Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 796 (2003) 155–164
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development of a liquid chromatography method for the determination of linezolid and its application to in vitro and human microdialysis samples

Cornelia Buerger[a], Christian Joukhadar[b], Markus Muller[b], Charlotte Kloft[a,*]

[a] *Department Clinical Pharmacy, Institute of Pharmacy, Freie Universitaet Berlin, Kelchstrasse 31, D-12169 Berlin, Germany*
[b] *Department of Clinical Pharmacology, Vienna University School of Medicine, Vienna, Austria*

Received 20 February 2003; received in revised form 18 July 2003; accepted 14 August 2003

Abstract

Linezolid is a new, promising antibacterial agent to treat severe infections. A rapid HPLC assay using UV detection for the determination in microdialysate and human plasma was developed. After sample preparation, using acetonitrile for plasma and water for microdialysate, 20 μl was injected and separated on a RP-18 column. Overall, the assay exhibited good precision and accuracy. The diffusion properties of linezolid investigated in in vitro microdialysis experiments revealed a mean relative recovery of 77.5% (CV: 5.4%; delivery and recovery experiments). Following characterization of linezolid in in vitro microdialysis, the setting is suitable for application in clinical studies.

Keywords: Microdialysis; Linezolid

1. Introduction

Linezolid (Zyvoxid™) is the first member of a novel class of antimicrobial agents, the oxazolidinones, developed for the treatment of infectious diseases caused by gram-positive pathogens, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *Enterococcus* strains (VRE) [1]. Linezolid is considered to be a promising new agent in the management of severe infections [2] due to its innovative mode of action, i.e. preventing formation of the initiation complex of bacterial protein biosynthesis [3].

Linezolid is rapidly and completely absorbed following oral administration (bioavailability ∼ 100%) with a plasma protein binding of approximately 31%. The volume of distribution is about 40–50 l and corresponds to total body water. The drug is eliminated via renal and non-renal routes with a terminal plasma elimination half-life between 4.5 and 5.5 h [2]. For effective treatment not only of infectious diseases, it is extremely important to possess thorough knowledge about drug concentrations at the site of action [4]. However, currently pharmacokinetic data about tissue distribution of linezolid in humans is not entirely available. Tissue penetration following multiple

* Corresponding author. Tel.: +49-30-838-506-28; fax: +49-30-838-507-11.
E-mail address: ckloft@zedat.fu-berlin.de (C. Kloft).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.08.019

oral doses into inflammatory exsudate was investigated in healthy volunteers by means of skin blister fluid sampling [5]. The data suggested that penetration of linezolid into inflammatory fluids is highly variable between individuals. Other studies evaluated the distribution of linezolid into tissues by use of the biopsy method and measured concentrations in homogenized samples from osteo-articular tissues [6] as well as from bone, fat and skeletal muscle [7]. These studies investigated either patients suffering from osteoarthritis [6] or the administration of linezolid as antibiotic prophylaxis during an orthopaedic surgery [6,7]. Available information on tissue penetration of linezolid is not yet complete, though it is increasingly recognized that effective drug tissue concentrations are fundamental to microbiological and clinical outcome. In order to extend the knowledge about penetration of linezolid into tissues in severely compromised patients, an innovative probe based sampling technique, microdialysis, was employed.

Microdialysis is a highly attractive method used to evaluate target site concentrations and minimizes the sampling burden on a patient in comparison to conventional tissue sampling methods. It is used to measure unbound drug concentration in the interstitial space fluid of several tissues, e.g. skeletal muscle or subcutaneous adipose tissue. The method has been described in more detail previously [8–11].

In brief, a microdialysis probe is constantly perfused with a physiological solution (perfusate) at a flow rate of 0.5–10 μl/min. Unbound drug molecules present in the medium around the microdialysis probe, at a concentration c_{medium}, are allowed to diffuse from the interstitial space through a semipermeable membrane and into the fluid perfusing the probe, achieving the concentration $c_{\text{dialysate}}$. For most substances, the diffusion equilibrium between surrounding medium and perfusion fluid is incomplete and $c_{\text{medium}} > c_{\text{dialysate}}$. The correlation factor between these two concentrations is termed relative recovery (RR). Therefore, to obtain absolute interstitial from dialysate concentrations, the microdialysis probes have to be calibrated for in vivo recovery rates according to, e.g. the retrodialysis method [11]. The principle behind this method is based on the assumption that the diffusion process is quantitatively equal in both directions through the semipermeable membrane.

