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Short communication

Hydrophilic interaction liquid chromatography–tandem mass spectrometry for the determination of levofloxacin in human plasma

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Abstract

A rapid, sensitive and selective hydrophilic interaction liquid chromatography–tandem mass spectrometric (HILIC–MS/MS) method for the determination of levofloxacin in human plasma was developed. Levofloxacin and ciprofloxacin (internal standard) were extracted from human plasma with dichloromethane and analyzed on an Atlantis HILIC Silica column with the mobile phase of acetonitrile-ammonium formate (100 mM, pH 6.5) (82:18 v/v). The analytes were detected using an electrospray ionization tandem mass spectrometry in the multiple-reaction-monitoring mode. The standard curve was linear ($r > 0.999$) over the concentration range of 10.0–5000 ng/ml. The lower limit of quantification for levofloxacin was 10.0 ng/ml using 20 μl plasma sample. The coefficient of variation and relative error for intra- and inter-assay at four QC levels were 2.9–7.8% and −7.3% to −2.2%, respectively. The recoveries of levofloxacin and ciprofloxacin were 55.2% and 77.3%, respectively. This method was successfully applied to the pharmacokinetic study of levofloxacin in humans.

Keywords: Levofloxacin; Human plasma; HILIC–MS/MS

1. Introduction

Levofloxacin, the active levo-isomer of racemic ofloxacin, possesses a wide spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria, as well as atypical pathogens such as *Mycoplasma*, *Chlamydia* and *Legionella* [1]. It exerts antibacterial activity via antagonism of the interaction between bacterial DNA gyrase and cell DNA [2]. Levofloxacin has been used in the treatment of community-acquired pneumonia, acute maxillary sinusitis and acute exacerbation of chronic bronchitis. Levofloxacin is well absorbed and is less metabolized than other fluoroquinolones, showing that 87% of an oral dose is excreted unchanged in the urine within 48 h [3].

Numerous methods for the determination of levofloxacin in plasma, urine, bile and/or tissues were reported using capillary electrokinetic chromatography [4–7], solid phase spectrofluorometry [8] and high-performance liquid chromatography (LC) methods with UV, fluorescence detection [9–17] or mass spectrometry (MS) [18–20]. Three reviews on analysis of quinolone antibacterials in dosage forms, biological fluids and edible animal products have been published to date [9–11]. The clean-up procedures for the extraction of fluoroquinolones from biological matrix consist of protein precipitation, solid-phase extraction (SPE), liquid–liquid extraction (LLE), combinations of LLE with SPE, dialysis or ultrafiltration [4–20]. Those methods use a large amount of biological samples (0.25–2 ml) or include time-consuming extraction procedures and relatively long run time.

Polar compounds are poorly retained on a reversed-phase (RP) column even with high aqueous mobile phases. For RPLC–MS methods with electrospray ionization, poor analyte on-column retention may result in detrimental matrix effects and high aqueous content mobile phases are also not conductive to achieving the good spray conditions. To overcome this fundamental mismatch between RPLC and MS detection, LC–MS/MS methods using hydrophilic interaction liquid

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chromatography (HILIC) on bare silica with low aqueous/high organic mobile phase has been used for the determination of polar compounds in biological fluids [21–26]. In this study, the use of HILIC–MS/MS on a silica column with high organic/low aqueous mobile phase is presented to analyze the polar levofloxacin in human plasma. The rapid, robust and sensitive HILIC–MS/MS method using LLE with dichloromethane was validated for the quantitative analysis of levofloxacin using 20 μl human plasma and the present method has been successfully applied to the evaluation of levofloxacin pharmacokinetics in humans.

