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Capillary electrophoresis–laser-induced fluorescence (CE-LIF) assay for measurement of intracellular D-serine and serine racemase activity

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ABSTRACT

An enantioselective capillary electrophoresis-laser-induced fluorescence (CE-LIF) method for the analysis of D-serine (D-Ser) in cellular matrices has been developed. The assay involves derivatization with FITC followed by CE-LIF using 0.5 mM hydroxyl propyl-β-cyclodextrin in borate buffer [80 mM, pH 9.3]. The method was able to resolve D-Ser and L-Ser with an enantioselectivity (α) of 1.03 and a resolution (R_s) of 1.37. Linearity was established from 0.25 to 100.00 μM. The assay was also able to enantioselectively resolve 6 additional amino acid racemates. The method was applied to the determination of intracellular D-Ser concentrations in PC-12, C6, 1312N1, and HepG2 cell lines. This method was used to determine the concentration-dependent increases in D-Ser and associated EC_{50} values produced by L-Ser and the concentration-dependent decreases in D-Ser and associated IC_{50} values produced by glycine, a competitive inhibitor of serine racemase (SR). Western blot analysis determined that the PC-12 and C6 cell lines contained monomeric and dimeric forms of SR while the 1321N1 and HepG2 cells contained only the monomeric form. Although the SR dimer has been identified as the active form of the enzyme, all four of the tested cell lines expressed enzymatically active SR.

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D-Serine (D-Ser)[2] is an *N*-methyl D-aspartate (NMDA) receptor coagonist that plays a key role in neurotransmission [1]. D-Ser is found in the mammalian brain [1,2] and elevated and depressed endogenous levels of the compound have been associated with a number of central nervous system (CNS) diseases and pathological states [2,3], including schizophrenia, aging, Alzheimer's disease, convulsion, anxiety, cerebellar ataxia, Parkinson's disease, neuropathic pain, and depression. For example, increased levels of D-Ser in the CNS have been linked to amyotrophic lateral sclerosis (ALS) and Alzheimer's disease [1] while decreased CNS concentrations have been associated with schizophrenia [2,3]. The relationship between decreased D-Ser levels and schizophrenia have led to clinical trials involving the administration of D-Ser and the initial studies indicated that the compound has positive effects in this disease [2].

The conversion of L-serine (L-Ser) into D-Ser by the pyridoxal-5′-phosphate-dependent serine racemase (SR) provides the primary source of endogenous D-Ser [2]. In mammals, SR is expressed in a number of central and peripheral tissues [1,2], and Western blot analysis has identified SR in the monomeric, dimeric, and tetrameric forms, although it has been assumed that human SR dimer is the active form of the enzyme [2,5]. The enzymatic activity of SR can be increased by increasing L-Ser levels [4] or intracellular Ca^{2+} levels using calcium ionophores, like A23187 [5], and decreased using competitive inhibitors such as glycine (Gly) [6] or by depletion of pyridoxal-5′-phosphate using sulfhydryl compounds [7]. SR expression can also be induced in vitro by incubation with amyloid β-peptide ($A\beta_{1-42}$) [8] or lipopolysaccharide (LPS) [9] and in vivo by single and chronic intraperitoneal administrations of morphine to male Wistar rats [10,11]. Since increased D-Ser concentrations in the CNS have been associated with ALS and Alzheimer's disease, the development of SR inhibitors is an emerging field in pharmaceutical research [2].

