A Competitive, Colorimetric ELISA for Quantification of 24(S)-Hydroxycholesterol, a Biomarker for Neurodegeneration

24(S)-Hydroxycholesterol (24-OHC) ELISA kit (ADI-900-210)

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ABSTRACT

Cholesterol imbalance is implicated in a variety of neurodegenerative diseases including Alzheimer's Disease, Huntington's Disease, and Multiple Sclerosis. Excess cholesterol is removed from the brain via the hydroxylation of C24 on cholesterol by CYP46 enzyme, a highly conserved member of the cytochrome P450 family, to the more soluble 24(S)-Hydroxycholesterol (24-OHC) which is able to cross the blood brain barrier. Levels of 24-OHC in the cerebral spinal fluid (CSF) may give insight into the homeostasis of cholesterol within the central nervous system, and may serve as a valuable biomarker for neurodegenerative disease. We have developed a competitive colorimetric ELISA for the measurement of 24-OHC that can be used in both *in vivo* and *in vitro* studies. The assay is highly specific to 24-OHC, exhibiting less than 0.02% cross-reactivity to cholesterol, 22-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol and dehydroepiandrosterone (DHEA). The assay returns 24-OHC levels in CSF consistent with levels reported in the literature, and is also suitable for tissue homogenates and tissue culture supernatants, requiring 100 µL or less of sample volume. The assay has a dynamic range from 0.78 to 100 ng/mL and a time to answer of 2 hours, making it a convenient alternative to more labor- and instrument-intensive methods such as GC-MS, LC-MS and HPLC-MS.

BACKGROUND

The homeostasis and trafficking of cholesterol is an essential component of both the central and peripheral nervous system in the maintenance of neuronal tissues,[1,2] and disturbances in this homeostasis may be due to the onset of various neurological diseases such as Alzheimer's Disease (AD), Huntington's Disease (HD) and multiple sclerosis (MS).[3,4,5] Apolipoprotein E and Cyp46 (also known as 24S-Cholesterol Hydroxylase) are both important in the homeostasis of cerebral cholesterol[6] and thus are of clinical interest in understanding the relation of these molecules to the pathogenesis of these, and potentially other, neurodegenerative diseases.

24-OHC, an enzymatically-generated side chain-hydroxylated derivative of cholesterol, is a pivotal marker in the study of cerebral cholesterol homeostasis. Cholesterol is unable to cross the blood-brain barrier (BBB) however, Cyp46 enzyme converts cholesterol to the more soluble 24-OHC, and this hydroxylated form of cholesterol is able to cross the BBB.[7,8] This conversion allows for the reduction of cholesterol in the brain and the efflux of 24-OHC from the brain into cerebral spinal fluid and blood. The flux of 24-OHC has been observed in patients with a variety of neurodegenerative diseases.[3,4,5] In the instance of Alzheimer's disease, the change in 24S-hydroxycholesterol concentrations may be indicative of different pathogenetic mechanisms and/or the progression of the disease.[3] As in the case of multiple sclerosis, concentrations of 24-OHC have been shown to decrease, likely due to the loss of neuronal cells responsible for the synthesis.[5]

Current 24-OHC measurements are performed using GC-MS, LC-MS and HPLC-MS methodologies, all of which carry high initial instrumentation costs and routing maintenance. We have developed a competitive colorimetric ELISA for the measurement of 24-OHC for use in both *in vivo* and *in vitro* studies.

ASSAY VALIDATION

Principle

The 24(S)-Hydroxycholesterol ELISA is a competitive format immunoassay. Standards and samples are added to wells coated with a goat anti-rabbit IgG antibody (Figure 1). A solution of biotinylated 24(S)-Hydroxycholesterol (conjugate) is then added, followed by a solution of rabbit polyclonal antibody to 24(S)-Hydroxycholesterol. During a simultaneous incubation at room temperature for 1 hour, the antibody competitively binds 24(S)-Hydroxycholesterol in the sample or conjugate. The plate is washed, leaving only bound 24(S)-Hydroxycholesterol. A solution of streptavidin conjugated to horseradish peroxidase is added to each well and incubated for 30 minutes at room temperature, binding biotinylated 24(S)-Hydroxycholesterol. The plate is washed to remove excess HRP conjugate, and developed with TMB substrate solution for 30 minutes at room temperature, which generates a blue color in the solution. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is

