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Penicillin and Ciprofloxacin as Internal Standards for Piperacillin and Levofloxacin: An in-vitro Microdialysis Validation Study

Research Article

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Abstract

Microdialysis allows the measurement of free interstitial drug concentrations. Calibration of microdialysis probes is crucial during in-vivo microdialysis and is most commonly achieved by retrodialysis using either the investigational drug itself or an "internal standard". The internal standard should possess similar physicochemical properties with respect to the investigational drug without significant drug-drug interactions. This *in-vitro* microdialysis study was performed to investigate, whether penicillin G and ciprofloxacin are appropriate internal standards for piperacillin/tazobactam and levofloxacin, respectively.
Three forward and retrodialysis experiments were performed using three microdialysis probes with membrane cutoff of 20kDa and a membrane length of 30mm perfused at 2µl/min. Forward dialysis and retrodialysis were performed using piperacillin/tazobactam, levofloxacin and piperacillin/tazobactam + levofloxacin at three concentrations. Loss and gain ratios (LR, GR) were calculated.

LR and GR for piperacillin/tazobactam and levofloxacinalone and in combination were >0.7 and >0.8, respectively. LR of penicillinand ciprofloxacin were >0.7 and >0.8. LR of piperacillin, levofloxacin, penicillinand ciprofloxacin slightly overestimated GR of piperacillinand levofloxacin. Bland-Altman analysis showed good agreement between LR of piperacillin/penicillin and levofloxacin/ciprofloxacin.

This *in-vitro* microdialysis study showed that penicillin and ciprofloxacin may be used as internal standards for piperacillin and levofloxacin for *in-vivo* calibration of microdialysis probes.

Keywords: Microdialysis; Retrodialysis; Calibration; Internal Standard; Antibiotics.

Introduction

Microdialysis allows the measurement of free interstitial drug concentrations and emerged as an elegant technique for determining target site penetration of antimicrobial drugs [1, 2]. Calibration of microdialysis probes is an important aspect of *in-vivo* microdialysis experiments. During sampling, equilibration between the interstitial compartment and the perfusate is usually not complete and concentrations measured in the dialysate may not reflect interstitial drug concentrations. The relative proportion of drug that diffuses across the MD membrane and is subsequently measured in the dialysate fluid is termed "relative recovery". Relative recovery is probe-specific and depends on various factors, such asflow rate, membrane surface, temperature, composition of the perfusion medium, physicochemical properties of investigational drugs and conditions of the interstitial environment [3]. Therefore, calibration of individual microdialysis probes is essential. Calibration is most often achieved by the retrodialysis (RD) method [2]. RD relies on the assumption that analytes diffuse across the semipermeable membrane similarly in either direction. Thus, gain ratios (GR) should be equal to loss ratios (LR) for specific analytes. For RD, either the parent compound (RD by drug) or a

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calibrator with similar physicochemical properties (RD by calibrator orinternal standard) may be used [4]. Although RD by drug is most commonly used, this technique may not be appropriate when analytes of interest are still present in the interstitium, for example after short wash-out periods or when measuring endogenous compounds, or if substantial variations in individual probe recovery are expected throughout an experiment[5]. Alternatively, the internal standard methodmay be used for continuous probe calibration irrespective of interstitial drug concentrations. The calibrator (= internal standard) is added to the perfusion solution during the experiment. The internal standard should have similar physicochemical properties with respect to the investigational drug without significant drug-drug interactions. In in-vitro studies, the recovery of both drugs may be investigated and, assuming that the ratio of both recoveries does not differ between *in-vivo* and *in-vitro, in-vivo* recovery may be calculated [4, 6-8].

Although previous in-vivo microdialysis studies used penicillin as an internal standard for piperacillin [9, 10], the validity of this approach has not been formally investigated to the best of our knowledge. A combined *in-vitro* and *in-vivo* microdialysis study in rats used ciprofloxacin as an internal standard for levofloxacin, showing good agreement in relative recoveries [11].

A planned *in-viv*o microdialysis study investigating pulmonary pharmacokinetics of piperacillin/tazobactam and levofloxacin prompted us to validate the use of internal standards penicillin and ciprofloxacin for piperacillin and levofloxacin, respectively. We sought to investigate the following questions: First, does the loss ratio of internal standard predict the gain ratioof the reference substance during forward dialysis? Second, does the loss ratio of the internal standard differ from the loss ratio of the reference substance during RD? Third, are there relevant interactions between internal standards and reference substances?