The disappearance rate of the drug added to the perfusate ($c_{\text{perfusate}}$) through the membrane is taken as the in vivo recovery rate which can be calculated using the following equation:

$$\text{RR}\,(\%) = \left(1 - \frac{c_{\text{dialysate}}}{c_{\text{perfusate}}}\right) \times 100 \quad (1)$$

Previously reported bioanalytical methods for linezolid determine the concentration in plasma and/or urine samples [12–16]. We here report the development of a high-performance liquid chromatography (HPLC) method for microdialysate and plasma. Although the microdialysate matrix is not as complex as plasma, the analytical challenge derives from the very low sample volume of microdialysate, usually only a few microliters. The assay was validated according to an international FDA-guideline [17] in terms of stability, specificity, linearity, precision and accuracy for both matrices. In addition, in vitro microdialysis experiments were performed to explore the permeability of the semipermeable membrane for linezolid. These investigations are a prerequisite for the utilization of the microdialysis technique for linezolid in human pharmacokinetic studies.

2. Experimental

2.1. Chemicals

Linezolid standard (purity > 99.9%) was kindly provided by Pharmacia (Kalamazoo, USA). The following drugs were used for specificity evaluation in their licensed form: cefotiam·2HCl (Takeda, Aachen, Germany), dexamethasone-21-di-hydrogen phosphate (Merck, Darmstadt, Germany), sodium flucloxacillin (SmithKline Beecham, Munich, Germany), potassium canrenoate and midazolam·HCl (Hoffmann-La Roche, Grenzach-Wyhlen, Germany), ketamine·HCl (Parke-Davis, Berlin, Germany), levofloxacin (Hoechst Marion Roussel, Frantfurt/M, Germany), sodium phenytoin (Desitin, Hamburg, Germany), physostigmine salicylate (Dr. Franz Koehler Chemie, Alsbach-Haehnlein, Germany), procaine·HCl (Jenapharm, Jena, Germany), promethazine·HCl (Bayer Vital, Leverkusen, Germany), propranolol·HCl (Isis Pharma, Zwickau, Germany), ranitidine·HCl (Ratiopharm, Ulm, Germany), sodium rifampicin (Fatol,

Schiffweiler, Germany) and ropivacaine·HCl (Astra Zeneca, Wedel, Germany). Acetonitrile (ACN), HPLC gradient grade, was purchased from Acros Organics (Geel, Belgium), Ringer's solution from Serumwerk Bernburg (Bernburg, Germany), di-sodium hydrogen phosphate anhydrous and sodium acetate anhydrous from Merck (Darmstadt, Germany). Water for preparation of all solutions was obtained from a Milli-Q^{TM} Plus water purification system (Millipore, Bedford, MA, USA). The mobile phase was filtered through 0.22 μm membrane filters (Sartorius, Goettingen, Germany) and degassed prior to use in an ultrasonic bath.

2.2. High performance liquid chromatography (HPLC)

All HPLC experiments were performed on a Kontron HPLC (Kontron Biotech, Neufahrn, Germany) with UV detection at 251 nm.

Samples were separated on a Spherimage-80 ODS2 5 μm column, 125 mm × 4 mm, with an integrated pre-column (Knauer, Berlin, Germany) as stationary phase and 80/20 sodium acetate buffer (25 mM, pH 5)/ACN (V/V) as mobile phase at a flow rate of 1 ml/min (isocratic).

2.3. Preparation of stock solution, calibration samples and quality control samples

2.3.1. Stock solutions

Two stock solutions were prepared separately for the purposes of calibration and quality control (QC). For each solution, 7.5 mg linezolid was accurately weighed and dissolved in water yielding concentrations of 1.5 mg/ml each. One stock solution was diluted with water to obtain working solutions of 2, 5, 10, 50, 100 and 200 μg/ml for plasma or 8, 15, 30, 60, 120 and 200 μg/ml for microdialysate calibration samples. Working solutions for QC samples were prepared by diluting the second stock solution with water to yield linezolid concentrations of 2, 5, 8, 20, 100 and 150 μg/ml. Aliquots of stock and working solutions were frozen at −70 °C.

2.3.2. Calibration samples

Plasma calibration samples were prepared prior to each analytical run by mixing 10 μl aqueous working solution with 90 μl empty human plasma to yield linezolid concentrations of 0.2, 0.5, 1, 5, 10 and 20 μg/ml.

The preparation of microdialysate calibration samples was as follows: 5 μl aqueous working solution was diluted with Ringer's solution to obtain final linezolid concentrations of 0.8, 1.5, 3, 6, 12 and 20 μg/ml.

