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Development and validation of a fast isocratic liquid chromatography method for the simultaneous determination of norfloxacin, lomefloxacin and ciprofloxacin in human plasma

Joana Sousa,[a,b] Gilberto Alves,[b,c] Ana Fortuna,[a,b] Angelina Pena,[a,d] Celeste Lino[a,d] and Amílcar Falcão[a,b]*

ABSTRACT: A simple and fast liquid chromatographic method coupled with fluorescence detection (LC-FD) is reported, for the first time, for the simultaneous quantification of norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) in human plasma, using levofloxacin as internal standard (IS). Sample preparation consists of a single-step precipitation of plasma proteins followed by vortex-mixing and centrifugation. Chromatographic separation was achieved within 7 min on a reversed-phase C_{18} column with a mobile phase consisting of 0.1% aqueous formic acid (pH = 3.0, triethylamine)–methanol (82:18, v/v) pumped isocratically at 1.2 mL/min. The detector was set at excitation/emission wavelengths of 278/450 nm. Calibration curves were linear ($r^2 \geq 0.994$) in the range of 0.02–5.0 μg/mL, and the limit of quantification was established at 0.02 μg/mL for all analytes (NOR, CIP and LOM). The overall precision did not exceed 8.19% and accuracy was within ±10.91%. NOR, CIP and LOM were extracted from human plasma with an overall mean recovery ranged from 90.1 to 111.5%. No interferences were observed at the retention times of the analytes and IS. This novel LC-FD method enables the reliable determination of NOR, CIP and LOM in a single chromatographic run, which may be suitable to support human pharmacokinetic-based studies with those antimicrobial agents. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: norfloxacin; lomefloxacin; ciprofloxacin; method validation; human plasma

Introduction

Norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) (Fig. 1) are synthetic antibiotics developed from fluorination of the nalidixic acid, the first quinolone introduced for clinical use (Wise, 2000; Oliphant and Green, 2002). Since the discovery of nalidixic acid in the early 1960s, numerous quinolone derivatives have emerged, which are currently classified into four generations based on their spectrum of activity and pharmacokinetic properties. From a pharmacological point of view, the latter generations of quinolones have several advantages over the earlier ones, mainly a broader spectrum of activity and improved pharmacokinetics with an appropriate tissue penetration that allows tissue drug concentrations equal to or greater than those attained in plasma (Ball, 2000; Zhanel and Noreddin, 2001; Emmerson and Jones, 2003). However, despite the better pharmacokinetic and pharmacodynamic profiles of the newer fluoroquinolones, it should be noted that those of the second generation, such as NOR, CIP and LOM remain in clinical use as valuable antimicrobial agents (Oliphant and Green, 2002; Rafalsky *et al*., 2006). In particular, CIP is extensively used in outpatients and in hospitalized patients with severe infections. In addition, CIP is commonly used as part of antibiotherapy regimens prescribed to critically ill patients in intensive care units (Wallis *et al*., 2001; Bellmann *et al*., 2002; van Zanten *et al*., 2008).

Taking into account the concentration-dependent antibacterial activity characteristic of the fluoroquinolone antibiotics, it is evident that intra- and inter-individual pharmacokinetic variability may result in inadequate antibiotic concentrations and therapeutic inefficacy. Nowadays, two relevant parameters are considered as good predictors of treatment success, the 24 h area under the concentration–time curve (AUC_{24})/minimum inhibitory

* Correspondence to: A. Falcão, Pharmacology Department, Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal. E-mail: acfalcao@ff.uc.pt

[a] Faculty of Pharmacy, University of Coimbra, 3000-548 Coimbra, Portugal

[b] Pharmacometrics Group, Centre for Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal

[c] Health Sciences Research Centre (CICS), University of Beira Interior, 6200-506 Covilhã, Portugal

[d] Group of Health Surveillance, Centre of Pharmaceutical Studies, Faculty of Pharmacy, University of Coimbra, 3000-548 Coimbra, Portugal

Abbreviations used: CIP, ciprofloxacin, LOM, lomefloxacin; NOR, norfloxacin; TDM, therapeutic drug monitoring.

