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Method Article

The concentration of brain homogenates with the Amicon Ultra Centrifugal filters

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A B S T R A C T

Accurately measuring the brain concentration of a neurotherapeutic is critical in determining its pharmacokinetic profile *in vivo*. Biologics are potential therapeutics for neurologic diseases and biologics fused to an antibody targeting a transcytosis receptor at the Blood-Brain Barrier, designated as antibody-biologic fusion proteins, are Blood-Brain Barrier penetrating neurotherapeutics. The use of sandwich immunosorbent assays to measure concentrations of antibody-biologic fusion proteins in brain homogenates has become increasingly popular. The raw brain homogenate contains many proteins and other macromolecules that can cause a matrix effect, potentially interfering with the limit of detection of such assays and reduce the overall sample signal. Further, the low sample loading volumes while running these assays can reduce the sample signal. Our aim was therefore to optimize the existing tissue sample preparation and processing to concentrate the sample to elevate the signal of the analyte. Here, we present a protocol for concentrating and increasing the signal of transferrin receptor antibody-biologic fusion proteins in mouse brain homogenates using the Amicon Ultra Centrifugal filters.

- The presented method uses the Amicon Ultra Centrifugal filters to concentrate mouse brain tissue homogenates.
- The concentrated brain tissue homogenates are then assayed using standard sandwich enzyme-linked immunosorbent assay (ELISA) protocols.
- This method improves upon the traditional brain homogenization procedure and ELISA measurements for antibody-biologic fusion proteins by effectively concentrating brain tissue homogenates.

Abbreviations: (Asn), Asparagine; (BBB), Blood-Brain Barrier; (CNS), Central Nervous System; (ELISA), Enzyme-Linked Immunosorbent Assay; (EPO), Erythropoietin; (EPOR), EPO Receptors; (Gly), Glycine; (cTfRMAb), Chimeric Monoclonal Antibody Targeting the Mouse Transferrin Receptor; (PBS), Phosphate Buffered Saline; (SEM), Standard Error of the Mean; (T-PER), Tissue Protein Extraction Reagent; (TBS), Tris Buffered Saline.

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Resource availability	N/A

Introduction

The Blood-Brain Barrier (BBB), formed by the brain microvascular endothelial cells that are sealed together by tight junctions, is a highly selective semipermeable membrane that regulates the movement of ions, molecules, and nutrients into the brain, maintaining homeostasis and protects the brain from toxins and pathogens [1]. The BBB also restricts the entry of drugs into the central nervous system (CNS) and acts as a major obstacle to brain drug delivery [2]. *In vivo* techniques provide the highest sensitivity and more reliable reference information for testing and validating other BBB permeability models [3]. Determining the brain pharmacokinetic profile of a CNS permeant drug is dependent on accurately measuring the drug concentration in the brain. Over the last decade, there has been an increase in the number of studies using sandwich immunoassays to measure brain concentrations of BBB-penetrating antibody (IgG)-biologic fusion proteins [4–6]. These studies utilize sandwich immunoassays to measure transferrin receptor antibody-biologic fusion protein concentrations in brain homogenate-derived supernatants. However, homogenization of the brain tissue in the homogenization buffer dilutes the detection signal. Furthermore, the brain contains many proteins and other macromolecules that, if excluded before retrieving the supernatant containing the CNS therapeutic of interest, may increase the assay signal. These proteins and macromolecules can cause the matrix effect, adversely impacting the limit of the detection of the assay [7]. To increase the signal-to-noise ratio of the sandwich ELISA to measure BBB-penetrating transferrin receptor IgG-biologic fusion proteins, we developed a method to concentrate the brain homogenate supernatants using the Amicon Ultra Centrifugal filters. This method concentrates the sample volume and improves the overall signal in the ELISA. This entire process is shown in Fig. 1.

This protocol was tested with the fusion protein of a chimeric transferrin receptor monoclonal antibody and erythropoietin (cTfRMAb-EPO) and a mutant form of the fusion protein (cTfRMAb-N292G-EPO). The cTfRMAb-N292G-EPO fusion protein was engineered by removing the Fc N-linked glycosylation site at position 292 in the heavy chain of the TfRMAb with substitution of the amino acid asparagine (Asn) with glycine (Gly) [8].

Materials

1. 4mL glass scintillation vials (#03-339-23B or #03-339-25B; Fisherbrand, Pittsburgh, Pennsylvania, USA).