2.3.3. Quality control samples

Spiked matrix samples were prepared from aqueous working solutions by dilution with either plasma or Ringer's solution. Plasma QC samples contained 0.2, 0.5, 10 and 15 μg/ml and microdialysate QC samples contained 0.8, 2, 10 and 15 μg/ml linezolid, respectively. QC samples at the lower limit of quantification (LLOQ) were only used for pre-study validation. Aliquots of QC samples for pre- and in-study validation were stored at −70 °C until analysis.

2.4. Sample preparation

Plasma samples were prepared by mixing a 50 μl aliquot with 200 μl ACN. The mixtures were allowed to rest at ambient temperature for 10 min and centrifuged at 10,000 × *g* for 5 min (Eppendorf Centrifuge 5417 R, Eppendorf-Netheler-Hinz, Hamburg, Germany). Two-hundred microliter of the supernatant were evaporated to dryness by Speed-vac^{TM} (Savant Instruments, Inc., Farmingdale, USA) and redissolved in 50 μl 80/20 H_2O/ACN (V/V). Twenty microliter were injected into the HPLC system.

For microdialysate, a simple one-step dilution preparation procedure was developed due to the lack of proteins. To 10 μl of microdialysate 30 μl of water was added. Ten microliter of probe calibration solution or retrodialysis samples were diluted with 390 μl water. Following dilution, a volume of 20 μl was injected into the HPLC system.

2.5. Stability

Linezolid stability was assessed in plasma and microdialysate, reflecting situations likely to be encountered during actual sample collection, storage, preparation and analysis [17]. Low and high QC concentrations were investigated in triplicate under three different conditions for both matrices. Three sets of QC samples were assayed after one, two or three freeze-thaw cycles and compared to freshly

prepared QC samples, using Eq. (2) to determine the freeze-thaw stability of linezolid in plasma and microdialysate, respectively.

$$\text{Stability}\,(\%) = 100 \times \frac{\text{result}_{\text{stored sample}}}{\text{result}_{\text{freshly prepared sample}}} \quad (2)$$

To evaluate stability at room temperature, QC samples were thawed at ambient temperature and kept under these conditions for 4 or 24 h. The data were compared to results from freshly thawed QC samples as described above.

To determine the stability of the drug in prepared samples, two sets of QC samples were prepared as described above. One set was stored in the sample tray of the autosampler for 8 h. The other set was frozen after preparation for at least 24 h. These results were compared with those of QC samples measured immediately after preparation using Eq. (2).

2.6. Specificity

In order to evaluate the specificity of the analytical method, linezolid-free matrices, plasma and microdialysate from six different healthy human sources were investigated for compounds influencing linezolid during analysis. In addition, to investigate interference of drugs commonly used in patients, a broad variety of drugs that are possibly co-administrated with linezolid were assayed in each matrix. For this purpose, aqueous solutions were diluted with either empty plasma or di-sodium hydrogen phosphate buffer pH 7.4, 0.1 M, 1 + 9 (V/V), to yield final concentrations which were at least within clinically relevant ranges: cefotiam·2HCl 50 mg/l, dexamethasone-21-di-hydrogen phosphate 20 mg/l, sodium flucloxacillin 10 mg/ml, potassium canrenoate 10 mg/ml, ketamine·HCl 0.1 mg/ml, levofloxacin 0.17 mg/ml, midazolam·HCl 0.8 mg/ml, sodium phenytoin 40 mg/l, physostigmine salicylate 50 mg/l, procaine·HCl 5 mg/ml, promethazine·HCl 2 mg/ml, propranolol·HCl 100 mg/l, ranitidine·HCl 1 mg/ml, sodium rifampicin 2 mg/ml and ropivacaine·HCl 67 mg/l. Samples were prepared and assayed as described above.

2.7. Accuracy and precision

Accuracy, or more precisely inaccuracy, was assessed by calculating the mean percentage deviation (RE) of measured concentration of QC samples from their nominal concentration (c_{nom}). For pre-study validation, six QC samples per concentration and matrix were analyzed for 3 days. Precision, or more precisely imprecision, was evaluated using the coefficient of variation (CV) of multiple determinations. For both parameters, the within- and between-day results were determined. In each instance, four concentrations covering the whole concentration range were investigated.

2.8. Linearity and determination of LLOQ

Linearity was evaluated using freshly prepared, spiked matrix samples in a concentration range from 0.2 to 20 μg/ml for plasma samples and 0.8 to 20 μg/ml for microdialysate samples, respectively ($n = 3$). Each calibration curve consisted of six calibrator concentrations.

