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Experimental Insights and Recommendations for Successfully Performing Cerebral Microdialysis With Hydrophobic Drug Candidates

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ABSTRACT

Cerebral microdialysis in rodents represents a robust and versatile technique for quantifying the pharmacologically relevant unbound fraction of drugs in the brain. When this unbound fraction is simultaneously determined in plasma, it facilitates the calculation of the corresponding unbound plasma-to-brain partition coefficient ($K_{p,uu}$) for a given compound in vivo. This coefficient is critical for understanding the penetration and distribution of drugs across the blood–brain barrier (BBB). However, obtaining valid and accurate microdialysis data can be particularly challenging for hydrophobic drugs due to their pronounced non-specific interactions with the components of the microdialysis system. The present study reports the outcomes of comprehensive microdialysis investigations in rodents, focusing on three hydrophobic compounds: actinomycin D, selinexor, and ulixertinib. These compounds exhibited varying degrees of non-specific binding to the surfaces of the microdialysis apparatus, leading to low recovery rates and substantial carry-over effects. To diminish these limitations, strategies such as surface coating and the use of optimized materials were employed to enhance the reliability of the microdialysis system. To ensure the robustness and reproducibility of microdialysis-related research outcomes, our experimental findings were supplemented with a narrative literature review. This review encompassed keyword-driven PubMed-indexed publications on microdialysis from 1970 to 2024, providing a broader context for the challenges and solutions associated with the technique. By integrating empirical results with practical recommendations, this study offers a comprehensive resource aimed at advancing the application of cerebral microdialysis in preclinical drug development, particularly for compounds with challenging physicochemical properties.

Julia K. Sundheimer and Julia Benzel contibuted equally to this work. Senior authors Max Sauter and Kristian W. Pajtler contributed equally to this work.

Summary

- What is the current knowledge on the topic?
  - Cerebral microdialysis has been successfully applied to various drugs. However, hydrophobic compounds, which have a greater likelihood of crossing the BBB, pose unique challenges due to their propensity for non-specific binding. A comprehensive overview addressing these challenges is currently lacking.
- What question did this study address?
  - This study focused on the challenges associated with non-specific binding of hydrophobic drugs to microdialysis equipment. It provides practical recommendations to minimize these issues and ensure the successful execution of microdialysis experiments, enabling reliable calculation of the $K_{p,uu}$ for unbound drugs.
- What does this study add to our knowledge?
  - This study addresses drug-specific, calibration-related, and model-related challenges encountered in cerebral microdialysis experiments. It presents practical solutions for hydrophobic compounds and introduces a decision tree to guide the selection of appropriate microdialysis equipment, including tubing and probe materials, tailored to the specific characteristics of the drug.
- How might this change clinical pharmacology or translational science?
  - Microdialysis is increasingly applied in both preclinical and clinical settings for various applications, such as neurophysiological and cancer research. The findings of this study are readily translatable to clinical contexts, as similar cerebral microdialysis setups are already employed in human studies, until now they have primarily been applied to measure endogenous metabolites [1]. By adapting microdialysis methods for hydrophobic drugs, this work provides a framework for obtaining accurate and clinically relevant data, thereby advancing clinical pharmacology and translational science.

1 | Introduction

The blood–brain barrier (BBB) imposes a significant limitation on the penetration of most drugs from the bloodstream into brain tissue. Consequently, evaluating drug concentrations within the brain is a critical component in the development of therapeutic candidates targeting central nervous system (CNS) diseases. Cerebral microdialysis is a highly effective technique for this purpose, as it enables the continuous measurement of unbound drug concentrations, which represent the pharmacologically active fraction of the drug [2]. These measurements can be utilized to assess a compound's ability to achieve therapeutically relevant concentrations at the anatomical site of action and to characterize brain tissue-specific pharmacokinetics (PK) in vivo [3]. This methodology aligns with the reduction principle of the 3R concept (replace, reduce, refine) in animal experimentation, underscoring its ethical and scientific value.

By concurrently measuring unbound drug concentrations in plasma and brain tissue, cerebral microdialysis facilitates the calculation of the unbound plasma-to-brain partition coefficient ($K_{p,uu}$) [4]. This parameter is instrumental in elucidating the mechanisms of BBB transport, distinguishing between active influx/efflux processes and passive diffusion. Moreover, $K_{p,uu}$ serves as the critical factor for estimating unbound brain drug concentrations based on plasma measurements, further enabling mechanistic insights into BBB permeability and drug distribution [5].

The microdialysis apparatus comprises a pump system that delivers a perfusate—commonly artificial cerebrospinal fluid (aCSF) or Ringer's solution—at an optimal flow rate through tubing and into a probe containing a semipermeable membrane, which is positioned in either healthy or pathological brain tissue [6]. Following systemic drug administration, the unbound drug within the extracellular fluid (ECF) passively diffuses along the concentration gradient across the probe's membrane into the dialysate (see Figure 1A). The dialysate is subsequently collected in small fractions over defined time intervals using a fraction collector. Drug concentrations in the dialysate are quantified via highly sensitive analytical techniques such as ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). The accurate determination of unbound drug concentrations in the brain necessitates a calibration methodology to evaluate the recovery rate of each probe, which reflects the ratio between the dialysate drug concentration and the actual concentration in the ECF. Proper setup and calibration of the microdialysis equipment are paramount to achieving precise and reliable measurements [4].

