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Determination of levofloxacin in plasma, bronchoalveolar lavage and bone tissues by high-performance liquid chromatography with ultraviolet detection using a fully automated extraction method

S. Djabarouti [a,b], E. Boselli [c], B. Allaouchiche [d], B. Ba [a,b], A.T. Nguyen [a,b], J.B. Gordien [a,b], J.M. Bernadou [a,b], M.C. Saux [a,b], D. Breilh [a,b,*]

[a] *Pharmacy Haut-Lévêque Hospital, Magellan Avenue, 33604 Pessac, France*
[b] *Clinical Pharmacokinetic Department, Victor Segalen Bordeaux 2 University, Bordeaux, France*
[c] *Intensive Care Department, Hotel Dieu Hospital, Lyon, France*
[d] *Intensive Care Department, Edouard Herriot Hospital, Lyon, France*

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Abstract

The aim of this study was to develop a specific and sensitive high-performance liquid chromatographic (HPLC) assay for the determination of levofloxacin in human plasma, bronchoalveolar lavage and bone tissues. The sample extraction was based on a fully automated liquid–solid extraction with an OASIS cartridge. The method used ultraviolet detection set at a wavelength of 299 nm and a separation with a Supelcosil ABZ+ column. The assay has been found linear over the concentration range 0.25–25 μg/ml for levofloxacin in plasma, 1–6 μg/ml in bronchoalveolar lavage and 0.5–10 μg/g for bone tissues and it provided good validation data for accuracy and precision. The assay will be applied to determine the penetration of levofloxacin in human bronchoalveolar lavage (BAL) and bone tissues during pharmacokinetic steady state.

Keyword: Levofloxacin

1. Introduction

Levofloxacin is the active L-isomer isolated from the racemic ofloxacin. Its antibacterial action appears to be twice as active as the racemate ofloxacin in vitro [1]. Levofloxacin exerts antibacterial activity via antagonism of the interaction between bacterial DNA gyrase and DNA. The spectrum of activity includes Gram-positive aerobic organisms such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, Gram-negative bacteria (*Escherichia coli*, *Moraxella catharralis*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and intracellular pathogens responsible for atypical pneumonia [1,2]. These broad-range activity pathogens, based on in vitro data on minimum inhibitory concentration (MIC), time-kill kinetics and on post-antibiotic effect suggest that levofloxacin may be use in the treatment of community-acquired pneumonia (CAP), acute maxillary sinusitis and acute exacerbation of chronic bronchitis [2–5]. *S. pneumoniae*, *H. influenzae* and *Mycoplasma pneumoniae* are the most common bacterial pathogens in CAP. Because of the rising prevalence of resistance to penicillin in *S. pneumoniae* world-wide, especially multiply resistant strains, the choice of antimicrobial therapy must be carefully considered, including investigation of therapeutic alternatives [6]. Several studies present levofloxacin like a safe and effective treatment for CAP and at least equivalent to cephalosporins as ceftriaxone and cefuroxime [3–5]. The pharmacokinetic profile of levofloxacin supports once-daily administration and because of its high tissue distribution, levofloxacin may be also suitable in bone diseases. In order to assess human pharmacokinetics of levofloxacin in bronchoalveolar lavage (BAL) and bone tissues, a sensitive and specific assay is required to quantify levofloxacin concentrations in these different biological matrixes. Microbiological assays, microdialyse, high-performance liquid chromatography (HPLC) with fluorescence detection were described in the literature, especially for levofloxacin measurements in serum, urine and

* Corresponding author. Tel.: +33-5-57-65-68-12; fax: +33-5-57-65-68-23.
E-mail address: dominique.breilh@chu.bordeaux.fr (D. Breilh).

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different tissues but data on levofloxacin concentrations in cortical and cancellous bone are sparse, even non-existent in BAL [7–10]. The aim of this study is to propose a new assay to quantify levofloxacin in other sites which could be potential sites of infection. It will then provide data on the level of distribution of levofloxacin from serum to these sites.

