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A reproducible, simple and sensitive HPLC assay for determination of ofloxacin in plasma and lung tissue. Application in pharmacokinetic studies

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Abstract: A high-performance liquid chromatographic method with fluorometric detection was developed for the analysis of ofloxacin in plasma and lung tissue. The detection was performed at 280 nm for excitation and 500 nm for emission. The procedure involves the addition of an internal standard followed by treatment of the samples with acetonitrile and dichloromethane. The proposed technique is reproducible, selective, reliable and sensitive. Linear detector response was observed for the calibration curve standards in the range of 0.1–5 μg ml^{-1} for plasma and 0.025–2.5 μg g^{-1} for lung tissue. The limit of quantitation is 5 ng ml^{-1} or 5 ng g^{-1}. The accuracy of the method is good; that is, the relative error is <10%. This method was applied to the pharmacokinetic study of ofloxacin in 24 chronic obstructive pulmonary disease patients.

Keywords: *Ofloxacin; HPLC method; fluorescence detection; plasma; lung tissue.*

Introduction

Ofloxacin is a fluoroquinolone derivative with excellent *in vitro* activity against many Gram-positive and Gram-negative organisms. It has shown a large potency against many common bacterial pathogens and also a good activity against various mycobacteriacae, legionella species, rickettsiaceae and even multiple drug resistant nosocomial isolates.

Several methods for detection and quantitation of ofloxacin in biological fluid and tissues have been developed. These methods included microbiological assay [1], reversed-phase HPLC with UV detection [2–4] or fluorescence detection [5–9]. One method compared microbiological assay and HPLC [1]. Two methods were developed to quantify the ofloxacin enantiomers [10–11]. Liquid–liquid extraction and solid phase extraction were used for sample preparation.

The purpose of this study was to develop a rapid, sensitive and selective method for the determination of ofloxacin in plasma and lung tissue in order to use it routinely in clinical practice for therapeutic drug monitoring. As the marketed drug was the racemic form, the proposed HPLC method has been focused on racemic drug quantitation. This method was validated according to good laboratory practice guidelines, and was successfully used to determine the pharmacokinetic profile and lung tissues levels of ofloxacin in patients with chronic obstructive pulmonary disease (COPD).

Experimental

Materials and reagents

Ofloxacin ((±)9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7h-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid was obtained from Diamant Laboratories (Puteaux, France). The internal standard, 4844P an N-allyl derivative of pefloxacin was obtained from Roger Bellon Laboratories (Paris, France). The chemical structure of these compounds is illustrated in Fig. 1.

Stock solutions of ofloxacin and 4844P (1 g l^{-1}) in purified water (Aguettant Laboratories,

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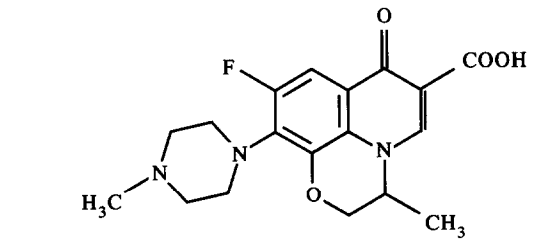
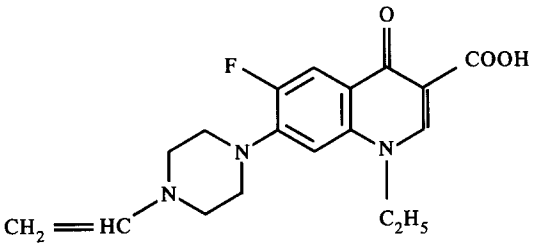


Ofloxacin



N-allyl pefloxacin

Figure 1
Structural formulae of ofloxacin and the internal standard used for the analytical method.

Lyon, France) were diluted 10 and 100 fold (only 10 fold for the internal standard) with purified water when appropriate.

Acetonitrile and dichloromethane were Chromasol grade (SDS, France) and used without further purification. Formic acid, hydrochloric acid, citric acid, triethylamine and sodium acetate were all analytical grade. Hydrochloric acid 0.5 M was prepared in water.

The buffer (pH 4.8) consisted of 1 g citric acid, 2 g sodium acetate and 1 ml triethylamine in 1 l of purified water adjusted to pH 4.8 with formic acid.

The validation samples for plasma assay were prepared in pooled human plasma samples obtained from human blood samples collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes from each patient prior to the injection of ofloxacin. The validation samples for bronchial mucosa assay were prepared in mixed bovine lung tissue (Ultraturax, 20000 revolutions per min); the spiked samples for calibration curves and quality-control were incubated at 20°C for 8 h then at 4°C for 48 h before treatment in order to allow a steady-state tissue protein binding.

