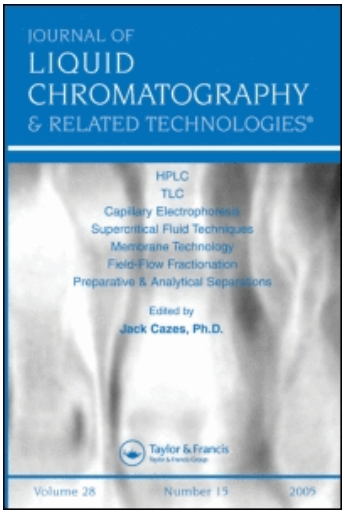

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Development and Validation of an HPLC Method for Determination of Lomefloxacin in Seminal Plasma Involving Solid-Phase Extraction (SPE)

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ABSTRACT

The development and validation of a reversed-phase liquid chromatographic method for the determination of lomefloxacin in seminal plasma is described. Lomefloxacin was extracted on a solid-phase cartridge and separated on a reversed-phase column with acetonitrile in phosphate buffer at pH 7.0 as mobile phase. The solid-phase extraction showed high recovery for lomefloxacin from seminal plasma samples. The chromatographic peaks height ratio of lomefloxacin and internal standard,

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obtained by fluorimetric detection, was used for quantitative analysis. The proposed method was validated with respect to accuracy, precision, linearity, and specificity. Also, the method was determined to be robust with regards to the following parameters: mobile phase, apparent pH; mobile phase organic content. The percent recoveries of lomefloxacin, the limit of detection (LOD) and limit of quantitation (LOQ) for the HPLC method have been determined. This high-performance liquid chromatographic method has been successfully used in medical laboratories to assay seminal plasma samples for studies on the treatment of chronic bacterial infections with lomefloxacin.

Key Words: Lomefloxacin; HPLC; Biological fluids; Seminal plasma; SPE; Validation.

INTRODUCTION

Lomefloxacin [Fig. 1(A)], or 1-ethyl-6,8-difluoro-1,4-dihydro-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid, is a difluorinated quinolone antimicrobial agent with a piperazinyl group on the quinolone ring, which has been demonstrated to have a broad antibacterial spectrum in vitro and in vivo against clinically important Gram-positive and Gram-negative bacteria,[1,2] including those resistant to beta-lactam and aminoglycosides antibiotics[3] and those that frequently cause urinary tract infections.[4]

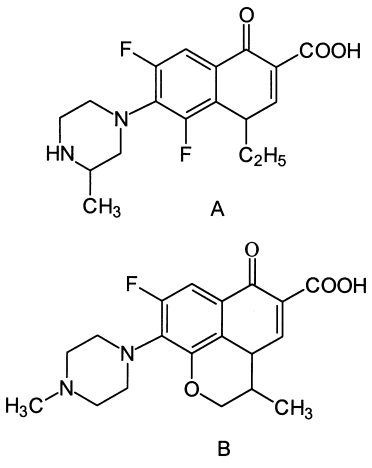

Figure 1. Chemical structures of lomefloxacin (A) and internal standard ofloxacin (B).

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The antibacterial activity of lomefloxacin is related to its ability to inhibit bacterial DNA synthesis by inhibition of DNA gyrase.[5]

The determination of lomefloxacin in biological fluids has been carried out by liquid chromatography coupled with electrospray ionization tandem mass spectrometry[6] and, especially, through high-performance liquid chromatography.[7–13] A procedure also has been described for the estimation by HPLC of lomefloxacin in human erythrocytes in vitro.[14]

So far, no method for the determination of lomefloxacin in seminal plasma has been reported.

Bacterial infections involving the male genital tract are potential causes of infertility in man. Epididimitis, orchitis and infections, which are subclinical or difficult to diagnose, have been reported as being responsible for infertility.[15,16] For these reasons, it seems very useful to have a procedure of quantification of lomefloxacin in seminal plasma, where the drug could represent a very important chemotherapeutic agent against a wide variety of pathogens.

In this work, we have developed and validated a sensitive and precise analytical method for lomefloxacin incorporating an internal standard, which can be applied for the determination of lomefloxacin concentrations in seminal plasma samples.

