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Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 765–771

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Rapid stereospecific high-performance liquid chromatographic determination of levofloxacin in human plasma and urine

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Received 12 February 1996; accepted 3 July 1996

Abstract

A rapid high-performance liquid chromatographic (HPLC) method for the determination of levofloxacin in human plasma and urine has been validated. A single-step liquid–liquid extraction procedure was used to isolate levofloxacin from the biological matrix prior to quantitative analysis. The compound was separated on an Inertsil C_{18} reversed-phase HPLC column and quantified by measuring the UV absorbance at 330 nm. The stereospecificity was achieved in the ligand-exchange mode by incorporating chiral reagents directly into the HPLC mobile phase. Ciprofloxacin was used as the internal standard. The method was linear from 0.08 to 5.18 μg ml^{-1} of levofloxacin in plasma and from 23 to 1464 μg ml^{-1} in urine. The overall utility of the method is reflected in its high sample throughput and easy adaptability to robotic automation, thus making the procedure suitable for pharmacological and pharmacokinetic studies of levofloxacin. © 1997 Elsevier Science B.V.

Keywords: High-performance liquid chromatography; Levofloxacin; Ligand exchange; Plasma; Stereospecific; Urine

1. Introduction

Levofloxacin or L-ofloxacin, (*S*)-(−)-9-fluro-2, 3,-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)--7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid, the bacteriologically active L-isomer of the racemic fluoroquinolone ofloxacin, is a broad-spectrum antimicrobial agent currently under investigation by The R.W. Johnson Pharmaceutical Research Institute for the management of a variety of bacterial infections. Following oral administration to man, the drug is extensively absorbed and rapidly eliminated renally with peak urinary levofloxacin concentrations being significantly higher than the MIC_{90} values for usual pathogens. Levofloxacin undergoes limited metabolism and is mainly eliminated unchanged. Within 24 h of oral administration, intact drug is recovered in the urine (80–85%) and in the feces (2%). The major urinary metabolites, desmethyllevofloxacin and levofloxacin *N*-oxide, each account for only 2% of the dose [1–4].

Several HPLC assays have been reported for the determination of ofloxacin or its stereoisomers in human biological fluids [2,5–9]. Of these, the

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PII S0731-7085(96)01890-0

assays pertaining to levofloxacin have generally involved the use of either liquid–liquid extraction coupled with subsequent chemical derivatization to form a diastereometric adduct and subsequent acidic back-extraction [2,5] or solid-phase extraction followed by fluorescene detection [8].

In this paper, a simple reversed-phase HPLC method employing a single-step liquid–liquid extraction and UV detection for the rapid stereospecific determination of levofloxacin in both human plasma and urine is reported. The assay demonstrates excellent specificity, linearity, precision and accuracy for levofloxacin.

2. Experimental

2.1. Materials

Levofloxacin was obtained as the hemihydrate from Daiichi Pharmaceutical (Tokyo, Japan). The internal standard, ciprofloxacin, was obtained as the hydrochloride salt from Sigma (St. Louis, MO, USA). The structures of levofloxacin and ciprofloxacin are shown in Fig. 1. Stock standard solutions of both levofloxacin and ciprofloxacin (103.5 and 1.4 μg ml^{-1}, respectively) were prepared in methanol. Dichloromethane and methanol were of HPLC grade (Burdick and Jackson, Muskegon, MI, USA). Sodium phosphate dibasic and potassium phosphate monobasic were obtained from Mallinckrodt (Paris, KY, USA). Chiral reagents, copper(II) sulfate pentahydrate and L-isoleucine were obtained from Aldrich (Milwaukee, WI, USA). All chemicals and solvents were of the highest grade commercially available Pooled drug-free human plasma and urine were obtained from healthy volunteers, stored at −20°C and allowed to thaw at ambient temperature prior to use.

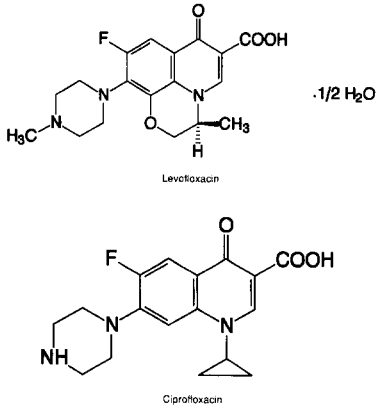

Fig. 1. Structures of levofloxacin and ciprofloxacin.