Tissue and plasma samples for pharmacokinetic study

The study was carried out in 24 COPD patients. Ofloxacin was administered orally (200 mg b.i.d.) for 10 days to obtain steady-state concentrations, with and without ambroxol treatment (30 mg twice daily). Ambroxol is a drug which increases the mucociliary transport and the synthesis of surfactant. It increases the penetration of several antibiotics in lung tissue [12]. The last day of treatment, 3 h after drug administration, ofloxacin concentrations in plasma, bronchial tissues and broncho-alveolar lavage (BAL) were determined.

Bronchial mucosa biopsies specimens of about 0.5–10 mg were obtained by fiber-optic bronchoscopy. The specimens were washed for 30 s in 0.9% sodium chloride solution in order to limit blood contamination, dried in gauze, put in a glass tube and stored at −20°C until analysis.

Blood samples were collected in EDTA-coated tubes, after centrifugation, plasma samples were pipetted into a 5 ml glass tube and stored at −20°C until analysis.

The study protocol was in accordance with legal requirements and the Declaration of Helsinki and was approved by the local Hospital Ethics Committee. Each patient gave a written informed consent to participate in this study.

Instrumentation

Analysis by HPLC was performed using a Gilson model 302 pump with a Rheodyn valve fitted with a 20-μl loop, an automatic sample injection system (Gilson 231), a stainless-steel column (150 × 4.6 mm i.d. SFCC, Neuilly Plaisance France) packed with 5 μm Ultrabase RP 8 and a guard column (20 × 4.6 mm i.d. SFCC, Neuilly Plaisance, France) packed with 10 μm Spherisorb C8 placed just before the inlet of the analytical column.

The column effluent was monitored with a fluorescence detector (model LS-1 Perkin–Elmer, France) operated at an excitation wavelength of 280 nm and an emission wavelength of 500 nm.

Chromatographic conditions

The mobile phase, containing 15 parts of acetonitrile and 85 parts of citrate buffer (pH 4.8) was degassed prior to use and was deaerated with helium during use. Acetonitrile and the aqueous phase were prefiltered through a membrane filter (0.45 μm, Millipore, Molsheim, France). The oven tempera-

ture was 50°C and the flow rate was 1.5 ml min^{-1}, which corresponds to a pressure of about 85 bars.

The signal was recorded and the peak heights were determined with a LCI 100 Perkin–Elmer laboratory computing integrator (chart speed of 2 mm min^{-1}).

Extraction procedure

After thawing, plasma samples (0.5 ml) were spiked with internal standard solution (10 μl) and homogenized. Acetonitrile (0.5 ml) was added to all samples and the mixture was vortex mixed for 10 s, then all of the vials were centrifuged at 1500*g* for 10 min. A volume of 0.8 ml of the supernatant was pipetted into a 10 ml glass tube then 5 ml of dichloromethane and 25 μl of 0.5 M hydrochloric acid solution were added. The samples were vortex mixed for 10 s then the tubes were centrifuged for 10 min and kept at −20°C for 30 min in a freezer. The aqueous solution was separated and 20 μl of this solution were injected into the chromatograph.

After thawing, the biopsy specimens were homogenized by micro crushing in 90 μl of purified water until total dilaceration then 10 μl of internal standard solution and 100 μl of acetonitrile were added. The mixture was homogenized by micro crushing for 10 min. After a 10 min centrifugation 170 μl of the supernatant was transferred into a microvial; 75 μl of water and 25 μl of 0.5 M hydrochloric acid were added to all samples and the mixture was vortex mixed for 10 s. Then 1 ml of dichloromethane was added and the assay procedure completed as for the plasma samples described above.

Instruments calibration

Calibration standards for control plasma and biopsy tissues were prepared using concentrations of 0.1, 0.25, 0.5, 0.75, 1, 2.5 and 5 mg l^{-1} in human plasma and 0.025, 0.05, 0.1, 0.5, 1 and 2.5 mg g^{-1} in 10 mg bovine lung tissue. The standard samples were prepared by adding appropriate volumes of ofloxacin solutions. The volume added was always smaller than or equal to 2% of the total volume of the sample, so that the integrity of the sample was maintained.

These standards were treated concurrently and in the same manner as the samples to be analysed.

Data analysis

For plasma and lung tissue assays, the ratio of the peak height of ofloxacin to that of internal standard was used as the assay parameter. Peak height ratios were plotted against theoretical concentrations. Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data.

