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Protocol to filter free steroids from serum using ultrafiltration and extract steroids via liquid-liquid extraction

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We use this protocol and it's working

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Abstract

Direct measurement of free glucocorticoids in small volumes of mouse and rat serum using ultrafiltration and liquid chromatography-tandem mass spectrometry

Guidelines

Recommended schedule:

- Day 1: Prep and conduct ultrafiltration on serum samples
- Day 2: Prep and extract steroids from ultrafiltered serum samples using liquid-liquid extraction

Materials

Materials and equipment explicitly listed on these pages:

- Ultrafiltration device (#4104, Sigma-Aldrich, Oakville, Ontario, Canada)
- Bead ruptor (BR) tubes (Fisher Scientific, Cat # NC0934160) containing five 1.4 mm ceramic oxide beads per tube (2 BR tubes per serum sample: 1 for free glucocorticoids, 1 for total glucocorticoids)
- Avanti J-15R centrifuge (Beckman Coulter) with rotor adaptors that fit the ultrafiltration devices
- Water bath set to 37°C
- Bucket with wet ice
- Pipettes and appropriate tips (able to pipette 5 µl, ≥30 µl, 10 µl, 50 µl, and 1 mL)
- Red reservoir caps for ultrafiltration devices
- Collection tubes for ultrafiltrate
- Biohazard waste container for discarded ultrafiltration devices
- Standards and quality control samples as in Table 2 (steroid/glucocorticoid standards at pg/10 µl): Standard 1 = 0.4; 2 = 0.8; 3 = 2; 4 = 5; 5 = 12.5; 6 = 50; 7 = 100; 8 = 200; 9 = 500; 10 = 1000; 11 = 2000 pg/10 µl
- Quality control — Low concentration x3 = 2 pg/10 µl; High concentration x3 = 200 pg/10 µl
- Blanks: Blank x2; Double blank x2
- BR tubes for filtered and non-filtered serum aliquots (stored at -70°C)
- Oven (preheat to 60°C)
- 0.6 mL polypropylene microcentrifuge tubes (labelled to correspond with BR tubes)
- 2 mL glass LC-MS/MS vials (with glass inserts) corresponding to BR tubes
- Caps for LC-MS/MS vials
- 12×75 mm glass culture tubes (MeOH-rinsed and oven-dried) — prepare two distinct glass culture tubes per sample (labelled e.g., 1a, 2a,... and 1b, 2b,...). Cover with aluminum foil until use
- 20 mL scintillation vials and a Duran bottle for MilliQ water
- HPLC-grade Ethyl Acetate, Methanol
- Fresh Milli-Q water
- Ability to prepare 50% MeOH and 25% MeOH solutions in rinsed 20 mL scintillation glass vials
- Small water bath (preheat to 65°C in fume hood)
- Vortex mixer
- Homogenizer/bead ruptor capable of 4 m/s
- Centrifuge capable of 16,100 g
- Glass culture tubes labelled to collect supernatant (e.g., 1a, 2a, 3a, ...)
- Aluminum foil for covering glass tubes
- Manifold with N₂ gas supply (nitrogen gas manifold for drying Ethyl Acetate under N₂)
- Tools for transferring ~150 µl to LC-MS/MS vials (pipettes/tips)
- Storage freezer set to -20°C (for extracted samples prior to injection)

Safety warnings

- ! Safety and caution notes present on these pages:
- Keep serum samples at physiological body temperature (i.e., 37°C for mice) during processing to preserve free glucocorticoid distribution.
 - It will take at least 30 min for the centrifuge to stabilize at 37°C — ensure devices are prewarmed before use.
 - Discard used ultrafiltration devices in biohazard waste.
 - Handle samples and disposables using appropriate biosafety precautions.
 - Note to leave sufficient serum for non-filtered aliquots (5 µl) before filtering.
 - Only work with ethyl acetate (highly volatile) in the fume hood using appropriate personal protective equipment (PPE)
 - Work quickly to minimize ethyl acetate evaporation when added to BR tubes.
 - Follow institutional safety procedures when working with organic solvents (e.g., Ethyl Acetate, Methanol)
 - Use caution when operating the N₂ gas manifold and ensure proper ventilation and secure connections to prevent leaks.
 - Cap glass LC-MS/MS vials carefully and ensure there are no air bubbles in the samples prior to storage/injection.

