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A novel microflow LC–MS method for the quantitation of endocannabinoids in serum

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ABSTRACT

Endocannabinoids (ECs) represent a class of endogenous, small molecules that bind and activate the G-protein coupled EC receptors. They are involved in a variety of fundamental biological processes and are associated with many disease states. Endocannabinoids are often present in complex matrices and at low concentrations, complicating their measurement. Here we describe a highly sensitive method for the quantitation of the following ECs in serum: *N*-arachidonoylethanolamine (anandamide), *N*-oleoylethanolamine, *N*-palmitoylethanolamine, 2-arachidonoylglycerol, and its inactive isomer 1-arachidonoylglycerol. On-line sample trapping coupled with separation via microflow liquid chromatography and detection by tandem quadrupole mass spectrometry results in the necessary sensitivity for accurate quantitation of ECs in less than 50 μL of serum, without the need for off-line solid phase extraction. Limits of quantitation between 1.2 and 13.4 pg/mL were achieved, representing a significant increase in sensitivity compared to previous methods using analytical flow rates. An additional benefit of microflow chromatography is the reduction of solvent consumption by more than two orders of magnitude. The experimental utility of the assay is demonstrated through the analysis of serum from hibernating bears to assess seasonal changes in circulating EC concentrations.

1. Introduction

Endocannabinoids (ECs) are endogenous, lipid-derived small molecule ligands of the G-protein coupled EC receptors CB1 and CB2. Together the EC receptors, the ECs, and the enzymes responsible for the production and degradation of the ECs constitute the EC system [1]. The EC system is involved in a plethora of biological processes and disease states including thermogenesis, pain, aging, cancer, and the 'runner's high' [2–5]. Endocannabinoids are typically present at low concentrations (low nM range) and in complex bio-matrices. Thus, accurate quantitation of ECs requires an analytical platform with high sensitivity and selectivity.

Analytical flow liquid chromatography (i.e. flow rates >100 μL/min) coupled to tandem quadrupole mass spectrometry (LC–MS/MS) is a common approach for the quantitation of ECs [6]. However, it is well documented that the use of lower flow rates (<10 μL/min) can result in sensitivity gains due to more efficient ion transmission to the mass spectrometer [7]. Indeed, most proteomics applications utilize nanoflow chromatography (nanoL/min flow rates) to achieve the sensitivities required for sample limited analyses. While sensitivity gains are significant in the nanoflow regime, technical challenges discourage its application in non-sample limited analyses. Microflow chromatography (i.e. flow rates of 2–10 μL/min) represents an intermediate flow regime that offers the benefits of increased sensitivity combined with the ease of use and robustness expected with higher flow techniques.

Here, we present a novel method utilizing microflow chromatography coupled with tandem quadrupole mass spectrometry (μLC–MS/MS) for the sensitive and selective quantitation of ECs: *N*-arachidonoylethanolamine (anandamide; AEA), *N*-oleoylethanolamine (N-OE), *N*-palmitoylethanolamine (N-PE), 2-arachidonoylglycerol (2-AG), and its inactive isomer 1-arachidonoyl glycerol (1-AG). The assay offers multiple advantages compared to previously published assays, most notably increased sensitivity, decreased solvent and sample consumption, and automated on-line sample cleanup. The utility of the assay is demonstrated through the quantitation of serum EC levels in black bears during hibernation [8].

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2. Materials and methods

2.1. Materials

N-arachidonoylethanolamine (anandamide; AEA), *N*-oleoylethanolamine (N-OE), *N*-palmitoylethanolamine (N-PE), 2-arachidonoyl glycerol (2-AG), 1-arachidonoyl glyercol (1-AG), AEA-d4, and 2-AG-d5 were from Cayman Chemical (Ann Arbor, MI, USA). Toluene, LC–MS grade acetonitrile, and LC–MS grade water were from Fisher Scientific (Lafayette, CO, USA). LC–MS grade formic acid was from Sigma (St. Louis, MO, USA).

2.2. Animal handling and sample collection

Wild black bears were captured in Virginia and held in pens at the Virginia Tech Center for Bear Research from fall through spring, representing one full hibernation season. Upon completion of the study, the bears were released. The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (#98-069-F&WS). To facilitate blood sample collection during the study, bears were anesthetized via pneumatic dart with a 2:1 mixture of ketamine (100 mg/mL):xylazine (100 mg/mL); the dosage was 1 cc of the mixture per 45.5 kg of body mass. Blood samples were drawn from the femoral vein while the bears were anesthetized. The blood was spun to isolate the serum, which was partitioned into aliquots and frozen at −80 °C. Blood samples were collected from each bear every 10 days from the beginning of October until the end of May. Hibernation began in early January and ended in early April. Thus, the collection dates encompassed a pre-hibernation period, a period of transition into hibernation during which bears gradually reduce their metabolic rate, a hibernation period, and a period of transition out of hibernation during which bears gradually increase their metabolic rate while becoming physically active following hibernation.