The linearity of the method was confirmed using classical statistical tests, that are a comparison of intercept with zero and correlation coefficients.

Determination of the limit of quantitation (LOQ)

The LOQ was determined from the peak and the standard deviation of the noise level, S_N. The LOQ was defined as the sample concentration of ofloxacin resulting in a peak height of 10 times S_N. The estimation of S_N was determined by extrapolation to zero. To determine the analytical error on the LOQ, spiked plasma and lung tissue were used.

Stability study

The stability of ofloxacin was assessed during all the storage steps and during all steps of the analytical method.

During the first days of the study, quality control samples (in plasma and bovine lung mucosa) were spiked with standard solutions of ofloxacin (0.1–0.5 mg l^{-1}). Then the aliquoted quality control samples were placed in a freezer at −20°C and randomly into each analytical sequence over a 2 month period. The ambient stability in the autosampler was assessed for all concentrations of the calibration curve after 12 and 24 h.

Results

Retention times

Observed retention times were 4.9 and 10.8 min for ofloxacin and the internal standard, respectively. The capacity factors k' were 3.88 for ofloxacin and 9.61 for the internal standard. The resolution between the two compounds was 11.5 and the selectivity was 2.48. Representative chromatograms are shown in Fig. 2. There were no significant interfering peaks in control plasma or control biopsy (Fig. 3) at the retention time of the respective analytes.

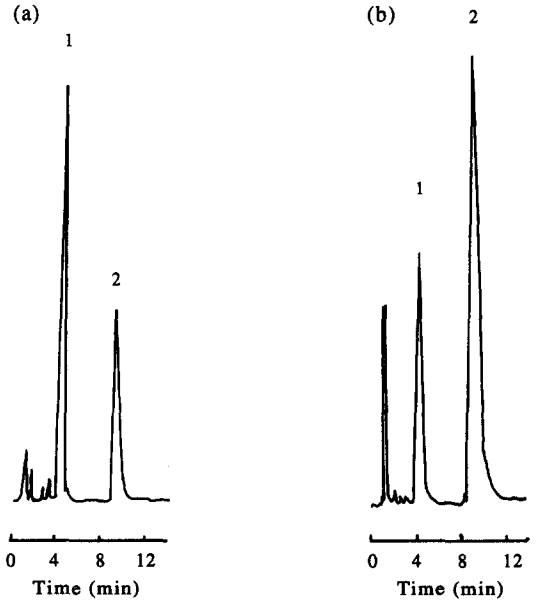


Figure 2
Typical chromatogram of plasma (a) and lung tissue (b) spiked with ofloxacin (1 mg ml^{-1} and 0.1 mg g^{-1}, respectively). Peaks: 1 ofloxacin, 2: internal standard. For chromatographics conditions, see text. Fluorometric parameters: (a) control factor: 650, fix scale: 20, response: 3; (b) control factor; 850, fix scale: 20, response: 4.

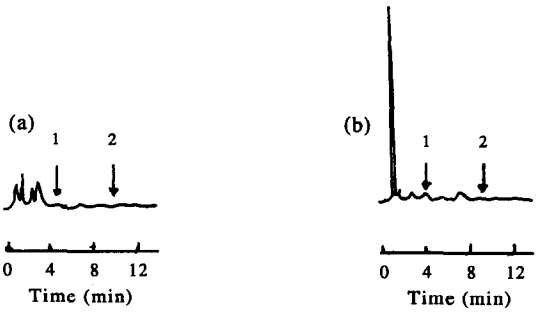


Figure 3
Representatives chromatograms of blank plasma (a) and blank lung tissue (b).

Linearity

In plasma, peak height ratio of ofloxacin over the internal standard varied linearly with concentration over the range used (0.1–5 mg l^{-1}). The correlation coefficients (r) for calibration curves were equal or better than 0.9985. Intra-assay reproducibility was determined for calibration curves prepared the same day in replicate (n = 6) using the same stock solutions. The intraday average slope of the fitted straight lines was 3.23 ± 0.061 (relative standard deviation, RSD = 1.89%), the correlation coefficient was 0.9996 ± $5.5.10^{-4}$ (RSD = 0.055%) and the mean intercept was 0.032 ± 0.031. For calibration curves prepared on different days (n = 11), mean results were as follows: slope = 2.96 ± 0.41 (RSD = 13.8%), r = 0.9999 ± $3.67.10^{-5}$ (RSD = 0.0037%) and intercept = 0.0055 ± 0.028.