Ethics statement

The procedures complied with the Canadian Council on Animal Care, and protocols received approval from The University of British Columbia Animal Care Committee (Protocols A23-0023 and A22-0191). All researchers were trained and received required approval for conducting the procedures by UBC Animal Care Services.

Before start

Only use HPLC- or LCMS- grade reagents.

Only use fresh MilliQ water (i.e., do not use water stored for a long time, or in container that has been opened/closed multiple times).

Only use polypropylene-grade plastics.

Rinse all glassware (except for LC-MS/MS vials/inserts) with HPLC-grade methanol two times.



Preparation for ultrafiltration

- 1 Label 1 ultrafiltration device (#4104, Sigma-Aldrich, Oakville, Ontario, Canada) per serum sample
- 2 Label 1 set of bead ruptor (BR) tube (Fisher Scientific, Cat # NC0934160) containing 5 1.4mm ceramic oxide beads for all serum samples for **free** steroid measurement
- 3 Label second set of bead ruptor (BR) tube (Fisher Scientific, Cat # NC0934160) containing 5 1.4mm ceramic oxide beads for all serum samples for **total** steroid measurement
- 4 Obtain ice bucket with wet ice
- 5 Thaw serum samples gently on wet ice
- 6 Prewarm centrifuge (Avanti J-15R, Beckman Coulter) **and** rotor adaptors (to fit the ultrafiltration devices) to 37°C (physiological body temperature of mice). You may need to spin centrifuge (i.e., 2000 g) for device to warm, follow manufacturer's instructions.
- 7 Warm water bath to 37°C (physiological body temperature of mice)

Ultrafiltration protocol

1h 10m

- 8 Once serum samples are thawed, place serum samples in water bath at 37°C (physiological body temperature of mice) for 10 min
- 9 Remove red reservoir caps off all labelled ultrafiltration devices
- 10 Pipette at least 30 µl serum of each sample into its respective ultrafiltration device, ensuring that you keep >5 µl of serum for total steroid measurement
- 11 Cap ultrafiltration devices

10m



- 12 Spin ultrafiltration devices at 2000 g at 37°C (physiological body temperature for mice) for 1 hr
- 13 While serum samples are being centrifuged, add 5 µl of remainder of each serum sample to respective labelled BR tubes labelled for total steroid measurement (i.e., non-filtered serum samples)
- 14 Store non-filtered serum samples for total steroid measurement (5 µl) at -70°C until extraction
- 15 Store any remaining serum that was not used for free and total serum measurement in -70°C
- 16 After spinning ultrafiltration devices for 1hr, transfer the entire ultrafiltrate from filter collection tubes to corresponding new BR tubes (i.e., filtered serum samples)
- 17 Store filtered serum samples for free steroid measurement (entire volume) at -70°C until extraction
- 18 Discard used ultrafiltration devices in appropriate waste according to institutional policies (e.g., biohazard waste)

1h

Preparation for liquid-liquid extraction

10m 2s

- 19 Preheat oven to 60°C
- 20 Obtain and label 1 set of bead ruptor (BR) tube containing 5 1.4mm ceramic oxide beads per standard curve/quality control/blank/double blank samples (filtered/non-filtered serum samples should already be in BR tubes)
- 21 Obtain 2 sets of 12×75 mm glass culture tubes
- 22 Add 1mL of HPLC-grade methanol to all 12×75 mm glass culture tubes
- 23 Vortex 12×75 mm glass culture tubes containing 1mL HPLC-grade methanol to rinse glassware for 2s
- 24 Discard HPLC-grade methanol from all 12×75 mm glass culture tubes

2s



- 25 Repeat steps 22-24 to rinse 12×75 mm glass culture tubes a second time
- 26 Place rinsed 12×75 mm glass culture tubes (rinsed twice) upside down in metal tube rack
- 27 Dry rinsed 12×75 mm glass culture tubes (rinsed twice) upside down in oven at 60°C for 10min
- 28 Invert rinsed and dried 12×75 mm glass culture tubes to be right-side up in tube rack
- 29 Label first set of rinsed and dried 12×75 mm glass culture tubes 1a, 2a, 3a, ..., *na*
- 30 Label second set of rinsed and dried 12×75 mm glass culture tubes 1b, 2b, 3b, ..., *nb*
- 31 Cover rinsed and dried 12×75 mm glass culture tubes with aluminum foil under ready to use
- 32 Label sealed 0.6 mL polypropylene microcentrifuge tubes to correspond with all BR tubes
- 33 Label 2 mL glass LC-MS/MS vials to correspond with all BR tubes
- 34 Place glass inserts into glass LC-MS/MS vials
- 35 Cover glass LC-MS/MS vials with aluminum foil to store until ready to use
- 36 Rinse 2 20 mL scintillation vials, a 500mL Duran bottle for MilliQ water, and 3 500mL beakers, with HPLC-grade methanol
- 37 Discard of methanol glassware rinse waste

