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

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Abstract

The aim of this study was to develop a specific and sensitive high-performance liquid chromatographic assay for the determination of linezolid in human plasma, and bronchoalveolar lavage. The sample extraction was based on a fully automated solid-phase extraction with an OASIS HLB cartridge. The method used ultraviolet detection set at a wavelength of 254 nm and a separation with a Zorbax Eclipse XDB C8 column. The assay has been found linear over the concentration range 0.02–30 μg/ml and 0.04–30 μg/ml for linezolid, respectively, in plasma and bronchoalveolar lavage. It provided good validation data for accuracy and precision (CV <4.64% and 5.08%, accuracy in the range 96.93–102.67% and 97.33–105.67%, respectively, for intra- and inter-day). The assay will be applied to determine the penetration of linezolid in human bronchoalveolar lavage during pharmacokinetic steady-state.

Keywords: Linezolid; Oxazolidinones; Pharmacokinetics

1. Introduction

Antimicrobial resistance has become a significant nosocomial problem and contributed to the increasing importance of community-acquired infections. This phenomenon required the development and employment of new antimicrobial agents effective against resistant strains. Linezolid is an antimicrobial agent of the oxazolidinone class, which inhibits the initiation of bacterial protein synthesis [1]. The mechanism of inhibition is different from other current available antibiotics so that cross-resistance phenomenon has not been reported to date. Linezolid is effective against Gram-positive organisms such as methicillin-resistant staphylococci, penicillin-resistant pneumococci and vancomycin-resistant *Enterococcus* species. This spectrum of activity explains why intravenous linezolid appeared as effective as intravenous vancomycin 1 g in the treatment of acquired pneumonia when administered twice daily [1]. Data suggest that C_{max}/MIC ratios of linezolid and percentages of time linezolid remains above the MIC in plasma determine the efficacy of linezolid in the treatment of human Gram-positive infections [2]. The pharmacokinetic parameters are highly variable between individuals so that determination of linezolid plasma concentrations is necessary to ensure treatment effectiveness [2]. Moreover, clinical efficacy in respiratory infections due to Gram-positive pathogens depends on the degree of intrapulmonary penetration of linezolid evaluated by the bronchoalveolar lavage (BAL)/plasma concentrations ratio. As linezolid is approved for the treatment of nosocomial and

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community-acquired pneumonia, the in vivo penetration of linezolid into respiratory tract must be studied [3]. However, currently pharmacokinetic data about tissue distribution of linezolid in humans is not entirely available. Two studies determined the mean concentrations of linezolid in plasma, alveolar macrophages and epithelial lining fluid (ELF) in healthy volunteers between 2 and 8 h post dose interval in order to compare them with the MIC and to evaluate the intrapulmonary penetration of linezolid [3,4].

The present paper reports the development and validation of a high-liquid chromatographic (HPLC) method coupled with ultraviolet (UV) detection for determination of linezolid in human plasma and bronchoalveolar lavage (BAL). This method will be applied to determine the intrapulmonary pharmacokinetic parameters of intravenously administered linezolid in intensive care unit patients.

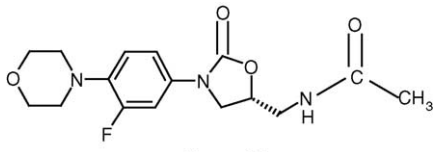
2. Experimental

2.1. Chemicals

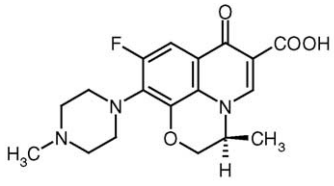
Linezolid and levofloxacin, the internal standard, were obtained, respectively, from Pharmacia & Upjohn (Kalamazoo, USA) and Aventis (Paris, France), (Fig. 1). Concentrated orthophosphoric acid and triethylamine were from Prolabo (Nogent sur Marne, France). Acetonitrile, Chromar HPLC quality, was purchased from Carlo-Erba (Val de Reuil, France).

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 Agilent 1100 series (Weldbronn, Germany) consisted of a model G1311A quaternary pump, a model G1365B UV detector and an Agilent Chemstation for LC systems.



Linezolid



Levofloxacin

Fig. 1. Molecular structures of linezolid and levofloxacin.

