL plasma using 0.4 mL of 0.05% acetic acid solution in methanol containing 0.5 µg/mL internal standard. Linear calibration curves were generated over the range of 25-5000 ng/mL with values for coefficient of determination greater than 0.999 and by using a weighted (1/c) linear regression. The lowest limit of quantification was 10 ng/ml. The values of precision (RSD%) and accuracy (relative error%) were less than 8.7% and 11.9%, for within- and between-run, respectively. The recovery of the analyte ranged between 82.5 and 91.1%. Ciprofloxacin demonstrated good stability in various conditions. The validated LC-MS method allows ciprofloxacin monitoring in human plasma during clinical treatment or other pharmacokinetics investigation.

Keywords: *ciprofloxacin, LC-MS, human plasma, pharmacokinetics, therapeutic drug monitoring*

INTRODUCTION

As it is known, ciprofloxacin (CPR) or 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid belongs to the second generation of fluoroquinolones with activity against both Gram-positive and Gram-negative bacteria and other microorganisms [1], having a large applicability in clinical practice.

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During ciprofloxacin treatment, the status of the patient's renal and hepatic function must be taken into consideration to avoid an accumulation that may lead to an overdose and the development of toxicity. Modification of the dosage is recommended for those patients with impaired kidney function by a precise monitoring of the plasma concentration.

Therapeutic drug monitoring (TDM) is an esential tool for a positive management therapy. The prefered laboratory strategy is immunoassay. Due to the cross-reactivity to other components, alternative approaches, such as liquid chromatography coupled with mass spectrometry (LC-MS), are continously under investigation. The main application area of LC–MS in the analysis of antibiotic and antibacterial compounds is the confirmation of their identity in animal food products for human consumption at maximum residue levels, set by the regulatory authorities.

However, in the past years there are many applications of LC-MS regarding fluoroquinolones determination in human plasma, but only a few are dedicated to ciprofloxacin.

In two articles, ciprofloxacin, together with other drugs, was LC-MS detected in human plasma. Ciprofloxacin and dexamethasone were monitorized in blood by a LC-MS method in order to describe the pharmacokinetics of ciprofloxacin and dexamethasone after administration of CIPRODEX Otic Suspension into the middle ears of children [2]. Tigecycline and ciprofloxacin were employed as the model compounds to study the effect of the anticoagulant ethylenediamine tetra-acetic acid (EDTA) on the determination of pharmacokinetic parameters [3], drug concentrations being quantified by LC-MS/MS analysis.

There are also two works, published only as abstracts, in which LC-MS analysis of ciprofloxacin after protein precipitation is described. Bugge and col. [4] validated a LC-MS/MS method with lomefloxacin as internal standard. Acetonitrile was used to precipitate plasma proteins, but a portion of the extract was evaporated and reconstituted in mobile phase. The detection was in positive ion multiple reaction monitoring (MRM) mode. In the other paper, a rapid, sensitive, selective, accurate LC-MS method for the determination of ciprofloxacin in human plasma was developed and validated after protein precipitation [5], the detection being also in MRM mode.

Taking in account to those present above, we attempted to develop and validate a new and fast LC-MS method able to quantify ciprofloxacin in human plasma during drug therapy or pharmacokinetic investigations by applying a simple and consistent plasma sample preparation by protein precipitation, ofloxacin (OFL) being the internal standard.

RESULTS AND DISCUSSION

No significant interference at the retention time of CPR (3.19 min) and OFL (2.3 min) was observed in different plasma blank samples chromatograms due to the specificity of the selected signals against endogenous compounds.

The analytes were well separated under the proposed chromatographic conditions in less than 3.5 min (Figure 1). The analyte carryover was verified using a blank injection made right after the injection of the calibration solution with the most elevated level of concentration (Figure 2, curve a). The interference at the retention times of analytes due to carryover was less than 1/10 of the signal corresponding to the lower limit of quantification - LLOQ (Figure 2, curve b).