A large concentration range (0.025–2.5 mg g^{-1}) was chosen for the spiked lung tissue. The peak height ratio of ofloxacin over the internal standard varied linearly with the concentration and the correlation coefficients were equal or greater than 0.9983. The intraday (n = 5) results of the unweighted least-squares linear regression analysis were as follows: r = 0.9995 ± $6.94.10^{-4}$ (RSD = 0.07%), slope = 5.58 ± 0.27 (RSD =4.8%) and intercept = −0.036 ± 0.029. The interday variability of calibration standards (n = 8) was: r = 0.9995 ± $5.31.10^{-4}$ (RSD = 0.05%), slope = 5.81 ± 0.4 (RSD = 5.33%) and intercept = −0.056 ± 0.024.

The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero.

For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves (experimental concentrations) and the percent relative standard deviations (RSD%) were computed. Interday and intraday variability in plasma and lung tissue at concentration of calibration standards are presented in Tables 1 and 2, respectively.

Precision and accuracy

In human plasma, within-run precision was assessed by performing five replicate analyses of three standard solutions of ofloxacin at 0.1, 0.5 and 2.5 mg l^{-1} and 25 replicate analyses of a solution at 5 mg l^{-1}. Results are shown in Table 3. Within-run precision was also determined in lung tissue for the concentrations of 0.05 (n = 7), 0.5 (n = 5) and 2.5 (n = 5) mg g^{-1}. Results, expressed as a percentage of the theoretical concentrations with the relative error, are presented in Table 3.

Between-run precision of the method was assessed by analysing quality control samples, prepared in human plasma and lung tissue, on different days for different concentrations. Results are shown in Table 4.

Limit of quantitation and limit of detection

The limit of quantitation was 5 ng ml^{-1}. At this level the analytical error ranged from 20 to

Table 1
Intra-assay reproducibility of the HPLC analysis

	Plasma		Lung tissue	
Theoretical concentration ($\mu g\ ml^{-1}$ or $\mu g\ g^{-1}$)	Experimental concentration ($\mu g\ ml^{-1}$) (mean ± SD)	RSD (%)	Experimental concentration ($\mu g\ g^{-1}$) (mean ± SD)	RSD (%)
0.025	—	—	0.035 ± 0.004	11.4
0.05	—	—	0.0057 ± 0.0053	9.3
0.10	0.12 ± 0.0091	7.6	0.10 ± 0.0039	3.9
0.25	0.26 ± 0.0097	3.7	0.25 ± 0.0046	1.9
0.50	0.49 ± 0.015	3.1	0.49 ± 0.023	4.7
0.75	0.74 ± 0.020	2.7	—	—
1.00	1.00 ± 0.022	2.2	0.97 ± 0.028	2.9
2.50	2.49 ± 0.037	1.5	2.51 ± 0.0051	0.20
5.00	5.01 ± 0.015	0.30	—	—

Table 2
Inter-assay reproducibility of the HPLC analysis

	Plasma		Lung tissue	
Theoretical concentration ($\mu g\ ml^{-1}$ or $\mu g\ g^{-1}$)	Experimental concentration ($\mu g\ ml^{-1}$) (mean ± SD)	RSD (%)	Experimental concentration ($\mu g\ g^{-1}$) (mean ± SD)	RSD (%)
0.025	—	—	0.030 ± 0.0034	11.3
0.05	—	—	0.053 ± 0.0042	7.9
0.10	0.10 ± 0.017	17.0	0.10 ± 0.0019	1.9
0.25	0.26 ± 0.015	5.8	0.24 ± 0.014	5.8
0.50	0.50 ± 0.021	4.2	0.48 ± 0.031	6.5
0.75	0.76 ± 0.0037	0.49	—	—
1.00	1.01 ± 0.047	4.7	1.01 ± 0.042	4.2
2.50	2.46 ± 0.099	4.0	2.49 ± 0.030	1.2
5.00	5.02 ± 0.048	0.96	—	—

Table 3
Within-run precision of the HPLC method

Theoretical concentration	n	Experimental concentration (mean ± SD)	RSD (%)	Mean recovery	Relative error (%)
Human plasma ($\mu g\ ml^{-1}$)					
0.1	5	0.096 ± 0.0040	4.2	96.3	−3.68
0.5	5	0.51 ± 0.0071	1.4	102.4	2.42
2.5	5	2.50 ± 0.018	0.72	99.9	−0.13
5	25	4.83 ± 0.27	5.6	96.6	−3.38
Lung tissue ($\mu g\ g^{-1}$)					
0.05	7	0.055 ± 0.0055	10.0	110.3	10.3
0.5	5	0.47 ± 0.027	5.7	94.0	−5.96
2.5	5	2.50 ± 0.11	4.4	100.1	0.12

30%. The limit of detection which represents a signal noise of 2:1 was about 1 ng ml^{-1}. These limits were in the same range of magnitude for plasma and lung tissue.