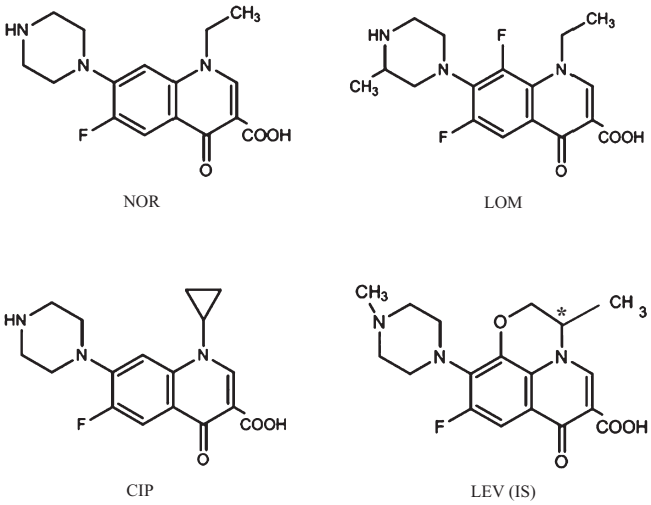

Figure 1. Chemical structures of norfloxacin (NOR), lomefloxacin (LOM), ciprofloxacin (CIP) and levofloxacin (LEV) used as internal standard (IS). The asterisk denotes the chiral center on LEV.

concentration (MIC) ratio and the peak concentration (C_{max})/MIC ratio. Therefore, there is a growing body of evidence suggesting the usefulness of therapeutic drug monitoring (TDM) of fluoroquinolones in critically ill hospitalized patients. Target values for AUC_{24}/MIC and C_{max}/MIC ratios have been established for CIP (Pea *et al*., 2006; Conil *et al*., 2008; van Zanten *et al*., 2008). Thus, the availability of a simple, fast and selective bioanalytical method is required to enable routine TDM and dosage individualization of fluoroquinolones.

Until now, a lot of bioanalytical methods have been reported in the literature for the simultaneous determination of a series of fluoroquinolones. However, due to the widespread use and/or misuse of fluoroquinolones in the veterinary field, the majority of such methods have been developed in non-human matrices, targeting the presence of fluoroquinolone residues in foodstuffs of animal origin and in water (Posyniak *et al*., 1999; Schneider and Donoghue, 2002; Wan *et al*., 2006; Dufresne *et al*., 2007; Seifrtová *et al*., 2008; Zhu *et al*., 2008; Herrera-Herrera *et al*., 2009; McMullen *et al*., 2009; Pearce *et al*., 2009; Tang *et al*., 2009; Cho *et al*., 2010; Lombardo-Agüí *et al*., 2010; Pena *et al*., 2010). On the other hand, few bioanalytical methods have been developed for the concomitant analysis of more than one fluoroquinolone in human plasma/serum or urine matrices (Liang *et al*., 2002; Samanidou *et al*., 2003; Torriero *et al*., 2006; Kumar *et al*., 2008; Liu *et al*., 2008; Srinivas *et al*., 2008; Yang *et al*., 2008; De Smet *et al*., 2009). Moreover the liquid chromatography (LC) method developed by Cañada-Cañada *et al*. (2007) enables the simultaneous determination of NOR, CIP and LOM, but it has been applied to the analysis of such drugs in human and veterinary pharmaceuticals. Accordingly, up to date, to the best of our knowledge, no method has been reported for the determination of NOR, CIP and LOM in the matrices of human plasma or serum.

The proposed research work describes the first LC method developed and validated to quantify NOR, CIP and LOM in human plasma. The method was validated in a wide concentration range for each compound (0.02–5.0 μg/mL) and therefore it can be applied to routine TDM and also to other pharmacokinetic-based studies intended to investigate, for instance, the bioavailability/bioequivalence of drug formulations of NOR, CIP and LOM.