2.2. Instrumentation

HPLC analysis was performed using a Hitachi LC-6200 pump (Hitachi, Danbury, CT, USA), an automatic sample injection system (Shimadzu, Columbia, MD, USA) and an ABI Model 783 absorbance detector (Applied Biosystems, Foster City, CA, USA). The detector wavelength was set at 330 nm. Data acquisition and manipulation were performed on an HP Model 3350A laboratory automation system (Hewlett-Packard, Avondale, PA, USA). Quantitation was based on linear regression analysis of peak-area ratios of levofloxacin to the standard versus levofloxacin concentration.

Separation was accomplished on an Inertsil ODS-2 (5 μm; 250 × 4.6 mm i.d.) column (Keystone Scientific, Bellefonet, PA, USA). The column temperature was maintained at 35°C. The isocratic mobile phase was copper(II) sulfate pentahydrate (5 mM) containing L-isoleucine (10 mM)–methanol (87.5:12.5, v/v), and was deaerated by sonication prior to use. The flow rate was set at 1.0 ml^{-1}.

In subsequent investigations, plasma samples were extracted automatically using a Zymate 100 robotic system (Zymark, Hopkinton, MA, USA).

2.3. Sample preparation

Stock standard solutions of levofloxacin and ciprofloxacin were prepared in methanol. Further dilution steps were made in either plasma or urine. Working standard solutions were prepared by adding appropriate volumes of the levofloxacin solution. The volume added was always 2% or less of the final plasma volume, so that the integrity of the samples was preserved. Quality control samples were also prepared in the same way, using a

separately weighed stock solution. The final concentrations of levofloxacin in plasma standards were 0.081, 0.162, 0.323, 0.647, 1.294, 2.588, 4.19 and 5.18 μg ml^{-1}. Quality controls of approximately 0.081, 0.647 and 5.18 μg ml^{-1} were prepared separately. The final concentrations of levofloxacin in urine standards were 23, 46, 92, 183, 366, 732 and 1464 μg ml^{-1}. Quality controls of approximately 23, 183 and 1464 μg ml^{-1} were also prepared. After aliquoting, both plasma and urine controls were stored at −20°C until analysis. Buffer was prepared by mixing 40 parts of potassium phosphate monobasic solution (70 mM) and 60 parts of sodium phosphate dibasic solution (80 mM).

2.4. Extraction of samples

To an aliquot of human plasma (0.25 ml) or urine (0.01 ml) was added 100 μl of the internal standard stock solution and 250 μl of phosphate buffer. After the addition of dichloromethane (4.0 ml), each sample was vortex mixed for 30 s and centrifuged at 2000*g* for 5 min. The organic portion was separated. After evaporation under nitrogen and reconstitution in 100 μl of HPLC mobile phase, an aliquot (10 μl) was injected into the HPLC system.

The assay was evaluated in patients receiving oral doses of levofloxacin (500 mg). Blood samples (10 ml) were collected pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 5, 8, 12, 24, 30 and 36 h post-dose and were later centrifuged at 2500*g* for 10 min in order to harvest plasma. Urine samples were collected pre-dose and at 0–12, 12–24 and 24–36 h periods post-dose. Concentrations of levofloxacin were evaluated by determining the ratio of the peak area of levofloxacin to that of the internal standard and then calculated from the calibration curve obtained after linear regression analysis of the calibration standards.

2.5. Precision and accuracy

Precision and accuracy were assessed by performing replicate analyses of quality control samples against calibration standards. The precision and accuracy of the method were calculated as the relative standard deviation (RSD) and the percentage deviation of observed concentration from theoretical concentration, respectively.

2.6. Recovery

The extraction efficiency (recovery) was determined by calculating the ratio of the amount of extracted compound from drug-free plasma or urine spiked with known amounts of levofloxacin (quality control plasma and urine samples) to the amount of compound added at the same concentrations to water just prior to HPLC injection.

2.7. Stability study

The stability of levofloxacin was assessed during all the storage steps and during all the steps of the analytical method. During the first days of the study, quality control samples in plasma and urine were prepared from standard solutions of levofloxacin. These quality control samples were then placed in freezer storage at −20°C and randomly removed at various times in each analytical sequence during a 12–24 month period. Additionally, the freeze–thaw stability and the ambient stability in the autosampler after 24 h at room temperature were assessed in quality control samples for both matrices.

