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Journal of Chromatography A, 1111 (2006) 153–158

JOURNAL OF CHROMATOGRAPHY A

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Quantitation of nicotinamide and serotonin derivatives and detection of flavins in neuronal extracts using capillary electrophoresis with multiphoton-excited fluorescence

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Available online 22 August 2005

Abstract

Capillary electrophoresis (CE) with multiphoton-excited fluorescence detection (CE-MPE) allows low-background analysis of spectrally distinct fluorophores using a single long-wavelength laser. Extracts were prepared from immortalized rat raphe nuclei neurons, and were analyzed by CE-MPE. Native fluorescence was detected from reduced nicotinamide adenine dinucleotide (NADH) and its phosphorylated form (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), riboflavin, serotonin, and 5-hydroxytryptophan (5HTrp). Quantitation of exogenous serotonin (taken up by cells) and endogenous NADH and 5HTrp was possible using internal standards or standard addition. This system should be useful to study monamine oxidase inhibitors (MAOIs) and selective serotonin reuptake inhibitors (SSRIs).

Keywords: Vitamin B_3; Vitamin B_2; Antidepressant; Brain; Indole; RN46A-B14; Multiphoton

1. Introduction

Neuronal content of redox cofactors and neurotransmitter derivatives can reflect metabolism, biosynthesis, and regulation in normal and pathological states. The cytosolic and organelle-associated levels of these compounds can be influenced through the use of pharmacological agents, such as monoamine oxidase inhibitors (MAOIs) [1], classic antidepressants that alter the pathway summarized in Fig. 1. Metabolic degradation of serotonin (5HT) proceeds first through monoamine oxidase, which is covalently modified with a flavin cofactor, and then through aldehyde dehydrogenase, which uses a solution-phase nicotinamide cofactor [2]. The intermediate in this catabolism, 5-hydroxyindole-3-acetaldehyde, typically is degraded rapidly in vivo to the primary metabolite, 5-hydroxyindole-3-acetic acid (5HIAA) [2]. By preventing catabolism of serotonin to 5HIAA, monoamine oxidase inhibitors elevate serotonin levels and, as a consequence, effect positive modification on subjective mood in humans. In the human brain, the two isoforms of monamine oxidase (MAO_A, which preferentially deaminates serotonin and epinephrine, and MAO_B, which may preferentially deaminate dopamine) are differently distributed and are targets of dozens of MAOIs, each with its own selectivity, kinetics, and reversibility [2]. Therefore, each MAOI could uniquely affect levels of flavins (Vitamin B_2 derivatives) and nicotinamides (Vitamin B_3 derivatives). Beyond MAOIs, other drug therapies seek to elevate neurotransmitter levels by inhibiting cellular uptake [3] or by increasing availability of biosynthetic precursors [4], such as tryptophan [5] and 5-hydroxytryptophan (5HTrp, the immediate precursor to serotonin) [6]. These drug-targeted processes are complex, involve diverse analytes, and are sometimes studied in poor models, such as blood platelets [7], rather than in neuronal cells.

Various analytical approaches have been developed for analysis of the cofactors and metabolites involved in such pathways, commonly fractionating complex cytosolic or tissue homogenates using HPLC [8] or capillary electrophoresis (CE) [9–13]. Sensitive detection of components can be accomplished using electrochemical or fluorescence

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0021-9673/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.chroma.2005.07.067

