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Capillary electrophoresis-laser induced fluorescence (CE-LIF) assay for measurement of intra-cellular 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 μ M – 100.00 μ M. The assay was also able to enantioselectively resolve 6 additional amino acid racemates. The method was applied to the determination of intra-cellular 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 EC₅₀ values produced by L-Ser and the concentration-dependent decreases in D-Ser and associated IC₅₀ 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 1312N1 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.

Keywords

D-Ser; L-Ser; glycine; enantioselective resolution; serine racemase monomer; serine racemase dimer

Introduction

D-serine (D-Ser) is an N-methyl D-aspartate (NMDA) receptor co-agonist 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

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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 ($\text{A}\beta_{1-42}$) [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], brain tissue [12,14], as well as extracellular and intracellular matrices [4]. One experimental approach is based upon 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-butylloxycarbonyl-L-cysteine, *N*-isobutyl-L-cysteine and *N*-acetyl-D-penicillamine 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 diastereomeric derivatization methods have used dabsyl chloride, 4-fluoro-7-nitro-2,1,3-benzoxadiazole, 1,5-difluoro-2,4-dinitrobenzene and analogues, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, (+)- 1-(9-fluorenyl)ethyl chloroformate, O-tetraacetyl-/3-Dglucopyranosyl 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 L-Ser has also been reported based upon 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 intra-cellular 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- β -CD, 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 4 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). De-ionized 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 (EMEM) 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 μ g/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 μ m 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 H₃PO₄, H₂O and running buffer each for 4 min. Samples were injected with pressure at 0.5 p.s.i. for 10 s and separated using a voltage gradient in which separation voltage was 15 kV between 0 – 44 min, followed by 22 kV between 45 – 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 μ M.

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 μ M 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 μ M solution of D-Arg in H₂O was used as the internal standard solution.

Sample preparation—Cells were collected and centrifuged for 5 min at $200 \times g$ at 4 °C. The supernatant was discarded and the cells were suspended in 1.00 ml of H₂O, 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 $2500 \times g$ at 4 °C, 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 and 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, 0.10 ml of FITC solution was added to the 0.90 ml samples and the resulting solutions were placed in darkness for 12 h at room temperature.

Effect of L-serine and glycine on serine racemase activity and expression

Cells were seeded on 100 $\times$ 20 mm tissue culture plates and were maintained at 37 °C under humidified 5% CO₂ in air until they reached >70% confluence. The original media was replaced with media containing sequential concentrations of the test compounds and the plates were incubated for an additional 36 h. The medium was removed, and the cells collected for analysis. All of the studies were done in triplicate. When L-Ser was studied the concentrations used were: 0, 2, 4, 6, 8, 10, 15 and 20 mM; whereas the study of the effects of Gly was performed using the following concentrations: 0, 0.25, 0.5, 1, 2, 4, 6 and 8 mM.

Western Blotting

Cells were lysed with RIPA buffer containing ethylene glycol tetraacetic acid and ethylenediamine tetraacetic acid (Boston BioProducts, Ashland, MA, USA). The lysis buffer contained a protease inhibitor cocktail composed of 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma-Aldrich). Protein concentrations were determined using the bicinchoninic acid (BCA) reagent obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). Proteins (20 μ g/well) were separated on 4 to 12% precast gels (Invitrogen, Carlsbad, CA, USA) using SDS-polyacrylamide gel electrophoresis under reducing conditions and then they were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membrane (Invitrogen). Western blots were performed according to standard methods which involved blocking in 5% non-fat milk and incubated with the antibody of interest, followed by incubation with a secondary antibody conjugated with the enzyme horseradish peroxidase. The visualization of immunoreactive bands was performed using the ECL Plus Western Blotting Detection System (GE Healthcare, NJ, USA). The quantification of bands was done by volume densitometry using Image software and normalization to β -actin.