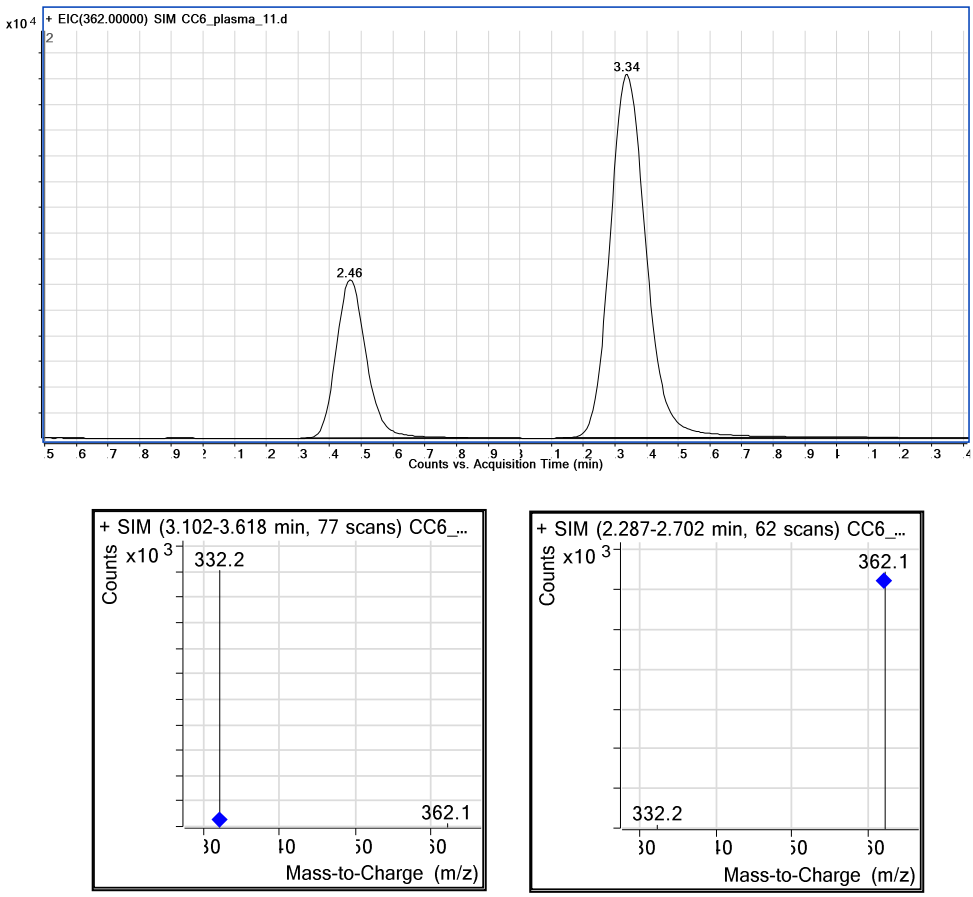

Figure 1. Chromatograms of a plasma standard with 5000 ng/ml CPR and 1000 ng/ml OFL (upper image) and SIM mass spectra of CPR and OFL (lower images)