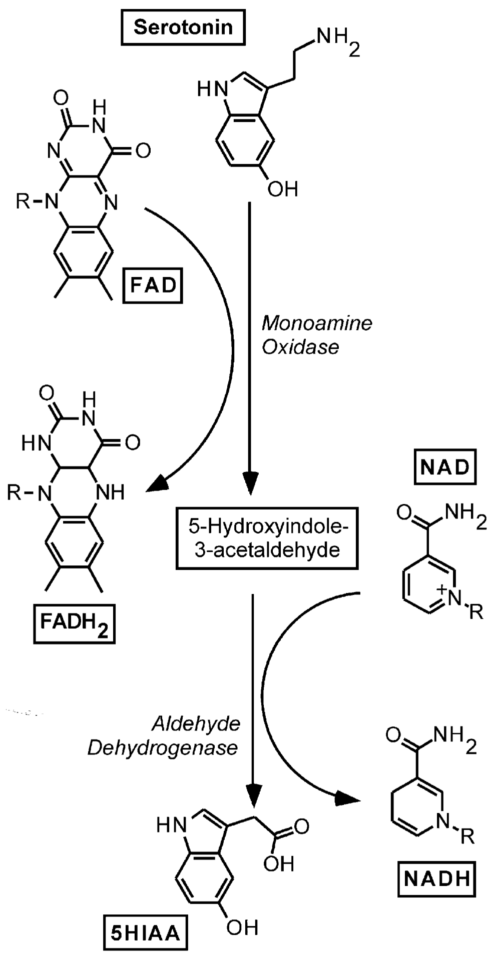

Fig. 1. Metabolic degradation of serotonin. R: ribose-diphosphate-ribose-adenine. FAD/NAD and $FADH_2$/NADH are the oxidized and reduced forms of flavin and nicotinamide adenine dinucleotides, respectively. Additional co-reactants and by-products are not shown.

methods, but often is not general enough to provide low detection limits for all species of interest. This limitation has been particularly challenging for laser-induced fluorescence detection. The excitation spectra of three representative analytes, shown in Fig. 2, demonstrate that no single one-photon laser wavelength could adequately excite all species. However, our laboratory has demonstrated that capillary electrophoresis with multiphoton-excited fluorescence detection (CE-MPE) allows low-background analysis of these spectrally distinct biological fluorophores using a single long-wavelength laser [14]. In this approach, a pulsed, femtosecond laser source – capable of generating nanojoules of near-infrared light in pulses of ∼100 fs duration – efficiently excites molecular transitions through the non-resonant absorption of two or more photons. Using a femtosecond titanium:sapphire laser, we have shown previously that compounds with visible to near-UV electronic energy spacings (e.g., oxidized flavins, reduced nicotinamides) can be excited through a two-photon absorption mechanism with the same laser output capable of exciting mid- to deep-UV chromophores (e.g., monoamine neurotransmitters) via three-photon absorption. In addition, we have demonstrated improved detection limits for hydroxyindoles by using the same titanium:sapphire wavelengths to generate emission in the visible spectral region through an unusual photochemical transformation process. In all cases, efficient detection of emission across most of the ultraviolet and visible spectral ranges (∼300–600 nm) can be performed virtually free from background caused by laser scatter due to the relative ease of filtering long-wavelength excitation light and the insenstivity of photomultipliers to scatter that does reach the detector [14].

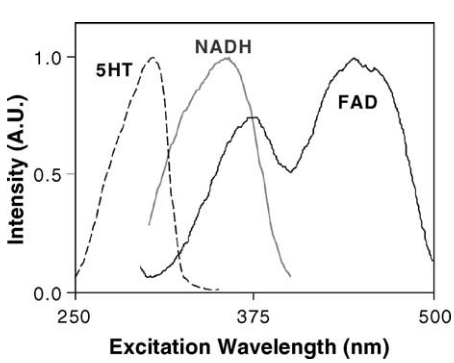

Fig. 2. One-photon excitation spectra of serotonin (5HT), NADH, and FAD. Each spectrum is normalized to yield a maximum intensity of 1.0.

Earlier studies in our laboratory have focused on development and optimization of this high-sensitivity analysis approach [14–16] and have demonstrated the utility of the technique for characterization of nicotinamide cofactors in cellular samples [17,18]. The current work extends these methods to determination of multiple analyte classes in real and complex neuronal samples.

2. Experimental

Unless otherwise noted, all chemicals and supplies were obtained from Fisher Scientific (Pittsburgh, PA), Sigma Chemical (St. Louis, MO), or Invitrogen (Carlsbad, CA) and were used as received.

2.1. Cell culture

RN46A-B14 cells were received as passage 27 from Professor Scott R. Whittemore (Department of Neurological Surgery, University of Louisville, KY) and were generally cultured as recommended [19–21]. These cells were originally immortalized from embryonic rat medullary raphe nuclei [22], the major serotonergic centers of the brain, and then were engineered to express a neurotrophic factor [20]. Cells were propagated at ∼1:3 splits on tissue-culture treated flasks incubated at 33 °C in 5% CO_2. Growth medium was a 50:50 mix of Dulbecco's minimum essential medium with Ham's F12 medium, supplemented with 10% fetal bovine serum and antibiotics (250 mg/L gentamycin, 100 U/mL penicillin, 100 mg/L streptomycin, and 100 mg/L