This assay is based on HPLC with UV detection using an extraction method with a polymeric cartridge. This new method will be applied to determine plasma, BAL, cortical bone and cancellous bone concentrations of levofloxacin, in order to evaluate the ratio of concentrations (tissue/plasma).

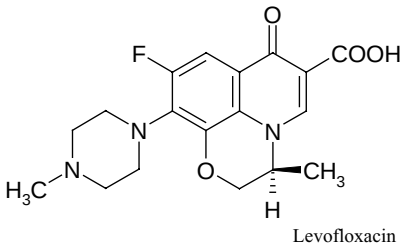
2. Experimental

2.1. Chemicals

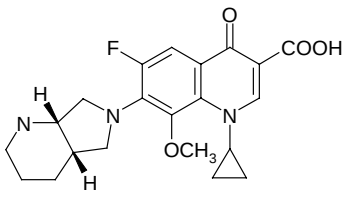
Levofloxacin and moxifloxacin (Fig. 1), the internal standard, were obtained, respectively, from Aventis (Paris, France) and Bayer Pharma (Puteaux, France). Concentrated orthophosphoric acid and triethylamine were from Prolabo (Nogent sur Marne, France). Acetonitrile, Chromar HPLC quality, was purchased from Carlo-Erba (Val de Reuil, France) and PBS (phosphate-buffered saline) Dubelco's was obtained from Gibco BRL (Cergy Pontoise, France).

2.2. Solid-phase extraction (SPE) and HPLC instrumentation

An automated SPE method on OASIS HLB extraction cartridges (Waters, MA, USA) was performed using an ASPEC Xli system (Gilson Medical Electronics France, Villiers le Bel, France). The Kontron HPLC system (Milan, Italy) consisted of a model 525 Pump, a model 360 autosampler and a model 332 UV detector and data acquisition was performed on a D450 software for signal treatment.



Levofloxacin



HCL

Moxifloxacin

Fig. 1. Molecular structures of levofloxacin and moxifloxacin.

2.3. Sample extraction procedure

An automatic sample processor was used for the extraction (ASPEC Xli, Gilson) as described in a previous manuscript about moxifloxacin [11].

2.4. Chromatography

The mobile phase consisted of water containing 0.4% of triethylamine, adjusted to pH 3 with concentrated orthophosphoric acid and mixed with acetonitrile (83:17, v/v). It was filtered through a 0.45 μm filter from Millipore (Saint Quentin en Yveline, France) and the flow rate was set at 1.2 ml/min. The elution conditions were isocratic. The analytical column was a Supelcosil ABZ+ (5 μm, 150 mm × 4.6 mm) from Supelco (Saint Quentin Fallavier, France). The sample injection volume was 20 μl. UV absorbance detection at 299 nm was carried out and the range of the detector was set at 0.050 AUFS (absorbance unit full scale) for plasma and 0.020 AUFS for BAL and bone tissues. The chromatographic run time was 12 min.

2.5. Preparation of standards and quality controls

2.5.1. Plasma calibration and quality controls

A working stock solution of 25 μg/ml of levofloxacin was prepared by appropriate dilution into distilled water. Then, it was diluted into free plasma to obtain a concentration range from 0.25 to 25 μg/ml of levofloxacin for calibration. Quality control concentrations represented 5, 9, and 18 μg/ml of levofloxacin in plasma. These three concentrations were different from those used for calibration.

2.5.2. Bronchoalveolar lavage calibration and quality controls

The working stock solution, the same as plasma, was diluted into free BAL to obtain a concentration range from 1 to 6 μg/ml of levofloxacin for calibration. Quality control concentrations representing 0.50, 3 and 5 μg/ml of levofloxacin were prepared in drug-free BAL.

2.5.3. Bone tissue calibration and quality controls

Bone tissue samples were first crushed into small pieces with an IKA-WERK grinder or pulverised with a mortar and a pestle, respectively, for cortical and cancellous bone tissue. Several cortical bone fragments of 200 mg were weighed precisely. A precise volume of a working stock solution in PBS is added to each bone samples to obtain a calibration concentration range from 0.50 to 10 μg/g. Samples were incubated for 15 min at 20 °C. We added the internal standard and 4 ml of PBS to each tube. The tissue suspension obtained was homogenised by shaking and stored for 24 h at 4 °C to ensure complete extraction of levofloxacin. After centrifugation (3000 × *g*, 10 min), the supernatant is ultrafiltered (11,000 × *g*, 10 min, 4 °C) and injected into the HLPC apparatus. Quality control concentrations representing 0.50,

5 and 9 μg/g of levofloxacin were prepared with the same procedure.

