ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 1243–1248

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Stereospecific high-performance liquid chromatographic assay of lomefloxacin in human plasma ☆

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Received for review 18 November 1994; revised manuscript received 15 March 1995

Abstract

This report describes an HPLC assay developed for the quantification of the enantiomers of lomefloxacin (LFLX), a quinolone antibiotic, in plasma. Following addition of racemic acebutolol (internal standard, IS), plasma samples were extracted at pH 7 with a mixture of chloroform–isopentyl alcohol–diethyl ether (71.25:3.75:25, v/v/v). The organic layer was evaporated, and LFLX and IS enantiomers in the resulting residue were derivatized with chloroform solutions of 1% triethylamine and 1% (*S*)-(+)-(1-naphthyl)ethyl isocyanate, followed by 2% ethyl chloroformate (ECF) 1 min later. Ethanolamine was added 30 s after the addition of ECF. The enantiomers were separated as diastereomers on an 8 × 100 mm Radial Pak normal phase column using a mobile phase of hexane–chloroform–methanol (64.5:33:2.5, v/v/v) pumped at 2.0 ml min^{-1}. The IS was detected by fluorescence at 245 and 420 nm (excitation and emission, respectively) during the first 12 min, after which time the wavelengths were 280 and 470 nm for detection of LFLX. The method: (1) was sensitive and showed excellent linearity (10–1000 ng ml^{-1}, $r^2 > 0.99$) between added enantiomer concentrations and peak-area-ratio (LFLX/IS); and (2) separated LFLX and IS enantiomers within 25 min. The assay is suitable for the quantification of LFLX enantiomers in plasma samples.

Keywords: Stereospecific; Lomefloxacin; HPLC assay; Enantiomers; Plasma

1. Introduction

Lomefloxacin (LFLX, (±)-1-ethyl-6,8-difluoro-1,4-dihydro-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid) (Fig. 1) is a chiral quinolone carboxylic acid antibiotic which is marketed as the racemate. The enantiomers of LFLX possess equipotent antimicrobial activity in vitro [1a]. It has been reported that the (−)-enantiomer of another chiral fluoroquinolone antibiotic, ofloxacin, was more potent than the (+)-antipode [1b]. Although the enantiomeric potencies of LFLX appear to be similar, the pharmacokinetics of LFLX may be stereoselective, and it follows that stereospecific analytical techniques for the quantification of LFLX in biological samples must be developed if this possibility is to be examined.

Over the past few years, several methods for the quantification of LFLX have been reported

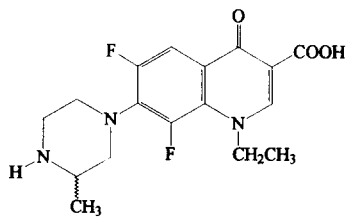

Fig. 1. Structure of (±)-lomefloxacin (LFLX).

☆ Presented at the Ninth Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists (Analysis and Pharmaceutical Quality Section), San Diego, CA, 6–10 November 1994.

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SSDI 0731-7085(95)01560-4

in the literature [2–4]. However, these methods are not able to discern the quantities of the individual enantiomers of LFLX in biological fluids. Hence, little is known regarding the enantiomeric disposition of LFLX in humans.

In this paper, we report an HPLC method suitable for the quantification of LFLX enantiomers in plasma. This method utilizes a two-step pre-column derivatization with subsequent separation of peaks corresponding to LFLX enantiomers on a silica stationary phase.

2. Experimental

2.1. Chemicals

Racemic LFLX hydrochloride as well as the pure enantiomers (*R*)-(+)- and (*S*)-(−)-LFLX hydrochloride were obtained from G.D. Searle (Skokie, IL, USA). Racemic acebutolol hydrochloride was used as the internal standard (IS) and was obtained from Rhone-Poulenc (Essex, UK). Ethyl chloroformate (ECF), ethanolamine (EOA) and (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) were obtained from Aldrich. HPLC grade triethylamine (TEA) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). ACS reagent grade isopentyl alcohol as well as HPLC grade hexane and water were obtained from Caledon Laboratories (Georgetown, Ont., Canada). ACS reagent grade monopotassium phosphate, disodium phosphate, sodium carbonate, and diethyl ether, as well as HPLC grade methanol and chloroform were obtained from BDH (Toronto, Ont., Canada).

