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Urea as an endogenous surrogate in human microdialysis to determine relative recovery of drugs: Analytics and applications

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Abstract

During in vivo microdialysis studies time-consuming and laborious bedside calibration methods, e.g. retrodialysis, have to be performed. To reduce the burden on the patient it would be desirable to establish a reliable, time-saving calibration technique to obtain the in vivo recovery describing the relative drug transfer across the membrane of the microdialysis probe. The performed study aimed to evaluate and validate the use of urea as an endogenous reference compound to determine relative in vivo recovery of anti-infectives, e.g. linezolid used herein as model drug. In order to meet the special requirements imposed by microdialysis to measure urea concentrations in very small sample volumes (~10 μL) a photometric assay in 96-well microtiter plates was established based on the method of Berthelot. Subsequently, concentration- and flow rate-dependence were evaluated in vitro to determine the relative recovery (RR) of urea. Finally, urea and linezolid concentrations in human microdialysis samples were measured. The developed assay was validated according to international guidelines and met all requirements. Relative in vitro recovery was found to be independent from concentration and dependent on flow rate. Subsequently, relative in vivo recovery of urea was correlated with relative in vivo recovery of linezolid obtained by the traditional retrodialysis method. In healthy volunteers, the mean ratio of the relative recovery of linezolid to the relative recovery of urea was 0.6 for the subcutaneous (s.c.: CV 33.4%, $n = 48$) and 0.7 for the intramuscular probe (i.m.: CV 18.8%, $n = 40$), respectively. In critically ill patients this ratio was 0.7 for both tissues (s.c.: CV 32.8%, $n = 18$; i.m.: CV 22.1%, $n = 17$). Successful calibration of the urea reference technique without the need to use in vitro data will further promote the application of microdialysis in clinical studies especially in critically ill patients, as it reduces the imposed burden to a minimum.

Keywords: Microdialysis; Linezolid; Urea; In vivo calibration; Relative recovery; Endogenous reference compound

1. Introduction

Whereas microdialysis has been extensively used in animal studies [1], first studies in humans were conducted only about 20 years ago [2]. The method and its applications have been described in detail previously [2–6]. Microdialysis, a minimally invasive tool, presents an excellent opportunity to measure interstitial drug concentrations in peripheral tissues. Hence, it provides valuable information on the distribution of a drug to the target site of, e.g. Gram-positive pathogens. In brief, a microdialysis probe is inserted into the interstitial tissue fluid and subsequently perfused with a physiological solution (perfusate) at a flow rate of 0.5–10 μL/min. Unbound drug molecules present in the probe surrounding medium at a concentration c_{medium} are allowed to diffuse into the perfusion fluid of the probe, achieving a concentration $c_{dialysate}$. For most substances, the diffusion equilibrium between surrounding medium and perfusion fluid is incomplete and consequently $c_{medium} > c_{dialysate}$. As a result, time-consuming and laborious bedside in vivo calibration methods, e.g. retrodialysis [3] or the no-net-flux technique [2], have to be performed. Most calibration methods are based on adding the respective analyte to the perfusate and measuring the disappearance rate through the semipermeable membrane. It is here assumed that diffusion processes are quantitatively the same in both directions through the membrane. To reduce the burden on the patient it would be desirable to establish a reliable, time-saving calibration technique to determine the relative recovery (RR) of an analyte of interest describing the relative drug transfer across the membrane of the microdialysis probe. Since urea equally distributes throughout the extracellular space, unbound

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tissue concentrations are expected to be very similar to plasma concentrations [7]. Hence, RR of urea was defined as the ratio of microdialysate and plasma concentration.

It was the objective of the present study to investigate and validate the use of urea as an endogenous reference compound to determine the RR of drugs. Linezolid, a promising novel antimicrobial agent, served as a model drug. Investigations were conducted in vitro as well as with samples from healthy volunteers and critically ill patients.

2. Materials and methods

2.1. Reagents/chemicals

Urea for biochemical purposes (purity 99.91%) was purchased from Merck (Darmstadt, Germany). Linezolid standard (purity >99.9%) was kindly provided by Pharmacia (Kalamazoo, USA). The following drugs were used for specificity evaluations in their licensed form: aciclovir (Hexal, Holzkirchen, Germany), sodium amoxicillin/clavulanic acid and sodium flucloxacillin (GlaxoSmithKline, Munich, Germany), ciprofloxacin (Bayer Vital, Leverkusen, Germany), clindamycin dihydrogen phosphate (Pharmacia, Erlangen, Germany), dobutamine·HCl and dopamine·HCl (Fresenius Kabi, Bad Homburg, Germany), glyceryl trinitrate (G. Pohl-Boskamp, Hohenlockstedt, Germany), potassium canrenoate and midazolam·HCl (Hoffmann-La Roche, Grenzach-Wyhlen, Germany), lorazepam (Wyeth Pharma, Munster, Germany), propranolol·HCl (Alpharma-Isis, Langenfeld, Germany), tobramycin·HCl and vancomycin·HCl (Lilly, Bad Homburg, Germany). A commercial reagent kit (Ecoline^{TM} S + Urea Berthelot) was purchased from DiaSys Diagnostic Systems (Holzheim, Germany) comprising of Reagent 1 (R1, phosphate buffer 120 mM, sodium salicylate 62 mM, sodium nitroprusside 3.15 mM, EDTA 1.3 mM), reagent 2 (R2, phosphate buffer 120 mM, sodium hydroxide 310 mM, sodium hypochlorite 40 mM) and reagent 3 (R3, urease $\geq$2 kU/mL). Water for the preparation of all solutions was deionized by a Milli-Q^{TM} Plus water purification system (Millipore, Bedford, MA, USA). Ringer's solution was purchased from Serumwerke Bernburg (Bernburg, Germany).