2.6. Sample treatments

2.6.1. Plasma samples

Plasma samples, received in a BD Vacutainer system™, were stored as soon as possible at −80 °C. After being thawed, they were extracted by the automated solid-phase process.

2.6.2. Bronchoalveolar lavage samples

BAL samples received were stored as soon as possible at −80 °C. Prior treatment of BAL consisted in reducing viscosity of samples. 500 μl of samples were mixed with 500 μl of Digest-Eur reagent (Eurobio, 91953 Les Ulis Cedex B, France). After 15 min of incubation at 20 °C, samples were centrifuged at 700 × *g* during 15 min. Then, the supernatants were collected and extracted exactly like plasma samples.

2.6.3. Bone tissue samples

The treatment of human bone tissue samples was exactly the same as described for bone tissue calibration and quality controls.

2.6.4. Blood contamination

The amount of levofloxacin in bronchoalveolar and bone samples due to blood contamination was measured by haemoglobin dosage on the supernatant obtained, using a spectrophotometer. This amount was calculated from the formula [12]:

$$\text{blood contamination (\%)} = \frac{\text{Hb in supernatant}}{\text{Hb in blood}} \times K \times (100 - \text{Ht})$$

where Hb is haemoglobin (g%), *K* the dilution factor and Ht blood haematocrit (%). In order to obtain the dilution correction factor, volume of water displaced by 1 g of bone was measured. A value of 0.66 ml was used for *K*.

2.7. Calibration and calculation procedures

Daily calibration curves were constructed using the ratios of the observed peak areas of levofloxacin and the internal standard. Unknown concentrations were computed from the unweighted linear regression equation of the peak area ratio against concentration for the calibration curve.

2.8. Accuracy, precision and recovery

The intra-day accuracy and precision of the method were estimated from the back-calculated concentrations from quality controls. The overall mean precision was defined by the relative standard deviation (R.S.D.) with relative errors from six standards analysed on the same day. Inter-day variability was estimated from the sixplicate analysis of six samples on 6 days.

2.9. Specificity and selectivity

Specificity was assessed in the presence of enoxacin, ciprofloxacin, ceftazidime, cefsulodine, cefepime, itraconazole, amphotericin B, fosfomycin, ofloxacin, cefixime, tazobactam, piperacillin, amikacin, rifampicin, vancomycin, norfloxacin, penicillin G, erythromycin, cloxacillin, amoxicillin, clavulanic acid, fusidic acid, imipenem, tobramycin, gentamicin, netilmicin, and cefazolin.

2.10. Limit of detection and limit of quantitation

The limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) is the minimum injected amount that gave precise measurements (accuracy and R.S.D. both less than 15%). If the required precision of the method at the LOQ has been specified, the Eurachem approach can be used [11]. A number of samples with decreasing amounts of the analytes are injected six times. The calculated R.S.D. of precision is plotted against the analyte amount. The minimum quantifiable concentration (MCQ) measuring the lowest concentration at which there is satisfactory a priori measures of accuracy and precision using the appropriate biological matrix was calculated.

3. Results

3.1. Chromatographic characteristics

Fig. 2(a)–(h) shows chromatograms used for validation analytical assay for blank and quality control spiked sample. Fig. 3(a)–(d) shows patient's chromatograms. The mean retention time of levofloxacin was 3.10 min. In BAL, an additional peak between levofloxacin and moxifloxacin was observed and corresponded to the reagent Digest-Eur used for decreasing viscosity of samples.