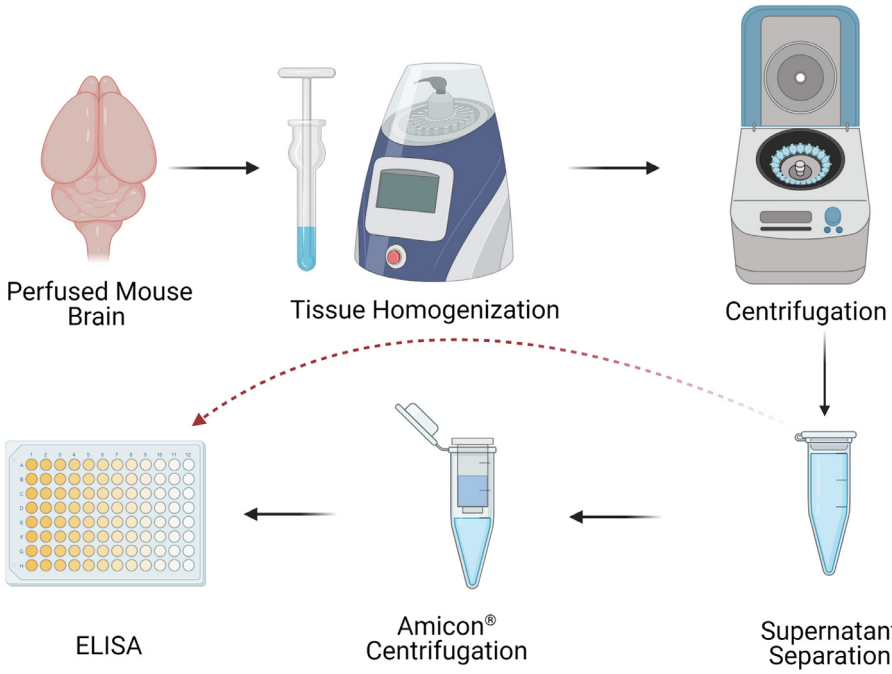

Fig. 1. Brain homogenization and Amicon concentration. The perfused mouse brain is homogenized and processed. The homogenized supernatant is separated from the brain pellet via centrifugation and either directly run on an enzyme-linked immunosorbent assay (ELISA) (represented by the pink dotted arrow) or put through the Amicon centrifugation. The Amicon retentate concentrate is further run on an ELISA. The figure was created using BioRender.com.

2. 2 mL Eppendorf® Safe-Lock microcentrifuge tubes (#EP022363344; Millipore Sigma, Burlington, Massachusetts, USA).
3. Pulverizer.
4. Handheld tissue homogenizer (#H100197; Waverly Scientific, Waverly, Iowa, USA).
5. T-PER™ Tissue Protein Extraction Reagent (#78510; Thermo Fisher Scientific, Waltham, Massachusetts, USA).
6. cOmplete™ Mini-EDTA-free protease inhibitor cocktail tablets (#4693159001; Sigma, St. Louis, Missouri, USA).
7. Shaker.
8. Dry Ice.
9. Ice.
10. Eppendorf® Centrifuge 5417R (Z366021; Sigma Aldrich, St. Louis, Missouri, USA).
11. Weighing scale for brain hemispheres.
12. Euthasol (stock concentration: 390 mg/mL. Each mL contains 390 mg pentobarbital sodium and 50 mg phenytoin sodium). Euthasol was diluted to 1:10 with sterile saline.
13. Perfused mice brain hemispheres.
14. Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filters (#UFC205024; Millipore Sigma, Burlington, Massachusetts, USA).
15. Eppendorf® Centrifuge 5810R with the swinging bucket rotor A-4-62 (#EP022628168; Sigma Aldrich, St. Louis, Missouri, USA).
16. Tris Buffer Saline (TBS): 0.05 M Tris/0.15 M NaCl/pH 7.4.
17. TBST: TBS + 0.05% Tween-20.