Although microdialysis has been successfully employed to measure hydrophilic compounds [7], its application to hydrophobic drugs poses significant challenges. Hydrophobic compounds, which often exhibit superior BBB permeability, tend to exhibit pronounced non-specific binding to microdialysis system components and are typically present at very low unbound concentrations in the ECF [8, 9]. This article provides a detailed examination of the critical considerations and experimental adaptations necessary for effective microdialysis of hydrophobic compounds. Based on results from our extensive experimental investigations with hydrophobic drugs such as actinomycin D, selinexor, and ulixertinib (Figure 1C)—identified in different studies as potential candidates for treatment of brain tumor patients [10–12]—along with a comprehensive keyword-driven literature review, challenges were categorized into drug-related, method-related, and model-related factors (Figure 1B). The discussion includes preparatory experiments and recommendations to optimize the accuracy and validity of microdialysis studies involving hydrophobic drugs.

2 | Methods

2.1 | Literature Search

A comprehensive keyword-dependent literature search for cerebral microdialysis studies was conducted by two independent researchers (JS and JBe), encompassing publications from 1970 to July 2024. The search was performed using PubMed, applying the search terms listed in Table S1, without imposing language restrictions.

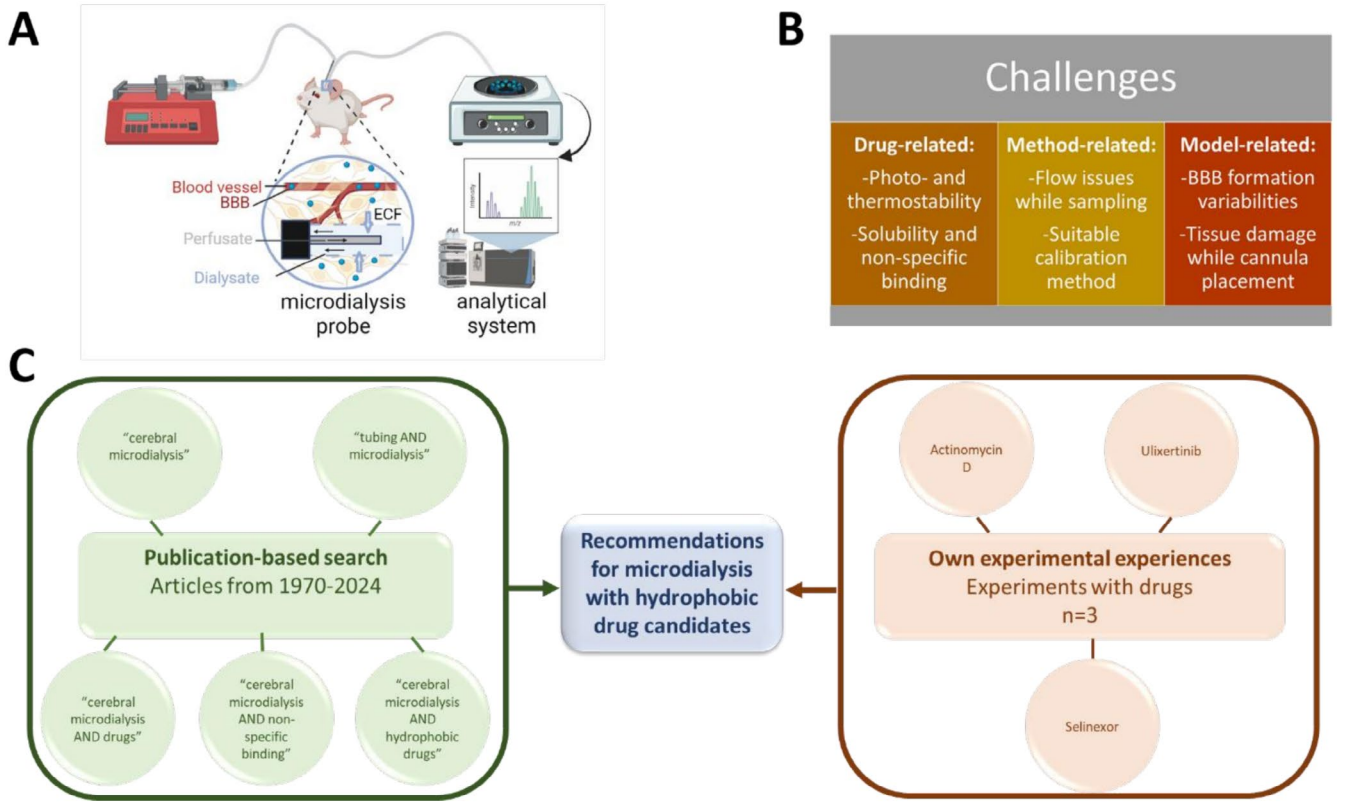


FIGURE 1 | Setup of cerebral microdialysis created in BioRender. Sundheimer, J. (2025) https://BioRender.com/a91j359 (A), main challenges occurring while experimental procedures (B), and design of the keyword-dependent publication-based and experimentally confirmed design of recommendations for hydrophobic drug candidates (C).

2.2 | Dark–Light and Temperature Experiments

The thermostability of actinomycin D (Recordati Rare Diseases) was assessed by storing known concentrations of the drug in Ringer's solution containing 1% bovine serum albumin (BSA) under specified conditions for 24 h at −20°C, 4°C, and room temperature. Photostability was evaluated by exposing drug solutions to light or keeping them in darkness. Additionally, drug solutions were subjected to 37°C using a water bath, heating blocks, or plates.

2.3 | In Vitro Probe Recovery Studies

Microdialysis recovery for each probe (MD-2211, Bioanalytical Systems Inc.; CMA7 and CMA8, CMA Microdialysis) was determined in vitro using retrodialysis recovery. The probes were immersed in stirred blank Ringer's solution containing 0.5%–1.5% BSA at 37°C. For ulixertinib, 0.01% or 0.1% Dimethylsulfoxid (DMSO) was added in addition to BSA. Each probe was perfused with a 100 ng/mL drug solution (actinomycin D, selinexor (Karyopharm), and ulixertinib (Biomed Valley Discoveries)) at a flow rate of 0.5 μL/min. After equilibration, three consecutive fractions were collected at 1 h intervals. Dialysate samples were stored at −80°C for subsequent analysis.