3.2. Calibration curve

The analysis of levofloxacin in plasma exhibited excellent linearity through the coefficient of correlation r^2 (0.9996, 0.9931, 0.9967 and 0.9971, respectively, for plasma, cortical bone, cancellous bone and BAL). Regression intercepts for the calibration curves were not statistically significant compared to zero. Daily calibration curves were used for calibration and calculation purposes. The results indicated that the assay of levofloxacin had acceptable precision and accuracy (<10% for plasma, cortical bone, cancellous bone and BAL).

3.3. Accuracy, precision and recovery

The overall mean precision as defined by the R.S.D., ranged from 1.03 to 1.13, 1.04 to 5.61, 0.97 to 2.67, and 3.23

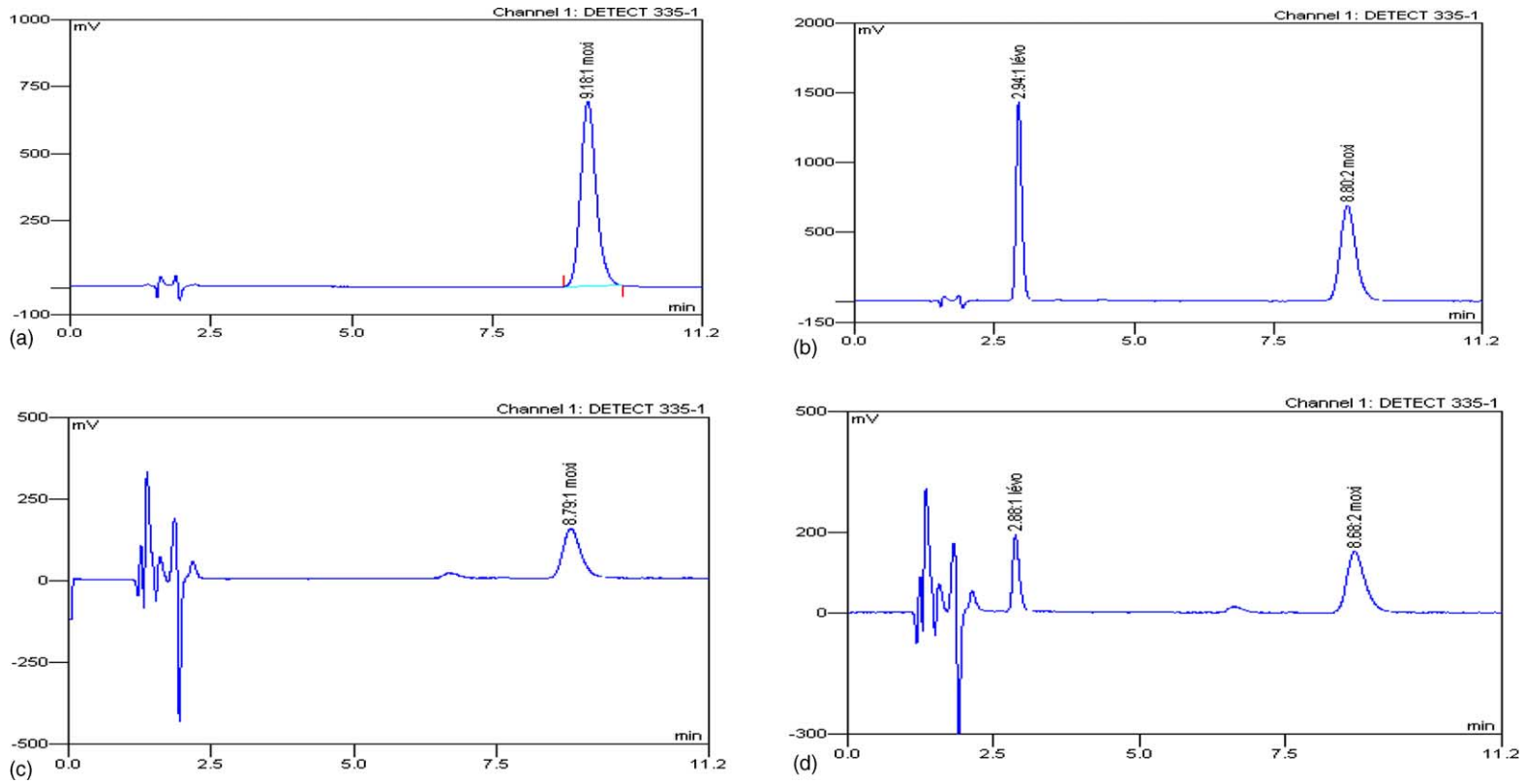


Fig. 2. Chromatograms used for validation analytical assay: chromatograms of blank human plasma sample (a), human plasma sample spiked with 9 μg/ml of levofloxacin and 12.5 μg/ml of moxifloxacin (b), blank human cortical bone (c), human cortical bone spiked with 9 μg/g of levofloxacin and 20 μg/g of moxifloxacin (d), blank human cancellous bone (e), human cancellous bone spiked with 9 μg/g of levofloxacin and 20 μg/g of moxifloxacin (f), blank human bronchoalveolar lavage (g), and human bronchoalveolar lavage spiked with 5 μg/ml of levofloxacin and 25 μg/ml of moxifloxacin (h).