2.8. Specificity study

The ability of the assay to quantify levofloxacin accurately in the presence of endogenous compounds and major metabolites (desmethyllevofloxacin and levofloxacin *N*-oxide) was confirmed through the analysis of blanks and spiked quality control samples, respectively. The chiral specificity of the assay was assessed by obtaining calibration curves for samples in which both the D- and L- (levofloxacin) enantiomers of ofloxacin were present.

3. Results and discussion

Typical chromatograms of blank human plasma and urine, plasma from a subject contain-

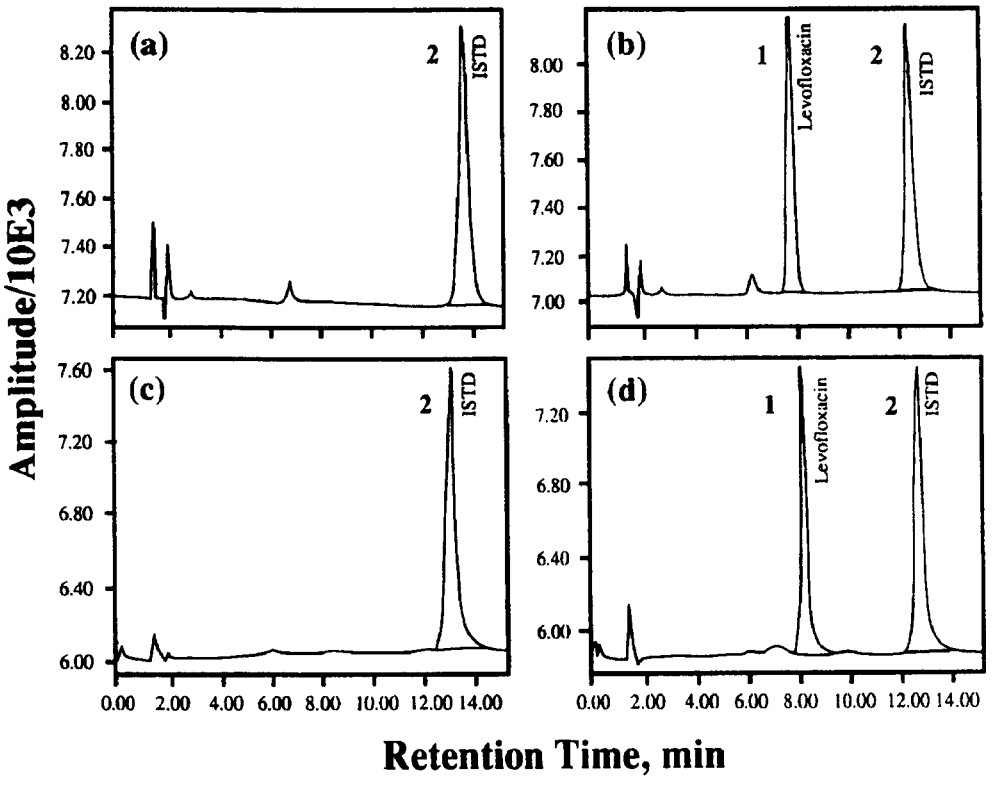

Fig. 2. Representative chromatograms of (a) blank plasma, (b) a plasma sample from a human volunteer containing 3.12 μg ml^{-1} of levofloxacin (c) blank urine and (d) a urine sample from a human volunteer containing 89 μg ml^{-1} of levofloxacin. Peaks: 1 = levofloxacin; 2 = ciprofloxacin (internal standard). For chromatographic conditions, see Experimental.

ing 3.12 μg ml^{-1} of levofloxacin and urine from a subject containing 88.5 μg ml^{-1} of levofloxacin are shown in Fig. 2. The retention times of levofloxacin and ciprofloxacin were approximately 8 and 13 min, respectively.

3.1. Precision and accuracy

Precision and accuracy were assessed in both plasma and urine by performing replicate analyses of spiked samples against calibration standards. The procedure was repeated on the same day and for different days on the same spiked standards at concentrations in range of the standard series. Assay linearity was demonstrated in both plasma and urine, as shown by regression analysis of calibration curves (Table 1). The method was shown to be linear for levofloxacin in the range 0.08–5.18 μg ml^{-1} in plasma and 23–1464 μg ml^{-1} in urine. The between-day precision and accuracy of the method are presented in Table 2. When the extraction was performed robotically, the r values were at least 0.9990 and the precision and accuracy were excellent with an RSD of less than 8%.