2.2. Analytical procedures

For the determination of urea in microdialysate a photometric assay in 96-well microtiter plates (Corning Costar^{TM} 3595, Schiphol-Rijk, The Netherlands) was developed based on the method of Berthelot [8]. All photometric experiments were performed on an Easyreader 400 AT (SLT-Labinstruments, Salzburg, Austria) with detection at 690 nm. For analysis 5 μL sample volume was transferred into the wells of the microtiter plate, followed by 100 μL R1A (R1-R3 (100:1, v/v)). After gentle mixing (10 s) and incubation at room temperature (10 min) 100 μL R2 was added. Again after gentle mixing (10 s) and incubation at room temperature (10 min) photometric measurement was performed against reagent blank ($n = 1$).

Urea concentrations in plasma were determined on an automated analyzer (Hitachi 917, Yokohama, Japan) for routine clinical chemistry. For the measurement of linezolid in microdialysate samples a validated HPLC assay using UV detection was employed [9].

2.3. Pre-study validation of urea assay in microdialysate

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

Two stock solutions were prepared separately for purposes of calibration and quality control (QC). For each solution 3 g urea was accurately weighed and dissolved in water yielding concentrations of 30 g/L each. One stock solution was diluted with water to obtain working solutions of 450, 250, 125, 62.5 and 30 mg/dL for microdialysate calibration samples. Working solutions for QC samples were prepared by diluting the second stock solution with water to achieve urea concentrations of 400, 200 and 30 mg/dL. The working solution containing 15 mg/dL was obtained in a two-step dilution. Aliquots of stock solution and working solution were stored at −70 °C.

Microdialysate calibration samples were prepared prior to each analytical run by mixing 10 μL aqueous working solution with 90 μL Ringer's solution to achieve urea concentrations of 45, 25, 12.5, 6.3, 3.0 and 1.5 mg/dL. QC working solutions were diluted accordingly yielding urea concentrations of 40, 20, 3.0 and 1.5 mg/dL.

2.3.2. Stability

Freeze–thaw stability, short-term ambient temperature stability and stock solution stability at room temperature were investigated according to the FDA guidance for bioanalytical method validation [10]. Three sets of QC samples were assayed after one, two or three freeze–thaw cycles and compared to freshly prepared samples. To evaluate stability at room temperature, QC samples were thawed at ambient temperature and stored under these conditions for 4 or 24 h. Aliquots of the QC stock solution were thawed at room temperature and kept under these conditions for 6 h. All stability tests were performed using high (=40 mg/dL) and low (=3 mg/dL) concentration QC samples and analyzed in triplicate.

2.3.3. Specificity

Interference of the analytical method was investigated with 16 different drugs possibly administered in critically ill patients. For this purpose, aqueous drug solutions were diluted (1:10, v/v) with di-sodium hydrogen phosphate buffer (pH 7.4, 0.067 M). Subsequently, diluted drug solutions were assayed by the developed microtiter assay as described above. In addition, diluted drug solutions were spiked with urea yielding concentrations of 30 mg/dL urea and subsequently assayed. Measurement was performed against reagent blank and a drug-free urea solution, respectively.

2.3.4. Accuracy and precision

Urea concentrations of QC samples in six replicates were determined daily for 3 days. In each instance, four concentrations covering the whole concentration range (see Section 2.3.1) were analyzed. Accuracy, or more precisely inaccuracy, was assessed

by calculating the mean percentage deviation (RE) of measured concentration of QC samples from their nominal concentration. 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.

2.3.5. Linearity and range

Linearity was evaluated using freshly prepared calibrator samples (concentration range: 1.5–45 mg/dL). Each calibration function consisted of six calibrator concentrations. On Day 1 of pre-study validation analysis of calibrator samples at each concentration was carried out in triplicate ($n = 1$ on Days 2 and 3).

2.4. In vitro microdialysis experiments

Prior to in vivo studies diffusion characteristics of urea through the semipermeable membrane of the microdialysis probe were investigated in vitro.