2.4 | Nominal Concentration Test

To assess potential drug loss due to non-specific binding, a solution with a known drug concentration was prepared in Ringer's solution and transferred to three different types of vials: polypropylene reaction tubes, plastic microdialysis reaction tubes, and glass tubes. Drug concentrations were measured after each transfer, and recovery was calculated using Formula 1 (Table S2).

2.5 | Adsorption to Tubing and Retention Test

Solutions with a pre-defined drug concentration (selinexor 100 ng/mL or ulixertinib 100 ng/mL) were prepared and loaded into a 1 mL microdialysis glass syringe. The solution was pumped through a 1 m-long tubing system made of either fluorinated ethylene propylene (FEP) or polyetheretherketone (PEEK) tubing. Samples were collected at three time points (T1–T3) over a 3 h period at a flow rate of 0.5 μL/min. Additionally, samples were collected directly from the syringe before (S1) and after (S2) perfusing the tubing. Subsequently, the syringes were cleaned, filled with Ringer's solution (without drug), and the sampling process was repeated (T4-T6, S3, S4). Recovery rates were calculated using Formula 2 for the first part of the experiment and Formula 3 for the second part (Table S2).

2.6 | Calculation

Relative recovery rates in calibration studies were calculated using the formula provided for in vitro retrodialysis (Table 3 II).

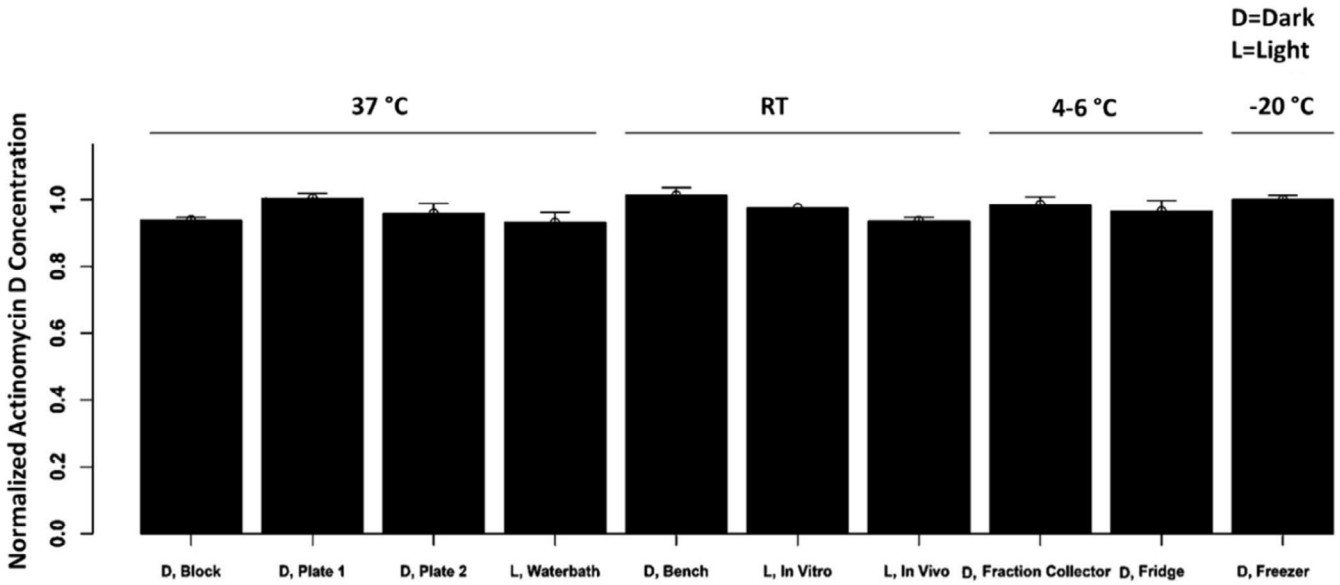


FIGURE 2 | Stability-based part of the decision tree with experimental investigation for actinomycin D. Actinomycin D in 1% BSA Ringer's solution was exposed to light (L) or darkness (D) for 24 h at various temperatures at different conditions: 37°C (calibration studies, water bath, heat block or plate), RT (bench, in vitro /in vivo studies), 4°C –6°C (fraction collector storage), −20°C (short-term storage). Drug concentrations measured by UPLC-MS/MS. Data presented as mean ± S.D.

2.7 | UPLC-MS/MS Analysis

Quantification of drug concentrations was performed using validated UPLC-MS/MS methods, as previously described for actinomycin D [13], selinexor [14], and ulixertinib [11].

2.8 | Data Visualization

All diagrams were generated using R Studio, while figures were created with BioRender.com.

3 | Results

The results section highlights the primary drug-related challenges observed in experimental setups using the three hydrophobic drugs: actinomycin D, selinexor, and ulixertinib.

3.1 | Tested Drugs Are Stable in Microdialysis Conditions

The stability of the tested drugs under experimental conditions was a critical factor. Among the three drugs, actinomycin D is known to be photosensitive. However, additional tests confirmed its stability at room temperature, 37°C, 4°C, and −20°C in dark and light conditions (Figure 2), eliminating the need for specific adaptations to the microdialysis setup. Achieving high recovery rates requires selecting an appropriate membrane tailored to the drug's chemical characteristics (molecular weight of actinomycin D is 1255.4 Da, for selinexor 443.3 Da, and for ulixertinib 433.3 Da). Testing different molecular weight cut-offs (MWCO, CMA7: 55 kDa, CMA8: 20kDA, MD2211: 35 kDa (Table S3)) for microdialysis probes led to the identification of a 55 kDa membrane (CMA7) that demonstrated retrodialysis recovery rates of 81.6% ± 9.9% for actinomycin D and 80.0% ± 7.25% for selinexor. However, ulixertinib showed only a recovery rate of 1.5% (Table 1) for the CMA7 probe, suggesting that the material of the probe is not suitable for ulixertinib.