10m



- 38 Repeat steps 36-37 to rinse glassware twice
- 39 Dry rinsed 20mL scintillation vials, 500mL Duran bottle, 3 500mL beakers (e.g., in fume hood)
- 40 Retrieve 500mL of fresh MilliQ water in rinsed 500mL Duran bottle
- 41 Preheat water bath to 65°C in the fume hood
- 42 Prepare 10 mL of 50% HPLC-grade methanol (diluted with MilliQ water) in rinsed 20 mL scintillation glass vial
- 43 Prepare 20 mL of 25% HPLC-grade methanol (diluted with MilliQ water) in rinsed 20 mL scintillation glass vial
- 44 Thaw standard curve standards (0.02 pg/μL - 400 pg/μL, depending on study) and internal standard mix (10 pg/μL) (stored at -20°C) on bench top and allow to come to room temperature
- 45 Thaw filtered, and non-filtered serum samples (in BR tubes) for free and total steroid measurement, respectively (stored at -70°C) gently on wet ice

Liquid-liquid extraction protocol

18m 37s

- 46 Add 10 μL of standard curve standards (0.02 pg/μL - 400 pg/μL, depending on study) to respective BR tubes
- 47 Add 10 μL of low concentration (0.2 pg/μL) and high concentration (20 pg/μL) quality controls to respective BR tubes
- 48 Add 10 μL of HPLC-grade 50% methanol into blank BR tubes to make volumes equal
- 49 Add 50 μL internal standard mix (10 pg/μL) to all BR tubes except double blank BR tubes



- 50 Add 60 μ L of HPLC-grade 50% methanol into double blank BR tubes to make volumes equal
- 51 Add 1 mL HPLC-grade Ethyl Acetate into all BR tubes (standard curve, blanks, double blanks, quality controls, samples) in fume hood
- 52 Vortex all BR tubes for 2 sec 2s
- 53 Homogenize samples at 4 m/s for 30 sec using a bead mill homogenizer (i.e., Fisherbrand™ Bead Mill 24 Homogenizer, Cat # 15-340-163) 30s
- 54 Centrifuge samples at 16,100 g for 5 min at room temperature 5m
- 55 Remove 1000 μ L of supernatant and place in respective 12×75 mm glass culture tubes (i.e., glass culture tubes labelled 1a, 2a, 3a, ..., na)
- 56 Add 500 μ L MilliQ water to all samples
- 57 Vortex all samples for 5 sec
- 58 Centrifuge samples at 3200 g for 2 min at room temperature 2m
- 59 Carefully remove top liquid layer (ethyl acetate) from samples (without disturbing tissue pellets at the bottom) and transfer to respective new glass culture tubes (e.g., tubes labelled 1b, 2b, 3b, ..., nb), in a fume hood
- 60 Discard the glass culture tubes containing MilliQ water waste
- 61 Dry samples (in ethyl acetate in glass culture tubes) in water bath at 65°C for 8 min with N₂ gas, in a fume hood 8m
- 62 Resuspend dried samples in glass culture tubes with 200 μ L 25% HPLC-grade methanol
- 63 Vortex samples in glass culture tubes for 5 sec to ensure resuspension



5s

- 64 Centrifuge samples in glass culture tubes at 3200 g for 1 min at room temperature
- 65 Transfer sample (whole volume) to respective 0.6 mL polypropylene microcentrifuge tubes
- 66 Centrifuge samples in 0.6 mL polypropylene microcentrifuge tubes at 16,100 g for 2 min at room temperature
- 67 Transfer 150 µl of supernatant to respective glass LC-MS/MS vial glass inserts
- 68 Cap glass LC-MS/MS vials and ensure no air bubbles in the samples
- 69 Store samples in glass LC-MS/MS vials in -20°C until injection into mass spectrometer (MS)
- 70 Inject sample into LC-MS/MS system according to manufacturer's guidelines (in our case, we injected 35 µl of each sample into LC-MS/MS)

1m

2m

Acknowledgements

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