2. Experimental

2.1. Materials

Levofloxacin (purity: 99.5 %) and ciprofloxacin (purity: 99.2%, internal standard) were the gifts from Hanmi Pharm. Co. Ltd. (Seoul, Korea) and Choongwae Pharm. Co. (Seoul, Korea), respectively. Acetonitrile and dichloromethane (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of HPLC grade or the highest quality available. Drug-free human plasma containing sodium heparin as the anticoagulant was obtained from healthy volunteers.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of levofloxacin and ciprofloxacin (1 mg/ml) were prepared in acetonitrile. Working standard solutions of levofloxacin were prepared by diluting each primary solution with acetonitrile. The working solution for internal standard (1000 ng/ml) was prepared by diluting an aliquot of stock solution with acetonitrile. All levofloxacin and ciprofloxacin solutions were stored at ca. 4 °C in polypropylene bottles in the dark when not in use.

Human plasma calibration standards of levofloxacin (10.0, 20.0, 50.0, 100, 200, 500, 1000, 2000 and 5000 ng/ml) were prepared by spiking appropriate amount of the working standard solutions into a pool of ten lots of drug-free human plasma. Quality control (QC) samples at 10.0, 30.0, 600 and 4000 ng/ml were prepared in bulk by adding 150 μl of the appropriate working standard solutions (0.2, 0.6, 12 and 80 μg/ml) to drug-free human plasma (2850 μl). The QC samples were aliquoted (20 μl) into polypropylene tubes and stored −20 °C until analysis.

2.3. Sample preparation

Twenty microliters of blank plasma, calibration standards and QC samples were mixed with 10 μl of internal standard working solution and 100 μl of 50 mM potassium phosphate buffer (pH 7.0). The samples were extracted with 800 μl of dichloromethane in 1.5 ml-polypropylene tubes by vortex-mixing for 2 min at high speed and centrifuged at 5000 × *g* for 5 min at 4 °C. The organic layer (600 μl) was pipette transferred and evaporated to dryness under nitrogen at 35 °C. The residues were dissolved in 80 μl of 100% acetonitrile by vortex-mixing for 2 min, transferred to injection vials, and 5 μl were injected onto the LC–MS/MS.

2.4. LC–MS/MS analysis

For LC–MS/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on an Atlantis HILIC Silica column (5 μm, 3 mm i.d. × 50 mm, Waters Co., Milford, MA, USA) using a mixture of acetonitrile-ammonium formate (100 mM, pH 6.5) (82:18 v/v) at a flow rate of 0.5 ml/min. The column and autosampler tray temperature were 30 °C and 4 °C, respectively. The analytical run time was 5.0 min. The eluent was introduced directly into the positive ionization electrospray source of a tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd, UK). The ion source and desolvation temperature were held at 120 and 350 °C, respectively. The optimum cone voltages were 35 and 33 V for levofloxacin and ciprofloxacin, respectively. The molecular ions of levofloxacin and ciprofloxacin were fragmented at collision energy of 23 and 31 eV using argon as collision gas. Multiple reaction monitoring (MRM) mode was employed for the quantification: *m/z* 362.7 → 261.2 for levofloxacin and *m/z* 332.3 → 231.1 for ciprofloxacin (internal standard). Peak areas for all components were automatically integrated using MassLynx version 3.5 software (Micromass UK, Ltd.).

2.5. Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 10.0, 30.0, 600 and 4000 ng/ml were assayed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision, respectively.

The absolute and relative matrix effect and recoveries of levofloxacin and ciprofloxacin were assessed by analyzing three sets of standards at three concentrations (30.0, 600 and 2400 ng/ml) according to the approach of Matuszewski et al. [27]. The absolute matrix effect for levofloxacin and ciprofloxacin was assessed by comparing mean peak areas of an analyte at three concentrations spiked after extraction into plasma extracts originating from five different lots (set 2) to mean peak areas for neat solutions of the analytes in acetonitrile (set 1). The variability in the peak areas of analytes spiked post-extraction into five different plasma extracts (set 2) expressed as CVs (%), was considered as a measure of the relative matrix effect. Recoveries of levofloxacin were determined by comparing mean peak areas of analyte spiked before extraction into the same five different sources as set 2 (set 3) with those of the analyte spiked post-extraction into different blank plasma lots at three concentrations (set 2).

To assess post-preparative stability, six replicates of QC samples at each of the low and high concentrations (30.0 and 4000 ng/ml, respectively) were processed and stored under autosampler conditions for 24 h were assayed.