2.2. Chromatography

The HPLC apparatus consisted of a multi-solvent delivery system model 600E System Controller attached to a WISP model 717 autosampler, a model 470 scanning fluorescence detector, and an NEC Powermate 486/33i computer with Millennium 2010 chromatography manager software (Waters Scientific, Mississauga, Ont., Canada). Separation of the diastereomers was accomplished an ambient temperature using an 8 × 100 mm Radial Pak cartridge column containing 4 μm Nova-Pak silica packing material (Waters Scientific, Mississauga, Ont., Canada). Detection of the fluorescent chromophores was accomplished by programming the detector to switch at 12 min from an initial setting of 245 and 420 nm for excitation and emission respectively, with the detector gain set at 1000 times, for detection of the IS to 280 and 470 nm for excitation and emission respectively, with detector gain set at 1000 times, for detection of LFLX. The mobile phase consisted of hexane–chloroform–methanol (64.5:33:2.5, v/v/v), pumped at a flow rate of 2.0 ml min^{-1}.

2.3. Standard solutions

To 5.0 ml of 0.01 M sodium carbonate was added 10 mg racemic LFLX hydrochloride (as the base), which was then diluted to a total volume of 100 ml with HPLC grade water. The IS solution consisted of 10 mg racemic acebutolol hydrochloride (as the base) dissolved in 10 ml of HPLC grade methanol and diluted to a total volume of 100 ml with HPLC grade water. These solutions were stored at 4 °C. To drug-free plasma was added LFLX to give final enatiomer concentrations of 10, 25, 50, 100, 250, 500 and 1000 ng ml^{-1}.

Solutions of NEIC (1:100, v/v), TEA (1:100, v/v), ECF (1:50, v/v) and EOA (1:40, v/v) were prepared in chloroform.

2.4. Sample preparation

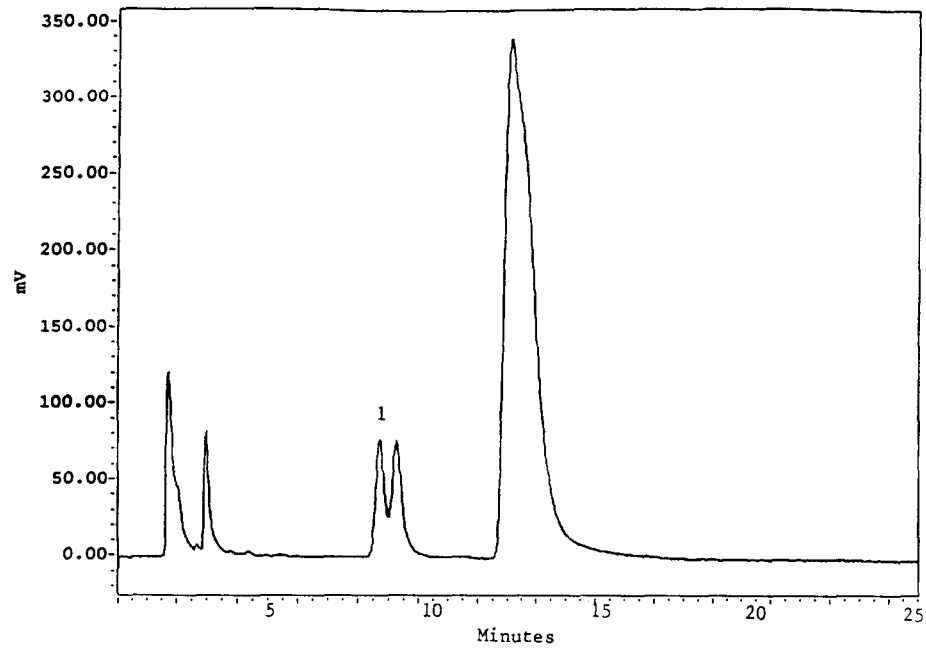
To 0.5 ml plasma containing LFLX was added 25 μl of IS solution and 100 μl of 0.07 M phosphate buffer, pH 7. Samples were then extracted with 4 ml of a mixture of chloroform–isopentyl alcohol–diethyl ether (71.25:3.75:25, v/v/v). The resultant mixture was vortexed for 30 s using a Vortex Genie 2 mixer (Fisher Scientific, Edmonton, Alta., Canada) and centrifuged for 5 min at 1800*g* using a Dynac II centrifuge (Becton Dickinson, Parisippany, NJ, USA). The upper aqueous layer was aspirated off and the remaining organic layer transferred to a clean tube. Samples were then evaporated to dryness using a Savant Speed Vac concentrator–evaporator (Fisher Scientific, Edmonton, Alt., Canada). The resultant residue was reconstituted in 100 μl of TEA in chloroform (1:100, v/v). To this was added 100 μl of NEIC in chloroform (1:100, v/v) and, 1 min later, 50 μl of ECF in chloroform (1:50, v/v). After 30 s, 50 μl of EOA in chloroform (1:40, v/v) was added react with excess ECF. Aliquots of 20 to 125 μl were injected into the HPLC. Sample preparation and analysis were both conducted at ambient temperature (22–25 °C).