2.4.1. Probes

For microdialysis investigations in vitro, commercially available microdialysis probes (CMA60, CMA Microdialysis AB, Solna, Sweden) with a membrane length of 30 mm, an outer diameter of 0.6 mm and a molecular weight cut-off of 20 kDa were employed. Probes were perfused with Ringer's solution (or urea in Ringer's solution) at different flow rates (see below) by use of a precision pump (CMA102, CMA Microdialysis AB, Solna, Sweden).

2.4.2. Relative recovery experiments

Two microdialysis probes were placed into two glass tubes containing Ringer's solution at room temperature. Perfusate consisted of urea in Ringer's solution at a concentration of 25 mg/dL. Recovery was assessed performing the retrodialysis method described by Stahle et al. [3], at flow rates of 0.8, 1.0, 1.5, 2.0, 2.5 μL/min to determine the dependence of relative recovery on the flow rate. Samples ($n = 3$) were collected at intervals of 10 min for flow rates of 1.5, 2.0 and 2.5 μL/min and at intervals of 20 min for flow rates of 0.8 and 1.0 μL/min, respectively. Relative recovery was calculated using Eq. (1), where $c_{\text{dialysate}}$ is the urea concentration in dialysate and $c_{\text{perfusate}}$ the corresponding concentration in perfusate.

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

Dependence of relative recovery on urea concentration was investigated by retrodialysis [3] and recovery experiments. In the latter, blank Ringer's solution was pumped through the microdialysis probe, which was placed into Ringer's solution containing urea (medium). Relative recovery was calculated employing the quotient of urea concentrations in dialysate and plasma (Eq. (2)).

$$\text{RR}\,(\%) = \frac{c_{\text{dialysate}}}{c_{\text{medium}}} \times 100 \quad (2)$$

Five different urea concentrations were investigated in this experiment: 8.0, 12.5, 25, 45 and 200 mg/dL at a flow rate of 1.5 μL/min. Dialysate samples were collected in triplicate at intervals of 10 min. At the beginning and after changing the urea concentration microdialysis probes were perfused at a flow rate of 10 μL/min for 1 min to prime the system. Afterwards, probes were perfused at a flow rate of 1.5 μL/min for 20 min prior to start of sampling with the new urea concentration in the medium.

2.5. Human microdialysis

2.5.1. Clinical study

In an ongoing multicenter study the pharmacokinetics of linezolid (600 mg bid) were investigated. Samples for determination of analytes were collected in plasma and in subcutaneous (s.c.) as well as intramuscular (i.m.) interstitial tissue fluid after single dose (study Day 1) and at steady state (study Day 2) using the microdialysis technique. Urea and linezolid concentrations were determined in microdialysis and plasma samples of healthy volunteers and critically ill patients. The characteristics of the participating individuals were as follows: nine healthy volunteers (five males, four females), age (median (range)): 54 (41–76) years, weight: 64 (51–75) kg and four critically ill patients (one male, three females), age: 64 (57–76) years, weight: 60 (57–76) kg.

2.5.2. In vivo calibration of microdialysis probes

Microdialysis probes were calibrated using the retrodialysis method [3]: Prior to administration of the first dose (study Day 1) and 8 h after administration (study Day 2) microdialysis probes were perfused with Ringer's solution containing 10 and 150 μg/mL linezolid, respectively ($n = 2$ each) to determine relative recovery of linezolid ($\text{RR}_{\text{linezolid}}$). Flow rate through the probe was set to 1.5 μL/min.

RR of urea (RR_{urea}) was calculated ($n = 1$–3 per study day) by the following equation at the beginning, in the middle and at the end of a study day:

$$\text{RR}_{\text{urea}}\,(\%) = \frac{c_{\text{dialysate}}}{c_{\text{plasma}}} \quad (3)$$

where $c_{\text{dialysate}}$ the urea concentration in the dialysate; and c_{plasma} the urea concentration in plasma at corresponding times. Subsequently, ratios of relative recoveries (RRR) of linezolid ($\text{RR}_{\text{linezolid}}$) and urea ($\text{RR}_{\text{urea}}$) were calculated according to Eq. (4).

$$\text{RRR} = \frac{\text{RR}_{\text{linezolid}}}{\text{RR}_{\text{urea}}} \quad (4)$$

3. Results

3.1. Analyte stability in microdialysate

Exposure of urea in Ringer's solution to one to three freeze–thaw cycles revealed a recovery of 103.6% (CV = 0.9%) to 111.1% (CV = 0.9%) on average compared to freshly prepared

Table 1
Within-day and between-day imprecision (expressed as coefficient of variation, CV (%)) and inaccuracy (as mean percentage deviation, RE (%)) of determined urea concentrations in microdialysate

c_{nom}[a] (mg/dL)	c (mg/dL) (mean ± S.D.[b])	CV (%)	RE (%)
Within-day variability ($n=6$)			
1.58	1.78 ± 0.10	5.78	12.72
2.96	2.89 ± 0.09	2.96	−2.55
19.95	20.56 ± 0.53	2.58	3.02
39.61	41.94 ± 0.68	1.62	5.88
Between-day variability ($n=18$)			
1.58	1.64 ± 0.20	12.34	3.74
2.96	2.79 ± 0.14	5.01	−5.82
19.95	19.87 ± 0.61	3.12	−0.44
39.61	42.01 ± 0.59	1.44	6.11

[a] Nominal concentration of urea.
[b] Standard deviation.