2.6. Application

The developed LC–MS/MS method was used in a pharmacokinetic study after an oral administration of levofloxacin to humans. Four healthy male volunteers, fasted for 10 h, received a single oral dose of levofloxacin (200 mg tablet) with 200 ml of water. Blood samples (1 ml) were withdrawn from the forearm vein at 0, 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 h post dosing, transferred to Vacutainer™ plasma glass tubes (sodium heparin, BD, NJ, USA) and centrifuged. Following centrifugation (3000 × *g*, 20 min, 4 °C), plasma samples were transferred to polypropylene tubes and stored at −20 °C prior to analysis. The peak concentration (C_{max}) and the time to peak concentration (T_{max}) were determined by visual inspection from each volunteer's plasma concentration–time plot for levofloxacin. Area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal method from 0 to 24 h.

3. Results and discussion

3.1. HILIC–MS/MS

The electrospray ionization of levofloxacin and ciprofloxacin produced the abundant protonated molecular ions ($[M+H]^+$) at *m/z* 362 and 332, respectively, under positive ionization conditions, without any evidence of fragmentation and adduct formation. $[M+H]^+$ ions from levofloxacin and ciprofloxacin were selected as the precursor ion and subsequently fragmented in MS/MS mode to obtain the product ion spectra yielding useful structural information (Fig. 1). The fragment ions at *m/z* 261 ($[M-CO_2-NC_3H_7+H]^+$) and *m/z* 231 ($[M-H_2O-NC_2H_5-C_3H_4+H]^+$) were produced as the prominent product ions for levofloxacin and ciprofloxacin, respectively. The quantification of the analytes was performed using the MRM mode due to the high selectivity and sensitivity of MRM data acquisitions: *m/z* 362.7 → 261.2 for levofloxacin and *m/z* 332.3 → 231.1 for ciprofloxacin.

HILIC–MS/MS methods operated with the silica column and low aqueous–high organic mobile phase have been proved to be ideal for the analysis of polar compounds in biological fluids [21–26]. Pitos et al. [28] reported that the solute-stationary phase non-hydrophobic interactions may be considerably responsible for the retention in RP-HPLC compared to the hydrophilic nature of zwitterionic ofloxacin, norfloxacin and ciprofloxacin. Because increasing the content of water, a stronger elution solvent in HILIC, in the mobile phase decreased the retention of levofloxacin and ciprofloxacin, a primary retention mechanism for levofloxacin and ciprofloxacin may be also the hydrophilic interaction between the analytes and the silica stationary phase [21]. The higher organic content in the mobile phase of HILIC resulted in the sensitivity improvement compared to RP-HPLC [21]. Because of the higher sensitivity of HILIC-MS/MS method compared to that of RPLC–MS/MS, the use of a small sample volume (i.e., 20 μl of human plasma) was allowed.