2.8.1. LLOQ

Lower limit of quantification was assessed by comparing the chromatograms of empty matrix with those obtained from five spiked matrix samples at each concentration. Linezolid working solution was added to empty plasma yielding concentrations from 0.05 to 0.2 μg/ml. Furthermore, linezolid was added to Ringer's solution to yield concentrations between 0.2 and 0.8 μg/ml. The LLOQ for each matrix was regarded as the lowest concentration within acceptable ranges of accuracy and precision which could be analysed.

2.9. Recovery of the analyte

Peak area data of six spiked matrix samples at three concentrations were compared to the results of three diluted aqueous solutions at the same nominal concentration, and the recovery calculated.

2.10. Microdialysis

The properties of linezolid in the microdialysis probes were evaluated in vitro prior to the use of the drug in human microdialysis.

2.10.1. Probes

For microdialysis investigations in vitro, commercially available microdialysis probes (CMA60, CMA

Microdialysis AB, Stockholm, Sweden) with a molecular cut-off of 20 kDa, an outer diameter of 0.6 mm and a membrane length of 30 mm were used. Probes were perfused with Ringer's solution at different flow rates (see below) by use of a precision pump (CMA102, CMA Microdialysis AB, Stockholm, Sweden).

2.10.2. Recovery experiments

Three microdialysis probes were placed in three vials containing Ringer's solution. Perfusion medium consisted of linezolid in Ringer's solution at a concentration of 10 μg/ml. Recovery was assessed performing the retrodialysis method described by Stahle et al. [11], at flow rates of 0.8, 1.0, 1.5, 2.0 and 2.5 μl/min, to determine an optimal flow rate for subsequent in vitro and in vivo experiments. Samples ($n = 3$) were collected at intervals of 10 min for flow rates of 1.5, 2.0 and 2.5 μl/min and every 20 min for flow rates of 0.8 and 1.0 μl/min, respectively. Relative recovery was calculated using Eq. (1).

To investigate the effects of concentration on RR, three probes were perfused with Ringer's solution containing linezolid concentrations of 5, 10, 20 or 50 μg/ml at a flow rate of 1.5 μl/min. The experiment was performed in two different settings for two possible directions of diffusion: (1) as delivery (retrodialysis) and (2) as recovery experiment (diffusion of drug from the surrounding medium into the probe). Samples ($n = 3$) were taken every 10 min.

As mentioned above, microdialysis probes have to be calibrated in vivo. For this purpose, the retrodialysis method is generally performed. Usually, the calibration procedure takes place prior to the administration of the respective drug. However, for situations where the drug has already been administered and steady state is already present, microdialysis probe calibration is a challenging task. In order to make sure that drug presence in tissue will not affect its diffusion out of the probe during retrodialysis, drug concentration in the perfusate (calibration solution) should substantially exceed expected tissue concentrations.

In our in vitro experiments, a linezolid concentration of 10 μg/ml was chosen for the medium surrounding the probe, to reflect maximum expected in vivo conditions. Linezolid solutions of 50, 100, 150 and 200 μg/ml were used as perfusate in order to determine the most suitable concentration for the calibration solution. The concentration at which recovery remains unchanged, despite increasing perfusate concentration, determines the concentration where linezolid will leave the probe unaffected. Dialysate samples were collected in triplicate at intervals of 10 min. Samples were unstirred and used at room temperature for all in vitro experiments.

2.11. Statistics

All statistical calculations were performed using SPSS™ for Windows, version 7.5.1 (SPSS, Inc., Chicago, USA). The Student's *t*-test or the Welch test were used to compare means. A *P*-value of <0.05 was considered statistically significant. If not stated otherwise results are given as mean (CV, %).

3. Results and discussion

3.1. Stability

Evaluating the stability of linezolid in plasma after one to three freeze-thaw cycles, 96.8% (2.5%) to 111% (3.6%) was found on average compared to stored and freshly prepared QC samples. This difference was not statistically significant. In addition, no tendency towards degradation or enrichment could be detected in relation to the number of freeze-thaw cycles.