QC samples. Furthermore, storing samples containing urea in Ringer's solution for 4 or 24 h did not cause any degradation. Mean urea concentrations ranged from 93.0 (CV = 0.8%) to 98.7 (CV = 1.2%). In addition, urea stock solution displayed an average stability of 102.1%. Statistical evaluation did not show any statistically significant differences between the exposed and freshly prepared samples (Student's t-test, $n=6$, $\alpha=0.05$) with the exception of urea samples at high concentration which yielded a mean recovery of 111.1 (CV = 0.9%) after two freeze–thaw cycles ($p<0.001$). This tendency towards higher concentrations after two freeze–thaw cycles did not continue after the third one. Hence, urea was considered to be stable in Ringer's solution under all circumstances investigated.

3.2. Specificity

Aciclovir (concentration in sample 2.0 mg/mL), ciprofloxacin (0.2 mg/mL), clindamycin dihydrogen phosphate (18.5 mg/mL), dobutamine·HCl (0.6 mg/mL), dopamine·HCl (1.0 mg/mL), glyceryl trinitrate (0.1 mg/mL), potassium canrenoate (2.0 mg/mL), linezolid (0.03 mg/mL), lorazepam (0.2 mg/mL), midazolam·HCl (0.5 mg/mL) and propranolol·HCl (0.1 mg/mL) did not interact with the signal of urea. Interference with the signal of the analyte could be observed for sodium amoxicillin/clavulanic acid, sodium flucloxacillin, tobramycin·HCl and vancomycin·HCl since measured absorbance of urea solutions spiked with these drugs exceeded that of comparison samples containing only urea by more than 15%.

3.3. Accuracy and precision

The results for within- and between-day accuracy and precision are listed in Table 1. CV and RE data ranged from 1.4 to 12.3% and from −5.8 to +12.7%, respectively. Thus, these data complied with the acceptance criteria for pre-study validation specified within the FDA guideline [10].

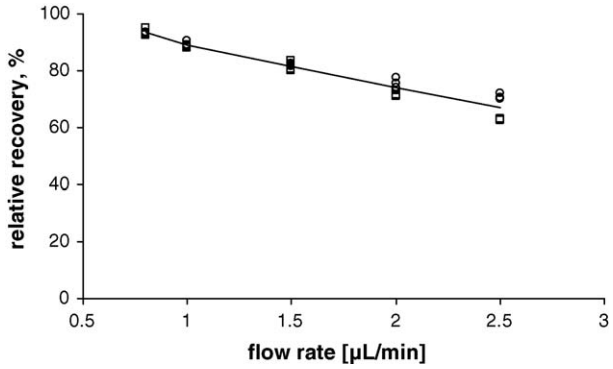

Fig. 1. Dependence of relative recovery on flow rate of perfusate (25 mg/dL urea) during retrodialysis experiments. Open squares and circles: individual results ($n=3$) of two probes; line: overall mean.

3.4. Assay linearity and range

A linear regression analysis was performed (absorbance versus nominal urea concentration) to describe the relationship between detector response and urea concentration using the reciprocals of squared concentrations as weighting factor. Calibration functions showed good linearity across the concentration range, with regression coefficients typically in the range of 0.999. Mean regression function was $y=(0.046\pm0.0005)x-(0.0084\pm0.0078)$ (x: urea concentration, y: absorbance, $n=3$). The intercept did not differ statistically significantly from 0 ($p=0.073$, one-sample t-test, $n=5$, $\alpha=0.05$). The validated concentration range was 1.5–45 mg/dL with RE and CV values within the FDA criteria [10]. The ability to dilute solutions yielding high urea concentrations into the validated concentration range could be demonstrated.

3.5. In vitro microdialysis experiments

In Fig. 1 the relationship between RR of urea and flow rate is depicted. In these retrodialysis experiments, increasing the flow rate from 0.8 to 2.5 µL/min resulted in a decrease of RR (interprobe CV, %; $n=6$) from 93.4% (0.9%) to 66.9% (6.5%). Elevated flow rates were associated with higher interprobe variability.