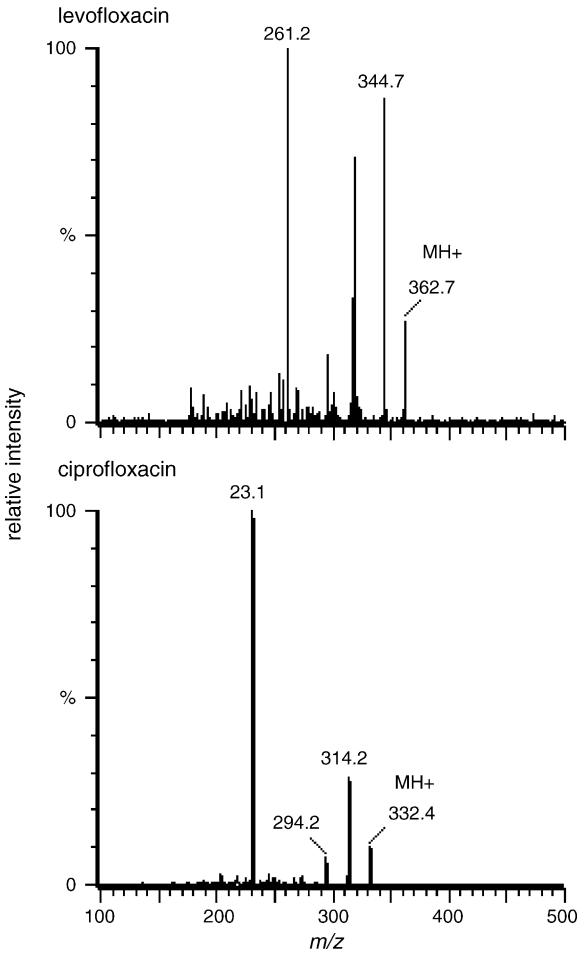

Fig. 1. Product ion mass spectra of (a) levofloxacin and (b) ciprofloxacin (internal standard).

No interference at the retention times of levofloxacin (1.9 min) and ciprofloxacin (2.0 min) was observed in any of the thirty different lots screened as shown in representative chromatograms of the extracted blank plasma sample, confirming the specificity of the present method (Fig. 2). The retention times of levofloxacin and ciprofloxacin were reproducible throughout the experiment and no column deterioration was observed after analysis of 900 human plasma samples. Sample carryover effect was not observed.

3.2. Method validation

This method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [29].

Calibration curves were obtained over the concentration range of 10.0–5000 ng/ml of levofloxacin in plasma. Linear regression analysis with a weighting of 1/concentration gave the optimum accuracy of the corresponding calculated concentrations at each level (Table 1). The low CV value for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and inter-batch precision and accuracy data for QC samples containing levofloxacin.

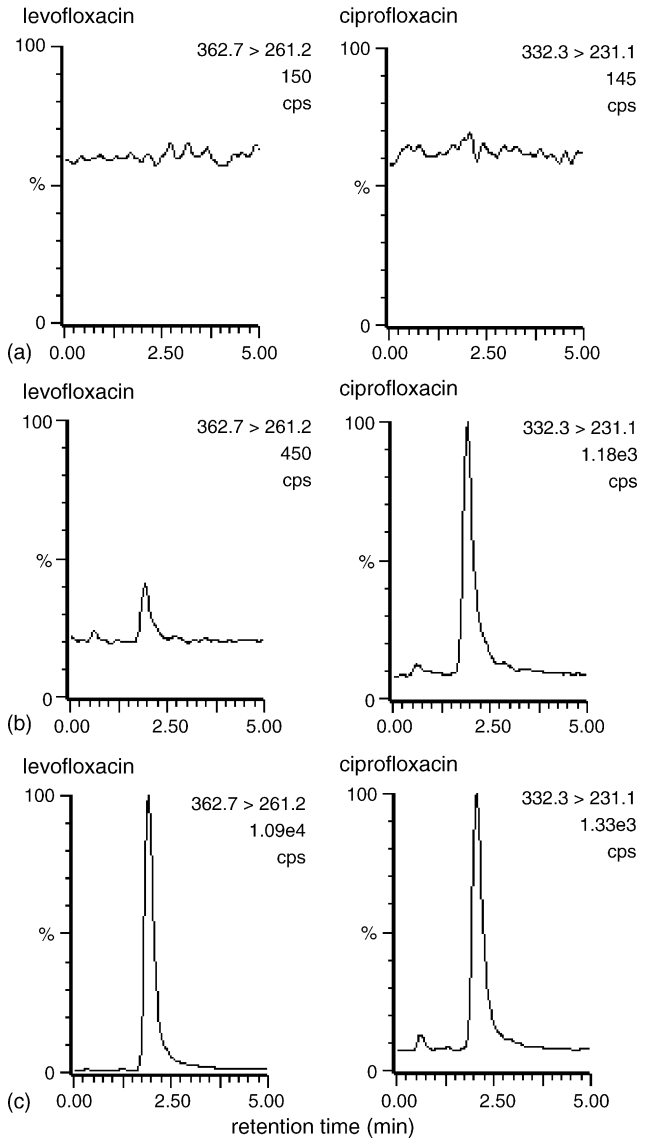

Fig. 2. MRM LC/MS/MS chromatograms of (a) a blank human plasma, (b) human plasma sample spiked with 10.0 ng/ml of levofloxacin and (c) a plasma sample obtained 2 h after oral administration of levofloxacin (200 mg) to a male volunteer.