Storing plasma samples containing linezolid at room temperature for 4 or 24 h did not affect drug concentrations. Mean linezolid concentrations ranged from 93.5% (3.0%) to 101% (6.5%). Linezolid was also stable after sample preparation where one set of QC samples was frozen after preparation for at least 24 h and another set was stored in the sample tray of the autosampler for 8 h. QC samples exhibited no significant difference in relation to the samples measured immediately after preparation. Average concentrations varied between 95.8% (7.3%) and 107% (6.9%). The stability of linezolid in microdialysate was comparable with its stability in plasma. After storage under freeze-thaw conditions samples contained between 90.4% (1.0%) and 106% (6.7%) of linezolid on average. Storage of microdialysate samples at room temperature for 4 or 24 h yielded mean concentrations between 102% (0.63%) and 111% (3.3%). QC samples stored at ambient temperature or in a freezer after sample preparation showed average results between 97.2% (0.66%) and 104% (3.1%). No

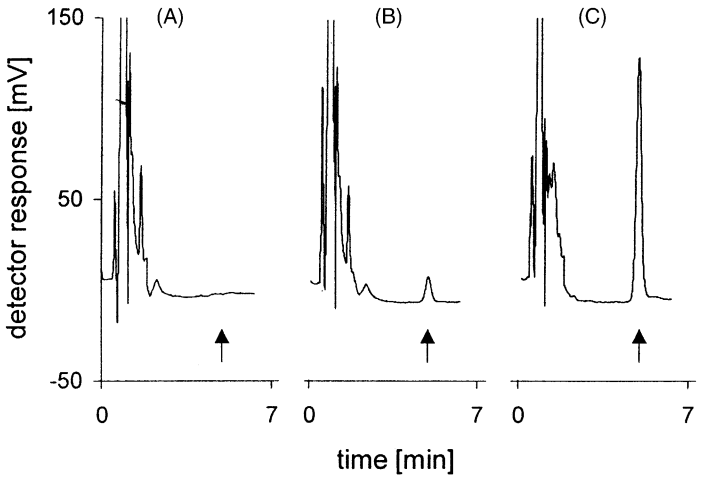

Fig. 1. Chromatograms of plasma. (A) Empty plasma; (B) plasma calibrator (0.501 μg/ml); (C) plasma of a volunteer (5.6 μg/ml). Arrows indicate the signal of linezolid (5.0 min). A detector response of 100 mV corresponds to an absorbance of 0.1 AU.

tendency towards degradation or enrichment related to the various storage conditions could be detected. Thus, linezolid was considered to be stable in plasma and microdialysate under the conditions investigated in the present experiments.

3.2. Specificity

In all linezolid-free plasma and microdialysate samples measured, no interference with the signal of the analyte could be detected (see Figs. 1 and 2). Furthermore, no interactions with linezolid, eluting at approximately 5 min, and matrix components were observed. Cefotiam·2HCl (retention time: t_R = 1.5 min) and sodium flucloxacillin (t_R = 1.5 min), sodium phenytoin (t_R = 25.8 min), ranitidine·HCl (t_R = 32.7 min), sodium canrenoate (t_R = 2.2 min) and ropivacaine·HCl (t_R = 6.6 min) were eluted without interfering with the signal of linezolid. All other substances tested were not detectable due to the

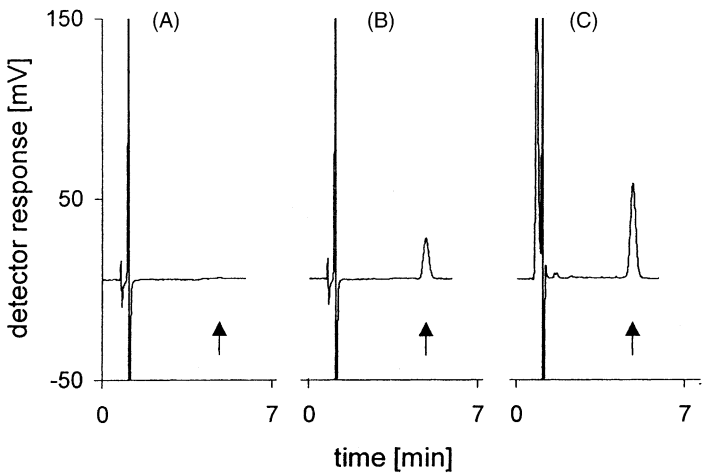

Fig. 2. Chromatograms of microdialysate. (A) Empty microdialysate; (B) microdialysate calibrator (3.01 μg/ml); (C) microdialysate of a volunteer (6.7 μg/ml). Arrows indicate the signal of linezolid (4.8 min). A detector response of 100 mV corresponds to an absorbance of 0.1 AU.