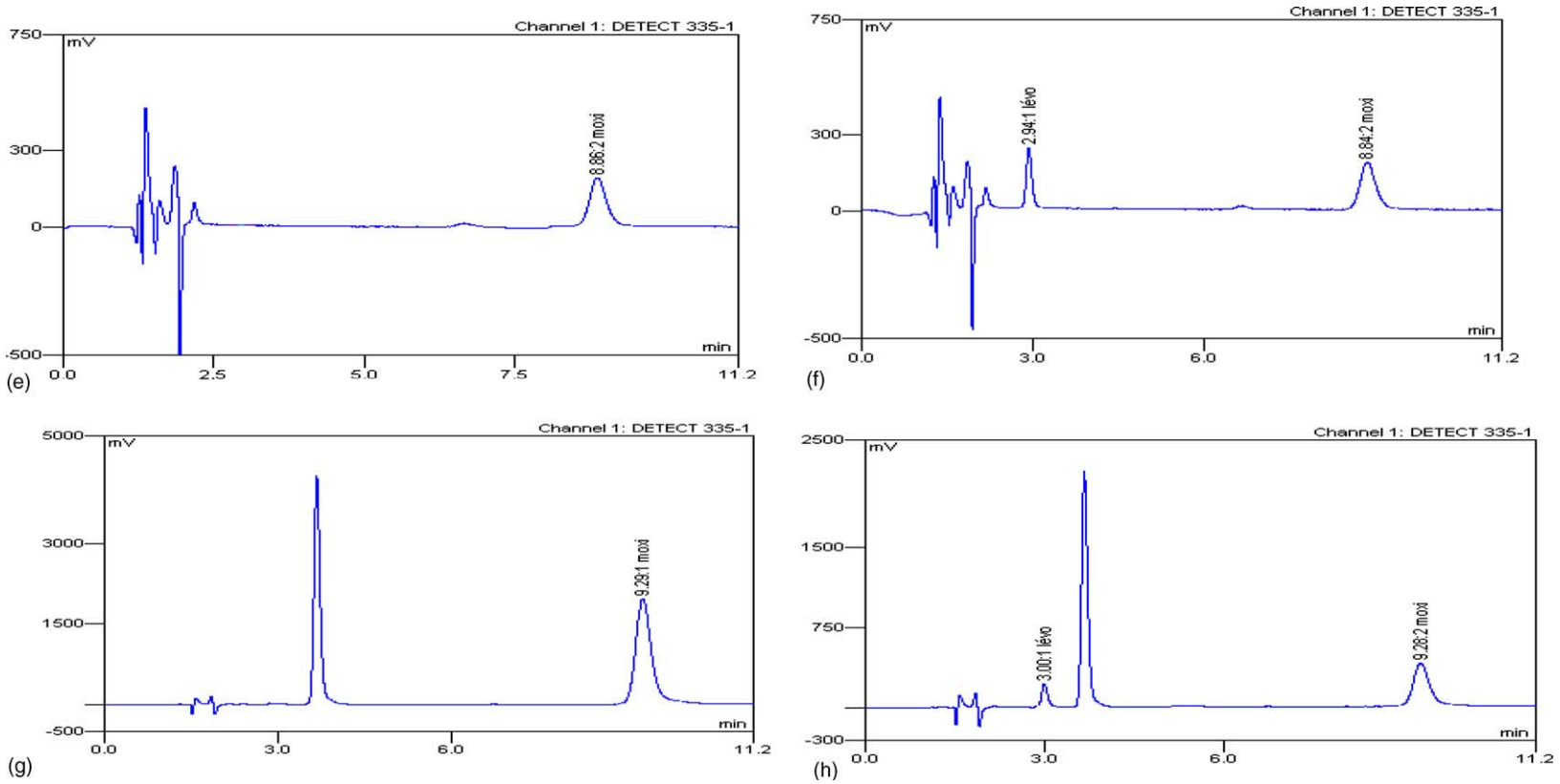


Fig. 2. (*Continued*).