2.5. Quantification

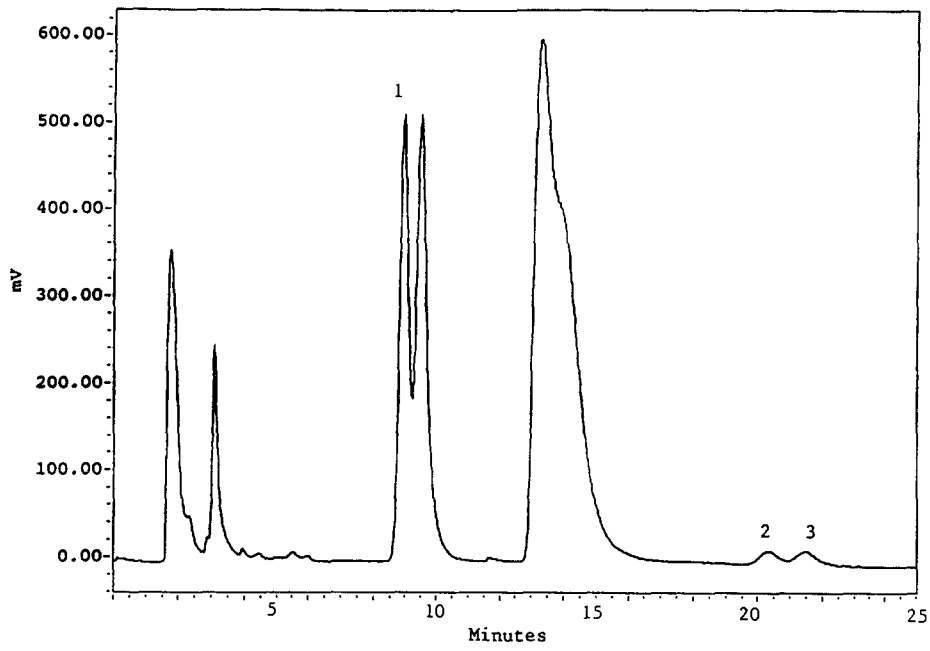
Weighted $(1/x)$ calibration curves for LFLX enantiomers in plasma were constructed by plotting the peak area ratios (LFLX/IS) obtained by analysis of the drug versus their corresponding spiked plasma concentrations. The first eluting peak of the IS was used for quantification of the peak area ratio. Measured concentrations of LFLX enantiomers were generated from the respective weighted $(1/x)$ regression curves. The results are reported as mean ± standard deviation (SD).