Table 1
Within- and between-day imprecision (expressed as coefficient of variation, CV, %) and inaccuracy (expressed as mean percentage deviation, RE, %) of determined linezolid concentrations (μg/ml) in plasma

c_{nom} (μg/ml)	c (μg/ml) (mean ± S.D.)	CV (%)	RE (%)
Within-day variability ($n = 6$)			
0.207	0.212 ± 0.0076	3.6	2.6
0.518	0.518 ± 0.025	4.9	−0.0064
10.3	10.34 ± 0.22	2.1	−0.028
15.5	15.49 ± 0.58	3.8	−0.27
Between-day variability ($n = 18$)			
0.207	0.214 ± 0.013	6.1	3.4
0.518	0.521 ± 0.029	5.5	0.65
10.3	10.48 ± 0.39	3.7	1.3
15.5	15.11 ± 0.64	4.3	−2.7

sample preparation procedure, e.g. low recovery, or the particular wavelength required for linezolid. In conclusion, none of the possibly co-administered drugs investigated showed any interference with the signal of linezolid.

3.3. Accuracy and precision

The results for within- and between-day accuracy and precision are listed in Tables 1 and 2. CV and RE data ranged between −2.7 and 6.1% under all circumstances investigated and thus met the acceptance criteria for pre-study validation specified within the FDA guideline [17].

Table 2
Within- and between-day imprecision (expressed as coefficient of variation, CV, %) and inaccuracy (expressed as mean percentage deviation, RE, %) of determined linezolid concentrations (μg/ml) in microdialysate

c_{nom} (μg/ml)	c (μg/ml) (mean ± S.D.)	CV (%)	RE (%)
Within-day variability ($n = 6$)			
0.827	0.865 ± 0.042	4.8	4.6
2.07	2.08 ± 0.054	2.6	0.72
10.3	10.6 ± 0.22	2.1	2.1
15.5	15.6 ± 0.25	1.6	0.23
Between-day variability ($n = 18$)			
0.827	0.864 ± 0.051	5.9	4.4
2.07	2.05 ± 0.087	4.2	−0.73
10.3	10.3 ± 0.48	4.7	−0.65
15.5	15.1 ± 0.61	4.0	−2.7

3.4. Linearity and determination of LLOQ

A linear regression analysis was performed (peak area versus linezolid concentrations), to describe the relationship between detector response and concentration using the reciprocals of squared concentrations as weighting factor. Calibration curves for both matrices showed good linearity across each concentration range, with regression coefficients typically ranging from 0.999 to 1. Details are listed in Table 3.

The validated concentration ranges were 0.2–20 and 0.8–20 μg/ml for plasma and microdialysate, respectively. For plasma samples containing potentially more than 20 μg/ml of linezolid, the final re-dissolving step of the sample preparation procedure was modified: after evaporation, the residue was redissolved in 150 μl instead of 50 μl 80/20 H_2O/ACN (V/V) to yield concentrations within the validated range. Plasma samples with a nominal linezolid concentration of 40.2 μg/ml resulted in a mean back-calculated concentration of 41.5 μg/ml (3.8%), with an inaccuracy of 3.5% ($n = 5$). Microdialysate samples with a nominal linezolid concentration of 40.2 μg/ml were diluted with water 1 + 2 (V/V), and yielded an average back-calculated concentration of 40.6 μg/ml (1.8%), with a relative error of 1.0% ($n = 5$).

3.4.1. LLOQ

The lower limit of quantification was determined to be 0.2 μg/ml for plasma and 0.8 μg/ml for microdialysate. Concentrations of six back-calculated samples resulted in a CV of 3.6% (RE 2.6%) and 4.8% (RE 4.6%) for plasma and microdialysate samples, respectively. The LLOQ found in plasma conforms to other LLOQs reported for linezolid in bioanalytical assays [12,13,15,16]. In addition, the low plasma sample volume of only 50 μl required for this assay is highly advantageous in reducing the burden on the patient in clinical studies with frequent blood sampling. The higher LLOQ for microdialysate resulted from the very small sample volume available, i.e. 15 μl.

3.5. Recovery of the analyte

Recovery of spiked plasma samples was complete and ranged from 101.3 to 115.5%, with an average of 108.9% (3.2%). The comparison between peak area data obtained from spiked microdialysate and aqueous

Table 3
Regression parameters of the calibration curves of linezolid in plasma and microdialysate expressed as mean ± S.D. ($n = 3$)

	Slope (mV·min·ml/μg)	Intercept (mV·min)	R
Plasma	6.65 ± 0.14	−0.039 ± 0.028	0.9992 ± 9.0E−05
Microdialysate	1.93 ± 0.028	−0.25 ± 0.086	0.9996 ± 2.1E−04

solution produced a mean recovery of 100.5% (2.9%). Values ranged from 96.1 to 107.0%.