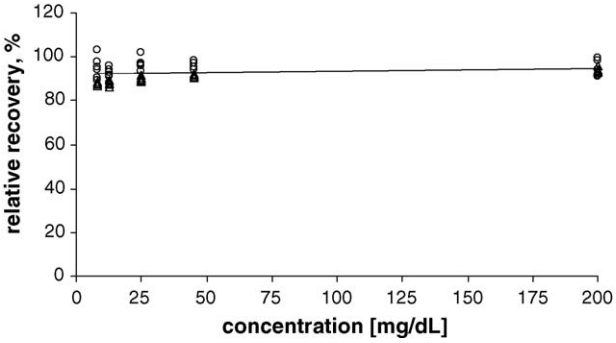

Fig. 2. Dependence of relative recovery on concentration of urea in perfusate or surrounding medium, respectively, during retrodialysis (triangles, $n=30$) and recovery experiment (open squares, $n=30$), each experiment two probes, flow rate 1.5 µL/min. Line: linear regression (weight $c^{-0.5}$).

Table 2
Mean RR_{urea}[a] and mean RRR[b] (=mean ratio of $RR_{linezolid}$ to RR_{urea}) in healthy volunteers and critically ill patients in subcutaneous and muscle interstitial fluid

	s.c.					i.m.				
	Mean RR_{urea}	CV (%)	Mean RRR	CV (%)	*n*	Mean RR_{urea}	CV (%)	Mean RRR	CV (%)	*n*
Healthy volunteers ($n=9$)	54.3	30.6	0.6	33.4	48	78.3	18.3	0.7	18.8	40
Patients ($n=4$)	51.0	43.9	0.7	32.8	18	71.4	20.7	0.7	22.1	17

[a] RR: relative recovery.
[b] RRR: relative recovery ratio.

Dependence of RR on concentration is displayed in Fig. 2. In retrodialysis experiments RR was, on average, 90.1% (CV = 3.0%), ranging from 86.2 to 96.0% and covering a concentration range from 8 to 200 mg/dL. Results of the recovery experiments were comparable with a mean RR of 94.9% (CV = 3.8%), a minimum at 88.2% and a maximum at 103.4%. Linear regression (weight $c^{-0.5}$) between RR, achieved in retrodialysis or recovery experiments, and concentration of surrounding or perfusion medium, yielded a regression line with a slope of 0.016% dL/mg (standard deviation (S.D.): 0.05% dL/mg) and an intercept of 91.6% (S.D.: 4.86%). The line did not run parallel to the abscissa as the 95% confidence interval of slope did not include zero.

3.6. Human microdialysis

Urea concentrations in humans were successfully determined in all microdialysis samples by the assay developed with 1–3 samples per individual and study day. For healthy volunteers the mean RR of urea for the subcutaneous and the intramuscular probe was 54.3% (CV = 30.6%) and 78.3% (CV = 18.3%), respectively (Table 2) whereas in critically ill patients the respective average RR was 51.0% (CV = 43.9%) and 71.4% (CV = 20.7%) (Table 2). Relative recoveries >100% (nine out of 95) were excluded from analysis since urea concentrations in dialysate could not be higher than corresponding plasma and hence unbound tissue concentrations.

The mean ratio of the relative recovery of linezolid to the relative recovery of urea was 0.6 (CV 33.4%, $n=48$) for subcuanteous tissue and 0.7 (CV 18.8%, $n=40$) for skeletal muscle in healthy volunteers, respectively. In critically ill patients the respective ratios were 0.7 for both tissues (s.c.: CV 32.8%, $n=18$; i.m.: CV 22.1%, $n=17$). Additionally, all individual ratios of healthy volunteers and patients are presented in Figs. 3 and 4. The mean ratio of all calculations with a ±20% interval was defined as a quality criterion of these relative recovery ratios. For healthy volunteers, 56.3% of the subcutaneous RR ratios and 80.0% of the skeletal muscle RR ratios were within this interval. Despite the relatively small number of patients, 66.7% (s.c.) and 82.3% (i.m.) of all individual ratios fell within this ±20% interval. Contrary to all other patients and healthy volunteers, where RR of linezolid were always lower than RR of urea, leading to an RRR of ~0.7, in one patient with ID-No. 4 (most right in Fig. 4) relative recovery of linezolid was very similar to that of urea which resulted in RRR of about 1. Interestingly, this value was attributable to different reasons in s.c. or i.m. application: (a) the relatively low s.c. relative recoveries