18. Chimeric transferrin receptor antibody-biologic fusion protein therapeutic: cTfRMAb-EPO (0.7 mg/mL; Genscript, Piscataway, New Jersey, USA).
19. Chimeric transferrin receptor antibody-biologic fusion protein therapeutic: cTfRMAb-N292G-EPO (0.78 mg/mL; Genscript, Piscataway, New Jersey, USA).
20. Transferrin receptor antibody-biologic fusion protein therapeutic vehicle: 96 mM glycine, 28 mM Tris, 148mM NaCl, 0.01% Polysorbate 80, pH=5.5.
21. Recombinant human EPO receptor (EPOR)/Fc fusion protein (R&D System #963-ER-050).
22. 0.1M $NaHCO_3$/pH 8.5.
23. TBSB: 0.01M Tris/0.15M NaCl/pH 7.4/1% bovine serum albumin (BSA).
24. Goat anti-mouse light chain (kappa) antibody conjugated to alkaline phosphatase (GAM-AP) (# A90-119AP; Bethyl, Montgomery, Texas, USA).
25. P-nitrophenyl phosphate (PNPP) (#P5994; Sigma Aldrich, St. Louis, Missouri, USA).
26. Glycine Buffer/pH 10.4.
27. 1.2M NaOH.
28. 96 well NUNC Maxisorp flatbottom 96 TS (#501123685; Fisher Scientific, Waltham, Massachusetts, USA).
29. SpectraMax Plus 384 Microplate Reader (Molecular Devices; San Jose, California, USA).
30. SoftMax Pro Software (Molecular Devices; San Jose, California, USA).
31. GraphPad Prism 8 Software (GraphPad Software Inc.; San Diego, California, USA).

Methods

Mouse animal study

All animal procedures were approved by the University of La Verne Institutional Animal Care and Use Committee (Protocol No. LV0012d). Eight-week-old male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were provided constant access to food and water and were maintained on a 12 h light/12 h dark cycle.

The mice were injected intravenously with 0.3 mg/kg (n=3) or 3 mg/kg (n=4), or intraperitoneally with 3 mg/kg (n=6) of cTfRMAb-EPO. A separate group of age-matched mice were injected intravenously with 0.3 mg/kg (n=3) or 3 mg/kg (n=6), or intraperitoneally with 3 mg/kg (n=3) of the mutant cTfRMAb-N292G-EPO fusion protein. Control mice were dosed intraperitoneally with saline (n=4). Twenty-four-hours after the initial injection, the mice were anesthetized with a lethal dose of Euthasol (150 mg/kg, intraperitoneally) and transcardially perfused with ice-cold phosphate buffer saline (PBS). The brain hemispheres were harvested and stored at -80°C until use.

Brain pulverization protocol

1. Prepare the 4 mL glass scintillation vials to store each individual pulverized brain hemisphere. Pre-weigh and label the scintillation vials clearly.
2. Place the handheld pulverizer in powdered dry ice for 3 min. This process ensures that the equipment is cold and prevents the frozen brain hemispheres from warming up and sticking to the pulverizer.
3. Place frozen brain hemisphere in the chilled pulverizer and pulverize into a fine powder with a hammer. This process is done over dry ice to maintain the cold temperature.
4. Separate the powdered brain hemisphere into two groups: the brain homogenate group and the Amicon Ultra Centrifugal Filter separated group.

NOTE: The brain homogenate group consists of the raw brain homogenate samples that have not been processed using the Amicon Ultra Centrifugal Filter but for which the brain homogenate supernatants are directly run on the ELISA (Fig. 1). The brain homogenate group was compared to the Amicon retentate concentrate sample to compare the relative signal between the two samples.

The Amicon Ultra Centrifugal Filter separated group consists of the raw brain homogenate supernatant samples that run through the Amicon Ultra 2 mL 50 kDa Protein Purification and

Concentration Filter Tubes (Fig. 1). This sample group is further separated between the relevant Amicon retentate concentrate samples and the extraneous Amicon filtrate samples. The retentate concentrate sample is the sample that is retained by the Amicon centrifugation process, while the filtrate sample is the sample that is filtered through and typically discarded.

1. Collect the powdered brain using a pre-chilled metal spatula and place it in pre-chilled scintillation vials.
2. Quickly weigh the scintillation vials again and note the weight. The weight difference in the pre-weighed vials and the vials containing the powdered brain is the total brain weight, which is important for the brain homogenization calculations below.
3. Place the 4 mL scintillation vials with the pulverized brains at -80°C until brain homogenization.

