Determination of neopterin and biopterin by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in rat and human plasma, cell extracts and tissue homogenates

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Method Article

Keywords: tetrahydrobiopterin, biopterin, chromatography, tandem mass spectrometry, GTP cyclohydrolase, pain, dorsal root ganglion, haplotype, single nucleotide polymorphism, whole blood assay

Posted Date: October 13th, 2006

DOI: https://doi.org/10.1038/nprot.2006.298

Abstract

Introduction

The rate limiting enzyme in tetrahydrobiopterin \(BH4) synthesis, GTP cyclohydrolase, is upregulated in DRGs following peripheral nerve injury or inflammation. The excess tetrahydrobiopterin synthesis contributes to the manifestation and persistence of pain. Using this protocol we find increased BH4 levels in the L4/5 DRGs and spinal cord tissue. Normalization of excess BH4 production reduces the nociceptive behaviour in various rodent models of neuropathic and inflammatory pain. In humans we identified single nucleotide polymorphisms \(SNPs) in the gene encoding GTP Cyclohydrolase \(GCH1) that are associated with low pain scores. Carriers of a specific pain protective haplotype show reduced upregulation of GTP Cyclohydrolase expression and activity. Using this protocol we showed that BH4 measured as its oxidation product biopterin, is reduced in forskolin-stimulated whole blood and white blood cells in carriers of the haplotype as compared to non-carriers. The "forskolin whole blood assay" for biopterin might be a useful diagnostic tool in combination with GCH1 genotyping to assess the individual patient's risk for chronic pain.

Reagents

Whole blood assay: Heparinized blood tubes \(e.g. BD VacutainerTM). Solid phase extraction: Extraction cartridges containing a mixed mode polymeric patented sorbent with reversed-phase and cation-exchange functionalities, 1 ml volume, 30 mg sorbent; e.g. Oasis MCX extraction cartridges, Waters. Chromatographic separation: Gemini C18 column \(150 × 2 mm i.d., 5 µm particle size, 110 Å pore size; Phenomenex). Reagents: Forskolin \(e.g. Sigma) Neopterin \(e.g. Sigma) Biopterin \(e.g. Sigma) Rhamnopterin \(internal standard) \(e.g. Fluka) 0.1 M hydrochloric acid \(e.g. Merck KGaA) 1 M hydrochloric acid \(e.g. Merck KGaA) Iodine \(e.g. AppliChem) Potassium iodide \(e.g. Merck KGaA) Ascorbic acid \(e.g. AppliChem) Ammonia solution 25% \(e.g. Merck KGaA) Formic acid 89-91% \(e.g. Merck KGaA) Methanol gradient grade \(e.g. Merck KGaA) Water LC-MS grade \(e.g. Roth GmbH)

Equipment

Extraction device: Visiprep vacuum manifold \(Supelco) attached to a diaphragm vacuum pump \(MZ2C, Vacuubrand GmbH) Chromatographic separation: Agilent 1100 Series binary pump \(G1312A) and degasser \(G1379A) connected to an HTC PAL autosampler \(e.g. Chromtech) MS/MS analysis: 4000 Q TRAP triple quadrupole mass spectrometer with a Turbo V source \(Applied Biosystems) Analyst software 1.4 \(Applied Biosystems)

Procedure

Whole blood assay: 1. Collect blood into two heparinized blood tubes \(for unstimulated control and forskolin). 2. Add forskolin stock to one tube, 10 µM final concentration. 3. Incubate control and forskolin