This is the first *in-vitro* study investigating whether penicillin and ciprofloxacin are suitable internal standards for piperacillin (in the presence of tazobactam) and levofloxacin, respectively.

Materials and Methods

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies[12].

Experimental setup

For each experiment three microdialysis (MD) catheters (M Dialysis, Sweden) with a membrane cutoff of 20kDa and a membrane length of 30mm were used. MD catheters were perfused at 2µl/min using battery-driven portable MD pumps (107 Microdialysis Pump, M Dialysis, Sweden). Microvials were used for collection of microdialysates and aliquots of applied test solutions (Ref. No. P000001, M Dialysis, Sweden). Borosilicate glass tubes (10ml) were used for immersion solutions (Ref. No. 99445-13, Corning Inc., NY 14831). Throughout all experiments, the hole of each glass tube was covered with Parafilm M in order to avoid evaporation. In order to ascertain that catheters and probes are functioning properly, pre-weighed test-vials were connected during the equilibration period and weighed after disconnection (these vials were not analyzed). Each vial was weighed before and after sampling. All experiments were performed in a shaking water bath at approx. 37°C (Type 1083, GFL, Burgwedel, Germany). Microvials were stored and frozen at -80°C in Microvial Racks after collection (Ref. No. P000028, M Dialysis, Sweden). EPPi Box 45 Cryo boxes were used for further storage until final analysis (Ref. No. KEA45-V81NA, National Lab, Germany).

Substances

Piperacillin/Tazobactam "Kabi"® and Levofloxacin "Kabi"® were purchased from Fresenius Kabi (Fresenius Kabi Austria GmbH, Graz, Austria), Penicillin G-Natrium 1 Mega IE was purchased from Sandoz (Sandoz GmbH, Kundl, Austria), Ciprofloxacin "Hikma"® was purchased from Hikma (Hikma Pharma GmbH, Germany), physiological 0.9% saline solution was purchased from Medica Medicare, Kufstein, Austria. Microdialysis catheters were purchased from M Dialysis, Stockholm, Sweden.Piperacillin/Tazobactam, Levofloxacin,

The following solutions were used:

- normal saline(NS): 0.9% normal saline
- A1: piperacillin/tazobactam 20/2.5µg/ml in normal saline
- A2: piperacillin/tazobactam 50/6.25µg/ml in normal saline
- A3: piperacillin/tazobactam 200/25µg/ml in normal saline
- A4: penicillin G 50µg/ml in normal saline
- B1: levofloxacin1µg/ml in normal saline
- B2: levofloxacin3µg/ml in normal saline
- B3: levofloxacin10µg/ml in normal saline
- B4: ciprofloxacin3µg/ml in normal saline
- C1: piperacillin/tazobactam 20/2.5µg/ml + levofloxacin1µg/ml in normal saline
- C2: piperacillin/tazobactam 50/6.25µg/ml + levofloxacin3µg/ml in normal saline
- C3: piperacillin/tazobactam 200/25µg/ml + levofloxacin10µg/ml in normal saline
- C4: penicillin G50µg/ml + ciprofloxacin3µg

Experiment A: piperacillin/tazobactamand internal standard penicillin G

Forward dialysis with piperacillin/tazobactamwith internal standard penicillin G

3 MD catheters were placed separately in glass vials containing [A1] and were perfused with [A4] at a flow rate of 2µL/min. After a run-in period of at least 60min, three consecutive microdialysate samples were collected at intervals 0-30, 30-60min and 60-90min from each catheter. Thereafter, these steps were repeated with immersion solutions [A2] and [A3].

Reverse dialysis with piperacillin/tazobactam

3 MD catheters were placed separately in glass vials containing [NS] and were perfused with [A1] at a flow rate of 2µL/min. After a run-in period of at least 60min, three consecutive microdialysate samples were collected at intervals 0-30, 30-60min and 60-90min. Thereafter, these steps were repeated with perfusion solutions [A2] and [A3].

Experiment B: levofloxacin and internal standard ciprofloxacin

FWD and RD were performed analogously to experiment A with solutions B1-4.