2.3. Sample extraction procedure

An automatic sample processor was used for the extraction (ASPEC Xli, Gilson). During the first step, the automate conditioned the OASIS HLB cartridge with 1 ml of methanol and 1 ml of distilled water. Internal standard prepared in water (500 μg/ml) was spiked directly in plasma and BAL samples. Then, 500 μl of sample were loaded. After a washing step with water containing 5% of methanol, the compounds were eluted with 0.6 ml of methanol twice. The solvent was evaporated at 65 °C under a stream of nitrogen. The residue was reconstituted in 100 μl of the mobile phase A and 20 μl were injected into the chromatographic system.

2.4. Chromatography

The mobile phase consisted of a gradient of water containing 0.4% of triethylamine (TEA), adjusted to pH 4 with concentrated orthophosphoric acid (phase A) and mixed with acetonitrile (phase B). It was filtered through a 0.45-μm filter from Millipore (Saint Quentin en Yvelines, France) and the flow rate was set at 0.4 ml/min. The analytical column was a Zorbax Eclipse XDB-C8 (3.0 mm × 100 mm, 3.5 μm) from Agilent Technologies (Melrose, USA). The gradient began with 90% and 10%, phase A and B, respectively, from 0 to 8 min, became 70% and 30%, phase A and B, respectively, from 8 to 14 min and came back to original conditions at 14 min. The sample injection volume was 20 μl. Ultraviolet absorbance detection was set at 254 nm and the chromatographic run time was 15 min.

2.5. Preparation of standards and quality control (QC) samples

2.5.1. Plasma calibration and quality control (QC) samples

A stock solution of 1000 μg/ml of linezolid was prepared by dissolution of linezolid powder in distilled water. A working solution of 50 μg/ml was prepared by diluting the stock solution into free human plasma. The latter was used to prepare a concentration range from 0.02 to 30 μg/ml of linezolid for calibration in plasma. Quality control (QC) concentrations were different from those used for calibration and represented 0.05, 3, 15, and 25 μg/ml of linezolid in plasma.

2.5.2. Bronchoalveolar lavage calibration and QC samples

The same aqueous stock solution of 1000 μg/ml was diluted into free BAL to obtain a working solution of 50 μg/ml. Then, it was diluted in free BAL to obtain the concentration range from 0.04 to 30 μg/ml of linezolid for calibration. Quality control concentrations representing 1, 3, 15 and 25 μg/ml of linezolid were prepared in drug-free BAL.

2.6. Sample treatment

2.6.1. Plasma samples

Plasma samples, received in a BD Vacutainer system™ (Becton-Dickinson, Le Pont-de-Claix, France), were stored at −80 °C until analyzed. After being thawed, 50 μl of the internal standard aqueous solution (500 μg/ml) were added to each patient's sample, calibration standard and QC sample. Then, they were extracted by the automated solid-phase process.

2.6.2. Bronchoalveolar lavage samples

The BAL samples came from "mini BAL" procedure, as previously described [5]. The average duration of the procedure was 5 min. The volume of the aspirated BAL was measured and recorded and was immediately spun at 700 × *g* during 15 min in a refrigerated centrifuge. The supernatant was separated and frozen at −80 °C until assay. When the analysis started, a prior treatment of BAL consisted in reducing the viscosity of samples. Then, 500 μl of samples were mixed with 500 μl of Digest-Eur reagent (Eurobio, Les Ulis, France). After 15 min of incubation at 20 °C, samples were centrifuged at 700 × *g* during 15 min. Then, the supernatant was collected and extracted exactly like plasma samples.

2.6.3. Blood contamination

The amount of linezolid in BAL samples due to blood contamination was measured by haemoglobin dosage on the supernatant obtained, using a spectrophotometer. This amount was calculated from the Roncoroni's formula as followed [6]:

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

where Hb is haemoglobin (g%) and Ht is blood haematocrit (%).

2.7. Assay validation

2.7.1. Calibration and calculation procedures

Linearity was evaluated using freshly prepared spiked matrix samples in a concentration range from 0.02 to 30 μg/ml for plasma and 0.04–30 μg/ml for BAL. Each calibration curve consisted of a blank sample, a zero sample and six calibrator concentrations. Daily calibration curves were constructed using the ratios of the observed peak areas of linezolid to the internal standard, to describe the relationship between detector response and concentration using reciprocals of squared concentrations as weighting factor. Unknown concentrations were computed from the linear regression equation of the peak area ratio against concentration for the calibration curve.