The primary antibodies for SR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), sc-48741, and Abcam (Cambridge, MA, USA), ab45434, and the primary antibody for β -actin was obtained from Abcam, ab6276. The antibodies were used at a dilution recommended by the manufacturer.

Statistical Analysis

Graphpad Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA) running on a personal computer was used to perform all the statistical data analysis including EC₅₀ and IC₅₀ value calculations.

Results

Optimization of the CE-LIF method for D-Serine and L-Serine analysis

Lu and Chen [20] have previously reported the development of a micellar CE-LIF method for the enantioselective separation of FITC-derivatized amino acids in which the optimum chiral separations were obtained using a 3 : 2 molar ratio of sodium taurocholate : β -CD or a 3 : 2 molar ratio of sodium taurocholate : HP- β -CD. We have adapted this approach to the study of the intracellular concentrations of D-Ser. In the current study, the enantioselective resolution of FITC-derivatized D-Ser and L-Ser was achieved without the addition of sodium taurocholate to the running buffer using either β -CD or HP- β -CD as the chiral selector. The enantioselectivity (α) and resolution (R_s) of D-Ser and L-Ser were optimized by altering the concentrations of the selectors in borate buffer [80 mM, pH 9.3], Fig 1A,B. With HP- β -CD, the optimum α and R_s values, ~1.04 and 1.65, respectively, were observed at a selector concentration of 1 mM, and resolutions of $R_s \geq 1.20$ were obtained at HP- β -CD concentrations between 0.5 and 2 mM, Fig. 1A. When β -CD was used as the selector, higher enantioselectivities and resolutions were obtained with the optimum α and R_s values of 1.09 and 3.15, respectively, observed at a β -CD concentration of 2 mM and baseline resolutions of $R_s \geq 1.50$ were obtained at all β -CD concentrations used in the study, Fig. 1B. The effect of buffer concentration was determined using 100 mM borate buffer and HP- β -CD concentrations of 0.25 to 8.0 mM and no significant differences between the data obtained with 80 mM buffer were observed (data not shown).

The electrophoretic buffer composed of 1 mM HP- β -CD in borate buffer [80 mM, pH 9.3] was chosen for further development. A group of 8 additional amino acids, including 6 racemic mixtures, were examined as potential interferences with the D-Ser determination. Under these conditions, enantioselective separations were achieved for each of the 7 D,L-amino acid standards investigated in this study with α values ranging from 1.03 (D,L-Ile) to 1.60 (D,L-Leu), Table S1 (Supplemental Data). However, there was no resolution between D-Ser and D-Ala and only a partial resolution of L-Ser from L-Ala and Gly, as L-Ala and Gly co-migrated, Fig. 2A. Resolution between D-Ser and D-Ala ($R_s = 1.21$) was achieved by reducing the HP- β -CD concentration to 0.5 mM without significant loss in the enantioselective separation of D-Ser and L-Ser, $\alpha = 1.03$; $R_s = 1.85$. Under these conditions, the resolution between L-Ser and L-Ala was improved while Gly was completely separated from L-Ser and L-Ala. A baseline separation between L-Ser and L-Ala was achieved using a voltage step-gradient of 15 kV from 0-44 min and 22 kV from 45-120 min, Fig. 2B. Under these conditions, the total run time for the separation of all of the standards was 120 min. Since the objective of the assay was the determination of the intra-cellular concentrations of D-Ser and L-Ser, the run time was reduced to 76 min by decreasing the voltage to 0 kV at 60 min followed by consecutive 4 min washes of 0.1 M NaOH, 0.1 M phosphoric acid, H₂O and running buffer. Under these conditions, the limit of detection (LOD) and limit of quantitation (LOQ) of this method for D-Ser were 0.1 μ M and 0.25 μ M, respectively, based on 'signal to noise' ratio, LOD (3:1) and LOQ (13:1). The linearity of the method was $r^2 = 0.998$ established between 0.25 – 100 μ M and the method was reproducible with %CV values ranging between 0.7% and 2.7% (Interday, $n = 3$). Relative migration factors of D-Ser and L-Ser were calculated relative to the migration time of D-Arg (internal standard), calculated using 10 experiments per day over 3 days ($n = 30$). The average relative

migration factor of D-Ser was 1.02 ± 0.02 , %CV = 2.36, and for L-Ser the average was 1.05 ± 0.03 , %CV = 2.46.