Experimental

Chemicals and Reagents

NOR (lot no. 028K1480), CIP (lot no. 1396107) and levofloxacin (LEV) (lot no. 1395156), used as internal standard (IS), were purchased from Sigma-Aldrich (St Louis, MO, USA). LOM hydrochloride (lot no. 62277) was obtained from Molekula (Shaftesbury, Dorset, UK). Methanol (HPLC gradient grade) was purchased from Fisher Scientific (Leicestershire, UK) and ultra-pure water (HPLC grade, >15 MΩ) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Reagents like fuming hydrochloric acid (37%), formic acid (98–100%) and triethylamine (TEA) were acquired from Merck KGaA (Darmstadt, Germany), and trichloroacetic acid was obtained in solid state from Sigma-Aldrich (Steinheim, Germany).

Plasma Sampling

Blank human plasma samples from healthy donors were kindly provided by the Portuguese Blood Institute after written consent of each subject according to the Declaration of Helsinki.

Apparatus and Chromatographic Conditions

The LC system used for analysis consisted of a pump model 305 (Gilson Medical Electronics S.A., Villiers-le-Bel, France), a manometric module model 805 (Gilson Medical Electronics S.A., Villiers-le-Bel, France) and a manual injector model 7125 (Rheodyne, Cotati, CA, USA) with a 20 μL loop. Detection was performed with an LC 305 model fluorescence detector (LabAlliance, State College, PA, USA) and data acquisition was controlled by Data Apex Clarity software version no. 2.6.06.574 (Data Apex Ltd, Prague, Czech Republic).

The chromatographic separation of the three analytes (NOR, CIP and LOM) and IS was achieved in 7 min and it was carried out at room temperature, by isocratic elution with 0.1% aqueous formic acid (pH 3.0, TEA)–methanol (82:18, v/v) at a flow rate of 1.2 mL/min, on a reversed-phase LiChroCART® Purospher Star-C_{18} column (55 × 4 mm; 3 μm particle size) purchased from Merck KGaA (Darmstadt, Germany). The mobile phase was filtered through a 0.45 μm filter and degassed ultrasonically for 15 min before use. A sample volume of 20 μL was injected and the excitation and emission wavelengths ($\lambda_{ex}/\lambda_{em}$) selected for the detection of all compounds were 278 and 450 nm, respectively.

Stock Solutions, Calibration Standards and Quality Control Samples

Stock solutions of 1 mg/mL were individually prepared for NOR, CIP, LOM and IS. The stock solutions of NOR and CIP were prepared by dissolving appropriate amounts of each compound in a mixture of methanol–37% hydrochloric acid (99.5:0.5, v/v), while stock solutions of LOM and IS were prepared in methanol. Appropriate volumes of each of the stock solutions of NOR, CIP and LOM were combined and diluted in methanol to obtain two intermediate solutions, each containing the three fluoroquinolones at the concentrations of 200 and 20 μg/mL. Then, the combined intermediate solutions were adequately diluted in methanol to obtain six spiking solutions at 0.5, 1, 4, 15, 50 and 125 μg/mL, which were used to spike aliquots of blank human plasma in order to prepare six plasma calibration standards at 0.02, 0.04, 0.16, 0.6, 2 and 5 μg/mL. An aqueous IS working solution at 10 μg/mL was also prepared daily by appropriate dilution of the corresponding stock solution. All solutions were stored protected from light at approximately 4°C for one month, except the IS working solution which was prepared daily. Quality control (QC) samples were prepared independently in the same matrix (blank human plasma).

Plasma Sample Preparation

Each aliquot (500 μL) of human plasma samples was mixed with 100 μL of the IS working solution (10 μg/mL) and 100 μL of 20% trichloroacetic acid. The final sample was vortex-mixed for 30 s and centrifuged at

5800 rpm for 5 min to precipitate plasma proteins. An aliquot of the clear supernatant (20 µL) was directly injected into the LC system for analysis.

Method Validation

The method validation was performed according to the FDA guidelines for bioanalytical method validation (US DHHS, FDA and CDER, 2001) as well as on the updated recommendations of the Conference Report of the Washington Conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies (Shah *et al*., 2000). The method was validated by determination of selectivity, linearity, limits of quantification and detection, precision and accuracy, sample dilution, recovery and stability.