2.6. Accuracy and precision

Drug-free plasma was spiked with racemic LFLX at seven different concentrations corre-

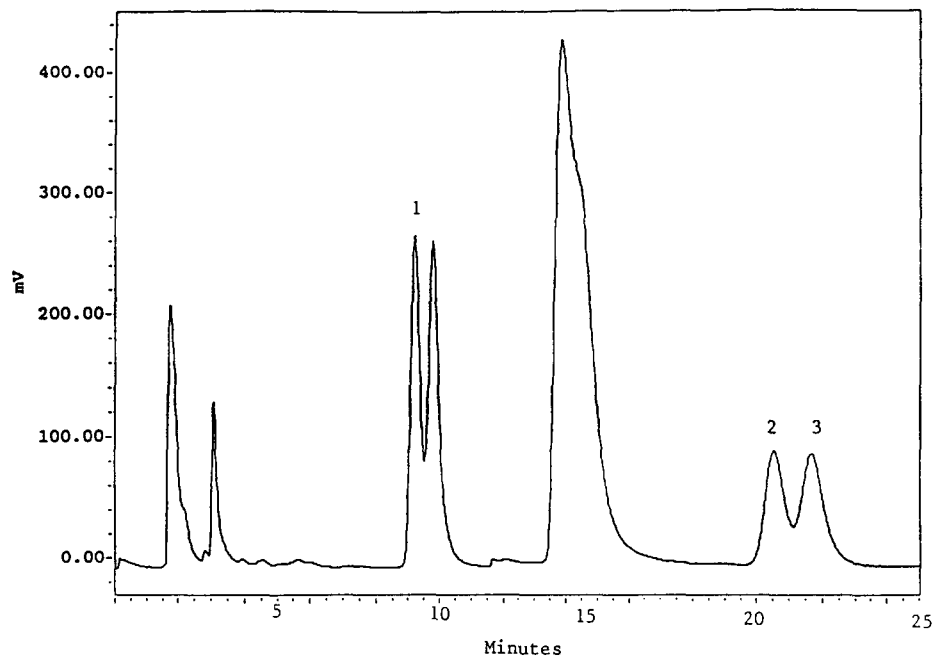


(A)



(B)

Fig. 2 (A) and (B).



(C)

Fig. 2. Chromatograms of (A) blank plasma, (B) plasma spiked with 20 ng ml^{-1} of racemic LFLX, and (C) plasma spiked with 200 ng ml^{-1} of racemic LFLX. All plasma samples were also spiked with 5 μg ml^{-1} of racemic IS Peaks: 1 = IS; 2 = (*S*)-(−)-LFLX; 3 = (*R*)-(+)-LFLX.

sponding to enantiomer concentrations ranging from 10 to 1000 ng ml^{-1} (as the base, $n = 9$). Samples at each concentration were analyzed in triplicate, on three consecutive days. The accuracy, expressed as percentage error, was determined by comparing the measured and added concentrations of LFLX enantiomers in each sample. The precision, expressed as relative standard deviation (RSD), was determined for measured LFLX enantiomer concentrations.

2.7. Extraction efficiency

Utilizing stock solutions of LFLX hydrochloride in acetonitrile, amounts of 10, 100, or 1000 ng (as the base) of each enantiomer were added to clean, dry glass tubes and evaporated to dryness ($n = 4$). After addition of 0.5 ml drug-free plasma, samples were extracted as described above. To compare these samples with those that were not extracted, another set of tubes containing the above amounts of drug were prepared and evaporated without the addition of plasma and subsequent extraction procedure. All samples were then derivatized and injected into the HPLC. The peak areas of the diastereomers corresponding to LFLX enantiomers for extracted drug versus unextracted equivalent drug concentrations were compared under identical chromatographic conditions.

3. Results and discussion

Although we have used chiral stationary phase columns for some enantiomeric separations (zopiclone [5], ketoprofen [6], verapamil [7]), pre-column derivatization can be advantageous in some situations. LFLX enantiomers have two potential sites for the occurrence of derivatization. Although derivatization with L-leucinamide via a coupling reagent has been reported for ofloxacin [1], this approach did not yield successful chromatographic resolution of the LFLX enantiomers. This failure was hypothesized to be due to the remoteness of the chiral center from the derivatized carboxylic acid group. It was thought that separation of LFLX enantiomers could be improved by derivatization of the secondary amine adjacent to the chiral carbon. We wanted to avoid using reagents that derivatized (possibly incompletely) both the amino and carboxyl functional groups, and that might therefore produce multiple reaction products and multiple chromatographic peaks. The unichiral