stimulated sample for 24 hrs at 37°C incubator or water bath. 4. Centrifuge in table top centrifuge at 3000 rpm for 10 min. 5. Store plasma at -80°C. **Tissue preparation:** 1. Euthanize and debleed animals and rapidly excise DRGs and spinal cord tissue, immediately freeze on dry ice or liquid nitrogen, store at –80°C. **Tissue homogenization, acidic oxidation \[1] and solid phase extraction:** 1. Add 200 µl of 0.1 M hydrochloric acid containing 20 ng/ml of the internal standard rhamnopterin to the defrosted tissue \(small pieces of about 2-3 mg) or plasma sample \(50 µl) in a 1.5 ml microcentrifuge tube. 2. Homogenize tissue with reusable pellet pestles and motor \(Kontes Glass Company, Vineland, New Jersey, USA) for 45 s with at least 10 strokes, remove 10 µl of the suspension for protein determination. 3. Add 100 µl of iodine solution \(1% \(w/v) iodine and 2% \(w/v) potassium iodide in 1 M hydrochloric acid), mix and incubate for 30 min in the dark at room temperature. CRITICAL STEP: Always prepare fresh iodine solution. 4. Stop the reaction by adding 200 µl of 5% \(w/v) ascorbic acid in water and vortex up to decolorization. CRITICAL STEP: Always prepare fresh ascorbic acid solution. 5. Activate the cation-exchange cartridge \(Oasis MCX extraction cartridge) with 1 ml methanol and 1 ml water before loading of the sample. 6. Load the completely decolorized sample onto the cartridge, allow binding and vacuum elute unbound substances, wash with 1 ml 0.1 M hydrochloric acid and 1 ml methanol. 7. Elute bound substances with 1 ml of 8% \(v/v) ammonia solution in 25% methanol. CRITICAL STEPS Points 5-7: Use a maximum speed of 1 ml per min for vacuum elution 8. Evaporate the organic solvent to dryness by a gentle stream of nitrogen at 40°C. 9. Reconstitute the residue with 200 µl water/0.002% formic acid \(v/v) and transfer the solution in a screw vial with a glass insert containing a Silikon/PTFE seals in the screw cap \(e.g. Macherey-Nagel). **Chromatography** 1. Use a Gemini C18 column \(150 × 2 mm i.d., 5 µm particle size, 110 Å pore size) for chromatographic separation, connected to a pump and autosampler for injection of the samples \(e.g. Agilent 1100 Series binary pump \(G1312A) and degasser \(G1379A) connected to an HTC PAL autosampler). 2. Use as mobile phase eluent A \(water with 0.002% formic acid) and eluent B \(methanol with 0.002% formic acid) and apply the following gradient: 0 - 0.5 min A/B 100:0 0.5 - 4 min a linear gradient from 100:0 to 5:95 4 - 7 min A/B 5:95 7 - 8 min a linear gradient from 5:95 to 100:0 and finally 8 - 13 min A/B 100:0 3. Inject 10 µl of the sample onto the LC-MS/MS. 4. Set the flow rate to 0.3 ml/min and the runtime to 13 min. 5. Biopterin, neopterin and rhamnopterin elute after 5.7, 4.1 and 5.5 min, respectively. **MS/MS analysis** For determination of total biopterin and neopterin concentrations use liquid chromatography coupled to tandem mass spectrometry employing a 4000 Q TRAP triple quadrupole mass spectrometer with a Turbo V source. 1. Choose the negative ion mode with an electrospray voltage of -4300 V at 400 °C and set the auxiliary and nebulizer gas to 50 psi, the curtain gas to 10 psi and the collision gas to 5 psi Use pure nitrogen as gases. 2. Use precursor-to-product ion transitions of m/z 236 to 192 for biopterin \(collision energy -20 V), 252 to 192 for neopterin \(-22 V) and 265 to 192 for rhamnopterin \(-20 V) as quantifier for the multiple reaction monitoring \(MRM) with a dwell time of 150 ms. 3. Evaluate concentrations of the samples with calibration standards \(0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 30, 50 ng/ml) using Analyst software 1.4 \(Applied Biosystems). Every tenth sample in each run should be a quality control \(1, 10, 50 ng/ml).

Critical Steps

Iodine and ascorbic acid solutions must be freshly prepared shortly before use The speed of vaccum elution must not be > 1 ml/min

Anticipated Results

Validation: The calibration curve is linear from 0.1 to 50 ng/ml. Variations in accuracy and intraday and interday precision were < 15% over the range of calibration. **Concentration ranges:** Concentrations of biopterin in human unstimulated plasma of healthy subjects are in the range of 1 to 2 ng/ml. In naïve rats plasma biopterin concentrations are in the range of 55–65 ng/ml. Plasma neopterin is below the quantification limit in naïve rats but increases after paw inflammation reaching about 5-10 ng/ml at 24 hours. Concentrations of biopterin and neopterin in L4/5 DRGs of naïve rats are about 15–25 ng/mg and 3-6 ng/mg protein. In spinal cord concentrations are about 40–50 ng/mg and 1–2 ng/mg protein, respectively. Biopterin and neopterin concentrations in the DRGs increase after peripheral nerve injury.