A number of analytical methods have been reported for the measurement of D-Ser in the presence of L-Ser in plasma [12,13] and brain tissue [12,14], as well as extracellular and intracellular matrices [4]. One experimental approach is based on the conversion of D- and L-Ser into diastereomeric fluorescent derivatives using *o*-phthaldialdehyde in combination with a chiral thiol reagent, such as *N*-acetyl-L-cysteine, *N*-*tert*-butyloxycarbonyl-L-cysteine, *N*-isobutyryl-L-cysteine, and *N*-acetyl-D-penicillamine

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[2] *Abbreviations used*: $A\beta_{1-42}$, amyloid β-peptide; β-CD, β-cyclodextrin; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; CNS, central nervous system; CE–LIF, capillary electrophoresis–laser-induced fluorescence; DMEM, Dulbecco's modified Eagle medium; E-MEM, Eagle's minimum essential medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; ACN, acetonitrile; SR, serine racemase; LPS, lipopolysaccharide.

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followed by HPLC or GC analysis [12,15,16]. This approach has been utilized in the determination of D- and L-Ser levels in plasma [12,13] and brain tissue [12]. Other diasteromeric derivatization methods have used dabsyl chloride, 4-fluoro-7-nitro-2,1,3-benzoxadiazole, 1,5-difluoro-2,4-dinitrobenzene and analogues, l-fluoro-2,4-dinitrophenyl-5-L-alanine amide, (+)-1-(9-fluorenyl)ethyl chloroformate, *O*-tetraacetyl-/3-D-glucopyranosyl isothiocyanate, (1*R*,2*R*)-*N*-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide), and *R*(2)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [17]. In addition to HPLC-fluorometric analysis, HPLC with electrochemical detection, LC-MS, GC/GC–MS, and CE have been used to separate and quantify the diastereomeric D-Ser derivatives [2]. The direct enantioselective separation of D- and D-Ser has also been reported based on fluorescent derivatization with an achiral reagent, fluorescein isothiocyanate (FITC), followed by CE-LIF separation of the derivatives using two chiral selectors, 2-hydroxypropyl-β-cyclodextrin (HP-β-CD)/β-cyclodextrin (β-CD) and sodium taurocholate, in the electrophoretic buffer [18].

While there are many reported approaches to the determination of D- and L-Ser levels in plasma and tissues, only a few have been used to measure intracellular D- and L-Ser [4,12]. The objective of this study was to establish an assay that could measure small, but significant changes in intracellular D-Ser levels produced by changes in SR expression and activity. In order to accomplish this, we have adapted the previously reported CE-LIF method [18]. We now report the development of an enantioselective CE-LIF method which uses a single chiral selector, HP-βCD, and the application of this method to the determination of concentration-dependent changes in intracellular D-Ser production in PC-12, C6, 1321N1, and HepG2 cell lines produced by incubation with L-Ser, a SR substrate, and Gly, a SR competitive inhibitor. The corresponding EC_{50} values of L-Ser and IC_{50} values of Gly were also determined. The assay was sensitive and precise and SR activity was detected in all of the tested cell lines even though the initial Western blot analysis indicated that the SR dimer was not present in the 1321N1 and HepG2 cells. A second SR antibody capable of detecting the monomeric and dimeric forms of the enzyme was used and the results demonstrated that the SR monomer was present in all four cell lines, thus indicating that the SR monomer also mediates the conversion of L-Ser to D-Ser.

Materials and methods

Materials

D-Serine (D-Ser), L-serine (L-Ser), D-alanine (D-Ala), L-alanine (L-Ala), D-arginine (D-Arg), L-arginine (L-Arg), glycine (Gly), D-leucine (D-Leu), L-leucine (L-Leu), D-isoleucine (D-Iso), L-isoleucine (L-Iso), D-glutamic acid (D-Glu), L-glutamic acid (L-Glu), D-aspartic acid (D-Asp), L-aspartic acid (L-Asp), L-lysine (L-lys), β-cyclodextrin (β-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), methanol, acetonitrile (ACN), lipopolysaccharide (LPS), and fluorescein isothiocyanate (FITC) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Deionized water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals used were of analytical grade.