2.7.2. Accuracy, precision and recovery

The intra-day accuracy and precision of the method were estimated from the back-calculated concentrations from QC samples. Accuracy was determined by the mean of the measured QC concentration relative to the theoretical value and was reported in percentage. The overall mean precision was denoted by the coefficient of variation (CV) as defined by the FDA guidelines [7]. Precision was defined as the ratio of the standard deviation of the observed QC concentration to the mean observed QC concentration. Each QC sample was injected six times on four separate days, thus determining inter-day variability. Recovery of linezolid using the automated extraction procedure was evaluated by comparing the mean peak areas of the different QC samples post-extracted with those prepared by adding compound to post-extracted plasma and BAL blanks at corresponding concentrations. The variability of recovery results was determined.

2.7.3. Selectivity

Analysis of blank plasma samples were carried out from six different healthy human sources. Each blank sample was investigated for interference of endogenous matrix components. Potential interfering medication was assayed. Selectivity was also ensured at the lower limit of quantification of linezolid.

Selectivity was assessed in the presence of rifampicin (2.5 μg/ml), ciprofloxacin (1.5 μg/ml), fosfomycin (1.5 μg/ml), ofloxacin (1.5 μg/ml), vancomycin (10 μg/ml), fusidic acid (1 μg/ml), penicillin G (1 μg/ml), cloxacillin (8 μg/ml), ceftazidime (20 μg/ml), cefepime (10 μg/ml), cefixime (5 μg/ml), cefazolin (16 μg/ml), cefsulodine (10 μg/ml), amoxicillin (11 μg/ml), clavulanic acid (1.5 μg/ml), aztreonam (2 μg/ml), norfloxacin (1.5 μg/ml), enoxacin (4 μg/ml), tazobactam (0.5 μg/ml), piperacillin (1 μg/ml), amikacin (1 μg/ml), gentamicin (3 μg/ml), tobramycin (5 μg/ml), netilmicin (3 μg/ml), erythromycin (5.6 μg/ml), imipenem (2 μg/ml), itraconazole (0.5 μg/ml) and amphotericin B (1 μg/ml).

2.7.4. Limit of detection and lower limit of quantification

The limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. The lower limit of quantification (LLOQ) was determined by injecting six times a number of spiked samples with decreasing concentration of the analyte. For each matrix, LLOQ was regarded as the lowest concentration with an accuracy and precision of 20% that could be analyzed [7].

2.7.5. Linezolid stability

Stability of linezolid was investigated both in human plasma and BAL. The stability of the analyte was evaluated during sample collection, after long-term and short-term storage, and through several freeze and thaw cycles [7]. Low and high QC concentrations were prepared in triplicate from a freshly made stock solution in both matrixes and were investigated under different conditions. The aliquots were stored at −20 °C for 24 h and were thawed at room temperature. Then, they were assayed after two new freeze-thaw cycles under the same conditions. Linezolid stability was determined by

analysis of thawed low and high QC concentrations ($n = 3$) after storage at room temperature for 24 h. Drug stability was also assessed from extracted QC concentrations ($n = 3$) stored in the sample tray of the autosampler for 12 h. All data were compared with results obtained from freshly prepared and analyzed QC samples using the formula: [stability (%) = (stored QC concentration/freshly prepared QC concentration) × 100]. Linezolid stability was confirmed if less than 5% difference in concentration was observed.

The stability of stock solutions of linezolid and its internal standard were evaluated at room temperature for 8 h by comparing the instrument responses of these solutions with those of freshly prepared solutions.

3. Results

3.1. Chromatographic characteristics

Fig. 2(a–d) shows the chromatograms used for the validation of the analytical assay for blank and QC samples. Fig. 3(a and b) shows the patient chromatograms. The mean retention time of linezolid was 9.10 min.

3.2. Calibration curve

The analysis of linezolid in plasma exhibited excellent linearity through the coefficient of correlation r^2 (0.09992 and 0.9995, respectively, for plasma and BAL). Regression intercepts for the calibration curves were not statistically significant compared to zero.