TABLE 1 | Concentrations (ng/mL) and recovery rates from ulixertinib using retrodialysis with CMA7 probe.

In vitro retrodialysis	Probe 1 (0.01% DMSO with 0.5% BSA Ringer)	Probe 2 (0.1% DMSO with 1% BSA Ringer)
1 h	2.2	< 1
2 h	0.9	< 1
3 h	< 1	< 1
Stock	102	70
Recovery rate (%)	1.5%	1%[a]

[a]0.5 was assumed as the dialysate fraction to calculate recovery loss.

3.2 | Adding BSA to Ringer's Solution Decreases Non-Specific Binding

To ensure accurate concentration measurements, we systematically evaluated non-specific binding to various materials and components within the microdialysis system. Actinomycin D exhibited strong adhesion to standard polypropylene vials, necessitating pre-coating with different solutions (e.g., polylysine, triton X, heparin). As pre-coating did not stabilize concentrations, 1% bovine serum albumin (BSA) was added to the Ringer's solution and decreased non-specific binding [13]. Selinexor exhibited significant

TABLE 2 | Non-specific binding studies of selinexor comparing different vials.

	Measured selinexor concentration (ng/mL) in Ringer's solution	Percent difference to polypropylene tubes
Polypropylene tubes	2536 ± 175	0
Dialysate tubes	2554 ± 102	−0.007
Glass	2109 ± 23	−20.25

non-specific binding to glass tubes, which were excluded from subsequent experiments (Table 2). Conversely, ulixertinib showed no evidence of binding to the tested vials (data not shown).

Non-specific binding was also investigated for microdialysis tubing materials. For selinexor, binding was observed with both FEP and PEEK tubing. However, adding BSA to the Ringer's solution improved recovered selinexor concentrations (T1, T2, and T3) from 0 to around 80 ng/mL, with optimal performance observed using FEP tubing and 1% BSA (Figure 3A–D). For ulixertinib, FEP tubing demonstrated reduced binding compared to PEEK tubing, particularly when combined with a 0.5% BSA and 0.01% DMSO Ringer's solution (Figure 3E,F). Drug adherence to probe membranes was also evaluated. Various membrane materials, including cuprophane, polyacrylethersulphone (PAES), polyethersulfone (PES), polyurethane, and cellulose, were tested (Table S3). Among these, the PES membrane of CMA7 probes provided the best retrodialysis recovery rate for actinomycin D (81.6% ± 9.9%) [13].

3.3 | In Vitro Pre-Calibration Challenges in Microdialysis

We selected retrodialysis and in vitro dialysis as calibration methods. All given recovery rate values were calculated based on retrodialysis, and their reliability was ensured using in vitro dialysis recovery values. Calibration was performed prior to the in vivo experiment. However, calibration of the microdialysis probe before in vivo experiments was identified as a potential source of drug residue within the system, which could confound subsequent analyses. This effect was particularly evident for actinomycin D, as previously reported [13].

4 | Discussion

4.1 | Drug-Related Challenges

The ability of drugs to penetrate the BBB is influenced not only by active transport mechanisms mediated by drug transporters but also by intrinsic compound properties such as molecular weight, hydrodynamic radius, hydrophobicity, and stability [15]. These properties also govern non-specific binding and probe penetration, directly affecting the choice of microdialysis equipment and experimental parameters. Based on our findings with actinomycin D, selinexor, and ulixertinib, as well as insights derived from the literature, we identified a series of critical preparatory steps that are required to ensure robust and reliable microdialysis outcomes. These considerations have been synthesized into a decision tree (Figure 4).

4.1.1 | Accurate Concentration Measurements Depend on Photosensitivity and Temperature Stability

Drug stability under experimental conditions is crucial for achieving accurate concentration measurements. Stability considerations must account for both photo- and temperature-dependent factors, as drugs are routinely exposed to room temperature (20-25°C) and body temperature (36-37°C) during cerebral microdialysis experiments. For thermally unstable drugs, adaptations such as rapid transfer of samples to refrigerated or frozen conditions, or the use of a refrigerated fraction collector, can mitigate degradation (Figure 4).

For photosensitive drugs, implementing protective measures such as aluminum foil covers, opaque tubing, or darkened sample containers is essential to prevent degradation caused by light exposure. In instances where drug stability cannot be ensured, immediate sample processing or the inclusion of stabilizing additives is recommended.

4.1.2 | Molecular Weight and Hydrodynamic Radius Influence Microdialysis Setup and Probe Selection

The molecular weight of the analyte under investigation is a decisive factor in selecting the appropriate microdialysis membrane. Membranes are defined by their MWCO, typically ranging from 6 to 100 kDa to 1–3 MDa (Table S3). Approximately 80%–90% of molecules with a molecular weight comparable to the MWCO cannot pass through the membrane, while smaller molecules pass more efficiently. To maximize recovery, the analyte's molecular weight should ideally represent no more than 20% of the membrane's MWCO, as recovery efficiency declines exponentially for molecules nearing the MWCO threshold [16, 17]. For the low-molecular-weight compounds tested in this study (≤ 1.3 kDa), standard membranes with a 55 kDa MWCO proved sufficient. However, larger molecules may necessitate membranes with a MWCO of 100 kDa. In such cases, the use of an osmotic colloid like dextran 500 is recommended to prevent ultrafiltration-related fluid loss. For even larger proteins or antibodies (> 100 kDa), a push-pull system is critical to prevent perfusion fluid leakage into tissue (Figure 4, [18, 19]). Additionally, macromolecules are prone to aggregation, which can limit their ability to pass through the membrane. The hydrodynamic radius or diameter of these molecules further influences membrane selection. For example, 100 kDa PES membranes have a pore size of approximately 10 nm, which may restrict penetration of larger proteins [20–22]. The length of the membrane is another impact factor in selecting the most suitable probe, with longer probes (1–4 mm) commonly used for sampling deeper brain regions. As summarized in the decision tree (Figure 4), a standard microdialysis setup can be used for smaller molecules requiring membranes with a MWCO up to 100 kDa, whereas larger molecules typically require a push-pull system for accurate sampling.