EXPERIMENTAL

Chemicals and Reagents

Lomefloxacin hydrochloride and ofloxacin [internal standard; Fig. 1(B)] were purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile (HPLC grade) was obtained from Carlo Erba Reagenti (Milan, Italy). All other analytical-grade reagents were purchased from Fluka Chemika-BioChemika (Buchs, Switzerland). Water (HPLC grade) was obtained by passage through the ELIX 3 and Milli-Q Academic water purification systems (Millipore, Bedford, MA).

Apparatus and Chromatographic Conditions

HPLC analysis was carried out using a chromatographic system composed of the following: a Model 515 pump (Waters, Milford, MA) and a Model LS 30 luminescence spectrometer detector (Perkin-Elmer Italy, Rome, Italy). A Model 7725i sample injector (Rheodyne, Cotati, CA) equipped with a 20 μL loop was used. Chromatographic data management was automated using software Millennium[32] (Waters, Milford, MA).

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The analysis was performed on an analytical 250 × 4.6 mm i.d. reversed-phase Spherisorb S5 ODS1 (5 μm particle size) column (Waters, Milford, MA), protected by a 20 × 4.6 mm i.d. (40 μm particle size) disposable Pelliguard precolumn (Supelco, Bellefonte, PA). Separations were performed at room temperature.

The mobile phase consisted of a mixture of acetonitrile and phosphate buffer (pH 7.0; 0.05 M) (20 : 80, v/v). Phosphate buffer prior to use was filtered through an HA 0.45 μm filter, while acetonitrile through a FA 0.5 μm filter (Millipore, Bedford, MA). The mobile phase was prepared daily, degassed using an in-line degasser (Waters, Milford, MA), and delivered at a flow rate of 1.2 mL min^{-1}. Excitation wavelength: 280 nm; emission wavelength: 440 nm.

Standard Solutions and Samples

A stock solution containing lomefloxacin (2.0 mg mL^{-1}) was prepared by dissolving a weighed amount of substance in acetonitrile. Standard solutions were obtained by dilution of the above stock solution with mobile phase containing the internal standard (5 μg mL^{-1}) and by varying the concentration of lomefloxacinin in the range 0.1–2.0 μg mL^{-1}.

Standard solutions of lomefloxacin and standard and stock solutions of internal standard were stored at −20°C for over one month, with no evidence of decomposition. The biological matrix standard solutions for the calibration were prepared by adding 50 μL of each lomefloxacin standard solution in acetonitrile to drug-free biological matrix. The biological matrix solutions had lomefloxacin concentrations of 0.1, 0.2, 0.3, 0.5, 0.8, 1.0, and 2.0 μg mL^{-1}.

Extraction and Isolation Procedures

For verifying the extraction procedure, specimens of sperm were obtained from volunteer patients. They had no drug allergies and had not taken any antibacterial agent in the preceding 72 hours. All the samples were rapidly centrifuged at 2500*g* for 10 min and the supernatant frozen at −20°C until extraction.

Isolute C_{18} solid-phase cartridges from StepBio (Bologna, Italy) were used for samples extraction. Each cartridge contained 100 mg of sorbent with a total reservoir of 3 mL. The cartridges were processed on a Luer syringe that fitted the top of the Supelco vacuum manifold connected to a drying attachment (Supelco, Bellefonte, PA).

After defrosting, the samples were added with appropriate amounts of the stock solution of lomefloxacin so as to obtain drug concentrations in the range of the previously described standard solutions.

The cartridge was conditioned with 2 mL of methanol followed by 2 mL of water. The sample (500 μL) was slowly passed through the cartridge, followed by 1.0 mL of water. The effluent was discarded, 2 mL of acetonitrile were then applied to the cartridge, and the eluate collected. This fraction was evaporated to dryness with a nitrogen stream under vacuum by using the Supelco drying attachment. The sample was subsequently reconstituted with 500 μL of mobile phase containing the internal standard (5 $\mu g\,mL^{-1}$) and injected into the chromatograph.