Cell lines and cell culture

The cell lines selected for this study were PC-12 pheochromocytoma derived from rat adrenal medulla, human-derived 1321N1 astrocytoma, rat-derived C6 glioblastoma, and human-derived HepG2 hepatocellular carcinoma. All of the cell lines were obtained from ATCC (Manassas, VA, USA). The PC12 cells were maintained in RPMI 1640 with L-glutamine (L-Gln) supplemented with 10% horse serum (heat inactivated), 5% fetal bovine serum (FBS), 1% sodium pyruvate solution, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, and 1% penicillin/streptomycin solution. The 1321N1 and C6 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with L-Gln supplemented with 10% FBS and 1% penicillin/streptomycin solution. The HepG2 cells were maintained in Eagle's minimum essential medium (E-MEM) supplemented with 1% L-Gln, 10% FBS, 1% sodium pyruvate solution, and 1% penicillin/streptomycin solution.

DMEM with glutamine, E-MEM, RPMI 1640, trypsin solution, phosphate-buffered saline, FBS, sodium pyruvate solution (100 mM), L-Gln (200 mM), and penicillin/streptomycin solution (containing 10,000 units/ml penicillin and 10,000 μg/ml streptomycin) were obtained from Quality Biological (Gaithersburg, MD, USA), horse serum (heat inactivated) was obtained from Biosource (Rockville, MD, USA) and Hepes buffer (1 M) was obtained from Mediatech Inc. (Manassas, VA, USA).

CE-LIF analysis

Instrumentation

The CE separations were performed with a P/ACE MDQ system equipped with a laser-induced fluorescence detector (Beckman Instruments, Fullerton, CA, USA). The laser-induced fluorescence detection was carried out with excitation at 488 nm and emission at 520 nm. An uncoated fused-silica capillary, 50 μm i.d., 60.2 cm total length, with an effective length of 50 cm, was used and the running buffer was composed of 0.5 mM HP-β-CD in borate buffer [80 mM, pH 9.3]. The capillary was conditioned before each analysis by flushing successively with 0.1 M NaOH, 0.1 M H_3PO_4, H_2O, and running buffer each for 4 min. Samples were injected with pressure at 0.5 psi for 10 s and separated using a voltage gradient in which separation voltage was 15 kV between 0 and 44 min, followed by 22 kV between 45 and 60 min. The total run time was 76 min. Quantification was accomplished using area ratios calculated for FITC-D-Ser with FITC-D-Arg as the internal standard, where the concentration of the internal standard was set at 5 μM.

Standard solutions

A concentrated stock solution of 0.5 mM D-Ser in borate buffer [80 mM, pH 9.3] was used to prepare 0.25, 0.5, 1, 2, 4, 10, 20, 40, 80, and 100 μM solutions for the calibration curve. Standard solutions, 1 mM in borate buffer [80 mM, pH 9.3], of L-Ser, D-Ala, L-Ala, D-Arg, L-Arg, Gly, D-Leu, L-Leu, D-Iso, L-Iso, D-Glu, L-Glu, D-Asp, L-Asp, L-Lys were also prepared. A 100 μM solution of D-Arg in H_2O was used as the internal standard solution.

Sample preparation

Cells were collected and centrifuged for 5 min at 200*g* at 4 °C. The supernatant was discarded, the cells were suspended in 1.00 ml of H_2O, 0.050 ml of the internal standard was added, and the resulting mixture vortex mixed for 1 min. A 4.00 ml aliquot of ACN was added and the suspension was sonicated for 20 min. The mixture was then centrifuged for 15 min at 2500*g* at 4 °C, and the supernatant collected and stream-dried under nitrogen. The residue was dissolved in 0.90 ml of borate buffer [80 mM, pH 9.3].

FITC labeling

FITC solution (3 mg/ml) was prepared in acetone and stored at −20 °C until use. For the derivatization of standard amino acids, a 0.05-ml aliquot of the internal standard solution was added to a 0.85 ml of the standard solution, 0.10 ml of FITC solution was added, and the resulting solutions were placed in darkness for 12 h at room temperature. When cellular extracts were assayed,