2.3. Sample preparation

Liquid-liquid extraction was used to extract endocannabinoids from serum using a protocol adapted from Zoerner et al. [9]. Briefly, after thawing on ice, 50 μL of serum was aliquoted into 2 mL glass vials. 1000 μL of toluene containing internal standards at 100 pg/mL was added followed by 15 min of vortexing on medium speed at room temperature. 400 μL of water with 3% formic acid was added followed by an additional 5 min vortex. Samples were kept at −80 °C for 2 h to facilitate protein precipitation. Next, samples were allowed to equilibrate to 4 °C, then were centrifuged for 15 min at 4 °C at 3,000 x *g*. The top, organic layer was transferred to a new 2 mL glass vial and evaporated at room temperature under a gentle stream of nitrogen. To resuspend the dried residue, 40 μL of 50:50 acetonitrile:MeOH was added and vortexed for 2 min 60 μL water with 0.2% formic acid was then added followed by an additional 2 min vortex. Standards for the external calibration curve were prepared in neat solution and subjected to the extraction procedure described above. The resulting extract was subjected to μLC–MS/MS analysis.

2.4. LC–MS/MS

For all LC–MS/MS experiments, a Waters Xevo TQ-S tandem quadrupole mass spectrometer was coupled to a Waters M-class UPLC equipped with a trap valve manager. To accommodate the different LC flow rates tested, two Waters stationary phases with different internal diameters were used: iKey with a post-column addition (PCA) channel (Peptide BEH C18 130 Å, 150 μm × 50 mm, 1.7 μm) and a column (Atlantis dC18, 300 μm × 150 mm, 3 μm). The auxiliary pump used for the PCA experiments was a Waters nanoAcquity binary solvent manager. For trapping experiments, a Waters Symmetry C8 (300 μm × 50 mm, 5 μm) column was utilized at room temperature. In all experiments, the autosampler temperature was held at 6° C.

2.5. Final assay

In the final method, the iKey was operated at a flow rate of 2 μL/min. Mobile phase A was water and mobile phase B acetonitrile, both with 0.1% formic acid. The iKey was operated at 55° C and the gradient was as follows: time (t) = 0 min, 60% B; t = 7 min, 70% B; t = 8 min, 100% B; t = 9 min, 100% B. The iKey was re-equilibrated at starting conditions for 5 min at 4 μL/min. The loading time for trapping was 2 min at a flow rate of 15 μL/min at 10% B. The injection volume was 10 μL.

The mass spectrometer was operated in selected reaction monitoring (SRM) mode in positive ion polarity. Settings for each SRM transition, including precursor *m*/*z*, product *m*/*z*, cone voltage, and collision energy, were optimized for sensitivity. Capillary voltage was 3.6 kV and source temperature was 120° C. Cone gas (N_2(g)) flow as 150 L/h and nanoflow gas pressure was 0.2 bar. Argon was used as the collision gas.

2.6. Data analysis

Peak areas for each compound were normalized to their corresponding internal standard. Peak integration was performed using the open source software Skyline-daily [10]. Concentrations were determined using an external standard curve. Percent recovery was calculated as a ratio of the concentration of each analyte in unspiked matrix and the corresponding concentration in spiked matrix. Figures were generated and statistics performed in GraphPad Prism 6 software. Statistical analysis of seasonal EC levels in bears was performed by first determining if datasets were normally distributed for each EC. All datasets contained one group that was not normally distributed, thus, a Kruskal-Wallis test was performed followed by a Dunn's multiple comparison test.

3. Results and discussion

3.1. SRM optimization

Conditions for each selected reaction monitoring (SRM) transition were optimized for sensitivity by direct infusion into the mass spectrometer. Final SRM conditions for each EC are presented in Table 1. The selected precursor and product *m*/*z* values for each EC are in good agreement with previously published assays [9,11].

3.2. Increased sensitivity with microflow chromatography

To determine if lower LC flow rates offer gains in sensitivity, peak heights for the ECs were compared at two different flow rates. The first using a flow rate of 20 μL/min (column: Atlantis dC18, 300 μm × 150 mm, 3 μm) and the second using a flow rate of 2 μL/min (iKey: BEH C18 130 Å, 150 μm × 50 mm, 1.7 μm). To prevent artificial changes in sensitivity due to solvent composition, analyses were performed with an isocratic gradient at 70% B. Peak height was increased on average 3-fold with the lower flow of 2 μL/min (Fig. 1). The AG isomers were not separated under these isocratic conditions and thus are not represented individually (Fig. 1a). The LC–MS chromatograms for anandamide at the different flow rates are overlaid in Fig. 1b.