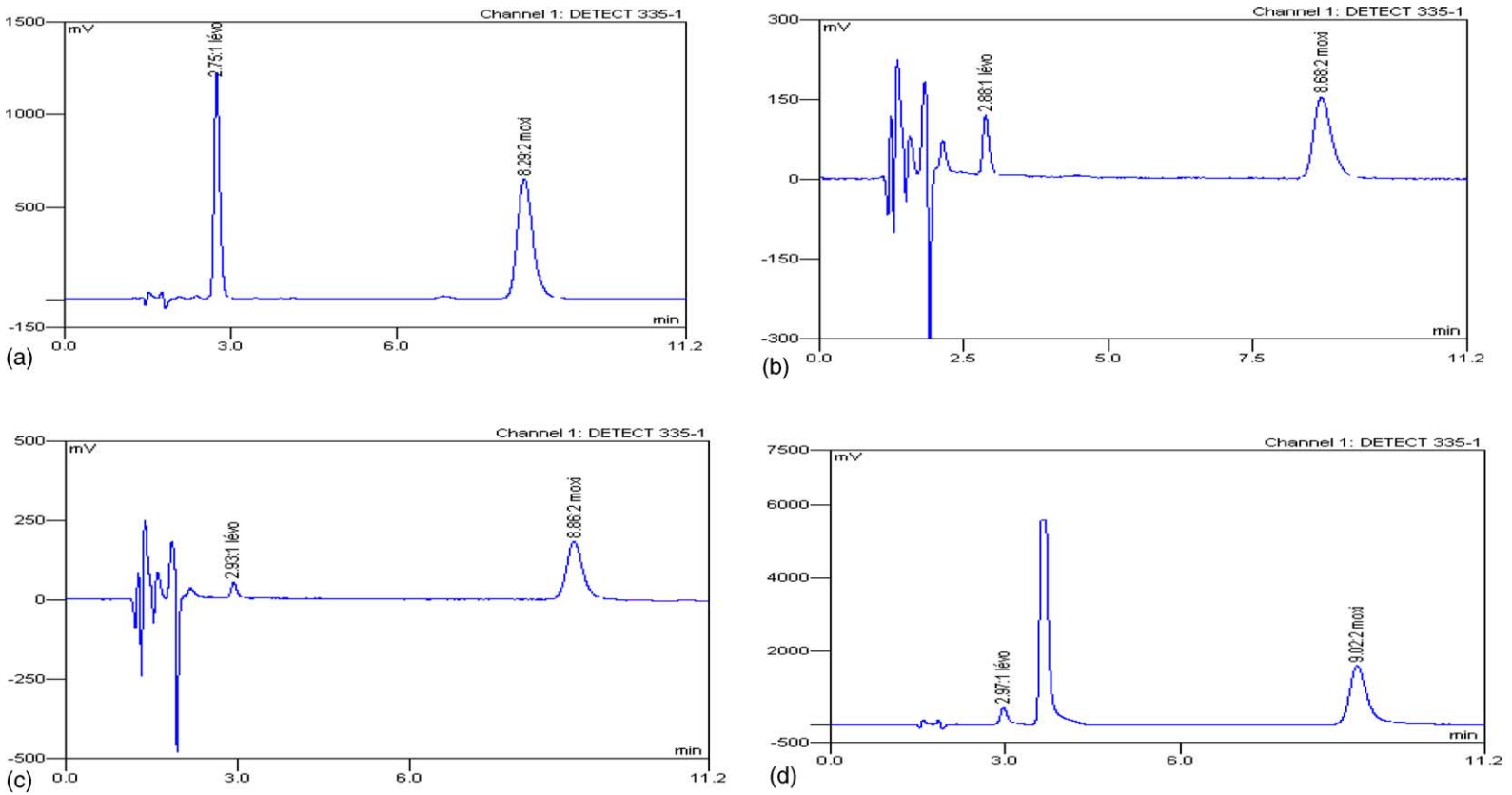


Fig. 3. Chromatograms used for determination of levofloxacin in patient's plasma and tissues: chromatograms of patient plasma sample (a), patient cortical bone (b), patient cancellous bone (c), and patient bronchoalveolar lavage (d).

Table 1
Intra-day and inter-day accuracy and precision from the determination of levofloxacin

Theoretical concentration	Intra-day				Inter-day			
	Concentration found (mean ± S.D.)	Accuracy (%)	R.S.D. (%)	*n*	Concentration found (mean ± S.D.)	Accuracy (%)	R.S.D. (%)	*n*
Plasma (μg/ml)								
5	5.10 ± 0.058	101.90	1.13	6	5.08 ± 0.075	101.70	1.48	36
9	9.18 ± 0.096	102.04	1.05	6	9.39 ± 0.215	104.39	2.29	36
18	18.28 ± 0.187	101.53	1.03	6	18.53 ± 0.245	102.96	1.32	36
BAL (μg/ml)								
0.5	0.51 ± 0.029	102.15	5.61	6	0.49 ± 0.025	97.33	5.17	36
3	2.96 ± 0.090	98.69	3.04	6	3.07 ± 0.100	102.22	3.28	36
5	4.93 ± 0.051	98.68	1.04	6	5.12 ± 0.200	102.40	3.92	36
Cortical bone (μg/g)								
0.5	0.53 ± 0.014	106.72	2.67	6	0.525 ± 0.025	105.21	4.79	36
5	4.9 ± 0.092	98.07	1.86	6	4.98 ± 0.22	100.01	4.46	36
9	8.78 ± 0.085	97.54	0.97	6	8.87 ± 0.25	98.53	2.81	36
Cancellous bone (μg/g)								
0.5	0.49 ± 0.028	98.00	5.77	6	0.48 ± 0.019	96.75	4.08	36
5	4.75 ± 0.311	95.00	6.55	6	4.76 ± 0.212	95.20	4.46	36