3.6. Microdialysis recovery experiments

In Fig. 3, the relationship between RR of linezolid and flow rate is depicted. In the delivery experiments, reducing the flow rate from 2.5 to 1.0 μl/min resulted in an increase in RR from 70.8% (13.1%) to 93.4% (1.3%). A decrease in the flow rate to 0.8 μl/min yielded an RR of 93.1% (2.9%) which did not significantly differ from RR at 1.0 μl/min. However, both flow rates require a longer sampling interval for sufficient sample volume. Thus, in order to comprehensively characterize the pharmacokinetic profile with still adequate recovery, a flow rate of 1.5 μl/min was chosen for all following in vitro recovery experiments and for clinical application.

Changing the concentration of the medium surrounding or perfusing a probe did not affect the relative recovery. In delivery experiments RR was, on average, 78.2% (3.6%), ranging from 73.4 to 86.2% and covering a concentration range from 5 to 50 μg/ml of linezolid. Results of the recovery experiments were comparable with a mean RR of 76.7% (7.9%), a minimum at 62.6% and a maximum at 89.9%. No statistically significant differences between the results of the delivery and recovery experiment could be detected. Linear regression between RR, achieved in the delivery and recovery experiments, and concentration of surrounding or perfusing medium, yielded a regression line with a slope of 0.0459%·ml/μg (standard error (S.E.): 0.029%·ml/μg) and an intercept of 76.6% (S.E.: 0.805%). The line was assumed to run parallel to the x-axis because the 95%-confidence interval of slope included zero (Fig. 4). It could be concluded that RR was not influenced by the concentration of linezolid used and that the diffusion process is quantitatively equal in both directions through the semipermeable membrane.

Performing the delivery experiment with probes placed in a linezolid solution of 10 μg/ml and different perfusate concentrations revealed an increase in RR from 39.3% (28.5%) at a perfusate concentration of 50 μg/ml, to 71.8% (8.0%) at 150 μg/ml of linezolid. The RR was 75.9% (6.1%) when perfusing the probes with a solution containing 200 μg/ml of linezolid and did not differ significantly from the

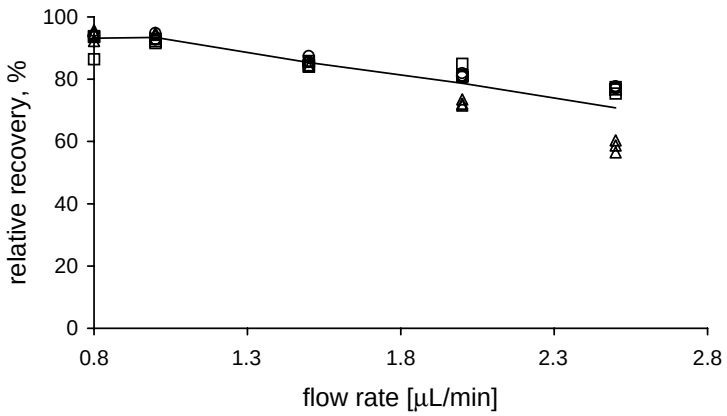

Fig. 3. Dependence of relative recovery (RR, %) on flow rate (μl/min) of microdialysis perfusate (10 μg/ml linezolid). The individual results ($n = 3$) of three microdialysis probes are depicted as open squares, cycles, triangles, the line represents the overall mean.

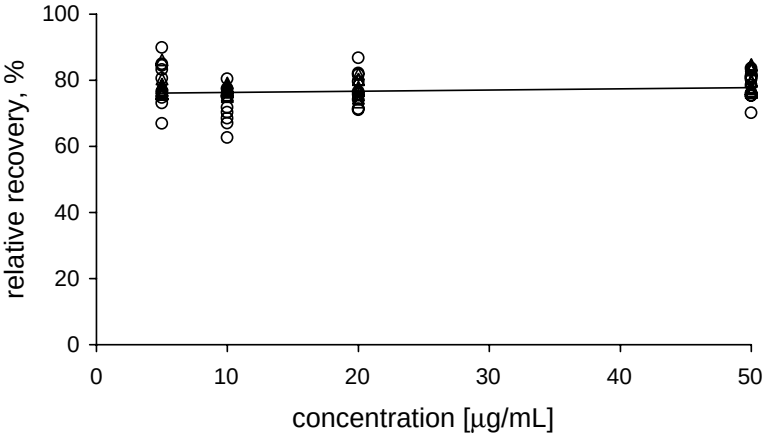

Fig. 4. Independence of relative recovery (RR, %) on concentration (μg/ml) of perfusion and surrounding medium, respectively, achieved in recovery ($n = 36$, open cycles) and delivery ($n = 44$, open triangles) experiment. See text for details.