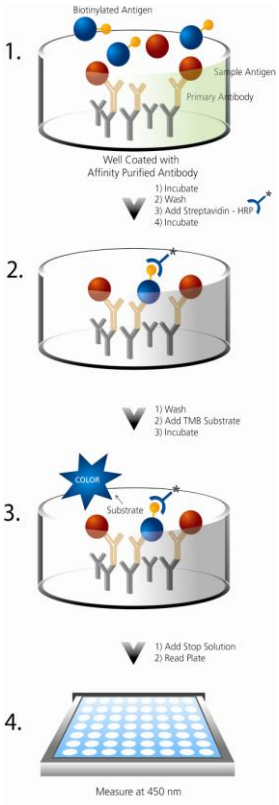

Figure 1: Assay Protocol

inversely proportional to the level of 24(S)-Hydroxycholesterol in the sample.

Standard Curve

To determine a typical standard curve, 24(S)-Hydroxycholesterol was serially diluted in assay buffer and run in the assay. The standard curve was plotted as optical density (OD) and as percent of binding (%B/B_0) both as a function of antigen concentration (Figure 2). Sensitivity of the assay, defined as the concentration of analyte measured at 2 standard deviations below maximum binding, was determined to be 0.78 ng/mL.

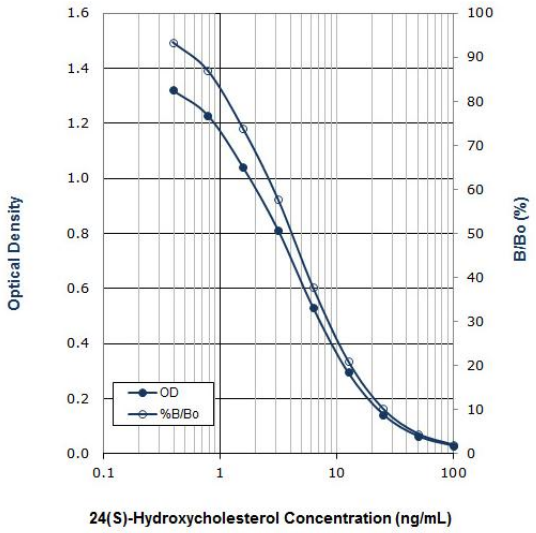

Figure 2: Typical standard curve produced using 24-OHC ELISA kit standards.

Assay Parallelism & Spiked Recovery in Various Matrices

Dose-response curves from human cerebral spinal fluid and rat brain tissue homogenate were diluted into assay buffer and compared to the 24(S)-Hydroxycholesterol standard curve. The parallel response (Figure 3) indicates the standard effectively mimics the native molecule.

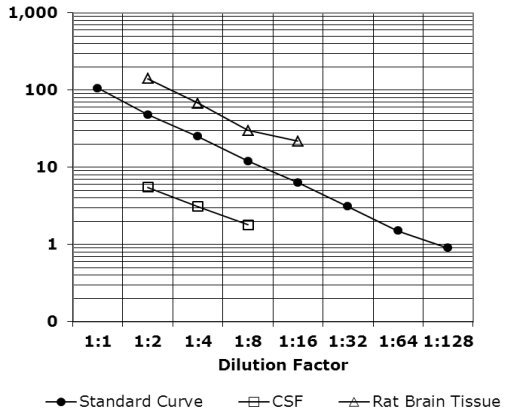

Figure 3: Graph displaying parallelism between the assay standard and native 24-OHC in human CSF and rat brain tissue.

Similarly, synthetic 24(S)-Hydroxycholesterol was spiked into CSF, brain tissue homogenate, and tissue culture medium containing various amounts of fetal bovine serum (FBS). Matrix background was subtracted from the spiked values and the average recovery was compared to the recovery of identical spikes in assay buffer. The average percent recovery for each matrix and minimum required dilutions are indicated in Table 1.

Sample	Recommended Dilution	Recovery of Spike
Cerebral Spinal Fluid	1:2	99.0%
Brain Tissue Homogenate	1:2	98.6%
TCM (serum free)	Neat	95.5%
TCM (1% FBS)	Neat	107.9%
TCM + 10% FBS	Neat	101.6%

Table 1: Assay recovery and required dilutions in various sample matrices.

Assay Specificity

To assess the specificity of the assay, cross reactivity for related compounds (Table 2) was determined by diluting the compounds in assay buffer at a concentration one hundred times the high standard. Samples were then measured in the assay, and percent cross reactivity determined.

Analyte	Cross Reactivity
Cholesterol	0.004%
22-Hydroxycholesterol	0.042%
25-Hydroxycholesterol	0.09%
27-Hydroxycholesterol	0.018%
Dehydroepiandrosterone	0.006%

Table 2: Cross-reactivity of 24-OHC-related compounds.