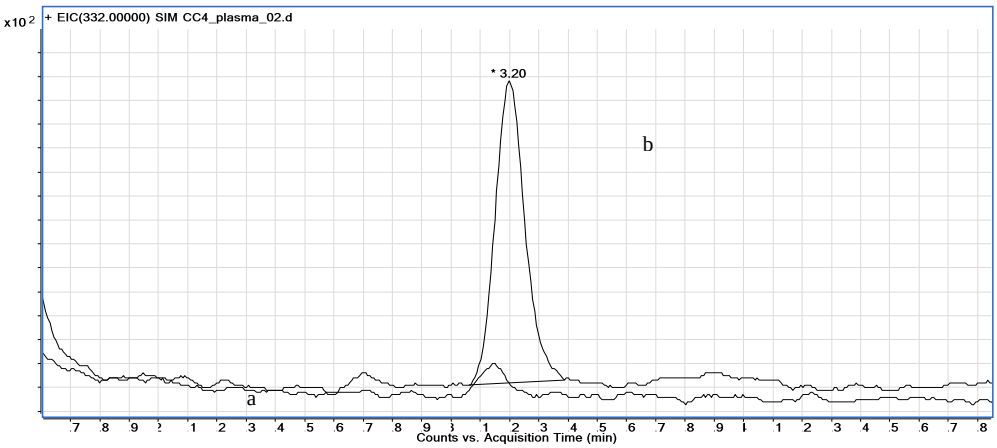

Figure 2. Chromatograms of blank plasma (a) and the LLOQ plasma standard with 25 ng/ml CPR (b)

The applied calibration curve model proved to be accurate over the concentration range 25 - 5000 ng/mL, with the coefficient of correlation greater than 0.999. The mean calibration curve, y = a(±SD) x + b (±SD), where SD is the standard deviation, was: y = 0.5655 (±0.0218) x + 0.0013 (±0.0031), N = 10 calibration points, n = 5 determinations for each calibration point. The residuals had no tendency of variation with concentration and were less than 13.4%.

The method had within- and between-run accuracy and precision (Tables 1 and 2), in agreement to the international regulations regarding bioanalytical methods validation [7-9]. The lower limit of the linearity domain was established at 25 ng/mL CPR, with accuracy and precision less than 5.2%. The lowest limit of quantification, corresponding to a signal to noise ratio of ten, was established at 10 ng/mL.

The recovery was consistent (87%) and reproducible (CV of 5%).

Table 1. Within-run accuracy and precision results

Level	QCA	QCB	QCC
Nominal concentration, ng/mL	100	2000	3200
Found mean, n=5	109	2040	3093
Accuracy, %	8.7	1.9	-3.4
CV, %	2.6	1.4	2.4

Table 2. Between-run accuracy and precision results

Level	QCA	QCB	QCC
Nominal concentration, µg/mL	100	2000	3200
Found mean, n=5	112	1979	3185
Accuracy, %	11.9	-1.1	-0.5
CV, %	5.7	5.6	5.2

The analytes proved their stability under various conditions, the Bias% of found concentration against concentration of the reference QC sample being less than 10%.

The method was finally verified by analyzing plasma samples obtained from a healthy volunteer after oral administration of a single dose of 500 mg CPR (Figure 3). As it can be seen, the proposed method is able to quantify CPR concentration with accuracy and precision after at least four half-times in order to obtain an adequate plasma concentration profile for pharmacokinetic or bioequivalence studies.

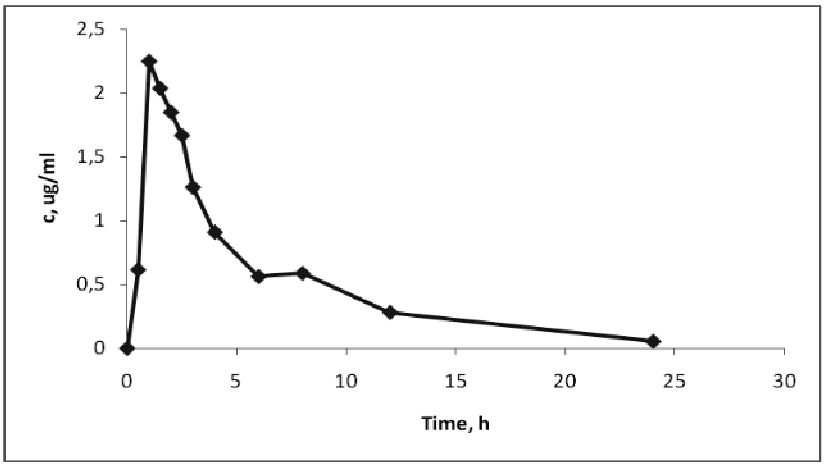

Figure 3. The time - concentration profile of ciprofloxacin in a healthy volunteer after oral administration of 500 mg CPR