The effect of L-Ser media concentration on intracellular D-Ser concentration

SR mediates the conversion of L-Ser to D-Ser with K_m values for L-Ser ranging from 4 mM, determined in purified recombinant human SR [19], to 18 mM, determined in SR purified from mouse brain [20]. In the present study we determined the effect of L-Ser, 2.0 to 20.0 mM, added to the incubation buffer on intracellular D-Ser levels. The CE-LIF assay was able to measure significant concentration-dependent increases in the intra-cellular D-Ser levels in all 4 cell lines, c.f. Fig. 3, which ranged from a 533% increase (C6) to a 50% increase (HepG2), Table 1. The data was used to calculate the EC_{50} value for the L-Ser-induced production of D-Ser in each cell line, Table 1, and the values ranged from 5.41 ± 0.76 mM (PC-12) to 9.37 ± 0.17 mM (C6).

The effect of Gly media concentration on intracellular D-Ser concentration

Previous studies have demonstrated that Gly is a competitive inhibitor of SR purified from mouse brain, and that the addition of 2 mM Gly to the incubation media produced an 80% reduction in the D-Ser production [6]. The effect was concentration-dependent with a calculated K_i value of 0.15 mM. Similar inhibitory activity has been reported for Gly with purified recombinant human SR, $K_i = 0.37$ mM [19]. In the present study we determined the effect on intracellular D-Ser levels produced by the addition of Gly, 0.25 to 8.0 mM, to the incubation buffer. The CE-LIF assay was able to measure significant concentration-dependent decreases in the intra-cellular D-Ser concentrations in all 4 cell lines, c.f. Fig. 3, which ranged from a 97% reduction (C6) to a 35% reduction (1321N1), Table 1. The data was used to calculate the IC_{50} value for the Gly inhibition of SR in each cell line, Table 1, and the values ranged from 0.68 ± 0.15 mM (C6) to 1.83 ± 0.07 mM (HepG2). The calculated IC_{50} values are consistent with an estimated IC_{50} value of 1.8 mM for the Gly inhibition of purified mouse brain SR using the data supplied by Dunlop and Neidle [6].

Determination of SR expression using Western blotting

The cell lines used in this study were probed for SR expression using the technique of Western blotting with sc-48741 as the primary antibody. The blots obtained using the cellular extracts from the PC-12 and C6 contained a band at 74 kDa consistent with the presence of the dimeric form of SR, while no bands were observed in the immunoblots from the HepG2 and 1321N1 cells, data not shown. On the basis of these results it was assumed that the HepG2 and 1321N1 cell lines do not express functional SR. However, when the cellular extracts were assessed for intra-cellular D-Ser concentrations using the CE-LIF assay, the results indicated that all of the cell lines had intrinsic SR activity and that this activity could be modified with varying concentrations of L-Ser and Gly in the incubation media, Table 1. Based upon these results, the cellular extracts were reexamined using a second primary SR antibody, ab45434. The resulting blots obtained with cellular extracts from the PC-12 and C6 cell lines contained two bands as 37 kDa and 74 kDa corresponding to the monomeric and dimeric forms of SR, respectively, Fig. 4. The relative ratios of the SR-dimer to the SR-monomer, normalized against β -actin, were ~5-fold and ~2-fold in the PC-12 and C-6 cells, respectively, Fig. 4. The extracts from the HepG2 and 1321N1 cell lines contained a single 37 kDa band corresponding to the monomeric form of SR, Fig. 4. Although the enzymatic activity of SR was affected by L-Ser and Gly (see above), the data from the Western blot experiments indicated that neither of these treatments produced a significant change in the SR dimer or SR monomer expression relative to control, Fig. 5A.