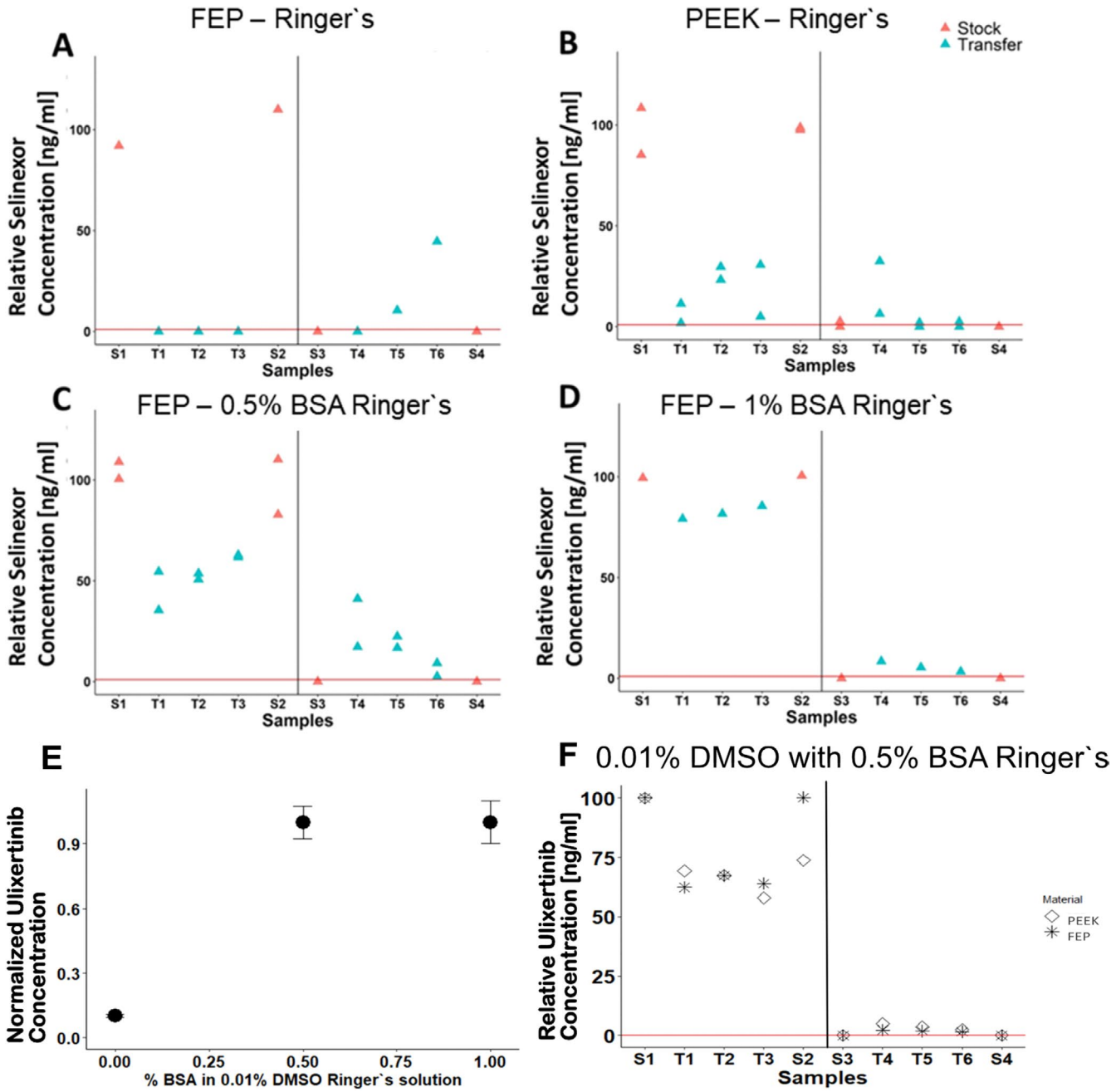


FIGURE 3 | Non-specific binding part of the decision tree and experimental investigation for selinexor and ulixertinib. Non-specific binding of selinexor to fluorinated ethylene propylene (FEP) (A) and polyetheretherketone (PEEK) tubing using known concentrated stocks of selinexor in Ringer's (B). Selinexor in 0.5% BSA Ringer's solutions (C), and 1% BSA Ringer's solution pumped through FEP tubing (D). Recovered ulixertinib concentrations in dependence to increasing BSA supplementation of Ringer's DMSO perfusate (mean ± S.D.) (E). Non-specific binding to FEP and PEEK tubing using known concentrated stocks of ulixertinib in BSA Ringer's DMSO solution (F). A defined drug stock solution (S1 and S2) was pumped (0.5 μL/min) by a syringe through the system. Transfer samples were collected every hour (T1, T2 and T3). After a cleaning procedure of the syringe, Ringer's solution without drug (S3 and S4) was pumped through the system. Transfer samples were collected (T4, T5 and T6) to identify potential contaminations in the system. Drug concentrations were measured by UPLC-MS/MS. The LLOQ of 0.1 ng/mL is indicated as the red lines.