Experiment C: Co-presence of piperacillin/tazobactam and levofloxacin with co-presence of internal standards penicillin Gand ciprofloxacin

FWD and RD were performed analogously to experiment A with solutions C1-4.

Microdialysis sample analysis

Drug concentrations were determined by HPLC with photometric (piperacillin, tazobactam, penicillin G) or fluorimetric (ciprofloxacin, levofloxacin) detection. The HPLC consisted of a Shimadzu Prominence modular system with a quaternary solvent pump (LC-20AD) with 3 channel degasser (DGU-20A3R), autosampler (SIL-20AC HT, set at 6°C), column oven (CTO-20AC, set at 40°C), and an SPD-M30A photodiode array detector equipped with a 10 mm optical path length cell set at 225 nm (piperacillin, penicillin G) or 210 nm (tazobactam), or an RF-10AXL fluorimetric detector set to ex/em 280/475 nm (levofloxacin, ciprofloxacin, all from Shimadzu, Duisburg, Germany). Separation was performed using a Cortecs T3 2.7µ 100x3 mm (piperacillin, penicillin G), an XBridge BEH C18 2.5µ 50x3 mm (tazobactam, both from Waters, Eschborn, Germany), or a Nucleodur RP18 HTec 3µ 125x4 mm analytical column (levofloxacin, ciprofloxacin, Macherey-Nagel, Düren, Germany). All columns were preceded by a Nucleoshell RP18 2.7µ 4x3 mm column protection system (Macherey-Nagel, Düren, Germany). The mobile phases were mixtures of 0.1 M H3PO4/acetonitrile (67:33% v/v for piperacillin, penicillin G, or 95:5% for tazobactam), or 0.015 M H3PO4/acetonitrile (85:15 v/v) with 1.6 g/L tetrabutylammonium hydrogen sulfate (for levofloxacin, ciprofloxacin). All mobile phases were adjusted to pH 3 with NaOH. The flow rates were 0.4 mL/min (piperacillin, penicillin G, tazobactam) or 0.8 mL/min (levofloxacin, ciprofloxacin), the retention times were 2.4 min (piperacillin), 3.0 min (penicillin G), 1.7 min (tazobactam), 1.8 min (levofloxacin) and 2.3 min (ciprofloxacin), respectively. Samples were injected directly. Injection volume was 1 µL. The linearity has been proven using dilution series of 1-300 mg/L (piperacillin, penicillin G), 0.125-37.5 mg/L (tazobactam) and 0.1-10 mg/L (ciprofloxacin, levofloxacin), respectively. The lowest concentration on the calibration curve has been defined as LLOD. Based on in-process quality controls in 0.9% saline (200/20 mg/L piperacillin and penicillin G, 25/0.25 mg/L tazobactam, 8/0.4 mg/L levofloxacin and ciprofloxacin) imprecision and inaccuracy were <5%.

Calculations and statistics

The gain ratio (GR) for forward microdialysis experiments was calculated according to the following equation:

$$GR = \text{concentration}_{\text{microdialysate}} / \text{concentration}_{\text{immersion solution}}$$

The loss ratio (LR) for retrodialysis was calculated according to the following equation:

$$LR = 1 - \text{concentration}_{\text{microdialysate}} / \text{concentration}_{\text{perfusion solution}}$$

Student's t-tests (paired and unpaired) and ANOVA were used where appropriate for comparison of GR and LR at different concentrations and drug combinations using a commercially available computer program (GraphPad Prism 8).

Results

Gain and loss ratios (GR, LR) were stable during the 90min sampling period, i.e. GR (piperacillin, tazobactam, levofloxacin) and LR (penicillin G, ciprofloxacin) during the 60-90min interval were 98.9 ± 2.6% (piperacillin), 98.9 ± 2.4% (tazobactam), 99.7 ± 3.3% (levofloxacin), 98.2 ± 1.4% (penicillin G) and 98.4 ± 2.2% (ciprofloxacin) of values obtained during the 0-30min interval. Results shown below are mean values obtained during the 0-30, 30-60 and 60-90min intervals from all three MD probes. Detailed results are given in figure 1 and table 1-3.