Table 1
Accuracy and precision of method ($n = 9$)

Enantiomer conc. added (ng ml^{-1})	Measured concentration [a]		Accuracy (error, %)		Precision (RSD, %)	
	S	*R*	*S*	*R*	*S*	*R*
10	10.4 ± 0.2	10.5 ± 0.2	4.4	4.9	2.2	1.4
25	22.7 ± 0.9	22.3 ± 0.5	9.3	9.5	4.3	2.1
50	48.0 ± 2.7	48.2 ± 2.7	5.2	5.1	5.7	5.5
100	101 ± 4	102 ± 4	3.5	3.3	3.5	3.9
250	240 ± 17	241 ± 18	5.6	5.8	7.2	7.3
500	506 ± 49	507 ± 49	6.7	6.8	9.7	9.7
1000	1098 ± 70	1099 ± 69	9.8	9.8	6.4	5.3

reagent, (*S*)-(+)-naphthylethyl isocyanate, was expected to preferentially react with the secondary amino function. The carboxylic acid group was then reacted with ethyl chloroformate to give the mixed anhydride. It was anticipated that this moiety would react with the excess ethanolamine added to quench the excess ethyl chloroformate.

Chromatographic resolution of derivatized LFLX enantiomers was accomplished using normal phase HPLC with a silica column. Fig. 2 depicts representative chromatograms obtained from blank plasma, plasma spiked with 20 ng ml^{-1} of racemic LFLX, and plasma spiked with 200 ng ml^{-1} of racemic LFLX. The peaks corresponding to LFLX enantiomers eluted at approximately 21 and 22 min. The first and second eluting LFLX peaks were identified as (*S*)-(−)-LFLX and (*R*)-(+)-LFLX, respectively. The order of elution was determined using pure enantiomer standards of LFLX hydrochloride. The IS, peaks corresponding to the enantiomers of acebutolol eluted at approximately 8.5 and 9.5 min. The first eluting peak of the IS was consistently used for quantitative purposes (peak area of LFLX/IS). The resolution (Rs) and selectivity (α) for the LFLX peaks were 1.13 and 1.07, respectively. As the LFLX peaks were not completely baseline separated, the integration of the peaks was from valley-to-valley, as opposed to forcing the baseline to zero. The resolution of the enantiomers was probably sufficient to accurately quantify disparate plasma concentrations of LFLX enantiomers, although this was not evaluated with samples abundant in one enantiomer.

Table 1 summarizes the accuracy and precision of the method in plasma. At the lowest calibration concentration, the RSD was approximately 2%. All other calibration concentrations had RSD values less than 10%. The accuracy was always within 10% of the true LFLX enantiomer concentration. A weighting factor of $1/x$, where x represents enantiomeric concentration, was found to result in more favorable accuracy and precision values than either $1/x^2$ weighting or equal weighting. The mean extraction yield was approximately 60% over the range of enantiomer concentrations examined. Although this yield was less than 100%, it was consistent and sufficient to allow for accurate and precise quantification down to 10 ng ml^{-1} of each enantiomer. Asymmetric induction was not observed during the derivatization of LFLX enantiomers, as the peak areas corresponding to the diastereomers was consistently equal. Further, analyses of both the racemate and individual enantiomers showed that the diasteromers of both LFLX and IS were stable for at least 24 h, as changes were not observed in the chromatograms upon repeated injection of the same samples at ambient temperature. The total run-time for analysis of LFLX enantiomers was within 25 min.

In summary, the assay developed achieved separation of the LFLX enantiomers which has not been previously reported. Sample preparation involved a sequential two-step derivatization with unichiral and achiral reagents. The assay is valid and is suitable for determination of LFLX enantiomers in human plasma with a run-time of approximately 25 min.

Acknowledgments

This project was supported by Searle US. We would also like to express our deepest heartfelt gratitude to Ms. C.H. Lemko who recently passed away and is sadly missed.

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