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Detection and Quantification of Calcitonin Gene-Related Peptide in Human Blood Plasma using a Modified Enzyme-Linked Immunosorbent Assay V.1

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Calcitonin gene-related peptide (CGRP) is a vasoactive neuropeptide that plays a putative role in the pathophysiology of migraine headaches and may be a candidate for biomarker status. Here, we present a protocol for purification and quantification of CGRP in human plasma. This protocol involves sample collection and preparation, extraction as a means of purification, and quantification via enzyme-linked immunosorbent assay (ELISA). After finding unrealistically high CGRP concentrations in spike-and-recovery experiments using a commercial CGRP ELISA kit protocol, we modified the existing protocol to produce satisfactory percent recoveries. This protocol can theoretically be used to quantify CGRP concentrations in plasma of individuals not only with migraine, but also with other diseases in which CGRP may play a role.

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Buffers

- TBS/Fish Gelatin (Bioworld, Catalog No. 50-199-167)
- Included in CGRP (human) ELISA kit (Bertin Bioreagent, Catalog No. A05481):
  - EIA Buffer (Bertin Bioreagent, Catalog No. A07000)
  - Wash Buffer (Bertin Bioreagent, Catalog No. A17000)

Equipment

- 6.0 mL Vacutainer EDTA collection tube (BD, SKU 367863)
- Allegra 64R benchtop centrifuge (Beckman Coulter, Inc., Product No: 367586)
- Precision micropipettes, multichannel pipettes, disposable tips
- 2 mL round bottom sterile cryovials (CRYO.S, Item No. 122263)
- Orbital Shaker (Bellco, SKU 7744-01010)
- Oasis HLB 3 cc Vac Cartridges (Waters, SKU WAT094226)
- 15 mL conical centrifuge tube (Falcon, Catalog No. 14-959-49B)
- 1.7 mL Safeseal microcentrifuge tube (Sorenson Bioscience, Inc., Catalog #11510)
- Vacufuge plus - Centrifuge Concentrator (Eppendorf, Catalog No. 022820109)
- SpectraMax M Series Multi-Mode Microplate reader (Molecular Devices, Part Number M2)

Materials

- 100% methanol
- Ultrapure water ELISA Grade (Bertin Bioreagent, Catalog No. A07001)
- 4% acetic acid
- Aprotinin (VWR, Catalog No. 76344-814)
- Ellman's Reagent (Bertin Bioreagent, Catalog No. A09000_49+1)

Sample Collection and Preparation

1 Collect whole blood from antecubital vein into 6.0 mL BD Vacutainer EDTA collection tube

2 Add 0.5 mL of aprotinin competitive serine protease inhibitor to tube after collection

3 Centrifuge at 3000 rpm for 4 minutes at 4°C, within 60 minutes of blood collection

4 Take off plasma fraction with an Eppendorf pipette and transfer to 2 mL round bottom sterile cryovials

5 Immediately store cryovials at -80°C for up to 2 weeks

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Extraction of Plasma Samples

6 Place an Oasis HLB Extraction cartridge inside a Falcon 15 mL conical centrifuge tube with the outer ridges of the cartridge supported by the outer ridges of the Falcon tube

7 Activate the cartridge by first passing 5 mL of 100% methanol and then 10 mL of ultrapure water through the cartridge. As fluid is passed through the cartridge, it will collect in the Falcon tube. Throw out excess fluid as it accumulates to ensure that the fluid does not engulf the cartridge. Ensure that the cartridge does not dry during the course of the experiment.

8 Dilute blood plasma at 1:4 with 4% acetic acid

8.1 Please note: The above ratio refers to dilution factor NOT dilution ratio (e.g. 1:4 refers to 1 volume of solute in 4 volumes of whole solution).

Per the manufacturer, each cartridge becomes saturated with 1 mL of blood plasma and acetic acid solution, therefore we dilute 250 uL of blood plasma with 750 uL of acetic acid. Theoretically, if one chooses to use more than 250 uL of blood plasma, one would need to dilute with the appropriate amount of acetic acid and pass this solution through more than one cartridge.

9 Pass 1 mL of blood plasma and acetic acid solution slowly through the cartridge at 1 mL per 30 seconds

10 Wash the cartridge with 10 mL of 4% acetic acid. As done previously, throw out excess fluid as it accumulates to ensure that the fluid does not engulf the cartridge.

11 After the last drop of acetic acid is released from the cartridge, remove the cartridge from the Falcon tube and place in a new Falcon 15 mL conical centrifuge tube.

12 Prepare a 3 mL solution of 100% methanol and 4% acetic acid (10:1) and elute the CGRP by passing this solution through the cartridge 1 mL at a time. Pause for 25 minutes between each mL of solution passed. Do NOT throw out the eluent.