Brain homogenization protocol

1. Make the homogenization buffer by mixing 10 mL of T-PER™ Tissue Protein Extraction Reagent with 1 tablet of cOmplete™ Mini-EDTA-free protease inhibitor cocktail tablet.
2. Take out the scintillation vials with the pulverized brains from -80°C and place them on ice.
3. Homogenize the pulverized brain in the homogenization buffer with the handheld tissue homogenizer for 30 sec. Wash the homogenizer with chilled ice-cold homogenization buffer between samples.
   a For homogenizing the brain homogenate group, put 4 brain volumes (4 μL/mg of the brain) of ice-cold homogenization buffer (T-PER) into the chilled scintillation vial with the pulverized brain. Over-dilution of the samples will lead to a lower signal.
   b For homogenizing the Amicon Ultra Centrifugal Filter separated group, put 7 brain volumes (7 μL/mg of the brain) of ice-cold homogenization buffer (T-PER) into the chilled scintillation vial with the pulverized brain. Dilution of the samples is not an issue because the sample will be concentrated with the Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filters. NOTE: the Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filters can hold a maximum volume of 2 mL. NOTE: the average weight of the pulverized hemisphere was about 200-220 mg and the average brain homogenate volume based on 7 μL/mg was 1400-1540 μL.
4. Mix the chilled scintillation vials on a shaker at 4°C for 1 h.
5. Pipette out all the brain homogenate into a chilled centrifuge tube. Collect everything to avoid protein loss and inaccuracies in the calculation.
6. Weigh the centrifuge tubes. Arrange and counterbalance the centrifuge tubes into weight-matched pairs in the rotor of the Eppendorf centrifuge 5417R. Centrifuge the brain samples at 14,000xg for 20 min at 4°C.
7. Collect and aliquot (to prevent repeated freeze-thaw cycles) all the supernatant in labeled 2 mL Eppendorf tubes and freeze all aliquots in the -80°C freezer for long-term storage. If the brain ELISA is run the following day, keep all tubes in the 4°C refrigerator to avoid freeze-thaw.

Amicon concentration & purification protocol

1. Pipette the Amicon Ultra Centrifugal Filter separated group samples into the Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filters and assemble the Amicon Centrifugation Tubes, as described by the vendor.
2. Place and balance Amicon Centrifugation Tubes into the A-4-62 swinging bucket rotor of the Eppendorf® Centrifuge 5810R and centrifuge at 3000xg for 20 min at 4°C.
3. Take each individual Amicon Centrifugation Tube out and pipette up & down vigorously along the membranes of the Amicon Filter to detach any macromolecules sticking and blocking the membrane. NOTE: do this step carefully so as not to damage the membrane.
4. Place the Amicon Centrifugation Tubes back into the swinging bucket rotor and centrifuge at 3000xg for 7 min at 4°C.
5. Take each individual Amicon Centrifugation Tube out and pipette up & down vigorously again along the membranes of the Amicon Filter to detach any macromolecules sticking and blocking

the membrane. Place the Amicon Centrifugation Tubes back into the A-4-62 swinging bucket rotor and centrifuge at 3000xg for 5 min at 4°C. This step is repeated until the volume of the Amicon Centrifugation retentate is below 300 μL.
6. Once the volume of the collected Amicon retentate concentrate sample is below 300 μL, remove the assembled device from the centrifuge and separate the Amicon Ultra Filter from the filtrate collection tube.
7. To recover the Amicon retentate concentrate samples, invert the Amicon Ultra Filter device and concentrate collection tube. Place the inverted Amicon Ultra Filter device back in the Eppendorf® Centrifuge 5810R with rotor A-4-62 and counterbalance each sample. Spin for 2 min at 1000xg to transfer the Amicon retentate concentrate samples from the filter device to the concentrate collection tube.
8. Aliquot and freeze the final samples in the -80°C freezer to prevent repeated freeze-thaw cycles, unless you are running the brain ELISA the following day. If running the ELISA the next day, place the Amicon Ultra Centrifugal Filter separated samples in the 4°C refrigerator with the brain homogenate samples.