3.3. Accuracy, precision and recovery

The overall mean precision as defined by the CV, ranged from 1.36 to 4.64 and 1.07 to 4.31, respectively, for plasma and BAL, from each QC concentration analyzed in six replicate within the same day (Table 1). Inter-day variability, as estimated from four samples six-fold injected on four separate days (Table 1), was low, with CV ranging from 3.86 to 4.76 and 2.38 to 5.08, respectively, for plasma and BAL. Accuracy and CV data met the acceptance criteria for validation specified within the FDA guidelines [7]. The extraction mean recoveries of linezolid from QC samples on four separate days ranged from 99.64% to 104.45% with an average of 102% (1.74%) for plasma and from 96.75% to 100.14% with an average of 98% (1.2%) for BAL.

3.4. Selectivity

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

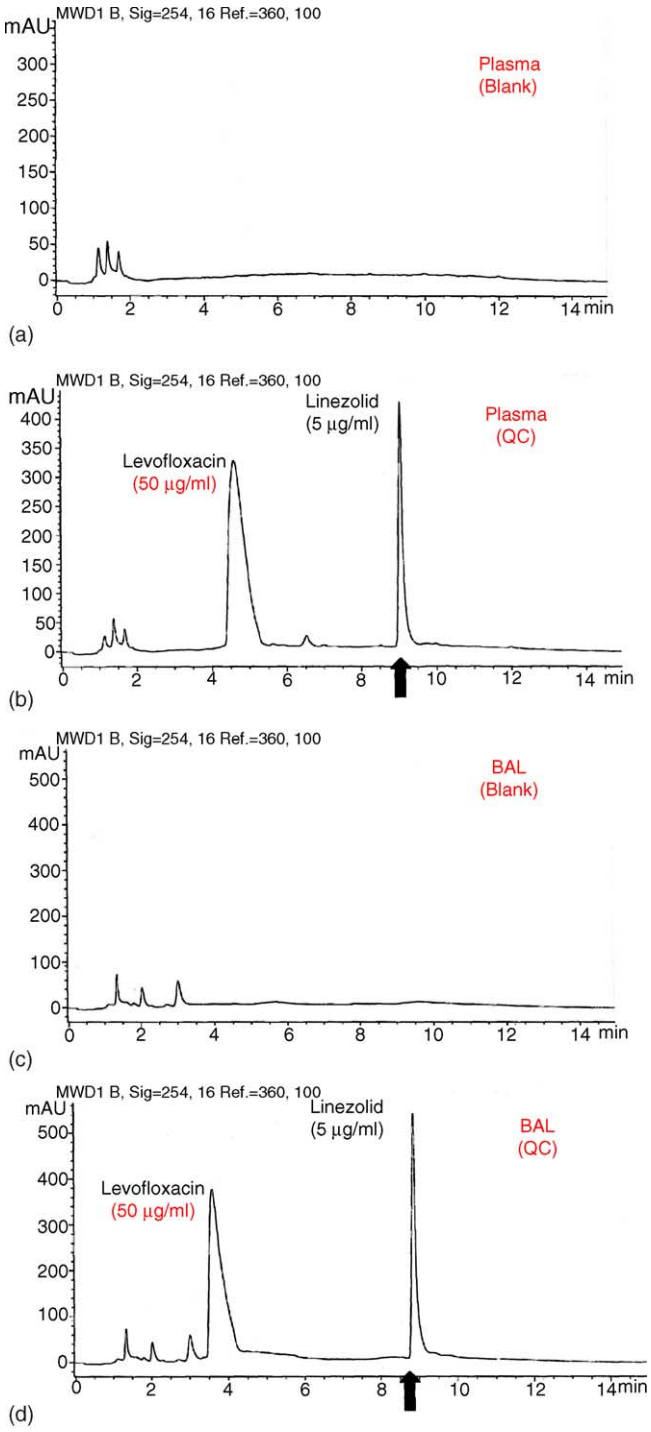


Fig. 2. Chromatograms used for the validation of the analytical assay: chromatograms of blank human plasma sample (a), human plasma sample spiked with 5 μg/ml of linezolid (QC) and 50 μg/ml of levofloxacin (internal standard) (b), blank human bronchoalveolar lavage (c), and human bronchoalveolar lavage spiked with 5 μg/ml of linezolid (QC) and 50 μg/ml of levofloxacin (d). Arrows indicate the signal of linezolid (retention time of 9.10 min).