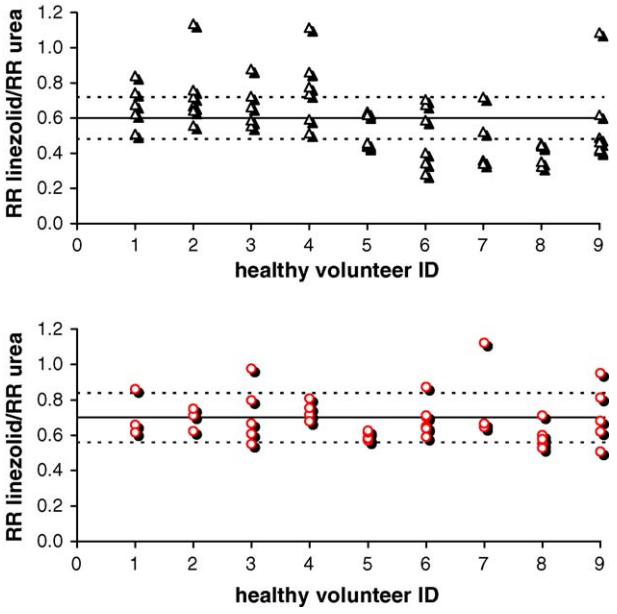

Fig. 3. Individual ratios of $RR_{linezolid}$ to RR_{urea} for healthy volunteers; line: mean ratio, dotted line: mean ratio ±20%; upper panel: s.c., lower panel: i.m.

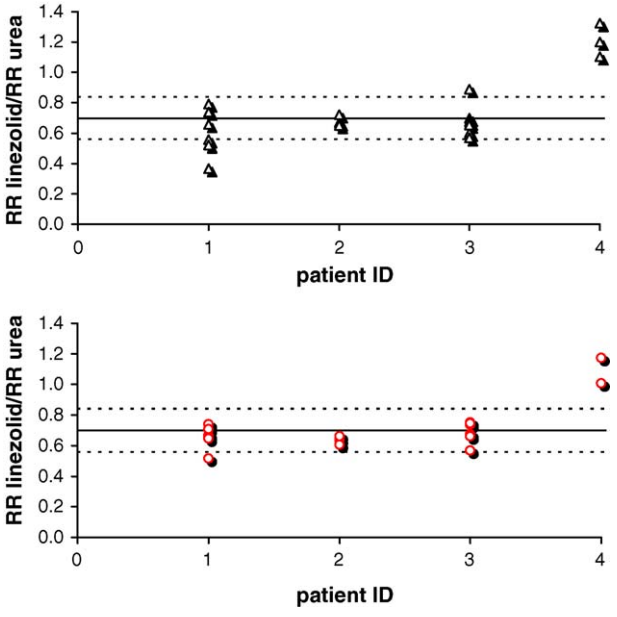

Fig. 4. Individual ratios of $RR_{linezolid}$ to RR_{urea} for critically ill patients; line: mean ratio, dotted line: mean ratio ±20%; upper panel: s.c., lower panel: i.m.

of urea (mean $RR_{urea} = 31.5\%$, $n = 3$) or (b) the relatively high i.m. relative recoveries of linezolid (median $RR_{linezolid} = 88.6\%$, $n = 2$), respectively.

4. Discussion

For the quantitative measurement of urea in microdialysate an assay was developed and validated to fulfill the following requirements: minimal sample volume for analysis, high throughput, economic use of reagents and validity according to international criteria for bioanalytical methods [10]. Since human microdialysate always contains urea, spiking of this matrix with urea is not accomplishable. Consequently, an appropriate proxy matrix, i.e. Ringer's solution, was taken as substitute. Particularly the small total volume that can be collected in microdialysis experiments (~10–30 μL) presented an analytical challenge. This volume was smaller than the minimum volume for sample containers of routine automated analyzers, e.g. Hitachi 917, which requires 50 μL in standard cups (personal communication of K Molter, Roche Diagnostics, Mannheim, Germany). Based on the standard procedure recommended by the manufacturer of the reagent kit (Ecoline™) which requires 10 μL sample and 1 mL reagent volume (R1A and R2) [11] in our new assay a 10-fold reduction in reagent volume as well as halving of sample volume could be achieved.

Relevant interferences of the assay with potentially administered drugs could be detected for sodium amoxicillin/clavulanic acid, sodium flucloxacillin, tobramycin·HCl and vancomycin·HCl. The marketed solution of tobramycin·HCl contained phenol [12] as a preservative which very likely reacted to an indophenol in addition to the assay substrate sodium-salicylate causing a relevant increase in absorption at 690 nm. Moreover, amoxicillin possesses a phenol partial structure and could react accordingly. In spite of these observations the relevance of the interference is most probably irrelevant since drug concentrations in specificity testing did by far exceed therapeutic drug concentrations in human body fluids (clavulanic acid ~20-fold to tobramycin·HCl ~600-fold).

The main goal of the in vitro microdialysis experiments was to demonstrate the independence of relative recovery from urea concentration for a wide range of concentrations as suggested by Stahl et al. [13]. Concentration independence in pilot in vitro studies would indicate that the probe would recover compound from the interstitial space in vivo in a linear fashion with a constant factor [13]. Urea concentrations investigated ranged from subphysiological to pathological levels (8–200 mg/dL). The average relative recovery obtained by the recovery method was 94.9% (CV = 3.8%) and by retrodialysis 90.1% (CV = 3.0%). Although both methods differed significantly (Student's *t*-test, $p < 0.05$, $\alpha = 0.05$), this, however, is not of any relevance to the practical implementation of microdialysis as in general RR variations of 20% are accepted under in vivo conditions [14]. More importantly, linear regression of RR versus concentration indicated an increase in relative recovery by only 1.6% when increasing the urea concentration by 100 mg/dL which is statistically significant but not at all relevant in scientific and practical terms. Hence, concentration independence in the in vitro setting was demonstrated for the large concentration range.