Selectivity. The potential chromatographic interference from endogenous compounds (matrix effects) at the retention times of NOR, CIP, LOM and IS was investigated by analyzing blank human plasma samples obtained from six different subjects. Interference from other commonly co-prescribed drugs such as paracetamol, salicylic acid, ibuprofen, teofiline, ranitidine, hydrochlorothiazide, furosemide, carbamazepine, amitriptyline, cefoxitin, dexamethasone, enoxaparin, propofol, ketamine, phenytoin, lamotrigine, erythromycin, penicillin-G, trimethoprim, neomycin, azithromycin, streptomycin, bromazepam and omeprazole at a concentration of 10 µg/mL was also tested.

Calibration curve. The linearity of the analytical method for NOR, CIP and LOM was assessed in the range of 0.02–5 µg/mL using calibration curves prepared on five separate days ($n = 5$) with spiked plasma calibration standards at six different concentrations levels. Calibration curves were prepared by plotting the peak-area ratios (peak area analyte/peak area IS) vs concentration, and fitted to the $y = mx + c$ using $1/x^2$ as weighting factor for all analytes. This weighting factor was selected because it yielded the best fit of peak-area ratios *versus* concentration for all compounds (Almeida *et al*., 2002).

Limits of quantification and detection. The limit of quantification (LOQ) was defined as the lowest concentration on the calibration curve which can be measured with precision (expressed as percentage of coefficient of variation, %CV) not exceeding 20% and accuracy (expressed as percentage of deviation from nominal concentration, %bias) within ±20%. The LOQ was evaluated by analyzing plasma samples which were prepared in five replicates ($n = 5$). The limit of detection (LOD), defined as the lowest concentration that can be distinguished from the noise level, was determined by analyzing plasma samples with known concentrations of NOR, CIP and LOM, after successive dilutions, and it was established by visual evaluation of the minimum level at which the analytes can be reliably detected.

Precision and accuracy. Intra and interday precision and accuracy were assessed by using QC samples analyzed in replicate ($n = 5$) at three different concentration levels representative of the entire range of the calibration curves (low, middle and high QC samples). The concentrations tested were 0.05, 2.5 and 4.5 µg/mL for all analytes (NOR, CIP and LOM). The acceptance criterion for precision was a CV less or equal to 15% (or 20% in the LOQ), and for accuracy a bias value within ±15% (or ±20% in the LOQ).

Sample dilution. The dilution effect (1:5) was also investigated with appropriate plasma QC samples at 10 µg/mL for NOR, CIP and LOM to ensure that plasma samples exceeding the highest concentration of the calibration range (0.02–5 µg/mL) could be diluted with blank human plasma and accurately quantified. The precision and accuracy of diluted plasma QC samples were determined intra- and interday by replicated analysis ($n = 5$).

Recovery. The recovery of NOR, CIP and LOM from human plasma samples submitted to protein precipitation was determined at three concentration levels 0.05, 2.5 and 4.5 µg/mL by repeated analysis ($n = 5$). The recovery of the analytes was calculated by comparing the analyte/IS peak area ratio of processed plasma samples with the corresponding ratio obtained from the processed aqueous solutions at the same concentrations. The recovery of the IS was also evaluated at the concentration used in sample analysis. It was also determined by calculating the peak area ratio of the IS from the spiked processed plasma samples and aqueous solutions at equivalent concentrations.

Stability. Human plasma stability of NOR, CIP and LOM was assessed, at low and high concentration levels, at room temperature for 3 h, at 4°C for 24 h and at −30°C for 30 days in order to simulate sample handling and storage time in the freezer before analysis. The effect of three freeze–thaw cycles on the stability of the analytes was also investigated in plasma. Aliquots of spiked plasma samples were stored at −30°C for 24 h, thawed unassisted at room temperature, and when completely thawed the samples were refrozen for 24 h under the same conditions until completing the three cycles. The stability of NOR, CIP and LOM was also studied at 4°C during 24 h in the supernatant of processed spiked plasma samples, simulating the time that sample can be in the auto-sampler before analysis. The stability was assessed by comparing the data of samples analyzed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). A stability/reference samples ratio of 85–115% was accepted as the stability criterion ($n = 5$).