9	9.42 ± 0.304	104.61	3.23	6	9 ± 0.343	100.06	3.81	36

to 6.55%, respectively, for plasma, BAL, cortical bone and cancellous bone from six series standards analysed within the same day (Table 1). Inter-day variability, as estimated from the sixplicate analysis of three samples on six separate days (Table 1), was low, with R.S.D. ranging from 1.32 to 2.29, 3.28 to 5.17, 2.81 to 4.79, and 3.81 to 4.46%, respectively, for plasma, BAL, cortical bone and cancellous bone. The extraction mean recovery of levofloxacin from three quality control samples on six separate days were 90.80, 92.93, 82.45 and 89.85%, respectively, for plasma, BAL, cortical bone and cancellous bone.

3.4. Specificity and selectivity

Blank plasma showed no interfering endogenous substances in the analysis of levofloxacin. Potentially co-administered drugs tested had retention times that were different from levofloxacin or were not detected.

3.5. Limit of detection, limit of quantification and minimal quantifiable concentration

The LOD was 0.050, 0.10 and 0.20 μg/g, respectively, for plasma, BAL and bone tissues. The LOQ was 0.20, 0.4 and 0.5 μg/g, respectively, for plasma, BAL and bone tissues. These values were defined as the minimal quantifiable concentrations.