References

1. Fukushima, T. & Nixon, J. C. Chromatographic analysis of pteridines. _Methods Enzymol_ **66**, 429-36 \(1980).

Figures

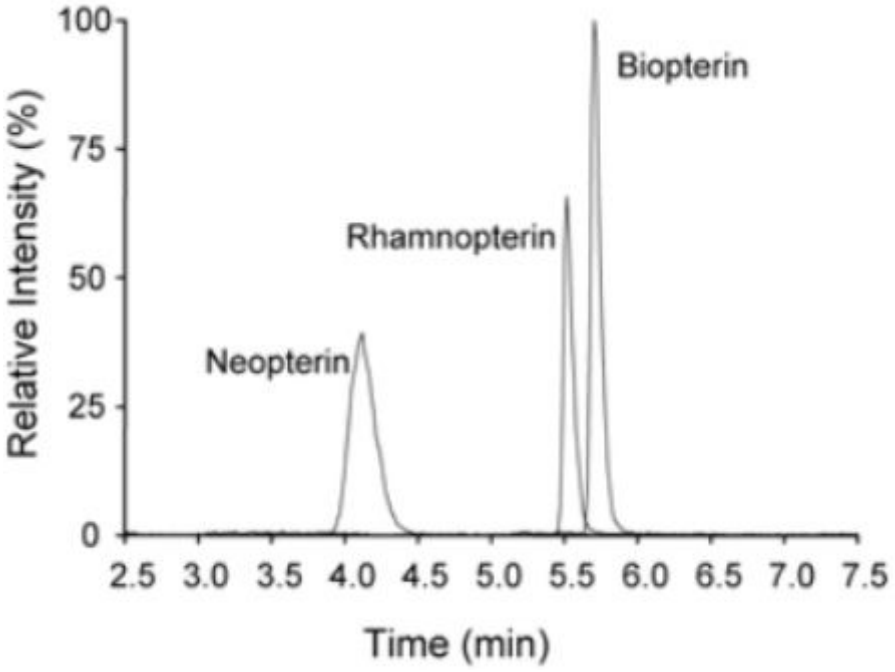

Figure 1

Chromatogram Representative chromatogram of a human plasma sample following solid phase extraction.

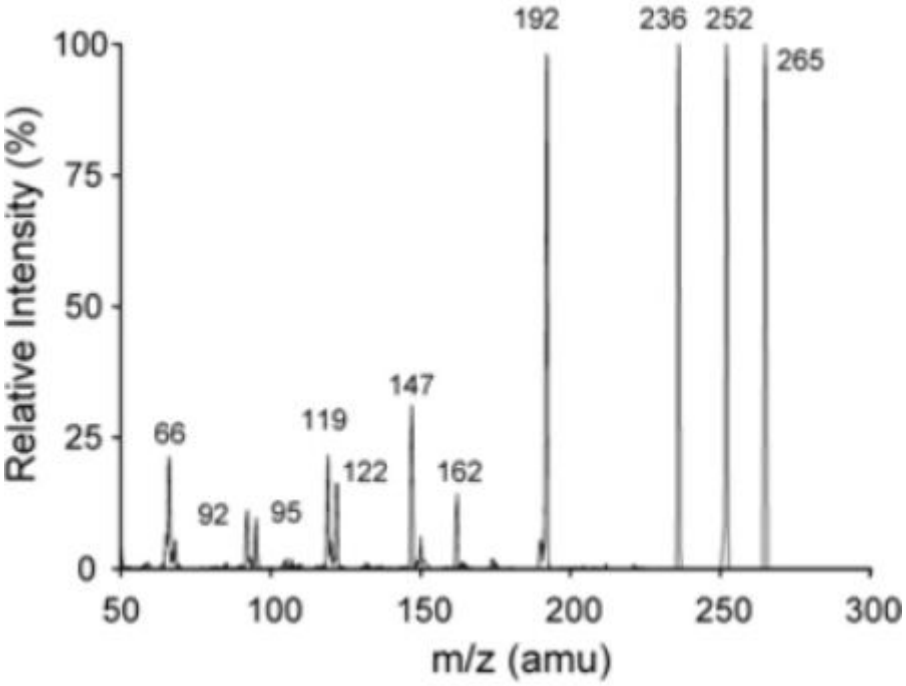

Figure 2

Fragment Spectra Fragment spectra of biopterin (236.0), neopterin (252.0) and rhamnopterin (265.0) in the negative ion mode. The analytes show almost identical fragments allowing depiction of mass spectra in one figure. Precursor-to-product ion transitions are m/z 236 to 192 for biopterin, 252 to 192 for neopterin and 265 to 192 for rhamnopterin.