3.2. Revovery

Upon analysis of quality control samples (triplicate) at concentrations of 0.081, 0.647 and 5.18 μg ml^{-1} (plasma) and 23, 183 and 1464 μg ml^{-1} (urine), the overall recoveries were 88–98% and 87–95%, respectively.

3.3. Stability

The stability of levofloxacin was assessed in quality control samples (triplicate) at concentra-

Table 1
Assay linearity

Matrix		Correlation coefficient ($r \pm$ S.D.)	Slope $\pm$ S.D.	Intercept
Plasma	Intra-assay	0.9995 ± 0.0002	$0.0008 \pm 8.2 \times 10^{-6}$	0.0014
	($n = 6$)	RSD = 0.02%	RSD = 1.0%	
	Inter-assay	0.9996 ± 0.0002	$0.0008 \pm 1.5 \times 10^{-5}$	−0.0028
	($n = 6$)	RSD + 0.02%	RSD = 1.9%	
Urine	Intra-assay	0.9993 ± 0.0005	0.0068 ± 0.0001	−0.0146
	($n = 6$)	RSD = 0.02%	RSD = 1.2%	
	Inter-assay	0.9993 ± 0.0002	0.0067 ± 0.0001	−0.0143
	($n = 6$)	RSD = 0.05%	RSD = 1.5%	

tions of 0.081, 0.647 and 5.18 μg ml^{-1} (plasma) and 23, 183 and 1464 μg ml^{-1} (urine). These samples were: (1) stored frozen at −20°C for at least 23 months (plasma) and 17 months (urine); (2) allowed to stand at ambient temperature in the autosampler for at least 24 h after extraction; and (3) subjected to five freeze–thaw cycles. Analysis of these samples consistently afforded values which were nearly identical with those of freshly prepared quality control samples, thus confirming the overall stability of levofloxacin in both matrices under long-term frozen storage, assay processing and freeze–thaw conditions.

3.4. Specificity

Endogenous compounds in plasma and urine did not interfere with the levofloxacin or the internal standard peaks. Standards to both desmethyllevofloxacin and levofloxacin *N*-oxide were added to plasma quality control samples at concentrations of 4.0 μg ml^{-1}, and their peaks were well resolved from those of levofloxacin and the internal standard (by the retention times of the desmethyl and *N*-oxide metabolites were approximately 9 and 24 min, respectively). Levofloxacin was reliably quantified in these quality control samples with an accuracy within 10% of the target concentration and an RSD of < 10%. Thus, the presence of high concentrations of both metabolites did not interfere with the determination of levofloxacin.

Additionally, in plasma spiked with racemate, ofloxacin, both the D- and the L-stereoisomers were reliably quantified with accuracy within 10% of the target concentration and an RSD < 10% over the range of the calibration standards. The retention times of the D- and the L-(levofloxacin) stereoisomers were approximately 10 and 8 min, respectively. A representative chromatogram of plasma spiked with the racemate is shown in Fig. 3.

Fig. 4 represents the plasma and urinary concentrations of levofloxacin over a 36 h period from a patient who had received an oral dose (500 mg) of the drug.

4. Discussion and conclusion

The method described involves a rapid and specific assay for the determination of levofloxacin in human plasma and urine. Additionally, information is provided regarding the stability of levofloxacin in both matrices under a variety of storage and treatment conditions.

Mobile phase additives are widely used in reversed-phase HPLC to regulate analyte retention behavior. For example, the phenomenon of ion-pair formation in organic media is utilized in order to partition a charged analyte (such as a protonated amine or a carboxylate anion) which is greatly influenced by its counter-ion. The use of an optically active counter-ion often results in the formation of diastereomeric ion pairs which can be easily separated on conventional reversed-phase columns. Specifically, the principle of ligand-exchange chromatography, as exemplified by the current assay, involves the use of a chiral ligand to form a cordination complex with a