Both intra- and inter-assay CV values ranged from 2.9% to 7.8 % at four QC levels. The intra- and inter-assay RE values for levofloxacin were −7.3% to −2.2% at four QC levels. These results indicated that the present method has an acceptable accuracy and precision. The lower limit of quantitation (LLOQ) was set at 10.0 ng/ml for levofloxacin using 20 μl of human plasma. Representative chromatogram of an LLOQ is shown in Fig. 2b and the signal-to-noise ratio for levofloxacin is about 20 at 10.0 ng/ml.

The mean absolute matrix effect, the ratio of mean peak areas of set 2 to those of set 1 multiplied by 100, was 96.1% and 92.0% for levofloxacin and ciprofloxacin, respectively (Table 3). A value of 100% indicates that the response in the solvent and in the plasma extracts were the same and no absolute matrix effect was observed. A value of <100% indicates an ionization suppression and a value of >100% indicates an ionization enhancement. The small ionization suppression for levofloxacin and ciprofloxacin was observed.

The assessment of a relative matrix effect was made based on direct comparison of the peak areas of levofloxacin and ciprofloxacin spiked post-extraction into extracts originating from five different sources of human plasma (set 2). The CVs of determination of set 2 at different concentrations varied from 3.9% to 4.6% for levofloxacin and 4.2% to 6.4% for ciprofloxacin (Table 4). This variability seemed to be comparable to the precision of determination of standards injected directly in acetonitrile (set 1) (3.0–4.5% for levofloxacin and 3.0–4.0% for ciprofloxacin, Table 4). These data confirm that the relative matrix effect for levofloxacin and ciprofloxacin was practically absent. The CV of the ratio of levofloxacin/ciprofloxacin for samples spiked post-extraction into extracts from five different lots of plasma varied from 2.2% to 4.4% at different concentrations and was slightly higher than the CV of the ratio of levofloxacin/ciprofloxacin injected directly in acetonitrile (2.0–3.0%, set 1 in Table 4), confirming that the absolute and relative matrix effects for ratio of levofloxacin and ciprofloxacin have practically no effect on the determination of levofloxacin spiked into five different lots of plasma.

As shown in Table 3, the overall extraction recovery of levofloxacin was 55.2%, which was consistent over the concentra-

Table 1
Calculated concentrations of levofloxacin in calibration standards prepared in human plasma ($n = 9$)

	Theoretical concentration (ng/ml)									Slope	Intercept	r
	10.0	20.0	50.0	100	200	500	1000	2000	5000			
Mean	9.8	19.1	46.9	95.2	196	469	935	1922	4724	0.00567	−0.00450	1.000
CV (%)	5.0	5.3	4.7	2.8	3.5	2.5	1.7	3.9	1.7	7.9		
RE (%)	−2.0	−4.5	−6.2	−4.8	−2.0	−6.2	−6.5	−3.9	−5.5			

Table 2
Precision and accuracy of levofloxacin in quality control samples

	Intra-batch ($n = 6$)				Inter-batch ($n = 18$)			
QC (ng/ml)	10.0	30.0	600	4000	10.0	30.0	600	4000
Mean (ng/ml)	9.5	27.8	550	3912	9.5	28.5	572	3904
CV (%)	7.4	5.4	3.0	2.9	7.8	5.9	7.4	3.6
RE (%)	−5.0	−7.3	−7.0	−2.2	−5.0	−5.0	−4.7	−2.4

Table 3
Matrix effect and recovery data for levofloxacin and ciprofloxacin (IS) in five different lots of human plasma

Nominal concentration (ng/ml)	Mean peak area[a]						Matrix effect[b] (%)		Recovery[c] (%)	
	Levofloxacin			Ciprofloxacin			Levofloxacin	IS	Levofloxacin	IS
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3				
30.0	171	164	87	734	672	528	95.9	91.6	53.0	78.6
600	3202	3090	1755	719	665	525	96.5	92.5	56.8	78.9
2400	12493	11981	6688	725	667	496	95.9	92.0	55.8	74.4
Mean							96.1	92.0	55.2	77.3

[a] In arbitary units, $n = 5$.
[b] Matrix effect expressed as the ratio of the mean peak area of an analyte spiked post-extraction (set 2) to the mean peak area of same analyte standards (set 1) multiplied by 100.
[c] Recovery calculated as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of an analyte spiked post-extraction (set 2) multiplied by 100.