As far as we are aware, there are only two works published as abstracts in which ciprofloxacin determination in human plasma by LC-MS is made after protein precipitation, alone or combined with other extraction procedure (Table 3). In comparison, the proposed method has some advantages: the sensitivity (the lowest limit of quantification of 10 ng/mL at 7 pg injected); a better recovery of the analyte (over 80%) in comparison with the cited method

in which only protein precipitation was applied [5]. This is the first full text paper about ciprofloxacin LC-MS determination in human plasma in which the sample preparation consisted of protein precipitation.

Table 3. Comparison of the actual work with already published methods in which the plasma protein precipitation was applied

Work	Method	Sample treatment	Recovery, %	Linearity domain, ng/ml / pg injected	LstLOQ, ng/ml / pg injected	Run time, min
Current	ESI+, SIM, isocratic elution	PP	87	25-5000 / 16	10 / 7	3.5
Bugge & col. (2005) [4]	ESI+, MRM, isocratic elution	PP + solvent evaporation	Extraction was quantitative	25-10000 / ?	?	2
Peng & col. (2008) [5]	ESI+, MRM, gradient elution	PP	53	25-2000 / ?	?	4.5

PP – protein precipitation; ? – no data available; LstLOQ - Lowest Limit of Quantification

CONCLUSIONS

The proposed method provides accuracy and precision for quantitative monitoring of ciprofloxacin in human plasma during therapy or pharmacokinetics investigation. The simple sample preparation by protein precipitation, while using less organic solvent with small amounts of sample plasma volume, the relatively short run time and the selected signals for monitoring allow a specific and efficient analysis of plasma samples, making the method more productive and thus more cost effective.

EXPERIMENTAL SECTION

Reagents

Ciprofloxacin and ofloxacin (OFL - internal standard) were analytical standards purchased from Fluka (Sigma-Aldrich Chemicals, UK) and Riedel-De Haën (Germany), respectively. Acetonitrile, methanol, acetic acid were Merck products (Merck KgaA, Germany) and formic acid was Scharlau (Scharlau Chemie S.A., Spain) reagent grade. Ultrapure, deionised water was produced by a Millipore Direct Q5 water system (Millipore SA, France). The human blank plasma was supplied from healthy volunteers.

Standard solutions

Two stock solutions of CPR and OFL, respectively, with concentration of 1 mg/mL were prepared by dissolving appropriate quantities of reference substances (weighed on an analytical balance AB54S, Mettler-Toledo, Switzerland) in 10 mL solution of 0.05% acetic acid in methanol. Ten calibration working solutions of 0.125 - 25 μg/mL CPR were then obtained by diluting specific volumes of stock solutions with the same solvent. Then these were used to spike 0.16 mL plasma blank, providing finally ten plasma standards with the concentrations ranged between 25 and 5000 ng/mL. Accuracy and precision of the method was verified using plasma standards with concentrations of 100, 2000 and 3200 ng/mL. Quality control samples (QC) with the same concentrations 100 (QCA), 2000 (QCB) and 3200 (QCC) ng/mL analyte will be used during clinical samples analysis.

Chromatographic and mass spectrometry systems and conditions

The HPLC system was an 1100 series model (Agilent Technologies, USA) consisted of a quaternary pump, an in-line degasser, an autosampler, a column thermostat, and a triple quadrupole mass spectrometer detector 6410 (Agilent Technologies). Chromatograms were processed using MassHunter software (Agilent Technologies).

The detection of the analyte was in single ion monitoring (SIM) mode using an electrospray positive ionization (ESI positive). The monitored ions were m/z 332 for CPR and m/z 362 for OFL. Other detector parameters: dry temperature 350 °C, nebulizer 50 psi, dry gas – nitrogen at 10 L/min.