Table 1
Intra-day and inter-day accuracy and precision (expressed as coefficient of variation, CV) for linezolid assay

Theoretical concentration	Intra-day				Inter-day			
	Observed concentration (mean ± S.D.)	Accuracy (%)	CV (%)	*n*	Observed concentration (mean ± S.D.)	Accuracy (%)	CV (%)	*n*
Plasma (μg/ml)								
0.05	0.05 ± 0.0023	100.67	4.64	6	0.049 ± 0.0019	98	3.86	24
3	3.04 ± 0.034	101.33	2.76	6	2.95 ± 0.12	98.33	4.07	24
15	15.40 ± 0.210	102.67	1.36	6	15.85 ± 0.755	105.67	4.76	24
25	24.42 ± 0.350	97.68	1.43	6	25.35 ± 1.15	101.40	4.54	24
BAL (μg/ml)								
1	1.003 ± 0.043	100.33	4.31	6	1.05 ± 0.025	105.00	2.38	24
3	3.09 ± 0.109	103.00	3.53	6	2.92 ± 0.105	97.33	3.59	24
15	14.54 ± 0.156	96.93	1.07	6	14.95 ± 0.688	99.67	4.60	24
25	25.60 ± 0.305	102.40	1.19	6	25.60 ± 1.30	102.40	5.08	24

3.5. Limit of detection and lower limit of quantification

The LOD was 0.01 and 0.02 μg/ml, respectively, for plasma and BAL. The LLOQ was 0.02 and 0.04 μg/ml, respectively, for plasma and BAL.

3.6. Linezolid stability

No trend towards degradation of linezolid was found in relation to freeze-thaw cycles, as average stability results ranged from 98.20% (1.6%) to 104.5% (3.6%) in plasma and from 97.3% (7%) to 102.5% (5.5%) in BAL, when stored QC samples were compared with freshly prepared QC samples. Storing QC samples at room temperature for 24 h had no influence on linezolid quantitation. Mean linezolid concentrations ranged from 96.4% (6.4%) to 99.4% (5.3%) and from 97.6% (2.5%) to 99.8% (1.2%), respectively, for plasma and BAL. Extracted QC samples stored in the sample tray of the autosampler for 12 h exhibited less than 5% difference in concentration comparing to samples measured immediately after preparation: average stability results ranged from 97.3% (4.7%) to 102.7% (6.5%) and from 96.8% (5.3%) to 103.6% (3.9%), respectively, in plasma and BAL. Stock solution stability was established as no difference in instrument response was found between solutions kept at room temperature for 12 h and freshly prepared solutions. Thus, linezolid was considered to be stable in plasma and BAL under the conditions encountered in the present assay.

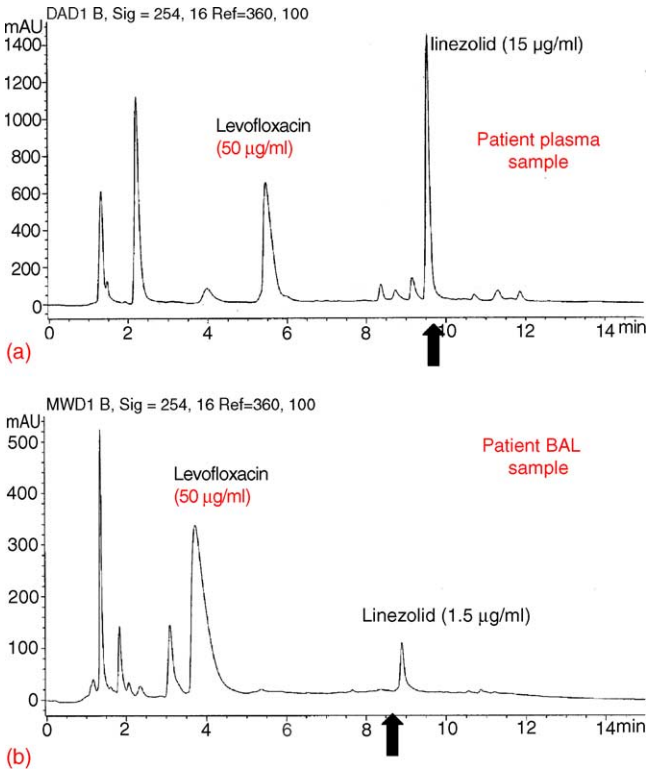


Fig. 3. Chromatograms used for the determination of linezolid in patient plasma and BAL: chromatograms of patient plasma sample (15 μg/ml of linezolid) (a) and patient bronchoalveolar lavage sample (1.5 μg/ml of linezolid) (b). Arrows indicate the signal of linezolid (mean retention time of 9.3 min).