Table 2
Precision and accuracy of the HPLC analysis

Matrix	Theoretical concentration (μg ml^{-1})	n	Experimental concentration (mean ± S.D.) (μg ml^{-1})	RSD (%)	Deviation from theoretical value (%)
Plasma	Within-day:				
	0.081	6	0.083 ± 0.005	5.6	2.5
	0.647	6	0.657 ± 0.024	3.6	1.6
	5.18	6	5.304 ± 0.065	1.2	2.5
	Between-day:				
	0.081	9	0.087 ± 0.007	7.7	7.7
	0.647	9	0.640 ± 0.025	3.9	1.1
	5.18	9	5.206 ± 0.098	1.9	0.6
Urine	Within-day:				
	23	6	25.5 ± 0.92	3.6	10.9
	183	6	179.3 ± 7.14	3.98	2.0
	1464	6	1477 ± 44	3.0	0.9
	Between-day:				
	23	9	24.6 ± 1.22	5.0	7.0
	183	9	183 ± 12.3	6.7	0.0
	1464	9	1484 ± 72	4.9	1.4

suitable transition metal ion, such as copper(II), nickel(II) or zinc(II). Ligand-exchange chromatography can be applied to compounds which possess electron-donating heteratoms or π-electron-donating double bonds which can be incorporated into the metal ion coordination sphere to complete the electron shell. During passage of a racemic mixture through the HPLC column, diastereomeric mixed-ligand complexes form by a displacement or exchange mechanism [10,11]. Generally, bivalent copper cations are preferred owing to the rapid formation and excellent stability of their diastereomeric complexes. Currently, the three major variants of ligand-exchange chromatography involve (1) specific chiral stationary phases, (2) conventional achiral column packings containing chiral metal chelates adsorbed on their surface and (3) a combination of achiral packings with an HPLC mobile phase containing the chiral

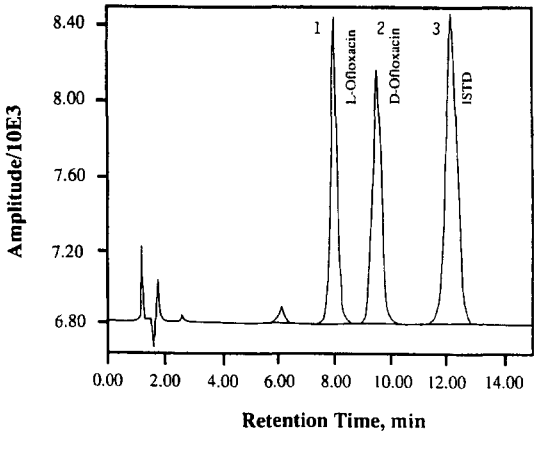

Fig. 3. Representative chromatograms of plasma spiked with racemic ofloxacin to contain the D- and L-stereoisomers at a concentration of 2.59 μg ml^{-1} each. Peaks: 1 = L-steroisomer (levofloxacin); 2 = D-stereoisomer; 3 = ciprofloxacin (internal standard). For chromatographic conditions, see Experimental.

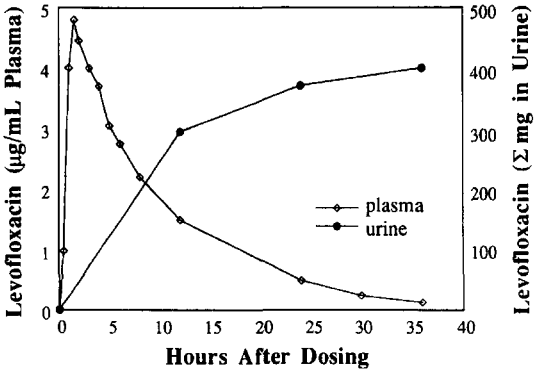

Fig. 4. Pharmacokinetic profile of levofloxacin in plasma and in urine following administration of a single oral dose of levofloxacin (500 mg) to human volunteer.

metal chelate additive [11]. The stereospecificity of the current assay was achieved through the incorporation of chiral ligand-exchange reagents, copper(II) sulfate and L-isoleucine, directly into the HPLC mobile phase. This expedient greatly simplified the overall procedure, thus resulting in rapid and efficient sample analysis while maintaining precision and accuracy. The sensitivity of this assay was adequate to monitor plasma and urinary concentrations of levofloxacin in all clinical investigations of the drug. Furthermore, the overall sample throughput in our laboratory improved dramatically upon switching from chemical derivatization to the current (ligand-exchange) assay mode. Additionally, the simplicity of the current method has allowed the facile adaptation of this methodology to laboratory robotics, thus resulting in a totally automated assay.

Acknowledgements

The authors thank Ms. Nika Anderson and Ms. Gennet Tegegne for their technical assistance. They also thank Dr. John Stubbs for a critical review of the manuscript.

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