Table 4
Precision[a] (CV, %) of determination of peak areas of levofloxacin and ciprofloxacin (internal standard), and peak area ratios (levofloxacin/ciprofloxacin) in sets 1[b], 2[c] and 3[d]

Nominal concentration (ng/ml)	Precision (CV, %)								
	Peak area of levofloxacin			Peak area of ciprofloxacin			Peak area ratio		
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
30.0	4.5	4.3	4.5	3.9	4.2	4.2	3.0	2.2	3.4
600	4.1	3.9	5.0	4.0	6.4	5.7	2.0	4.4	2.3
2400	3.0	4.6	6.5	3.0	4.2	6.3	2.8	3.6	2.4

[a] $n = 5$.
[b] Levofloxacin and ciprofloxacin standards in acetonitrile.
[c] Levofloxacin and ciprofloxacin spiked after extraction into extracts from five different plasma lots.
[d] Levofloxacin and ciprofloxacin spiked before extraction into extracts from five different plasma lots.

tion range of 30.0–2400 ng/ml. The recovery of ciprofloxacin was 77.3%. The LLE with dichloromethane at neutral pH has been successfully applied to the extraction of levofloxacin from human plasma.

Extracted QC samples were stable when stored at 4 °C for 24 h prior to injection, with <5 % difference from theoretical concentration.

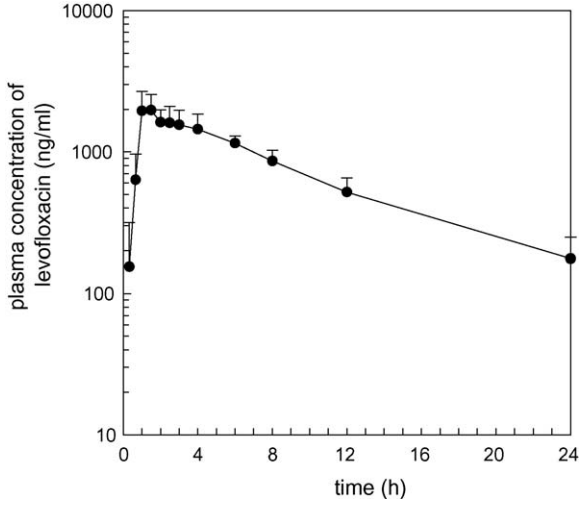

Fig. 3. Mean plasma concentration–time plot of levofloxacin after a single oral dose of levofloxacin (200 mg tablet) to four male volunteers. Each point represents the mean ± S.D.

3.3. Application study

This method has been successfully applied to the bioanalysis of 620 plasma samples in bioequivalence study of levofloxacin. Representative chromatograms of the extract of a plasma sample obtained 2 h after oral dosing of levofloxacin (200 mg) to human are shown in Fig. 2c. Fig. 3 shows mean plasma concentration profiles of levofloxacin obtained after a single oral dosing of levofloxacin (200 mg) to four healthy male volunteers. C_{max}, T_{max} and AUC of levofloxacin were 2.3 ± 0.6 μg/ml, 1.3 ± 0.3 h, 1.3 ± 0.3 h and 16.9 ± 3.5 μg h/ml, respectively.

4. Conclusion

The use of HILIC for the retention of zwitterionic levofloxacin and ciprofloxacin was demonstrated. HILIC-electrospray/MS/MS method for the analysis of levofloxacin in human plasma was developed and validated. The validated method was successfully applied to assay samples from the bioequivalence study of levofloxacin. The method was sensitive (LLOQ 10.0 ng/ml) using 20 μl of human plasma and fast.

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