Results

Chromatographic Separation and Selectivity

The chromatographic separation of NOR, CIP, LOM and LEV (IS) in spiked human plasma samples was successfully achieved using the chromatographic conditions previously described (Fig. 2). Under these analytical conditions the last-eluting analyte was LOM, with a retention time of approximately 6.30 min, and the order of elution of the compounds was the following: LEV (IS), NOR, CIP and LOM.

Representative chromatograms of blank and spiked human plasma samples are shown in Fig. 2. The analysis of blank human plasma samples showed no interfering peaks in the retention times of NOR, CIP, LOM and IS. Similarly, none of the tested drugs commonly co-prescribed with NOR, CIP and LOM were found to interfere with the peaks of the analytes or the IS.

Calibration Curve

The calibration curves prepared in human plasma for NOR, CIP and LOM were linear ($r^2 \geq 0.994$) over the concentration range 0.02–5 µg/mL. The calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the best-fit weighting factor for all compounds. Typical weighted regression equations ($n = 5$) of the calibration curves were $y = 3.945x + 0.018$ ($r^2 = 0.994$) for NOR, $y = 1.739x + 0.007$ ($r^2 = 0.996$) for LOM, and $y = 2.945x + 0.016$ ($r^2 = 0.996$) for CIP, where y represents the ratios of analytes/IS peak area and x represents the plasma concentrations. These results demonstrated a good linearity between peak area ratios and concentrations. The LOQ of the assay was set at 0.02 µg/mL for all analytes (NOR, CIP and LOM) with good precision (CV ≤ 8.19%) and accuracy (bias ±7.15%) (Table 1). The LOD was established at 0.001 µg /mL for NOR, CIP and LOM.

Precision and Accuracy

The results for intra- and interday precision and accuracy obtained from plasma QC samples at three different concentrations (0.05, 2.5 and 4.5 µg/mL) representative of the entire calibration range (0.02–5 µg/mL) are shown in Table 2. All the data