Since it has been previously demonstrated that the incubation of primary microglia with LPS (100 ng/ml) increased D-Ser levels in the incubation media and the expression of the

dimeric form of SR [9], we treated PC-12 cells with LPS and determined the effect on intra-cellular D-Ser concentration and relative expression of SR. The addition of 10 ng/ml and 100 ng/ml LPS to the incubation media produced 2.5-fold and 3.5-fold increases in intra-cellular D-Ser concentrations, respectively. Western blot analysis indicated that the expression of the SR dimer had increased relative to control by 1.2-fold and 1.6-fold, respectively, while the relative expression of the SR monomer was 0.8-fold and 0.5-fold of control, Fig. 5B.

Discussion

The objective of this project was the development of an analytical method capable of measuring changes in the intra-cellular concentrations of D-Ser in order to assess the effects of compounds on SR activity and expression. The experiments were carried out in 4 cell lines of various origins and the results clearly indicate that the CE-LIF assay developed in this study is capable of meeting these goals. This conclusion is supported by our data showing the concentration-dependent changes in D-Ser production by L-Ser and Gly in intact cells. To our knowledge, this study is the first to report on the calculated EC₅₀ and IC₅₀ values from cellular experiments and by the fact that these values are consistent with those from previous studies using recombinant SR.

A key element in the proposed analytical method was the comparison of the functional changes in SR, expressed as D-Ser concentrations, and the expression of the SR protein. To determine if L-Ser and/or Gly had affected SR expression and/or activity, we treated PC-12 cells with 10 mM L-Ser, which produced a 160% increase in intra-cellular D-Ser, and 6 mM Gly, which produced a 77% decrease in intra-cellular D-Ser, Table 2, without causing a significant change in the SR dimer or SR monomer expression relative to control, Fig. 5A.

The treatment of PC-12 cells with LPS produced a significant increase in expression of the SR dimer and what appears to be a reduction in the corresponding monomer. It has been suggested that the different forms of SR are in rapid equilibrium [5] and the results from the treatment with LPS may reflect a shift in this equilibrium. This phenomenon is currently being explored using the combined CE-LIF and Western blot approach and dose-response studies using the PC-12 and HepG2 cell lines, the latter expressing only the monomeric form of SR, Fig. 4. The results of these studies will be reported elsewhere.

It is generally assumed that human SR functions as a dimer, an assumption derived from recently determined crystallographic structures of the human and rat SR, c.f. [1,2]. Based on these reports, an antibody selective for the dimeric form of SR was chosen to screen potential cell lines for use as experimental and control cells; with PC-12 and C6 (SR dimer positive) chosen as the experimental cell lines, and 1321N1 and HepG2 (SR dimer negative) chosen as the control cell lines. However, the functional studies indicated that all four cell lines had intrinsic D-Ser production. The cell lines were re-probed using an antibody able to detect the SR monomer and dimer and the results indicated that the 1321N1 and HepG2 cell lines expressed monomeric SR, Fig. 4. Thus, the data from this study indicate that in the 1321N1 and HepG2 cell lines, the SR monomer mediates the racemization of L-Ser to D-Ser, is sensitive to L-Ser concentrations and is competitively inhibited by Gly, Table 1. Overall, the data from this study indicate that the CE-LIF assay developed in this project can be used to assess changes in intra-cellular D-Ser concentrations. Further, the results also demonstrate that this technique should be combined with Western blot analysis to obtain an accurate picture of the effect of substrate / inhibitors on SR activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

Aβ₁₋₄₂	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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Figure 1. Effect of the concentration of the chiral selector in the electrophoretic buffer on enantioselectivity (α) and chiral resolution (R_s). Where: A: Influence of HP- β -CD concentration on (1) α and (2) R_s ; B: Influence of β -CD concentration on (1) α and (2) R_s .

Figure 2.