4. Discussion and conclusion

We developed a specific HPLC assay to determine levofloxacin concentrations in plasma, BAL and bone tissue. The chromatographic parameters were UV detection ($\lambda = 299$ nm), LOD and LOQ were, respectively, 0.050 and 0.20 μg/ml for plasma samples, 0.10 and 0.40 μg/ml for BAL samples, and 0.20 and 0.50 μg/g for bone samples. Calibration range was based on human plasma levofloxacin concentrations in pharmacokinetic steady-state when levofloxacin was administered at 500 mg once or twice a day intravenously. These concentrations were different from previous described because all the studies were performed in single dose of levofloxacin [13,14]. Previous assays were described in the literature and concerned the measurement of levofloxacin using fluorescence detection but, for bone tissue and BAL, only few of the published methods give exact validation data [7–10].

Böttcher et al. [7] described an HPLC assay for levofloxacin and its metabolites desmethyl-levofloxacin and levofloxacin-*N*-oxide in serum, bile, soft tissue and bone. The LOD in serum and tissue was 0.010 μg/ml and the assay has been found linear over a concentration range 0.1–40 μg/ml in aqueous solutions. Precision and accuracy of the bioanalytical results were below 10% of theory in the whole working range of the method. Böttcher et al.'s aim was to compare the HPLC method with a microbiological assay and also apply it to a pharmacokinetic study in bile. Their lower concentration was 0.1 μg/ml and determined in aqueous solutions. In a second assay developed by Neckel et al. [8] using the same detection LOD and LOQ were, respectively, 0.0125 and 0.02 μg/ml in plasma samples. Neckel et al.'s aim was to correlate the assay to microdialyse and apply to clinical drug development studies. The comparison with these two assays showed that our results were similar for precision and accuracy but LOD and LOQ were higher in plasma and bone tissue. These results were justifiable by the fact that the matrixes of quantitation were different. Our objective was to propose a sensitive assay to

detect and quantify levofloxacin in a range of concentration adapted to previous pharmacokinetic results in plasma and bone tissue. As the metabolites appeared to be present in negligible amounts, we did not determine them. To take care of the different matrix effects, our validation was made in the different biological matrixes: human plasma, human BAL and human bone tissue. For BAL, we found no other assay focusing on the penetration of levofloxacin in BAL and consequently no other validation data in this matrix.

One important point to underline was the sample preparation presented in our assay. The quality of elution and chromatographic results depended on the quality of sample preparation. The plasma sample preparation used in the two other assays was a plasma protein precipitation with methanol and a direct injection of the supernatant after centrifugation. This procedure was very simple but had some inconveniences. First, the important proportion of methanol (80 and 40%, respectively) gave rise to some alterations of chromatographic parameters and showed interfering peaks. Secondly, the impurities contained in the supernatant could damage the column. The use of a pre-column is necessary to protect the column [8].We used a liquid–solid fully automated extraction procedure for the preparation of samples. On the one hand, it permitted to increase the selectivity of the assay. On the other hand, the injection of the internal standard in samples, the dilution of samples and the elution of analytes were monitored by an automated process which provided good data for reproducibility.

Finally, we took care of the quality of solid phase for liquid–solid extraction and of the chromatographic column. We chose the OASIS HLB cartridge for its large specific area ($800\,m^2/g$) which provided a good recovery of drugs [15]. The extraction recovery found in our assay for plasma is 90.80%, which is similar to recoveries obtained in Böttcher et al.'s assay. The optimal hydrophilic–lipophilic balance of the polymeric sorbent suits to amphoteric analytes like levofloxacin. The use of the Supelcosil ABZ + for the chromatographic column provided benefits of silica-based reversed-phase HPLC columns. The silanol deactivation, which is due to the incorporation of a polar group in the Supelcosil ABZ+ phase, gave good peak shape comparing to deactivated C18 reversed-phase columns [16].

In conclusion, the HPLC assay presented here allowed fast analyse of levofloxacin in human plasma, BAL and bone tissues in an isocratic mode within 12 min. It was applied to pharmacokinetic analysis of levofloxacin penetration in human infectious sites such as BAL or bone tissues.

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