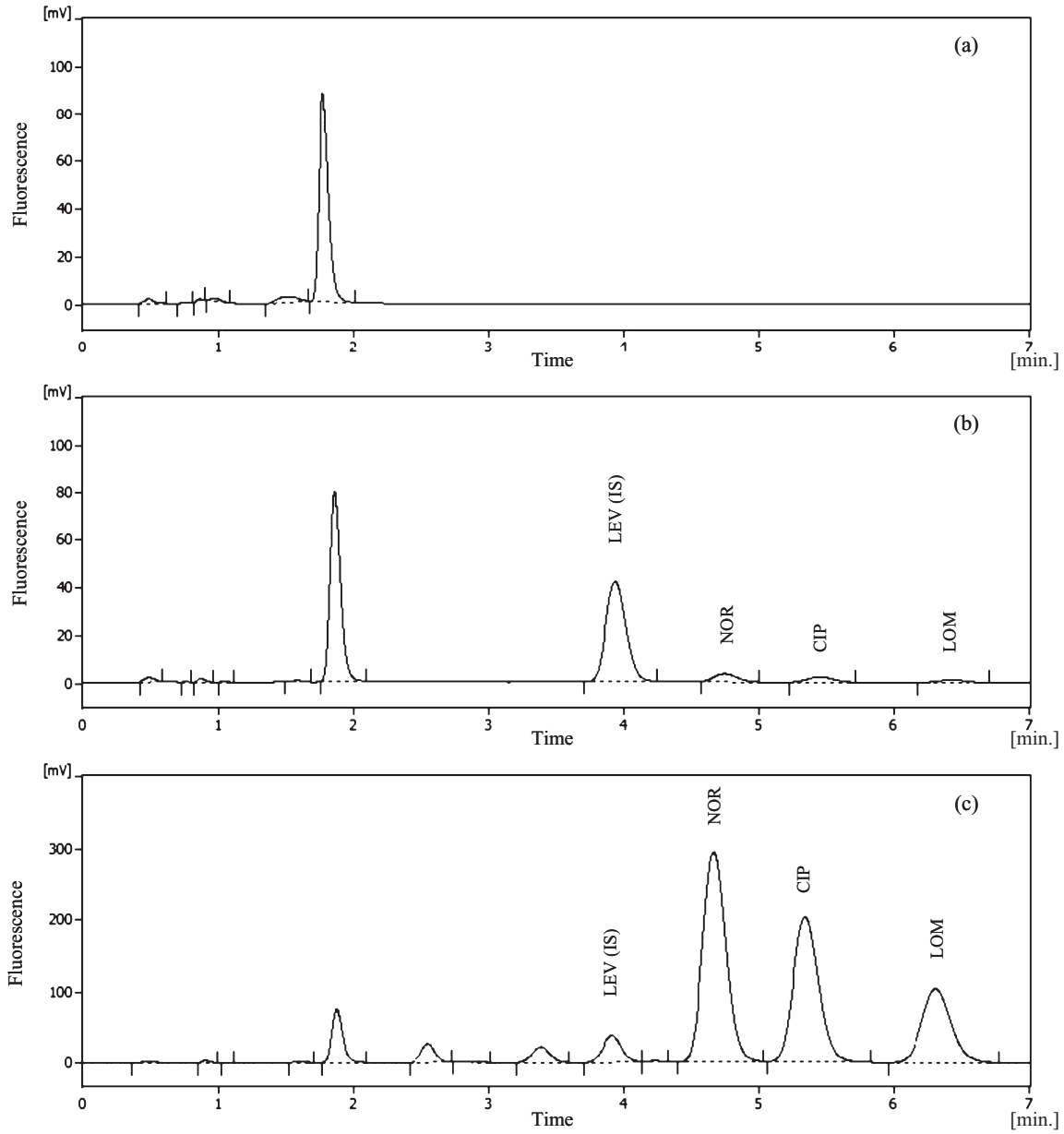

Figure 2. Typical chromatograms of extracted human plasma samples obtained by fluorimetric detection at $\lambda_{ex}/\lambda_{em}$ of 278/450 nm: (a) blank plasma; (b) spiked plasma with levofloxacin (LEV) used as internal standard (IS) and the analytes norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) at concentrations of the limit of quantification (0.02 μg/mL); (c) spiked plasma with LEV (IS) and the analytes NOR, CIP and LOM at concentrations of the upper limit of calibration range (5 μg/mL).

Table 1. Precision (% CV) and accuracy (% bias) for the determination of norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) in human plasma samples at the concentrations of the limits of quantification (n = 5).

Drug	Nominal concentration (μg/mL)	Measured concentration (mean ± SD, μg/mL)	Precision (% CV)	Accuracy (% bias)
Intraday				
NOR	0.02	0.020 ± 0.002	6.39	3.70
CIP	0.02	0.021 ± 0.002	8.19	2.78
LOM	0.02	0.019 ± 0.002	7.94	–7.15
Interday				
NOR	0.02	0.020 ± 0.001	3.51	0.05
CIP	0.02	0.020 ± 0.001	5.39	0.81
LOM	0.02	0.020 ± 0.002	7.21	1.12

Table 2. Precision (%CV) and accuracy (%bias) for the determination of norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) in human plasma samples at low, middle and high concentrations of the calibration range, and following a sample dilution (*) by a 5-fold factor ($n = 5$)