Experiment A: piperacillin/tazobactam with internal standard penicillin G

FWD of piperacillin/tazobactam showed slightly increasing GR upon switching from low (A1), medium (A2) to high (A3) concentrations of piperacillin/tazobactam. However, this effect was not observed during RD with piperacillin/tazobactam or penicillin G. GR ranged between 0.72-0.80 for piperacillin and 0.83-0.89 for tazobactam. During RD, loss ratios of piperacillin/tazobactam were 0.78-0.88 for piperacillin and 0.87-0.93 for tazobactam, showing good agreement with LR of the internal standard penicillin G (0.79-0.9) during FWD. Results of one MD probe during FWD with piperacillin/tazobactam were deemed implausible due to gain ratios of >2 and were excluded. GR and LR remained stable throughout the whole experiment with exception of MD probe 3 during RD with A3, where LR dropped ~25% after switching from A2 to A3. A reason for this observation could not be found and data were included in the final analysis.

Experiment B: levofloxacin with internal standard ciprofloxacin

FWD of levofloxacin showed consistent gain ratios across low (B1), medium (B2) and high (B3) concentrations of levofloxacin with GR between 0.85-0.88. During RD, LR of levofloxacin were 0.87-0.88, comparable to loss ratios of the internal standard ciprofloxacin (0.88-0.92). There was no significant difference between LR of levofloxacin and ciprofloxacin.

Experiment C: Co-presence of piperacillin/tazobactam and levofloxacin with co-presence of internal standards penicillin G and ciprofloxacin

The simultaneous presence of piperacillin/tazobactam and levofloxacin or penicillin G and ciprofloxacin in immersion and perfusion solutions did not affect GR or LR significantly at low, medium and high concentrations, suggesting no relevant physicochemical interaction. GR during FWD of piperacillin, tazobactam and levofloxacin were 0.85-0.86, 0.88-0.92 and 0.83-0.89, respectively. LR for piperacillin, tazobactam and levofloxacin were 0.79-0.84, 0.88-0.92 and 0.85-0.90, comparable to LR for penicillin G and ciprofloxacin (0.89-0.91 and 0.91.0.93) during FWD. Varying concentrations of piperacillin/tazobactam and levofloxacin had no effect on LR or GR.

Agreement between GR and LR

Table 1. Experiment A: FWD and RD with PIP/TAZ and PEN.

	A1			A2			A3		
FWD	PIP (GR)	TAZ (GR)	PEN (LR)	PIP (GR)	TAZ (GR)	PEN (LR)	PIP (GR)	TAZ (GR)	PEN (LR)
	0.728 ± 0.101	0.835 ± 0.07	0.799 ± 0.078	0.77 ± 0.004	0.859 ± 0.004	0.901 ± 0.016	0.807 ± 0.100	0.894 ± 0.068	0.863 ± 0.085
RD	PIP (LR)	TAZ (LR)		PIP (LR)	TAZ (LR)		PIP (LR)	TAZ (LR)	
	0.806 ± 0.0833	0.876 ± 0.059		0.883 ± 0.049	0.933 ± 0.028		0.786 ± 0.110	0,870 ± 0,077	
Data presented as mean ± SD. FWD = Forward Dialysis; GR = Gain Ratio; LR = Loss Ratio; PEN = Penicillin G; PIP = Piperacillin; TAZ = Tazobactam; RD = Retrodialysis.									

Table 2. Experiment B: FWD and RD with LEV and CIP.

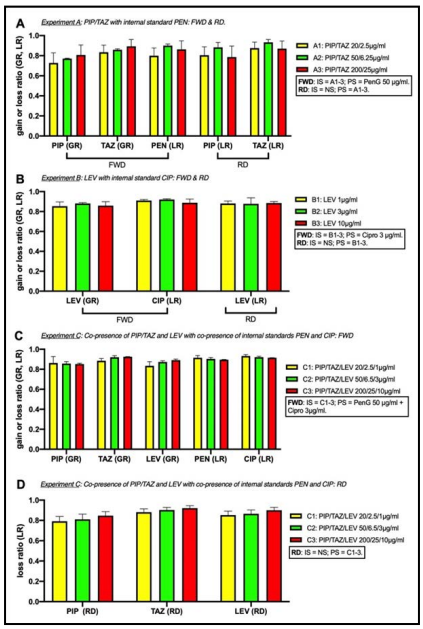
	B1		B2		B3	
FWD	LEV (GR)	CIP (LR)	LEV (GR)	CIP (LR)	LEV (GR)	CIP (LR)
	0.854 ± 0.042	0.910 ± 0.011	0.880 ± 0.010	0.920 ± 0.008	0.858 ± 0.040	0.888 ± 0.038
RD	LEV (LR)		LEV (LR)		LEV (LR)	
	0.880 ± 0.025		0.877 ± 0.060		0.885 ± 0.015	
Data presented as mean ± SD. CIP = Ciprofloxacin; FWD = Forward Dialysis; GR = Gain Ratio; LEV = Levofloxacin; LR = Loss Ratio; RD = Retrodialysis.						