Brunner et al. [15] investigating the concentration dependence of relative recovery of urea under slightly different circumstances obtained an average relative recovery of 94%. This result is in line with the current findings. Since the experimental set-up differed, e.g. probe with divergent membrane length, only the general tendency of high relative recoveries is comparable. Furthermore, both in vitro experiments demonstrated that no relevant adsorption processes to membrane or tubing took place which would complicate prospective in vivo studies.

In comparison to imaging techniques, e.g. positron emission tomography (PET) [16], microdialysis coupled with adequate analytics is a relatively simple method to gather information on the distribution of drugs to the target site. For the determination of absolute concentration profiles (and not only relative changes in concentrations) microdialysis probes need to be calibrated in vivo by an appropriate technique. Conventional calibration, e.g. retrodialysis, imposes additional burden on patient and study personnel as well as a reduction in sampling time. Hence, it is desirable to establish a calibration technique which enables calibration during sampling. A few research groups focused on the application of urea as an endogenous reference compound (=urea reference technique) to determine the relative recovery of drugs [15,17–19]. One experimental group described the use of urea as an endogenous reference compound to determine the relative recovery of glucose and paracetamol in muscle tissue of healthy volunteers ($n = 16$–18 RR determinations) [15]. The authors concluded that relative recovery calculated by urea reference technique was highly variable compared to retrodialysis. Moreover, Strindberg and Lonnroth determined relative recoveries of glucose, lactate and glycerol in subcutaneous and intramuscular tissue by means of this novel calibration technique (in each tissue 7–11 RR determinations). They observed significant correlations between relative recovery determined by urea reference technique and a standard method. Both groups assumed that the ratio of relative recoveries for two selected substances under in vivo to in vitro conditions should be constant over time. Subsequently, the in vivo recovery of any substance a ($RR_{a,\ in\ vivo}$) might be calculable from the relationship between the in vitro recoveries of the substance of interest ($RR_{a,\ in\ vitro}$) and urea ($RR_{urea,\ in\ vitro}$) multiplied by the relative in vivo recovery of urea ($RR_{urea,\ in\ vivo}$): $RR_{a,\ in\ vivo} = RR_{a,\ in\ vitro} \times RR_{urea,\ in\ vivo} \times (RR_{urea,\ in\ vitro})^{-1}$. Relative in vivo recovery of urea was defined as the ratio of the concentration in dialysate to the concentration in plasma. Urea is known to readily equilibrate between plasma and tissues and thus plasma concentrations can be regarded as a good predictor for tissue concentrations [20]. Whereas during in vitro experiments diffusion across the microdialysis membrane appears to limit relative recovery of compounds, during in vivo, analyte transport through the tissue interstitial fluid to the probe membrane has to be considered the limiting step of mass transport. For factors such as tissue tortuosity and limited volume fraction of extracellular fluid both increase the diffusion path length and subsequently reduce the effective analyte diffusion coefficient in tissue [6,15,21]. This characteristic was

also observed for urea in our experiments: the in vivo RR (s.c.) was ∼44% lower than the respective in vitro one. Additionally, binding to extracellular biological structures further diminishes the number of molecules available for diffusion [21]. If those in vivo influential factors were to affect diffusion characteristics of selected compounds equally, the ratios of in vivo and in vitro relative recovery should remain constant. However, this is often not the case due to the complexity of the tissue interstitial fluid and heterogeneity of compounds, respectively. Therefore, the basic idea of our experiments was to use a better concept devoid of in vitro data which correlated the relative in vivo recovery of urea to the relative in vivo recovery of a model analyte (i.e. linezolid). The in vivo recovery of linezolid was determined by retrodialysis, a standard calibration method for microdialysis probes. In contrast to Brunner et al. and Strindberg et al. the study population included healthy volunteers (with a 2.2–7-fold larger number of determinations) as well as critically ill patients. This means that we here present a valuable characterization of the urea reference technique under disease-state conditions which can be found in critically ill patients. Most importantly, both healthy volunteers and patients showed on average a fairly constant ratio of $RR_{linezolid}$ to RR_{urea} of ∼0.7. Although in critically ill patients drastic pathophysiological changes take place including alterations in organ perfusion and edema formation ('third spacing') [22] our results suggest that these changes might either not affect the diffusion characteristics of linezolid and urea or alter them relatively in the same magnitude and in the same direction. As a result, the relative recovery ratio was similar in healthy volunteers and patients. Our findings based on a small number of patients should be proven in a larger patient population. Moreover, a challenge to apply this calibration method were high coefficients of variation for the ratio of $RR_{linezolid}$ to RR_{urea} (∼35% s.c. and ∼20% i.m.). This can be explained by analytical as well as physiological variability. It was suboptimal that urea analytics in microdialysate and plasma were conducted with two different assays. Furthermore, it becomes apparent that relative recoveries of urea in subcutaneous (∼50% mean RR) and muscle (∼75% mean RR) tissue were different to a certain extent. This agrees with findings from Rosdahl et al. [23] who determined RR of urea by means of very slow flow rates. The variability of the ratio of $RR_{linezolid}$ to RR_{urea} is supposed to be due to a differing impact of above mentioned tissue effects (e.g. tortuosity) on the diffusion characteristics of urea and linezolid. Until now, no acceptance criteria for the interindividual and intraindividual precision of the urea reference technique have been described in the literature. According to Muller, by proper calibration intraindividual coefficients of variation for microdialysis measurements should range around 20% [14]. Following this, mean ratio of $RR_{linezolid}$ to RR_{urea} ±20% was defined as quality criterion for the interindividual and intraindividual precision of the urea reference technique. Practicability of urea reference technique could be assumed if 90% of all determined ratios were within this interval.