Drug	Nominal concentration (μg/mL)	Measured concentration (mean ± SD, μg/mL)	Precision (% CV)	Accuracy (% bias)
Intraday				
NOR	0.05	0.053 ± 0.004	6.32	5.35
	2.5	2.665 ± 0.083	3.11	6.58
	4.5	4.459 ± 0.292	6.54	–0.91
	*10	10.744 ± 0.692	6.43	7.44
CIP	0.05	0.052 ± 0.004	6.10	4.71
	2.5	2.729 ± 0.074	2.69	9.16
	4.5	4.589 ± 0.321	6.98	1.98
	*10	10.024 ± 0.676	6.74	0.24
LOM	0.05	0.051 ± 0.003	5.67	1.34
	2.5	2.773 ± 0.076	2.74	10.91
	4.5	4.767 ± 0.359	7.53	5.94
	*10	9.317 ± 0.635	6.81	–6.83
Interday				
NOR	0.05	0.051 ± 0.004	6.43	2.71
	2.5	2.573 ± 0.165	6.42	2.93
	4.5	4.383 ± 0.160	3.65	–2.61
	*10	10.459 ± 0.629	6.02	4.59
CIP	0.05	0.051 ± 0.003	5.73	1.71
	2.5	2.646 ± 0.180	6.80	5.83
	4.5	4.549 ± 0.239	5.26	1.10
	*10	10.056 ± 0.610	6.06	0.56
LOM	0.05	0.049 ± 0.003	6.38	–2.23
	2.5	2.640 ± 0.179	6.79	5.58
	4.5	4.701 ± 0.284	6.04	4.46
	*10	9.485 ± 0.652	6.87	–5.15

fulfil the acceptance criteria. The intra- and interday CV values did not exceed 7.53%. The intra- and interday bias values varied between –2.61 and 10.91%. These data indicate that the developed liquid chromatographic method coupled with fluorescence detection (LC-FD) for the quantification of NOR, CIP and LOM in human plasma is accurate, reliable and reproducible, since neither CV nor bias exceeded 15%, in agreement with literature recommendations. For the dilution of plasma samples (10 μg/mL) the precision and accuracy values varied from 6.02 to 6.87% and from –6.83 to 7.44%, respectively. These results show that a 5-fold dilution with blank human plasma can be rightly applied if the concentration of a trial sample exceeds the highest concentration of the calibration curve.

Recovery

The recovery of NOR, CIP and LOM from human plasma at three different concentrations (0.05, 2.5 and 4.5 μg/mL) was assessed and the results are listed in Table 3. The mean recoveries to NOR, CIP and LOM were 101.2–111.5, 91.2–98.5 and 90.1–97.2%, respectively, and showed low CV values. The recovery of the IS was also evaluated and a mean value of 68.9% was obtained.

Stability

The stability of NOR, CIP and LOM was evaluated under different conditions likely to be encountered during the analytical process and sample storage, by analyzing five replicates of low (0.05 μg/mL) and high (4.5 μg/mL) QC samples. The results of stability assays showed that no significant degradation occurred for NOR at the studied conditions both in unprocessed and in processed plasma samples. For LOM and CIP the stability criteria previously established were not fulfilled in plasma when samples were stored at –30°C for more than 7 days (in the case of LOM) and for more than 21 days (in the case of CIP). Stability data are shown in Table 4.

Discussion

To our knowledge there is no reported LC method in the literature that determines simultaneously NOR, CIP and LOM in human plasma. Therefore, the present paper describes the first LC-FD method developed and fully validated for the simultaneous quantification of NOR, CIP and LOM in the biological matrix of human plasma.

Although a significant number of bioanalytical methods for quantification of fluoroquinolones have been described in literature, only a few refer to human plasma and enable the simultaneous quantification of more than one fluoroquinolone (Liang *et al*., 2002; Samanidou *et al*., 2003; Srinivas *et al*., 2008; De Smet *et al*., 2009). Taking into account the fluoroquinolones of our interest (NOR, CIP and LOM), it is important to highlight the LC method with gradient elution developed by Cañada-Cañada *et al*. (2007) to quantify 15 quinolones, including NOR, CIP and

Table 3. Recovery (%) of norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) from human plasma samples ($n = 5$)