4. Discussion and conclusion

A sensitive and fast HPLC method for linezolid in human plasma and BAL was developed using an automated solid-phase extraction (SPE) and UV detection (λ = 254 nm). Previous assays were described in the literature about the analysis of linezolid in different biological matrixes. Two articles described the determination of linezolid using mass spectrometry [3,8]. Phillips et al. described the development of an assay based on liquid chromatography-tandem mass spectrometry and a SPE method [8]. Conte et al. measured linezolid in BAL by a combined HPLC–mass spectrometry technique while plasma concentrations were determined using HPLC and UV detection [3]. Our objective was to study the intrapulmonary penetration of linezolid in intensive care patients by comparing the observed plasma concentrations with those found in BAL. Therefore, we thought necessary to use the same extraction and detection of linezolid in BAL than in plasma.

The present analytical method was adapted from a previous report about levofloxacin, which described a HPLC assay with UV detection [9]. We used the same mobile phase and chose levofloxacin as internal standard. Mass spectrometry is characterized by a high degree of specificity and sensitivity so that this detection mode permits low quantitation of drugs in biological matrixes. However, sensitivity of UV detection was thought sufficient to quantify the lowest clinically relevant concentrations of linezolid observed in human plasma and BAL. Moreover, the use of mass spectrometry would have required changes of the mobile phase composition. Therefore, we chose HPLC coupled with UV detection for determination of linezolid both in human plasma and BAL.

Several analytical assays concerning the measurement of linezolid in plasma by HPLC used UV detection [2,3,10–16]. Some of them described a protein precipitation for sample clean-up [13–16]. The advantages of this sample preparation are a good extraction recovery with values close to 100% and a simple and less-time consuming procedure. However, compared with a SPE method, this purification mode is characterized by its weak selectivity. Indeed, it allows the extraction of linezolid combined with the extraction of endogenous compounds and potentially co-administered drugs. These compounds may co-elute with linezolid, giving rise to alterations of the chromatographic baseline and peak interferences. Co-elution phenomenon may be overcome by more chromatographic separation between linezolid and interfering compounds. This may be provided by an adequate mobile phase preparation, which becomes sometimes tedious [2,16]. Our assay describes a simple and fast preparation of the mobile phase. The use of SPE enhanced the selectivity and sensitivity of our method compared with the analytical assays, which used protein precipitation: our LOD and LLOQ in plasma were lower (respectively, 0.01 and 0.02 μg/ml).

Other assays using a SPE method had low values of LLOQ [2,3,10,11]. The objective of Peng et al. was different from ours since they developed a HPLC-UV assay for toxicological studies [11]. Analysis of linezolid was carried out in animal plasma. As our objective was to study the pharmacokinetic of linezolid in intensive care patients, we chose to work on human biological matrixes to take into account the matrix effect between human and animal.

Comparing to our assay, Conte, Stalker and Gee et al. used a higher percentage of organic solvent in the mobile phase (25% of methanol) while the retention times of linezolid and its internal standard, 7 and 10 min, respectively, were higher [2,3,10]. Ehrlich et al. used an extraction procedure carried out automatically by a HPLC-integrated-extraction system. The use of specific ADS-RP C8 columns required a frequent wash and was cost-effective so that it did not suit for our clinical routine use and analysis of a large number of samples per day [12]. Our SPE method was based on OASIS HLB cartridges. The optimal hydrophilic–lipophilic balance of the polymeric sorbent provided a good recovery of the drug. However, we used a higher plasma volume (500 μl) comparing to other methods, which described plasma volumes of 100 μl [14].

In conclusion, our assay provided a simple and fast analysis of linezolid in human plasma and BAL within 15 min. The limit of detection was 0.01 and 0.02 μg/ml, respectively, for plasma and BAL. The lower limit of quantification was 0.02 and 0.04 μg/ml, respectively, for plasma and BAL. Calibration range was based on human plasma linezolid concentrations during the pharmacokinetic steady-state when linezolid was administered at 600 mg twice daily intravenously. The assay may be applied to pharmacokinetic analysis of linezolid penetration in human infectious sites such as BAL.

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