Table 3. Experiment C: FWD and RD with combined PIP/TAZ + LEV and combined PEN + CIP.

	C1					C2					C3				
FWD	PIP (GR)	TAZ (GR)	LEV (GR)	PEN (LR)	CIP (LR)	PIP (GR)	TAZ (GR)	LEV (GR)	PEN (LR)	CIP (LR)	PIP (GR)	TAZ (GR)	LEV (GR)	PEN (LR)	CIP (LR)
	0.863 ± 0.064	0.885 ± 0.023	0.835 ± 0.041	0.916 ± 0.021	0.933 ± 0.014	0.857 ± 0.020	0.921 ± 0.016	0.873 ± 0.012	0.904 ± 0.014	0.921 ± 0.009	0.852 ± 0.009	0.925 ± 0.002	0.891 ± 0.011	0.895 ± 0.002	0.915 ± 0.001
RD	PIP (LR)		TAZ (LR)		LEV (LR)	PIP (LR)		TAZ (LR)		LEV (LR)	PIP (LR)		TAZ (LR)		LEV (LR)
	0.791 ± 0.047		0.881 ± 0.033		0.852 ± 0.040	0.811 ± 0.051		0.902 ± 0.026		0.865 ± 0.038	0.846 ± 0.040		0.921 ± 0.024		0.900 ± 0.028
Data presented as mean ± SD. CIP = Ciprofloxacin; FWD = Forward Dialysis; GR = Gain Ratio; LEV = Levofloxacin; LR = Loss Ratio; PEN = Penicillin G; PIP = Piperacillin; TAZ = Tazobactam; RD = Retrodialysis.															

Figure 1: Loss and gain ratios for experiments A-C.

A: Experiment A, FWD + RD. B: Experiment B, FWD + RD. C: Experiment C, FWD. D: Experiment C, RD.Data presented as mean values obtained during the 0-30, 30-60 and 60-90min intervals from all three MD probes. CIP = Ciprofloxacin; FWD = Forward Dialysis; GR = Gain Ratio; IS = Immersion Solution; LEV = Levofloxacin; LR = loss ratio; NS = Normal Saline; PEN = Penicillin G; PIP/TAZ = Piperacillin/Tazobactam; RD = Retrodialysis; PS = Perfusion Solution.



LR of piperacillin and levofloxacin slightly overestimated GR of piperacillin and levofloxacin. LR piperacillin was 106.8 ± 12.6% of GR piperacillin and LR levofloxacin was 102.1 ± 6.5% of GR levofloxacin. Similarly, LR of internal standards penicillin G and ciprofloxacin were greater than GR of piperacillin and levofloxacin, 110.7 ± 4.9% for LR penicillin G and 104.9 ± 4.9% for LR ciprofloxacin. Therefore, both RD methods show similar performance in FWD (Figure 2).

Agreement between LR of piperacillin-penicillin G and levofloxacin-ciprofloxacin

Bland-Altman analysis showed good agreement between RD by internal standards and RD by drug (Figure 3). The point outside the 95% limits of agreement in the piperacillin vs. penicillin G plot may be explained by an unexpected drop in LR for piperacillin of MD probe 3 after switching from solution A2 to A3. Excluding this potential outlier, loss rates obtained with internal standards penicillin G and ciprofloxacin would be ±7% and -13%/+6% of loss rates obtained by piperacillin and levofloxacin.

Discussion

This is the first in-vitro study specifically investigating whether

Figure 2: Comparison of loss ratios of piperacillin, levofloxacin, penicillin and ciprofloxacin with gain ratios of piperacillin and levofloxacin.