Drug	Nominal concentration (μg/mL)	*n*	Recovery (%)	
			Mean	CV (%)
NOR	0.05	5	101.2	8.33
	2.5	5	101.4	8.56
	4.5	5	111.5	5.80
CIP	0.05	5	93.0	8.78
	2.5	5	91.2	8.45
	4.5	5	98.5	6.31
LOM	0.05	5	90.1	11.20
	2.5	5	90.8	8.43
	4.5	5	97.2	6.78

Table 4. Stability (values in percentage) of norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) in unprocessed plasma samples left at room temperature for 3 h, at 4°C for 24 h, after three cycles of freeze (–30°C) and thaw, at –30°C for 30 days, and in processed plasma samples left at 4°C for 24 h ($n = 5$)

Drug	NOR		CIP		LOM	
Nominal concentration (μg/mL)	0.05	4.5	0.05	4.5	0.05	4.5
Plasma						
Room temperature (3 h)	95.1	98.5	96.0	99.4	85.6	95.2
4°C (24 h)	99.1	100.1	98.9	100.1	99.1	100.1
Freeze (–30°C)/thaw (three cycles)	107.1	101.4	96.1	102.4	95.7	103.0
–30°C (7 days)	107.5	106.3	108.8	109.4	107.1	110.9
–30°C (14 days)	90.4	109.1	92.5	114.4	90.2	117.2
–30°C (21 days)	92.5	106.6	92.7	113.8	93.8	123.6
–30°C (30 days)	101.5	107.3	102.3	118.6	92.2	120.3
Processed plasma sample						
4°C (24 h)	97.5	97.8	97.3	98.2	97.1	98.3

LOM; however, the method was validated to the analysis of the quinolones only in human and veterinary pharmaceuticals. Wan *et al*. (2006) also developed an LC method that makes possible the simultaneous determination of ofloxacin, NOR, CIP and LOM, but a chemiluminescence detection system was used and it was validated for analysis of prawn samples.

The method we have developed presents several important bioanalytical advantages. First of all, it was shown to be an accurate, precise, highly selective and sensitive LC method for the determination of NOR, CIP and LOM in human plasma, using very simple and economical chromatographic conditions. In particular, a good peak resolution of the different fluoroquinolones was achieved on a reversed-phase column using an isocratically pumped mobile phase essentially composed of water (82%), requiring only a small percentage of methanol as organic modifier. In addition, this method permitted a rapid analysis of samples since the elution of all analytes was achieved within 7 min; typical chromatographic run times for the determination of more than one fluoroquinolone are usually 10 min or even longer (Wan *et al*., 2006; Srinivas *et al*., 2008; De Smet *et al*., 2009). The coupling of fluorescence detection to the LC procedure that has been used enhances the sensitivity of the method. Indeed, the value of LOQ obtained herein (0.02 μg/mL) is lower than those achieved by methods using ultraviolet detection: 0.05 μg/mL (Liang *et al*., 2002) and 0.1 μg/mL (Srinivas *et al*., 2008). Moreover, a small volume of plasma (500 μL) is required and the sample preparation consists of a simple one-step deproteinization with trichloroacetic acid. A more complex manipulation involving either liquid–liquid extraction, solid-phase extraction or protein precipitation followed by evaporation and reconstitution of the residue is often referred to in the literature (Samanidou *et al*., 2003; Wan *et al*., 2006; Srinivas *et al*., 2008; De Smet *et al*., 2009). With our method, minimal sample handling is required, reducing time and error sources. In addition, no organic solvents are needed, making the procedure of sample preparation safer and less pollutant.

The validation of this method has demonstrated that it fulfills the international requirements and the linearity was demonstrated in a wide range of concentrations for all analytes (0.02–5.0 μg/mL). The feasibility of sample dilution for human plasma concentrations above the calibration range was also successfully assessed. No interferences were found between NOR, CIP or LOM, and human plasma endogenous compounds or commonly co-prescribed drugs.

In conclusion, a selective, accurate, reliable and reproducible new method for the simultaneous quantification of NOR, CIP and LOM has been developed and fully validated. Hence, it will be suitable to support the routine TDM of NOR, CIP or LOM, and it can be applied to other pharmacokinetic-based studies involving these fluoroquinolones.

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