Chromatographic separation was performed at 25°C on a Zorbax SB-C18, 100 x 3 mm, 3.5 μm column (Agilent Technologies), protected by an in-line filter.

Mobile phase

The mobile phase consisted of a mixture of water containing 0.1% formic acid and acetonitrile (70:30 v/v), each component being degassed, before elution, for 10 minutes in a Elma Transsonic T700 ultrasonic bath (Germany). The pump delivered the mobile phase at 0.5 mL/min.

Sample preparation

Standard and test plasma samples were prepared as follows in order to be chromatographically analyzed. In an Eppendorf tube 0.2 mL plasma with 0.4 mL internal standard methanolic solution (0.5 μg/mL OFL) was vortex-mixed for 30 seconds (Mix20, Falc Instruments, Italy). After 10 minutes, the tube was centrifuged for 10 minutes at 10000 rpm (Sigma 2K15 centrifuge, Germany). The supernatant was transferred in an autosampler vial and 2 μL were injected into the LC-MS system.

Validation

As a first step of method validation [7-9], specificity was verified using six different plasma blanks obtained from healthy human volunteers who had not previously taken any medication.

The concentration of analytes was determined automatically by the instrument data system using the internal standard method. Calibration was performed using singlicate calibration standards on five different occasions. The calibration curve model was determined by the least squares analysis. The applied calibration model was a linear one: $y = ax + b$, weight 1/x, where y – peak area ratio and x – concentration ratio. 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 ±20% at the lower limit of quantification (LLOQ) and within ±15% at all other calibration levels and at least 2/3 of the standards met this criterion, including highest and lowest calibration levels.

The lower limit of quantification was established as the lowest calibration standard with an accuracy and precision less than 20%. The lowest limit of quantification was investigated at a signal to noise ratio of ten.

The within- and between-run precision (expressed as coefficient of variation, CV%) and accuracy (expressed as relative difference between obtained and theoretical concentration, Bias%) of the assay procedure were determined by analysis on the same day of five different samples at each of the lower (100 ng/mL), medium (2000 ng/mL), and higher (3200 ng/mL) levels of the considered concentration range and one different sample of each on five different occasions, respectively.

The relative recoveries at each of the previously three levels of concentration were measured by comparing the responses of the final solutions obtained after preparation of plasma standards (N=3 replicates) with the responses of the standard solutions with the same concentrations of analytes.

The stability of stock solutions of CPR and OFL for four hours at room temperature was verified by comparing the responses of the diluted solutions (1000 ng/ml) with those obtained from stock solutions kept in the refrigerator ($t \leq 8$ ºC).

The stability of the analytes in human plasma was investigated in three ways, in order to characterize each operation during the process of bioanalytical studies: room-temperature stability (RTS), post-preparative stability (PPS) in the autosampler, freeze-thaw stability (FTS). For all stability studies, plasma standards at each of the lower (100 ng/mL), and higher (3200 ng/mL) levels were used. Five plasma standards at each of the three levels were prepared and let at room temperature four hours before processing (RTS study).

Other five pairs were prepared, immediately processed and stored in the HPLC thermostated autosampler (20 °C) (PPS study). The samples were injected after 22 hours, the expected longest storage times of the samples in autosampler before injection. For the freeze-thaw stability (FTS), aliquots at the same low and high concentrations were prepared. These samples were subjected to three cycles of freeze-thaw operations. After the third cycle the samples were analyzed against calibration curve of the day. The mean concentration calculated for the samples subjected to the cycles and the nominal ones were compared. For long-term stability (LTS), we appreciated it over 40 days as we verified previously in the same freezing device of the laboratory [6]. However, the same types of lower and higher concentration standards were used in order to evaluate analytes stability over two months of storage below -20°C. The requirement for stable analytes was that the difference between mean concentrations of the tested samples in various conditions and nominal concentrations had to be in ±15% range.

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