METHODS

Assessment of 24-OHC Levels in Human CSF

To determine the accuracy of the assay in reporting 24-OHC levels, normal human cerebral spinal fluid samples from male and female subjects of various ages were diluted 1:2 in assay buffer and analyzed using the ELISA for 24(S)-Hydroxycholesterol levels according to the assay protocol. Results are shown in Figure 4. The assay reported levels between 2 and 6 ng/mL (blue bars)

consistent with endogenous CSF 24(S)-Hydroxycholesterol levels as reported in the literature[9] (represented by dashed red lines).

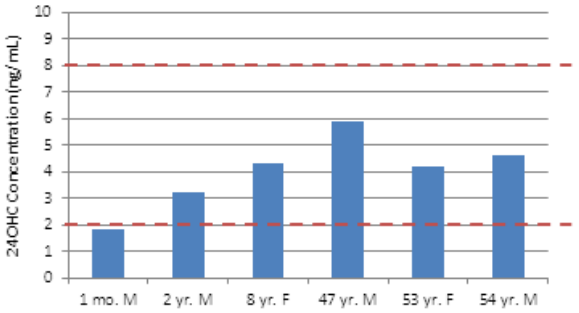

Figure 4. 24-OHC levels in human CSF determined by ELISA.

Assessment of 24(S)-Cholesterol hydroxylase (CYP46A1) Activity *In Vitro*

24(S)-Cholesterol hydroxylase (CYP46A1) converts cholesterol to 24(S)-hydroxycholesterol (24-OHC) in the brain. To display the potential of the ELISA for use in quantifying 24-OHC as a marker of CYP46A1 activity *in vitro*, supernatants from cells transfected with vector containing wild type CYP46A1 or a catalytically inactive CYP46A1 mutant [CYP46A1(mut)] were assessed. Cultured media was harvested from each transfection for analysis in the ELISA along with uncultured media to serve as a negative control. Samples were compared to the standard curve for determination of 24-OHC concentrations, and reported as 24-OHC level relative to uncultured media. Results are shown in Figure 5. Supernatants from cells transfected with active CYP46A1 enzyme displayed a greater than 2 fold increase in 24-OHC relative to CYP46A1 (mut) transfected, or untransfected cells.

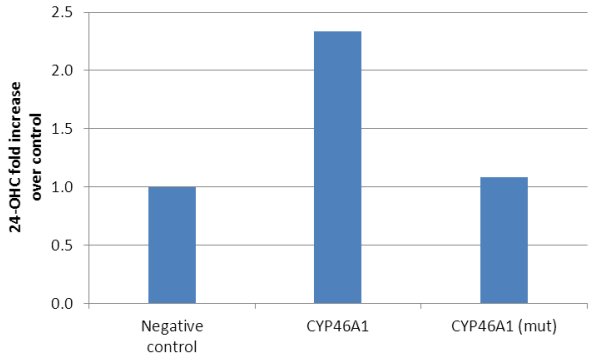

Figure 5. 24-OHC levels in supernatants of cells transfected with catalytically active or mutant (mut) CYP46A1.

RESULTS AND DISCUSSION

The 24-OHC ELISA is a competitive colorimetric immunoassay for the measurement of 24-OHC in both *in vivo* and *in vitro* studies. The assay is highly specific to 24-OHC, exhibiting less than 0.2% cross-reactivity to cholesterol, 22-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol and DHEA. The assay has been shown to accurately detect 24-OHC in CSF, tissue homogenates and tissue culture samples with no need for laborious sample work-up and only requires 100 µL or less of sample volume. The ELISA has a dynamic range from 0.78 to 100 ng/mL and a time to answer of 2 hours. Dose-response curves from CSF and tissue homogenates were compared to the standard curve and found to be parallel. Dilutional linearity (data not shown) and spike-and-recovery analysis determined minimal dilution requirements for CSF, tissue homogenates, and culture supernatants.

Analysis of 24-OHC levels in human CSF display the ability of the assay to return values consistent with 24-OHC levels reported in the literature. The assay is also able to detect 24-OHC metabolized and released by cultured cells into culture supernatants, demonstrating potential for monitoring 24-OHC as a marker of CYP46 activity in model systems. The assay provides a sensitive, high-throughput, and cost-effective alternative to methods such as mass spec and HPLC, which carry significant instrumentation costs and routing maintenance.

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