Brain ELISA protocol

1. Dilute the recombinant human EPOR/Fc fusion protein with 0.01M of PBS (pH 7.4) so that the stock is 100 μg/mL. For this, add 500 μL of PBS for the 50 μg of the lyophilized powder of the recombinant human EPOR/Fc fusion protein. Split this stock solution into 3 aliquots of 167 μL and freeze it in the -80°C freezer until use.
2. To make the working solution, remove and thaw one aliquot (16.7 μg) and dilute it with 8.35 mL of 0.1M $NaHCO_3$ (pH 8.5). The concentration is 2 μg/mL and will be enough for 85 wells with each well containing 0.2 μg of the EPOR/Fc fusion protein. Plate 100 μL per well in the 96 well Nunc maxisorp ELISA plate and incubate overnight at 4°C.
3. Aspirate off the EPOR/Fc solution from the ELISA plate after overnight incubation. Then wash each well 3 times with 150 μL of TBST per well.
4. Block each well with 150 μL of TBSB for at least 30 min at room temperature (RT). Prepare the standards during this blocking step. Both transferrin receptor antibody-EPO fusion proteins are diluted to 90,000 ng/mL, 30,000 ng/mL, 3,000 ng/mL, 300 ng/mL, 30 ng/mL, and 3 ng/mL for the standards.
5. Aspirate the TBSB and add 100 μL of cTfRMAb-EPO and cTfRMAb-N292G-EPO standards, 200 μL of the brain homogenate samples including the T-PER brain homogenate blank, and all the Amicon retentate concentrate sample volume per well and incubate for 2 h at RT.
6. Aspirate the samples from the ELISA plate and wash each well 3 times with 150 μL of TBST.
7. Dilute 17 μL (8.5 μg) of GAM-AP with 8,483 μL of TBST and add 100 μL of the diluted GAM-AP working solution per well (100 ng/well). This volume is enough for 85 wells. Incubate the ELISA plate for 45 min at RT.
8. Aspirate off the GAM-AP working solution from the ELISA plate and wash 3 times with 150 μL of TBST per well.
9. Dilute a 40 mg PNPP tablet in 40 mL of glycine buffer (pH 10.4). Add 100 μL of the PNPP working solution in each well and incubate for 20 min at RT in the dark. The color of the solution typically changes immediately, and saturation of the reaction may be reached before 20 min.
10. Add 100 μL of 1.2M NaOH to each well to stop the color reaction. Monitor the color of the solution in each well carefully. The reaction needs to be stopped before 20 min if the color changes rapidly.
11. Place the plate on a plate reader and read the absorbance at 405 nm. Compute the mean blank with the blank T-PER brain homogenate samples. Subtract the blank from each individual test sample and standard, and compute the mean of the blank-corrected test samples and standard for each group.

Brain concentration calculation

1. The blank-corrected standard curves (appropriate for the specific cTfRMAb-based fusion protein) were fit to the one site-specific binding nonlinear regression curve to determine the total density

of binding receptor (B_{Max}) and the equilibrium dissociation constant (K_D) values using GraphPad Prism 8.

2. The brain concentrations (ng/mL brain homogenate supernatant) of both TfRMAb antibody-based fusion proteins are calculated from the formula:

Concentration = (OD × K_D)/ (B_{Max} – OD)

The OD is the optical density measured from the plate reader at 405 nm that has been blank-corrected from the T-PER brain homogenate samples.

1 The amount (ng) of cTfRMAb-based fusion proteins (cTfRMAb-N292G-EPO and cTfRMAb-EPO) in the brain was calculated by multiplying the back-calculated concentration by the volume of the brain homogenate.

2 The amount of cTfRMAb-N292G-EPO and cTfRMAb-EPO per gram brain (ng/g brain) was calculated by dividing the amount (ng) of cTfRMAb-based fusion proteins in the brain by the weight of the brain (g).

Statistical analysis

All data are represented as mean ± standard error of the mean (SEM), and all statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA). To test the effect of two factors, two-way ANOVA with Holm-Sidak post-test was used. A two-tailed $p<0.05$ was considered statistically significant.

Methods validation

In our experiment, we used two bivalent, BBB-penetrating cTfRMAb-based fusion proteins as model BBB-penetrating IgG-biologic fusion proteins and evaluated their brain concentrations after intravenous and intraperitoneal routes of administration. The reason for selecting these two TfRMAb-based fusion proteins for method validation was the profound difference in their plasma concentrations and expected brain concentrations [8]. Saline-treated mice were used to determine the background signal in the brain homogenates (background of the assay) and limit of detection (LOD) of the assay as described previously [9].

Each set of Amicon Ultra Centrifugal Filter separated brain homogenates was paired with its brain homogenate group counterpart (Fig. 2). As expected, the background concentrations (ng/mL of brain homogenate supernatant) obtained from saline-treated mice for both the Amicon Ultra Centrifugal Filter separated group (n=4) and the brain homogenate group (n=4) were comparable and ranged between 0-0.6 ng/mL and 0-0.4 ng/mL (designated as saline concentration threshold), respectively, and LOD of the assay was 0.8 ng/mL.

For mice treated with the cTfRMAb-EPO (n=13), the concentrations (ng/mL of brain homogenate supernatant) resulting from the Amicon Ultra Centrifugal Filter separation ranged between 1-54 ng/mL, and those obtained using the regular brain homogenization method ranged between 0.1-4 ng/mL (Fig. 2). All the brain concentrations of mice treated with the cTfRMAb-EPO and separated using the Amicon Ultra Centrifugal Filters were above the saline concentration threshold. While using the regular brain homogenate method, eight of the cTfRMAb-EPO-treated mice had brain concentrations above and five had brain concentrations below the saline concentration threshold (Fig. 2). Amicon Ultra Centrifugal Filter separation elevated the brain homogenate concentration of the cTfRMAb-EPO in all thirteen brain samples tested.