4.1.3 | Hydrophobic Drugs Adhere to Microdialysis Tubing and Probes

Non-specific binding of hydrophobic drug candidates represents a major challenge, as it can lead to inaccurate quantification and carry-over effects from probe calibration. To address this, the solubility of the drug in the microdialysis buffer (mimicking extracellular fluid) must be verified, and the potential for adhesion to system components must be systematically evaluated.

Drug adhesion to collection vials is a well-documented issue, with polystyrene, glass, and polypropylene vials exhibiting varying non-adherent characteristics. Testing different vial materials is essential prior to microdialysis experiments. Similarly,

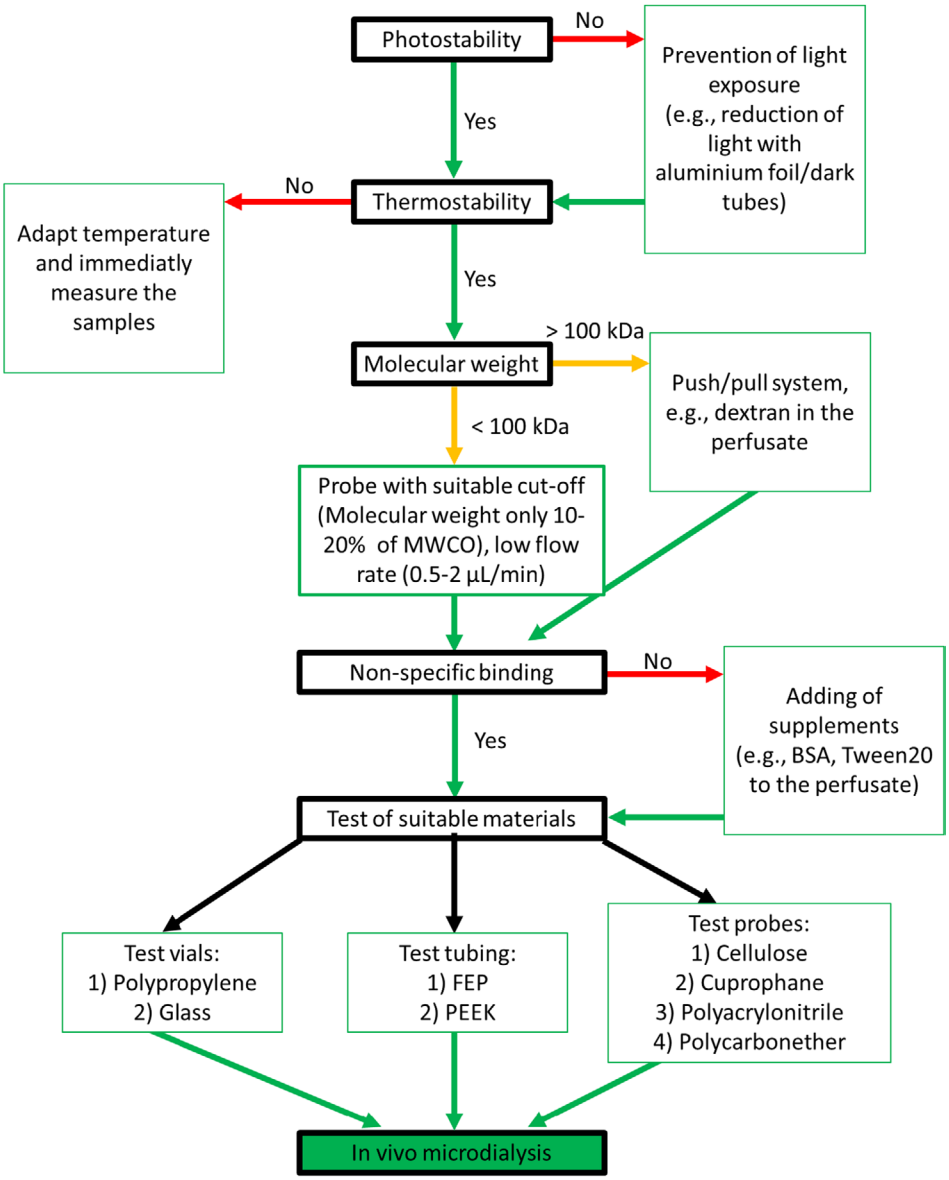


FIGURE 4 | Decision tree to successfully test hydrophobic drugs in microdialysis-based experiments.

tubing materials must be tested for non-specific binding. Both FEP and PEEK tubing are commonly available, with a swept volume of 1.2 µL/10 cm. FEP tubing, being elastic and transparent, facilitates the observation of flow issues, while PEEK tubing is opaque and has low oxygen permeability, making it suitable for highly oxidizable or light-sensitive compounds (Table S4).

Additionally, tubing length plays a significant role in reducing flow-related issues. Tubing should be as short as possible while maintaining sufficient length to accommodate animal movement and equipment flexibility. Flow dynamics within tubing are governed by parameters such as length, radius, flow rate, pressure, and fluid viscosity, as described by Poiseuille's law [23].