12.1 Please note: Per the definition of dilution factor, the 3 mL solution should contain 2.7 mL of 4% acetic acid and 0.3 mL of 100% methanol.

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13 25 minutes after the last mL of methanol and acetic acid solution is passed, you should find 3 mL of eluent in the Falcon tube. Place each 1 mL of eluent into a 1.7 mL Safeseal microcentrifuge tube. Thus, you will have 3 tubes from each cartridge.

14 Place each tube in a mini centrifuge for 1 second to ensure that all eluent is at the bottom of each tube.

15 Dry all samples by vacuum centrifugation

15.1 Please note: Time taken by the vacuum centrifuge to dry samples will depend on the type of eppendorf tube used and the concentrator. For example, the vacuum centrifugation step lasted 5 hours using the aforementioned Safeseal tubes in a Vacufuge plus - Centrifuge Concentrator. Using alternative eppendorf tubes in the same centrifuge caused the centrifuge step to last up to 11 hours.

16 Reconstitute the dried sample with a volume of EIA buffer equal to the original sample volume. EIA buffer should be previously warmed to 37°C.

16.1 Example: If using 250 uL of original sample (before diluting with acetic acid), reconstitute with 250 uL of EIA buffer.

Plate Coating

17 Add 250 uL of Bioworld TBS/Fish Gelatin blocking buffer to each well on the plate.

18 Place a cover sheet over the plate and incubate for 2 hours at room temperature.

19 Remove cover sheet and empty the plate by inverting it. Then blot the plate on a paper towel to discard any trace of liquid.

20 Dry the plate by leaving the plate in a laminar flow hood for 10 minutes

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21 After the plates are visibly dry, place the plates in a grip-sealed foil pouch with a silica gel dessicant sachet and store the pouch at -20°C.

Prior to Assay Procedure

22 Prepare reagents (EIA buffer, CGRP standard, CGRP quality control, CGRP tracer, wash buffer) per the kit instructions. Prepare Ellman's reagent only after 16-20 hour incubation period ends.

23 Let all samples and reagents reach room temperature before performing the assay

Assay Procedure

24 Rinse each well in the blocked plate 5 times with wash buffer (300 uL/well). For each rinse, pause 30 seconds after placing the wash buffer in the wells, then throw out the liquid.

25 After the final rinse, remove all buffer from the wells by inverting the plate and blotting the last drops onto a paper towel. Tap the inverted plate until all liquid is visibly removed from the wells.

26 Set aside at least 2 wells with no reagents or buffer to be known as "Blank" wells. Then set aside at least 2 other wells for non-specific binding (NSB).

27 Dispense 100 uL of EIA buffer into each NSB well.

28 Dispense 100 uL of the CGRP standards in duplicate to appropriate wells.

29 Dispense 100 uL of samples (reconstituted in EIA buffer) and quality control in duplicate to appropriate wells.

30 Dispense 100 uL of CGRP tracer to each well containing NSB, CGRP standards, samples, and

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quality control. Do not dispense tracer to the wells dedicated as "Blank."

31 Cover the plate with a cover sheet and incubate for 16-20 hours at 4°C.

32 After incubation is completed, reconstitute Ellman's reagent per kit instructions.

33 Invert the plate to remove all liquid. Rinse each well (as described above) 3 times with 300 uL of wash buffer.

34 After 3rd rinse, remove the liquid from the plates, and place the plate on a shaker plate and shake for 2 minutes.

35 Wash the plate an additional 3 times. After the final rinse, remove all buffer from the wells by inverting the plate and blotting the last drops onto a paper towel. Tap the inverted plate until all liquid is visibly removed from the wells.

36 Dispense 200 uL of Ellman's reagent into each well (not including the "Blank" wells).

37 Cover the plate with a new cover sheet and wrap in aluminum foil to prevent any light exposure. Incubate in the dark at room temperature for 1 hour.

38 Wipe the bottom of the plate with a paper towel to ensure that there is no liquid outside of the wells.

39 Read the plate for Absorbance at 405nm (yellow color)

Data Analysis

40 Calculate average absorbance for each "Blank," NSB, standard, quality control, and samples.

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41 Subtract "Blank" and NSB average absorbance values from standard, quality control, and sample absorbance values

42 Plot absorbance on the y-axis and concentration on the x-axis. Construct a standard curve using a Four-Parameter Logistic regression model.

43 Once the curve is constructed, use the curve's equation to determine the interpolated concentrations for quality control and samples, which can be read on the x-axis.

43.1 The standard curve is validated only if the calculated interpolated concentrations for the quality control is within 25% of the expected concentration (usually 125 pg/ml, but see label of the quality control vial).

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