Data presented as mean ± SD. Values were calculated as follows: e.g. for PIP (LR) and PIP (GR): % = (LR/GR)*100. CIP = Ciprofloxacin; GR = gain ratio; LEV = Levofloxacin; LR = loss ratio; PEN = Penicillin G; PIP = Piperacillin.

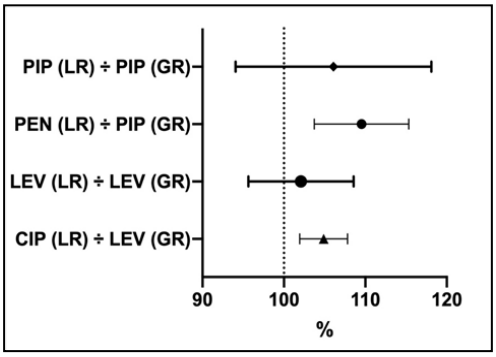
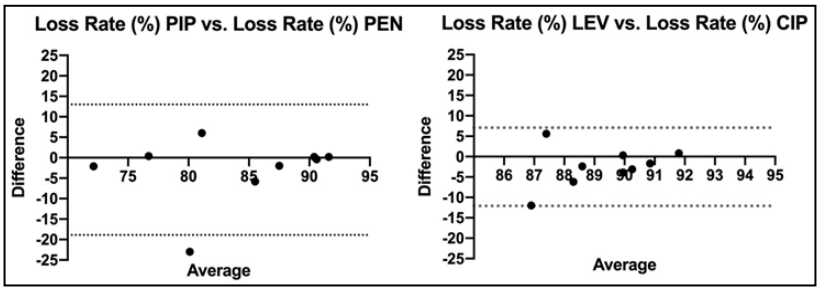


Figure 3: Bland-Altman plots of loss rates of piperacillin vs. penicillin G and levofloxacin vs. ciprofloxacin.

Absolute difference against averages of loss rates (in %) of PIP vs. PEN and LEV vs. CIP obtained during experiments A and B across all concentrations with upper and lower 95% limits of agreement. CIP = ciprofloxacin, LEV = levofloxacin, PEN = penicillin G, PIP = piperacillin.



penicillin G and ciprofloxacin are appropriate internal standards for piperacillin/tazobactam and levofloxacin, respectively. Three concentrations of piperacillin/tazobactam and levofloxacin were testedin FWD and RD experiments both alone and in combination. Concentrations were chosen based on expected plasma time-concentration curves with standard dosing regimens. Fixed concentrations of penicillin Gand ciprofloxacinwere used as internal standards during FWD for piperacillin and levofloxacin, respectively.

LR and GR were relatively high throughout experiments A-C for all substances and concentrations. High LR and GR were probably due to the membrane lengths of 30mm, low flow rates and absence of interfering factors encountered in in-vivo experiments. Reported recoveries obtained during in-vivo RD for piperacillin and levofloxacin, as well as the internal standard ciprofloxacin are generally well below values measured in the present study [8, 9, 11, 13]. However, one study reported 98% recovery for piperacillin, albeit at much lower flow rates of 0.3µl/min[14]. This underscores that recoveries determined in-vitro should not be used to calculate interstitial drug concentrations *in-viv*o.

There were no significant variations in LR or GR when study drugs were used in combination, suggesting no relevant physicochemical interaction *in-vitro*. GR increased minimally with increasing piperacillin/tazobactam concentrations. Likewise, RD increased slightly with increasing concentrations of piperacillin/tazobactam/LEVO in experiment C. However, the magnitude of this effect does not bear clinical significance. For all other experiments, a clear relationship between drug concentrations and LR or GR could not be observed.

LR of piperacillin, penicillin G, levofloxacin and ciprofloxacinall slightly overestimated respective GR by piperacillin and ciprofloxacin. Overall, RD by internal standards was in good agreement with RD by parent compounds.

Conclusion

In conclusion, this *in-vitro* microdialysis study showed that penicillin G and ciprofloxacin may be used as internal standards for piperacillin and levofloxacin for *in-vivo* calibration of microdialysis probes using the internal standard method.

Data sharing statement

The data that support the findings of this study are available on request from the corresponding author.

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