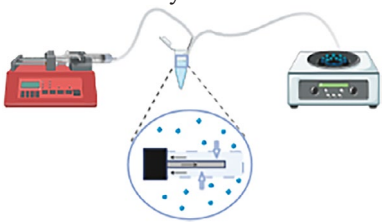
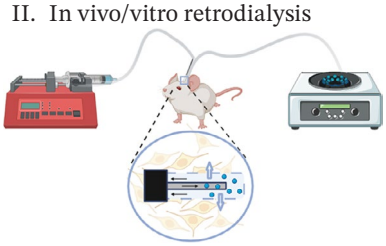
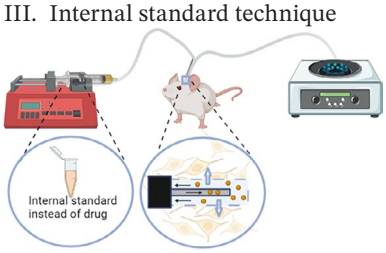
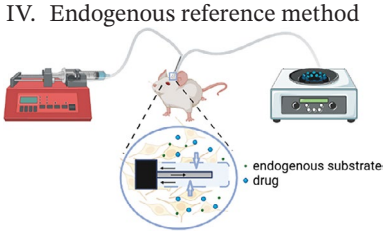
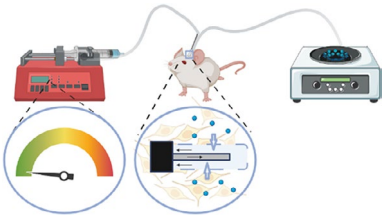
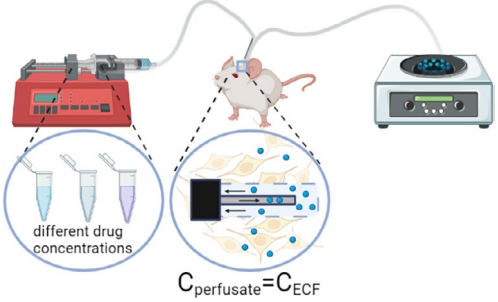
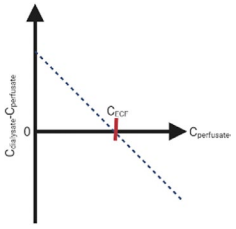
Non-specific binding may also occur at the probe membrane, which has a large surface area. While most membranes perform well with hydrophilic compounds [24], they are less effective for hydrophobic drugs. It is, therefore, advisable to evaluate probe penetration properties experimentally before beginning microdialysis. An additional potential non-specific binding that can occur in conjunction with the swivel, which is often made of stainless steel and is used to avoid the tangling of the animal in the tubing, should be considered. In summary, in addition to testing the compound's solubility in aCSF, its potential adherence to all parts of the microdialysis setup must also be considered and tested prior to starting microdialysis experiments (Figure 4).

To mitigate non-specific binding, pre-coating system components or incorporating additives into the microdialysis buffer is recommended. Commonly used additives include BSA, Tween20, and dextrans, which have been shown to reduce non-specific binding [24, 25].

4.2 | Method-Related Challenges

Cerebral microdialysis relies on achieving a stable equilibrium of drug diffusion between the perfusate and the ECF over the

TABLE 3 | Comparison of different calibration models including illustrations, advantages, disadvantages, and calculations. RR-relative recovery, Created in BioRender. Benzel, J. (2025) https://BioRender.com/d72w793.

Calibration method	Advantage	Disadvantage	Calculation
I. In vitro dialysis 	• No mice needed • Easy to control	• Differences between in vitro and in vivo	RR = C dialysate/C external (Epi)
II. In vivo/vitro retrodialysis 	• Mass transfer resistance considered	• Ensurance of drug-free brain necessary	RR = (C perfusate—C dialysate)/C perfusate $C_{ECF} = C_{dialysate}$/recovery
III. Internal standard technique 	• Continuous calibration during experiments	• Interaction between drug and standard	
IV. Endogenous reference method 	• Continuous calibration during experiments	• Different properties or interaction between drug and standard	
V. Zero/ultra slow flow rate method 	• Increased recovery rate	• Small sample volume	RR = (1 − exp. − PeAQ) × 100
VI. (Dynamic) no-net-flux method 	• Well investigated • Less time-consuming (dynamic)	• Steady state necessary • Large number of animals (dynamic)	

course of the experiment. This equilibrium is critical for accurately calculating the ECF drug concentration from the probe's relative recovery. The recovery rate is influenced by the flow rate and must be determined individually for each probe through proper calibration. Thus, the selection of an appropriate calibration method and flow rate is essential (Table 3).

4.2.1 | Flow Observation and Equilibrium

Maintaining a stable and consistent flow rate throughout the experiment is critical, as fluctuations can directly affect the recovery rate and compromise data quality. Clogging of the tubing or probe may result in reduced or interrupted flow, which can lead to inaccurate fraction collection. To mitigate these risks, it is recommended to use a liquid flow meter or regularly check the collected fractions for consistent volumes.

For efficient diffusion equilibrium across the probe, the flow rate should be kept as low as possible. However, in cases where high temporal resolution is required for short time-sampling, slightly higher flow rates may be necessary to preserve accuracy.

4.2.2 | Calibration Method

Calibration is essential for accurately determining the recovery rate of a microdialysis probe. The recovery rate depends on several factors, including molecular weight, temperature, flow rate, membrane cut-off, probe length, analyte gradient, and tissue-specific parameters such as diffusion rate and fluid volume [26]. For in vitro setups, these variables must be carefully controlled to simulate in vivo conditions. Calibration can be performed using in vitro dialysis or retrodialysis, and various in vivo methods are available, as summarized in Table 3.

4.2.2.1 | I in Vitro Dialysis. In vitro dialysis involves placing the probe in a large volume of solution to maintain constant drug concentrations (imitating the brain concentration) over time. The recovery rate is calculated based on the collected drug concentration in the dialysate $C_{dialysate}$ and the original drug concentration in the vial $C_{external}$. While this method allows accurate measurement of the drug concentration in the dialysate, the requirement for a large solution volume limits its practical application (Table 3I). Further, in vitro recovery rates do not always correspond to in vivo conditions [27].

4.2.2.2 | II Retrodialysis/Reverse Microdialysis. For the retrodialysis or reverse microdialysis, a defined drug concentration is perfused through the probe and the recovery rate is calculated by measuring the difference in drug concentration between the perfusate and dialysate. However, this method is affected by tissue-specific features in vivo, which can lead to discrepancies when compared to in vitro results [27]. In vitro retrodialysis typically uses large solution volumes to ensure sink conditions, enabling accurate probe penetration estimates (Table 3II).

