Published in final edited form as:

Neurosci Lett. 2008 November 7; 445(1): 53–57. doi:10.1016/j.neulet.2008.08.058.

D-Amino acids in rat brain measured by liquid chromatography / tandem mass spectrometry

Yaru Song^a, Yangzheng Feng^b, Xin Lu^c, Shulin Zhao^c, Cheng-Wei Liu^d, and Yi-Ming Liu^{a*}

^aDepartment of Chemistry and Biochemistry, Jackson State University, 1400 Lynch St., Jackson, MS 39217, USA.

^bDepartment of Pediatrics, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA.

^cCollege of Chemistry and Chemical Engineering, Guangxi Normal University, Guilin 541004, China.

^dDepartment of Anatomy, Guilin Medical College, Guilin 541004, China.

Abstract

Previous work has established that D-amino acids including D-serine (D-Ser) and D-aspartic acid (D-Asp) fulfill specific biological functions in the brain. In this work, the levels and anatomical distribution of D-amino acids in rat brain were determined by using an advantageous liquid chromatography-tandem mass spectrometric analytical method. The study was focused on D-Ser, D-Asp, and D-glutamic acid (D-Glu) because of the significance of L-Asp, L-Glu, and D-Ser in the nervous system. Prenatal, postnatal pups, and 90-day old rats were studied. Results indicated that D-Asp and D-Ser occurred in rat brain at the $\mu\text{g/g}$ tissue level. However, D-Glu was not detected ($< 110 \text{ ng/g}$ tissue). Through out the developmental stages D-Asp content in rat brain decreased rapidly from 9.42% of total Asp in 5-day prenatal rats to an undetectable level ($< 150 \text{ ng/g}$ tissue) in 90-day old rats. In contrast, D-Ser level increased gradually through out the developmental stages. D-Ser percentage (D-Ser / (D-Ser + L-Ser)) changed from 4.94 % in 5-day prenatal rats to 13.7 % in 90-day old rats. Regional levels of D-Ser were found to be significantly higher in cortex, striatum, and hippocampus than in thalamus. D-Ser was not detected in cerebellum ($< 172 \text{ ng/g}$ tissue).

Keywords

D-Amino acids; Rat brain; Anatomical distribution; Liquid chromatography - tandem mass spectrometry

Over the past years, evidence has been accumulating indicating that some D-amino acids occur and fulfill specific biological functions in mammals including humans [1–3]. In the brain, D-serine (D-Ser) has been recently identified as an endogenous neurotransmitter/modulator [4–7]. It is an effective co-agonist at the glycine-binding site of NMDA receptors. Incubation studies showed that rat brain tissue preferably uptakes D-Ser over L-Ser [8]. L-Glutamic acid (L-Glu) and L-aspartic acid (L-Asp) are major excitatory amino acids in the brain. Recent

Corresponding author: Yiming Liu, Ph.D., Department of Chemistry and Biochemistry, 1400 Lynch St. Jackson, MS 39217. Tel. 601-979-3491, Fax: 601-979-3674. E-mail: yiming.liu@jsums.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

studies have shown that D-Asp plays important roles in the neuroendocrine system, as well as in the development of central nervous system [9–13]. It has been shown that D-Asp promotes the synthesis of proteins involved in the development of the nervous system and regulates the hormonal release in the endocrine glands.

For a better understanding of the biological significance of D-amino acids, it is important to study their occurrence and metabolism in living systems. To quantify D-amino acids in the presence of L-amino acids, chemical assays with stereochemical selectivity are needed. Methods based on HPLC with UV, fluorescence, or electrochemical detection were developed for these purposes. However, most of these methods lacked the capability of peak identification [14–15]. HPLC coupled with mass spectrometric detection has become one of the most powerful techniques for bioanalysis. Sensitivity of HPLC-MS methods can be significantly improved by using capillary or nano-scale HPLC-MS regimes [16–18]. In this work, a sensitive and selective capillary HPLC- MS/MS method that we previously developed for simultaneous quantitation of D- and L-amino acids [19] was applied to measure the levels of D-amino acids in rat brain. Tissue samples taken from prenatal (5, 3, 1, and 0 day) and postnatal (1, 3, 7, and 90 day) rats were assayed. In addition, the regional distribution of D-Ser in 90 day-old rat brain was studied.