Sample Analysis

Seminal samples were stored at −20°C after taken, were thawed just before the extraction procedure, and centrifuged at 2500*g* for 10 min. Five hundred microlitre of the sample were then rapidly extracted and analysed as previously described.

RESULTS AND DISCUSSION

Extraction Efficiency

Recoveries of lomefloxacin and internal standard from spiked samples, were calculated by comparing the peak area of seminal plasma with standards at low, medium, and high concentration levels, and submitted to the sample preparation procedure; with those obtained from the analysis of corresponding directly-injected standards ($n = 3$). The extraction recoveries of lomefloxacin and internal standard in seminal plasma were $95 \pm 2.5\%$ and $93 \pm 1.6\%$.

Selectivity of Assay

The selectivity of the assay was determined by analysis of blank seminal plasma from 10 different subjects, with and without internal standard. Under these chromatographic conditions, no endogenous sources of interference were observed in seminal plasma, and the resolution between lomefloxacin and internal standard was satisfactory (Fig. 2).

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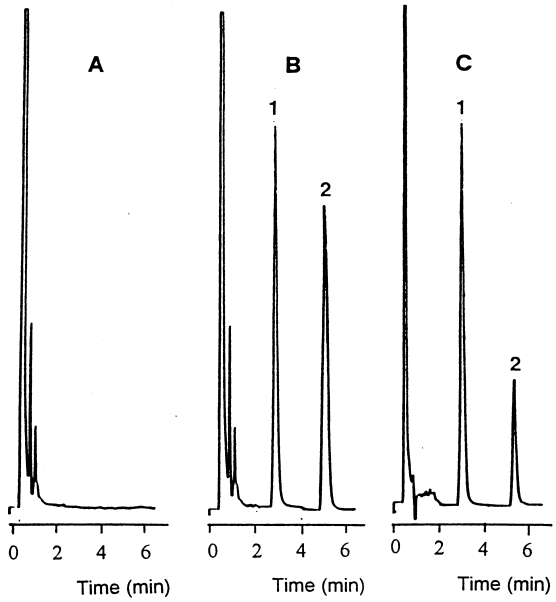

Figure 2. Typical chromatograms of seminal plasma samples after extraction. (A) Blank seminal plasma; (B) Blank seminal plasma spiked with internal standard (1) and lomefloxacin (2); (C) Sample obtained from a patient treated with 400 mg of drug ($0.85\,\mu g\,mL^{-1}$).

Sensitivity of Assay

The limit of detection (LOD) was calculated as three times the standard deviation of the analyte concentration determined in the blank. The calculated LOD was $0.02\,\mu g\,mL^{-1}$. The limit of quantitation (LOQ), defined in the present study as the lowest seminal plasma concentration in the calibration curve that can be measured routinely with acceptable precision and accuracy, was $0.05\,\mu g\,mL^{-1}$.

Linearity of Assay

Calibration curves were determined by linear regression. The assays exhibited linearity between the response (y) (peak-area ratio of lomefloxacin over the internal standard) and the corresponding concentration of lomefloxacin (x), over the $0.1–2.0\,\mu g\,mL^{-1}$ range in seminal plasma (typical equation: $y = 0.017x + 0.002$). The results of linear regression analysis show

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that the correlation coefficients of all standard curves are ≥ 0.9996. In addition, calculated standard points compared to nominal ones evaluated the quality of fit. The assays exhibited linearity ($r > 0.9997$), with a slope near to unity (0.9995) and an intercept not statistically different from zero.

Accuracy of Assay

The accuracy of the assay method was defined as the percentage of the systematic error, which is calculated as the standardised agreement between the measured value and the true value. To be acceptable, the measures should be within $\pm 12\%$ at all concentrations. The accuracy values in intra-day variation studies at low, medium, and high concentrations of lomefloxacin in seminal plasma were acceptable limits (Table 1).

Precision of Methods

The precision of a method is expressed as the percentage coefficient of variation (CV) of replicate measurements. To be acceptable, the measures should be within $\pm 12\%$ at all concentrations. In this work, precision of the method was tested by both intra-day and inter-day reproducibilities in seminal plasma.