For mice treated with the mutant cTfRMAb-N292G-EPO (n=12), the concentrations (ng/mL of brain homogenate supernatant) resulting from the Amicon Ultra Centrifugal Filter separation ranged between 0-6 ng/mL, and those obtained using the regular brain homogenization method ranged between 0-1 ng/mL (Fig. 2). Seven of the brains separated using the Amicon Ultra Centrifugal Filters, but only two of the brains separated using the regular brain homogenization method, were above the saline concentration threshold (Fig. 2). Notably, five of the brains treated with the cTfRMAb-N292G-EPO had brain concentrations within the saline concentration threshold while using either the Amicon or the regular brain homogenization separation method. This is an expected finding given the known

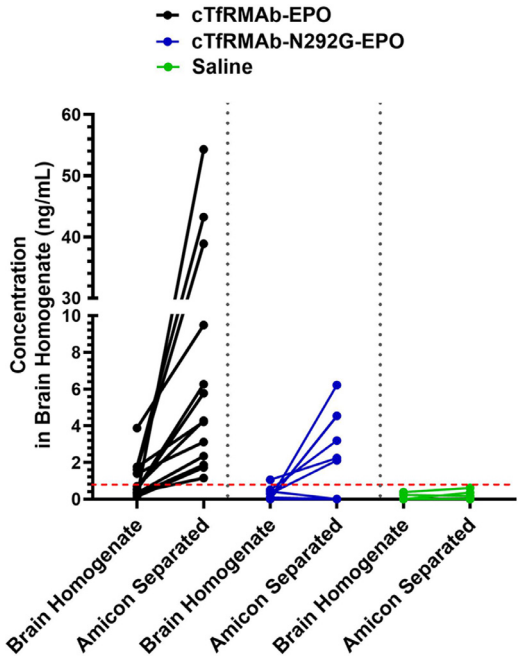

Fig. 2. The concentration of cTfRMAb-based fusion proteins in mouse brain homogenates. In the above-paired graph, each symbol-line-symbol represents the paired brain concentrations resulting post-ELISA by the brain homogenization and Amicon concentration method for the same brain. Each symbol-line-symbol represents an individual brain from a mouse treated either with cTfRMAb-EPO, cTfRMAb-N292G-EPO, or saline. The red dotted line represents the limit of detection of the assay (0.8 ng/mL).

profound differences in plasma clearance and thereby the resultant brain concentrations between the two cTfRMAb-based fusion proteins. The cTfRMAb-EPO has a higher plasma exposure and half-life with a lower clearance compared to that of the cTfRMAb-N292G-EPO [8]. As a result, the expected brain concentrations of the cTfRMAb-N292G-EPO are low at the doses used in the current study.

We then converted the ng/mL brain homogenate supernatant concentrations to brain concentrations in ng/g brain tissue. After intravenous administration, the Amicon Ultra Centrifugal Filter separated group displayed a significantly higher ($p < 0.0001$) brain concentration (ng/g brain) compared to the brain homogenate group for cTfRMAb-EPO, and a similar trend was seen for the cTfRMAb-N292G-EPO fusion protein, though these values did not reach statistical significance (Fig. 3A). As seen in Fig. 3A, as expected based on the plasma pharmacokinetics [8], the brain concentrations of the cTfRMAb-EPO were significantly ($p < 0.001$) higher than those of cTfRMAb-N292G-EPO and saline for the Amicon Ultra Centrifugal Filter separated group. The cTfRMAb-EPO group was 8-fold larger in brain concentration (ng/g brain) compared to the cTfRMAb-N292G-EPO group and was 642-fold larger in brain concentration compared to the saline group (Fig. 3A). The regular brain homogenization method was unable to detect any significant differences between the three groups (Fig. 3A). These trends are also confirmed following the intraperitoneal route of administration.