4.2.2.3 | III Internal Standard. In contrast to retrodialysis, this method employs an isotopically labeled version of the drug as an internal standard in the perfusate, allowing calibration of the probe recovery rate throughout the experiment. The recovery rate is calculated based on the loss of the labeled compound from the perfusate. This approach can be performed both in vitro and in vivo, providing accurate estimates for compounds with similar physicochemical properties as the utilized internal standard (14, Table 3III).

4.2.2.4 | IV Endogenous Reference Method. This method uses endogenous compounds, such as urea, as internal standards for probe calibration in vivo [28–31]. The probe's recovery rate can be followed over the full course of the experiment, similar to the internal standard method. Under the requirement that the endogenous substance distributes equally between plasma and interstitium and that recovery rates can be calculated from the in vitro recovery rate ratio of the drug and the endogenous standard. Although this approach allows differentiation between background signals and actual drug concentrations, potential interactions between the drug and the endogenous substrate may confound results. This method does not always provide an accurate recovery rate for the drug, as endogenous substrates often have different physicochemical and biological properties (Table 3IV) and rely heavily on prior in vitro—in vivo recovery correlations and endogenous compound concentrations determination.

4.2.2.5 | V Low-Flow-Rate Method. The low-flow-rate method relies on the correlation between increased recovery rate and decreased flow rates. Lower flow rates allow more time for equilibration between the ECF and perfusate, enabling direct measurement of true drug concentrations. However, reduced flow rates also necessitate ultrasensitive analytical techniques due to the smaller sample volumes, which can increase the likelihood of error ([32, 33] Table 3V).

4.2.2.6 | VI Flow Methods (Dynamic) No-Net-Flux. The no-net-flux method is a widely used in vivo calibration technique. It involves perfusing the probe with solutions of varying drug concentrations and measuring the point of equilibrium, where no net drug movement occurs between the perfusate and the dialysate (Table 3VI). This method, while accurate, is time-consuming and less practical for drugs with rapid elimination rates [27]. The dynamic no-net-flux method reduces time requirements by using separate animals for each perfusate concentration. However, this approach is associated with inter-individual variability, which can affect reproducibility [32].

In summary, each calibration method has distinct limitations, and it is advisable to use at least two complementary methods to validate recovery rate measurements and ensure data reliability.

4.3 | Model-Related Challenges

4.3.1 | Age

Age-related differences in BBB function can significantly influence microdialysis results. Animal models used to study pathological conditions should be carefully selected to account for variations in BBB permeability associated with age. For instance, BBB function tends to decline with age in both rodents and humans, which may complicate the use of adult animal models for predicting drug distribution in pediatric brain tumors [34–36].

4.3.2 | Tissue Damage

The surgical insertion of microdialysis probes can induce temporary tissue damage, leading to localized BBB disruption, bleeding, and inflammation. To minimize these effects, a recovery period of at least 24 h is recommended before beginning the experiment [37]. However, prolonged probe placement (> 5 days) may result in scar tissue formation, which can distort drug diffusion and confound data interpretation [38–40]. Visual inspection and histological analysis at the end of the experiment can help evaluate tissue integrity and validate results.

4.3.3 | Measurement of the Collected Plasma and Microdialysate Fractions

The ratio of unbound drug concentrations in plasma and brain ($K_{p,uu}$) is a critical parameter for understanding drug distribution (Table S2). In addition to microdialysis experiments, multiple blood samples should be collected from each animal to assess plasma PK and protein binding. Analytical techniques such as equilibrium dialysis, ultrafiltration, and biosensor-based assays are commonly used to determine the unbound drug fraction in plasma [41]. Highly sensitive methods, such as UPLC-MS/MS, are required to quantify the low drug concentrations typically present in microdialysate fractions. All methods should comply with bioanalytical validation guidelines (ICH M10) and demonstrate sufficient sensitivity and specificity to measure unbound fractions in plasma and brain tissue [42].

5 | Conclusion

Cerebral microdialysis enables the real-time, in vivo determination of unbound drug or metabolite concentrations in the brain of an individual awake model organism. This approach aligns with the 3R principles (replace, reduce, refine) in animal experimentation and facilitates the determination of the $K_{p,uu}$, making it a valuable tool in preclinical drug development. Hydrophobic drugs, which are more likely to penetrate the BBB, often pose additional challenges due to their high potential for non-specific binding to microdialysis system components.

In this study, all three tested hydrophobic drugs required the addition of BSA to the microdialysis buffer to reduce non-specific binding to vials and tubing. Among the tubing materials tested, FEP demonstrated superior recovery rates; however, the optimal setup must be confirmed for each drug individually. Accurate and sensitive quantification methods are crucial to ensure the reliability of microdialysis data, particularly for compounds present in low concentrations.

Given the varying strengths and limitations of calibration methods, it is recommended to employ multiple complementary techniques to address method-related challenges. Some drugs may need to be excluded from cerebral microdialysis studies due to technical limitations, such as insufficient sensitivity in drug quantification or significant issues with non-specific binding.

Model-related constraints, including tissue damage and bleeding at the probe site, can be mitigated through sufficient recovery periods following probe implantation and histological evaluations to confirm tissue integrity.

In summary, the selection of an appropriate microdialysis setup for each drug requires thorough preliminary investigations and careful selection of system components. This includes choosing the optimal vial, perfusate supplement, tubing material, and probe membrane based on the physicochemical properties of the drug. Additionally, establishing a robust calibration method is essential before commencing microdialysis experiments to ensure reliable and reproducible results.

Author Contributions

J.K.S., J.Be., K.W.P., and M.S. wrote the manuscript. All authors designed the research. J.K.S., J.Be., and M.S. performed the research. J.K.S. and J.Be. analyzed the data.

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Conflicts of Interest

The authors declare no conflicts of interest.

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