Recovery

In the range of calibration standards, the mean recovery of ofloxacin was 80% in plasma (n = 7) and 70% in lung tissue (n = 7).

Stability

The stability of ofloxacin in the autosampler was checked after 12 and 24 h at room temperature, for each point of calibration standards in plasma and lung tissue. For all concentrations, no significant difference appeared between t = 0, t = 12 and t = 24 h.

The stability of ofloxacin in the plasma frozen at −20°C at the concentration of 0.1 and 0.5 mg l^{-1} was inspected after 1, 2, 3 and 6 weeks of cold storage. The mean recovery was 101 ± 0.38% (range, 100.6–101.4%) for 0.1 mg l^{-1} concentration and 102.6 ± 7.34%

Table 4
Between-run precision of the HPLC method

Theoretical concentration $n = 5$	Experimental concentration ($\mu g\ ml^{-1}$ or $\mu g\ g^{-1}$)									Mean ± SD
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	
Human plasma ($\mu g\ ml^{-1}$)										
0.1	0.12 ± 0.017	0.12 ± 0.011	0.095 ± 0.0033	0.096 ± 0.0040	0.094 ± 0.0032	0.095 ± 0.0019	0.12 ± 0.0089	0.095 ± 0.0019	0.11 ± 0.0066	0.11 ± 0.0071
0.5	0.49 ± 0.030	0.52 ± 0.026	0.50 ± 0.022	0.49 ± 0.0044	0.49 ± 0.011	0.51 ± 0.0058	0.51 ± 0.0071	0.48 ± 0.0049	0.53 ± 0.019	0.51 ± 0.022
2.5	2.45 ± 0.11	2.47 ± 0.10	2.49 ± 0.055	2.50 ± 0.018						2.49 ± 0.062
Lung tissue ($\mu g\ g^{-1}$)										
0.5	0.47	0.45	0.50	0.47	0.49					0.48 ± 0.017
2.5	2.50	2.41	2.59	2.36	2.49					2.47 ± 0.087

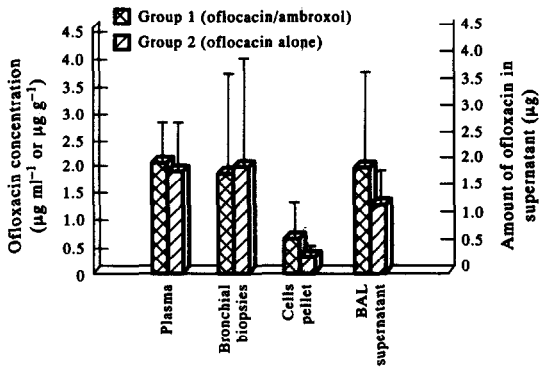


Figure 4
Ofloxacin concentrations in plasma, bronchial biopsies and BAL, 3 h after the last drug administration (200 mg b.i.d. during 10 days) with and without concomitant ambroxol treatment.

(range, 93.5–111.6%) for 0.5 mg l^{-1} concentration. No significant difference appeared.

Discussion

Most published methods to quantify ofloxacin in body fluids use tedious extraction, purification steps and sometimes evaporation under nitrogen stream. The present HPLC method involves a rapid assay for the determination of ofloxacin in plasma and lung tissue. This method which is extremely simple, has good reproducibility, accuracy and sufficient sensitivity. The separation between ofloxacin and endogenous substances was satisfactory. Moreover, this method has a good specificity from aminoglycosides (tobramycin, amikacin), cephalosporins (ceftazidime, ceftriaxone), sulphametoxazole and amoxicillin that could be co-administered to critically-ill patients.

This method was applied to determinations of ofloxacin in plasma, BAL and lung tissue collected during a pharmacokinetic study performed in 24 COPD patients. The aim of this study was to test whether the administration of ambroxol increases ofloxacin concentration in the lung tissue. Bronchial biopsies carried out by fiberoptic bronchoscopy, plasma sample and BAL were obtained on day 10 of treatment, 3 h after the last drug administration. Figure 4 illustrates the concentration of ofloxacin in plasma, BAL (supernatant and alveolar cells) and bronchial biopsy in the groups with and without ambroxol.

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