After intraperitoneal administration, the Amicon Ultra Centrifugal Filter separated group displayed a trend ($p = 0.054$) towards a higher signal compared to the brain homogenate group for cTfRMAb-EPO. This trend was not observed for cTfRMAb-N292G-EPO because of the below-detection brain concentrations of cTfRMAb-N292G-EPO following intraperitoneal administration at the doses used in the current study (Fig. 2). Again, as expected, the brain concentrations of the cTfRMAb-EPO were significantly ($p < 0.01$) higher than those of the cTfRMAb-N292G-EPO and saline for the Amicon Ultra

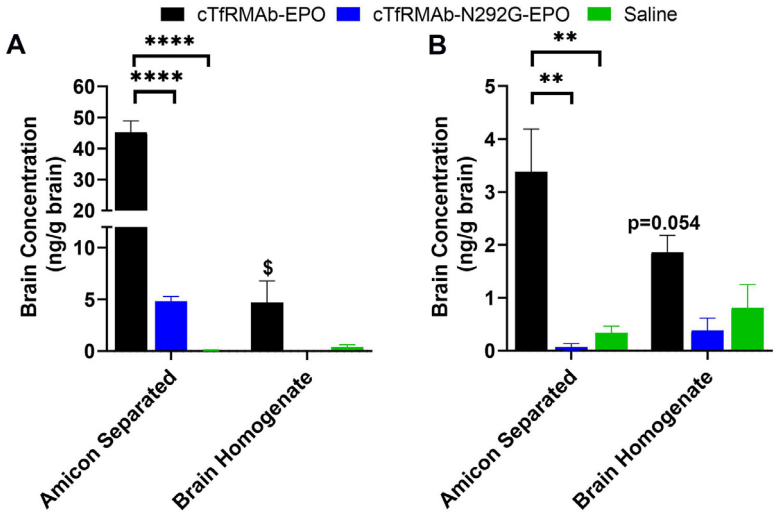

Fig. 3. The brain concentrations of the cTfRMAb-based fusion proteins following intravenous (A) and intraperitoneal (B) administration of a 3 mg/kg dose of each fusion protein comparing the Amicon Ultra Centrifugal Filter separated group and the regular brain homogenate group. The Amicon Ultra Centrifugal Filter data was presented in reference [8]. The data was analyzed using the two-way ANOVA with Holm-Sidak multiple comparisons test. All data are represented as mean ± standard error of the mean (SEM). ** $P < 0.01$ and **** $P < 0.0001$. $ $P < 0.0001$ in A and $p = 0.054$ in B represent comparisons to the corresponding Amicon Ultra Centrifugal Filter separated group.

Centrifugal Filter separated group (Fig. 3B). Similar to the intravenous administration, the regular brain homogenization method was unable to detect any significant differences between the three groups (Fig. 3B).

Overall, while comparing the Amicon Ultra Centrifugal Filter separated method with the regular brain homogenization method, the Amicon separation method concentrated the brain homogenate supernatant for the cTfRMAb-based fusion proteins tested in the current study. This concentration of the brain homogenate supernatant significantly increased the signal for the ELISA which allowed the detection of expected differences in the brain concentrations of the two different cTfRMAb-based fusion proteins tested herein using the Amicon separation method; these differences were not detected by the regular brain homogenization method. We attribute this improvement of the signal with the Amicon Ultra Centrifugal Filters to 1) the concentration of the brain homogenate volume which results in the enrichment of the brain homogenate for the analyte of interest, and 2) clean-up of the brain homogenate supernatant owing to the 50kDa molecular weight cut-off filters and therefore an expected reduction in the samples matrix-effect. The use of centrifugal filtration devices for sample clean-up has been previously reported for small molecule quantification in biological samples prior to liquid chromatography coupled with mass spectrometry (LC-MS) [10]. Besides the use of the Amicon Ultra Centrifugal Filters, there are other sample clean-up techniques including solid-phase extraction (SPE) that are extensively used for sample preparation to measure large molecules such as antibody-drug conjugates (ADCs) prior to LC-MS quantification [11]. However, the use of SPE is more time consuming than simple centrifugation-based separation, and its use for sample preparation to measure the brain concentrations of IgG-biologic fusion proteins has not been reported and requires further investigation. Further, though LC-MS quantification is widely used to measure ADCs in biological samples and can have a reported limit of detection of 1 ng/mL [12], the measurement of IgG-biologic fusion proteins is further complicated due to their even larger size and difficulty in interpretation of the mass signal [11]. Furthermore, measurement of *in vivo* brain concentrations of IgG-biologic fusion proteins with this method has not been reported yet but may be a viable approach [13] that needs to be further investigated.