RR at 150 μg/ml. These data indicate that no further increase in recovery was observed when a perfusate concentration of 200 μg/ml was applied. Thus, it can be concluded that linezolid molecules can leave the probe unaffected by the drug molecules present in the probe surrounding medium. Regarding tissue conditions as described above, linezolid concentrations in the interstitial fluid of 10 μg/ml or less would not interfere with probe calibration at steady state when using a perfusate concentration of at least 150 μg/ml of linezolid.

4. Conclusion

A rapid and reliable HPLC assay was established and validated for the determination of linezolid concentrations in microdialysate and plasma. Our method is characterized by simple preparation and uses common HPLC equipment. There is no need for an additional extraction column as described by Ehrlich et al. [13]. Decreasing the plasma sampling volume from 300 μl [12] to 50 μl reduces the sampling burden on study subjects. Compared with the method by Peng et al. [14], solid phase extraction was replaced by a less time-consuming method of sample preparation. All parameters met the criteria set in international guideline for bioanalytical methods. We have shown that linezolid is stable in these two matrices under several conditions investigated during sample collection, preparation and determination. Accuracy and precision were good for both matrices and all conditions investigated, with RE and CV values below |4.6%| and 6.1%, respectively. The lower limits of quantification allow for the measurement of antibiotic concentrations in small sample volumes (e.g. 15 μl microdialysate) in plasma and tissue down to the minimal inhibitory concentration (MIC_{90}) values reported for most relevant gram-positive pathogens, including MRSA and VRE [18].

In addition, in vitro microdialysis experiments revealed that linezolid concentrations achieved in the dialysate will be sufficient for in vivo investigation. Based on these results, optimal conditions for the in vivo microdialysis procedure were determined.

In conclusion, the bioanalytical method developed is suitable for clinical application. Initial results demonstrate its proficiency with regard to use in human samples.

References

[1] C.M. Perry, B. Jarvis, Drugs 61 (2001) 525.
[2] D.I. Diekema, R.N. Jones, Drugs 59 (2000) 7.
[3] S.M. Swaney, H. Aoki, M.C. Ganoza, D.L. Shinabarger, Antimicrob. Agents Chemother. 42 (1998) 3251.
[4] M. Muller, H. Stass, M. Brunner, J.G. Moller, E. Lackner, H.G. Eichler, Antimicrob. Agents Chemother. 43 (1999) 2345.
[5] T. Gee, R. Ellis, G. Marshall, J. Andrews, J. Ashby, R. Wise, Antimicrob. Agents Chemother. 45 (2001) 1843.

[6] B. Rana, I. Butcher, P. Grigoris, C. Murnaghan, R.A. Seaton, C.M. Tobin, J. Antimicrob. Chemother. 50 (2002) 747.
[7] A.M. Lovering, J. Zhang, G.C. Bannister, B.J. Lankester, J.H. Brown, G. Narendra, A.P. MacGowan, J. Antimicrob. Chemother. 50 (2002) 73.
[8] W.F. Elmquist, R.J. Sawchuk, Pharm. Res. 14 (1997) 267.
[9] P. Lonnroth, P.A. Jansson, U. Smith, Am. J. Physiol. 253 (1987) E228.
[10] M. Muller, O. Haag, T. Burgdorff, A. Georgopoulos, W. Weninger, B. Jansen, G. Stanek, H. Pehamberger, E. Agneter, H.G. Eichler, Antimicrob. Agents Chemother. 40 (1996) 2703.
[11] L. Stahle, P. Arner, U. Ungerstedt, Life Sci. 49 (1991) 1853.
[12] K. Borner, E. Borner, H. Lode, Int. J. Antimicrob. Agents 18 (2001) 253.
[13] M. Ehrlich, R. Trittler, F.D. Daschner, K. Kummerer, J. Chromatogr. B 755 (2001) 373.
[14] G.W. Peng, R.P. Stryd, S. Murata, M. Igarashi, K. Chiba, H. Aoyama, M. Aoyama, T. Zenki, N. Ozawa, J. Pharm. Biomed. Anal. 20 (1999) 65.
[15] O.A. Phillips, M.E. Abdel-Hamid, N.A. Al-Hassawi, Analyst 126 (2001) 609.
[16] C.M. Tobin, J. Sunderland, L.O. White, A.P. MacGowan, J. Antimicrob. Chemother. 48 (2001) 605.
[17] FDA, http://www.fda.gov/cder/guidance/4252fnl.pdf, 2001.
[18] R. Wise, J.M. Andrews, F.J. Boswell, J.P. Ashby, J. Antimicrob. Chemother. 42 (1998) 721.