In conclusion, an innovative, time-saving calibration technique for microdialysis probes was characterized using the relative recovery of urea as a predictor for the relative recovery of a model analyte. Further research, especially inclusion of more patients, is mandatory before this promising technique can be applied in clinical studies using microdialysis for the determination of relative recoveries of analytes.

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References

[1] U. Ungerstedt, J. Intern. Med. 230 (1991) 365–373.
[2] P. Lonnroth, P.A. Jansson, U. Smith, Am. J. Physiol. 253 (1987) E228–E231.
[3] L. Stahle, P. Arner, U. Ungerstedt, Life Sci. 49 (1991) 1853–1858.
[4] 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) 2703–2709.
[5] W.F. Elmquist, R.J. Sawchuk, Pharm. Res. 14 (1997) 267–288.
[6] N. Plock, C. Kloft, Eur. J. Pharm. Sci. 25 (2005) 1–24.
[7] A. Guyton, J. Hall, Textbook of Medical Physiology, ninth ed., W.B. Saunders, Philadelphia, 1996.
[8] J.K. Fawcett, J.E. Scott, J. Clin. Pathol. (1960) 156–159.
[9] C. Buerger, C. Joukhadar, M. Muller, C. Kloft, J. Chromatogr., B: Analyt. Technol. Biomed. Life Sci. 796 (2003) 155–164.
[10] FDA, http://www.fda.gov/cder/guidance/4252fnl.pdf, 2001.
[11] Reagent kit Ecoline™ S+ Urea Berthelot (Cat. no. 1 3115 99 90 305)—instructions for use, DiaSys Diagnostic Systems, Holzheim, Germany, 2003.
[12] Gernebcin® Injektionsloesung—product information, Lilly, Bad Homburg, Germany, 2002.
[13] M. Stahl, R. Bouw, A. Jackson, V. Pay, Curr. Pharm. Biotechnol. 3 (2002) 165–178.
[14] M. Muller, Br. Med. J. 324 (2002) 588–591.
[15] M. Brunner, C. Joukhadar, R. Schmid, B. Erovic, H.G. Eichler, M. Muller, Life Sci. 67 (2000) 977–984.
[16] A.J. Fischman, N.M. Alpert, R.H. Rubin, Clin. Pharmacokinet. 41 (2002) 581–602.
[17] S.N. Ettinger, C.C. Poellmann, N.A. Wisniewski, A.A. Gaskin, J.S. Shoemaker, J.M. Poulson, M.W. Dewhirst, B. Klitzman, Cancer Res. 61 (2001) 7964–7970.
[18] E.J. Eisenberg, W.M. Eickhoff, J. Pharmacol. Toxicol. Methods 30 (1993) 27–31.
[19] L. Strindberg, P. Lonnroth, Scand. J. Clin. Lab. Invest. 60 (2000) 205–211.
[20] S.P. Rastogi, T. Frost, J. Andersen, R. Ashcroft, D.N.S. Kerr, Proc. Eur. Dial. Transplant Assoc. 5 (1968) 102–112.
[21] C. Nicholson, J.M. Phillips, J. Physiol. 321 (1981) 225–257.
[22] M.M. Levy, M.P. Fink, J.C. Marshall, E. Abraham, D. Angus, D. Cook, J. Cohen, S.M. Opal, J.L. Vincent, G. Ramsay, Crit. Care Med. 31 (2003) 1250–1256.
[23] H. Rosdahl, K. Hamrin, U. Ungerstedt, J. Henriksson, Am. J. Physiol. 274 (1998) E936–E945.