The most widely used technique to sensitively measure brain concentrations of IgG-biologic fusion proteins is radiolabeling of the fusion protein. The use of sandwich immunoassays such as ELISA and the electrochemiluminescence-Meso Scale Discovery (ECL-MSD) platforms to measure brain concentrations of IgG-biologic fusion proteins has increased considerably in the last decade and offers the advantage of being non-radioactive approaches. However, sample preparation for these immunoassays can result in sample loss compared to using radioactive methods. Other non-radioactive approaches used to measure brain concentrations of IgG-biologic fusion proteins include near infrared fluorescence (NIRF) imaging and immunostaining techniques [14,15], however these techniques are semi-quantitative. The current study shows promise in the use of Amicon Centrifugal Filters to enhance the signal of a quantitative sandwich ELISA to measure the brain concentrations of IgG-EPO fusion proteins, and future work is needed to see if this technique can be widely applied to other IgG-biologic fusion proteins.

Declaration of Competing Interest

None.

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References

[1] R. Daneman, A. Prat, The blood-brain barrier, Cold Spring Harb Perspect Biol 7 (1) (2015) a020412.
[2] W.M. Pardridge, The blood-brain barrier: bottleneck in brain drug development, NeuroRx 2 (1) (2005) 3–14.
[3] U. Bickel, How to measure drug transport across the blood-brain barrier, NeuroRx 2 (1) (2005) 15–26.
[4] Y.J. Zuchero, X. Chen, N. Bien-Ly, D. Bumbaca, R.K. Tong, X. Gao, et al., Discovery of Novel Blood-Brain Barrier Targets to Enhance Brain Uptake of Therapeutic Antibodies, Neuron 89 (1) (2016) 70–82.
[5] J.A. Couch, Y.J. Yu, Y. Zhang, J.M. Tarrant, R.N. Fuji, W.J. Meilandt, et al., Addressing safety liabilities of TfR bispecific antibodies that cross the blood-brain barrier, Sci Transl Med 5 (183) (2013) 1–12 183ra57.
[6] G. Thom, M. Burrell, A.S. Haqqani, A. Yogi, E. Lessard, E. Brunette, et al., Enhanced Delivery of Galanin Conjugates to the Brain through Bioengineering of the Anti-Transferrin Receptor Antibody OX26, Mol Pharm 15 (4) (2018) 1420–1431.
[7] A. Wojnicz, J.A. Ortiz, A.I. Casas, A.E. Freitas, M.G. Lopez, A. Ruiz-Nuno, Data supporting the rat brain sample preparation and validation assays for simultaneous determination of 8 neurotransmitters and their metabolites using liquid chromatography-tandem mass spectrometry, Data Brief 7 (2016) 714–720.
[8] J. Yang, J. Sun, D.M. Castellanos, W.M. Pardridge, RK. Sumbria, Eliminating Fc N-linked glycosylation and its impact on dosing consideration for a transferrin receptor antibody-erythropoietin fusion protein in mice, Mol. Pharm. 17 (8) (2020) 2831–2839.
[9] D.A. Armbruster, T. Pry, Limit of blank, limit of detection and limit of quantitation, Clin. Biochem. Rev. 29 (Suppl 1) (2008) S49–S52.
[10] M. Sergi, C. Montesano, S. Napoletano, D. Pizzoni, C. Manetti, F. Colistro, et al., Analysis of bile acids profile in human serum by ultrafiltration clean-up and LC-MS/MS, Chromatographia 75 (9) (2012) 479–489.
[11] H. Cahuzac, L. Devel, Analytical methods for the detection and quantification of ADCs in biological matrices, Pharm. (Basel) 13 (12) (2020).
[12] T. Botzanowski, S. Erb, O. Hernandez-Alba, A. Ehkirch, O. Colas, E. Wagner-Rousset, et al., Insights from native mass spectrometry approaches for top- and middle- level characterization of site-specific antibody-drug conjugates, MAbs 9 (5) (2017) 801–811.
[13] A.S. Haqqani, G. Thom, M. Burrell, C.E. Delaney, E. Brunette, E. Baumann, et al., Intracellular sorting and transcytosis of the rat transferrin receptor antibody OX26 across the Blood-Brain Barrier *in vitro* is dependent on its binding affinity, J. Neurochem. 146 (6) (2018) 735–752.
[14] K.R. Ulapane, B.M. Kopec, T.J. Siahaan, Improving *in vivo* brain delivery of monoclonal antibody using novel cyclic peptides, Pharmaceutics 11 (11) (2019).
[15] D. Karaoglu Hanzatian, A. Schwartz, F. Gizatullin, J. Erickson, K. Deng, R. Villanueva, et al., Brain uptake of multivalent and multi-specific DVD-Ig proteins after systemic administration, MAbs 10 (5) (2018) 765–777.