(A) Electropherogram of FITC-labeled D,L-Arg, D,L-Ser, D,L-Ala and Gly obtained using 1 mM HP- β -CD in borate buffer [80 mM, pH 9.3] as running buffer; (B) Electropherogram of FITC-labeled D,L-Arg, L-Lys, D,L-Iso, D,L-Leu, D,L-Ser, D,L-Ala and Gly obtained

using 0.5 mM HP- β -CD in borate buffer [80 mM, pH 9.3] as running buffer. See Experimental section for assay conditions.

Figure 3.
The determination of D-Ser in PC-12 cell after treatment with L-Ser or Gly; where: (A) control; (B) with 10 mM Gly; (C) with 20 mM L-Ser.

Figure 4.

Expression of SR protein in PC-12, C6, 1321N1 and HepG2 cells. Left panel, Representative Western blot analysis using antibodies for SR shows the presence of dimer at 74kDa in PC-12 and C6 cells but not in 1321N1 and HepG2 cells. Conversely, monomer was detected at 37kDa in all 4 cell lines (left panel). Relative SR expressions after quantification of the immunoblots using ImageJ and normalization with β -actin are shown (right panel). Data represents the average $\pm$ S.D., n=2.

Figure 5.

Western blots showing expression of SR protein in PC-12 cells after a 36-h treatment with (A): L-Ser (10mM) and Gly (6mM) and (B) LPS (10ng/ml and 100ng/ml). Left panel, representative Western blots; right panel, relative changes of SR expression after quantification using ImageJ and normalization with β -actin. Data represents the average $\pm$ S.D., n=2.

Table 1

The effect of serial concentrations of L-Ser or Gly on the intra-cellular D-Ser production, n = 3.

	PC-12	C6	HepG2	1321N1
L-Ser (mM)				
0	13.56 ± 2.64	2.54 ± 0.15	17.57 ± 0.55	8.15 ± 0.32
2	16.48 ± 1.72	2.91 ± 0.17	15.48 ± 0.26	8.13 ± 0.14
4	21.97 ± 2.48	3.05 ± 0.08	16.21 ± 0.21	9.26 ± 1.00
6	29.51 ± 4.93	3.60 ± 0.14	17.53 ± 1.70	9.46 ± 0.87
8	31.46 ± 0.07	7.17 ± 0.27	18.50 ± 1.48	9.54 ± 0.82
10	36.49 ± 1.64	10.63 ± 0.45	20.10 ± 0.50	19.57 ± 0.77
15	38.01 ± 0.50	15.64 ± 0.10	21.81 ± 0.35	21.94 ± 0.18
20	39.13 ± 0.36	16.07 ± 0.16	26.28 ± 0.25	22.31 ± 1.27
EC₅₀ (mM)	5.41 ± 0.76	9.37 ± 0.17	7.87 ± 2.09	9.16 ± 0.27
Gly (mM)				
0	20.41 ± 2.64	3.86 ± 0.14	15.29 ± 0.44	9.61 ± 2.41
0.25	15.91 ± 1.72	3.74 ± 0.12	14.96 ± 0.91	9.05 ± 2.48
0.50	13.86 ± 2.02	3.49 ± 0.17	13.85 ± 0.22	8.34 ± 2.16
1.0	11.59 ± 2.43	3.15 ± 0.13	10.88 ± 0.17	7.38 ± 2.01
2.0	8.30 ± 0.51	1.69 ± 0.14	10.77 ± 0.14	6.69 ± 1.80
4.0	7.82 ± 0.49	0.51 ± 0.11	10.31 ± 0.32	6.53 ± 1.98
6.0	4.64 ± 1.36	0.41 ± 0.07	8.60 ± 0.12	6.32 ± 1.83
8.0	2.72 ± 0.44	0.11 ± 0.07	7.78 ± 0.27	6.29 ± 1.04
IC₅₀ (mM)	1.24 ± 0.39	1.83 ± 0.07	0.83 ± 0.27	0.68 ± 0.15