Intra-Day Variability of the Assay

The intra-day variability of the assay was determined by repeated analysis of quality control samples at low, medium, and high concentrations on the same day. Results are shown in Table 2. These data indicate that the assay method is reproducible within the same day.

Table 1. Accuracy of HPLC method for determining lomefloxacin in seminal plasma samples.

Concentration added ($\mu g\,mL^{-1}$)	Concentration found ($\mu g\,mL^{-1}$) (Mean $\pm$ SD)	Accuracy (%)
0.20	0.19 ± 0.01	-5.2
1.00	0.98 ± 0.02	-1.6
2.00	1.94 ± 0.04	-3.1

Note: $n = 5$.

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Table 2. Intra-day variability of HPLC method for determining lomefloxacin in seminal plasma samples.

Concentration added ($\mu g\,mL^{-1}$)	Concentration found ($\mu g\,mL^{-1}$) (Mean $\pm$ SD)	CV (%)
0.20	0.19 ± 0.02	4.5
1.00	0.98 ± 0.03	2.6
2.00	1.86 ± 0.04	2.7

Note: $n = 5$.

Inter-Day Variability of the Assay

The inter-day variability of the assay was determined by repeated analysis of quality control samples at low, medium, and high concentrations on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at $-20^{\circ}C$ until analysis. Results are shown in Table 3. These data indicate that the assay method is reproducible within different days. No changes in lomefloxacin concentration were detected in working standard solutions after 1 month of storage at $4^{\circ}C$. The standards in seminal plasma were stable for at least 8 h at room temperature. The extracted samples were stable for at least 24 h at ambient temperature. Lomefloxacin is stable in biological matrices when stored at $-20^{\circ}C$ for at least 6 months.

Figure 2 illustrates representative chromatograms of a drug-free seminal plasma, of a blank seminal plasma spiked with lomefloxacin and internal standard, and of a seminal plasma of a patient treated with the drug. The elution peaks are lacking in interferences deriving from other seminal plasma components and are characterized by retention times of 3.3 (internal standard) and 5.2 (lomefloxacin) min, respectively.

Table 3. Inter-day variability of HPLC method for determining lomefloxacin in seminal plasma samples.

Concentration added ($\mu g\,mL^{-1}$)	Concentration found ($\mu g\,mL^{-1}$) (Mean $\pm$ SD)	CV (%)
0.20	0.17 ± 0.02	5.7
1.00	0.95 ± 0.04	3.3
2.00	1.77 ± 0.06	4.2

Note: $n = 5$.

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Table 4. Effect of experimental parameters on the percent recoveries of lomefloxacin.

Parameter	Modification	Recovery (%)
Mobile phase ratio (v/v)	Buffer : ACN[a]	
	85 : 15	99.5
	80 : 20	100.0
	75 : 25	99.3
pH	6.8	98.8
	7.0	100.0
	7.2	100.0
Flow rate (mL min^{-1})	0.8	99.4
	1.0	100.0
	1.2	100.0
Column type	Used column	99.8
	New column	100.0

[a]acetonitrile.

The optimisation of the analytical procedure has been carried out by varying the following: reversed-phase column used, mobile phase composition, flow rate, pH, excitation, and emission wavelength. The degree of reproducibility of the results obtained through small deliberate variations in method parameters and by changing instruments and operators has been very satisfactory. Table 4 shows that the percent of recoveries of lomefloxacin were good under most conditions and did not show a significant change when the critical parameters were modified. The tailing factor for lomefloxacin and the internal standard was always less than 1.4 and the components were well separated under all the changes carried out.

Considering the modifications in the system suitability parameters and the specificity of the method, as well as carrying out the experiments at room temperature, one would conclude that the method conditions are robust. The solid-phase extraction procedure eliminates endogenous interference, which is frequently present in biological samples. The filtration of extracts before injection onto the chromatographic column avoids rapid obstruction of the precolumn, increasing its life. The HPLC assay method presented here is rapid, sensitive, specific, and robust, and should be of value for the quantitation of lomefloxacin in seminal plasma of patients treated with this drug for chronic bacterial infections of the urinary tract.

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