All animal experiments were performed in strict accordance with the protocol approved by the Institutional Animal Care and Use Committee at Jackson State University. Rats (Sprague-Dawley) were anesthetized with pentobarbital and sacrificed. The brain was immediately dissected out on ice. It was rinsed with PBS buffer (pH = 7.04), wiped dry, weighted, and ground in ice-cold (1+1) water/methanol containing 750 ng/mL L-Asp-d₃ as internal standard (1 : 3 w:v) with a Teflon-glass homogenizer on ice. The homogenate was sonicated for 5 min. Trichloroacetic acid solution (30% w:v) was added (30 µL /100 µL of homogenate). The mixture was vortexed and let stand for at least 1 h on ice before being centrifuged at 5000 g for 10 min. The supernatant was collected. To derivatize amino acids, 20 µL of borate buffer (100 mM, pH 9.0) and 60 µL of 7-fluoro- 4-nitrobenzoxadiazole (NBD-F) solution (10 mM in acetonitrile) were added to 10 µL of the supernatant. The mixture was vortexed and heated at 65 °C for 20 min with a dry heating block. After heating, the solution was cooled down in running tap water and kept at 4 °C until analysis. Portions (5 µL) of the derivative solution were injected into the capillary HPLC-MS/MS system for analysis.

The capillary HPLC-MS/MS system was previously described [19]. Samples were first stacked /enriched on a C₁₈ (50.0 × 0.25 mm i.d.) reversed-phase extraction column and then separated on a CHIROBIOTIC TAG (170.0 × 0.25 mm i.d.) chiral capillary. Injections were made by means of a Rheodyne 8125 injector equipped with a 5 µL sampling loop. The ion trap mass spectrometer (ThermoFinnigan LCQ Deca) was operated in positive ion mode. Tandem mass spectrometry (MS/MS) conditions were optimized with a solution of NBD-L-Asp as following: sheath gas flow, 40 units; auxiliary gas, 0 units; monitored precursor ion precursor isolation width 1u; capillary temperature, 220 °C; and spray voltage, 4.0 kV. For the MS/MS experiments, the relative energy was set at 30%. Other parameters were optimized by the autotune program in Xcalibur.

Amino acid enantiomers, L-aspartic-2,3,3-d₃ (L-Asp-d₃), 7-fluoro-4-nitrobenzoxadiazole (NBD-F), 2-isopropanol, formic acid, methanol (LC/MS grade), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). C₁₈ reversed phase silica particles (5 µm) used for packing extraction columns was obtained from Restek (Bellefonte, PA, USA). CHIROBIOTIC TAG CSP was from Astec (Whippany, NJ, USA). Milli-Q (Millipore Corp., Bedford, MA, USA) water was used throughout the work.

The HPLC-MS/MS method including the sample extraction procedure was validated. Because of the similarity in chemical structures of amino acids under study and L-Asp-d₃ (internal standard), the extraction recovery of amino acids was estimated by studying the recovery of L-Asp-d₃ added to the sample matrix that was determined to be $98.1 \pm 3.1\%$ at 750 ng/mL homogenate ($n = 9$). Typical chromatograms from separating a mixture of authentic Ser, Asp, and Glu enantiomers are shown in Fig.1. It's worth noting that the L-enantiomers were always eluted before the corresponding D-enantiomers. For quantitation, selected reaction monitoring (SRM) was used. Ion transitions, m/z 300 $\rightarrow$ 282 for NBD-L-Asp-d₃, m/z 297 $\rightarrow$ 279 for NBD-Asp, m/z 269 $\rightarrow$ 223 for NBD-Ser, and m/z 311 $\rightarrow$ 293 for NBD-Glu were monitored. Amino acid concentration was determined based on the peak height ratio of NBD-amino acid over NBD-L-Asp-d₃. Calibration curves (5-points) were prepared with standard solutions of authentic chemicals. Linear regression analysis was used to obtain the calibration equation. It is always a concern that, in some cases, ESI-MS/MS signals may be affected by sample matrix. Therefore, a stable isotope-labeled internal standard (L-Asp-d₃) was used in order to correct for the potential effects of ionization suppression or enhancement by the matrix, thus providing a reliable basis for quantitation. Fig.2. shows HPLC-MS/MS chromatograms from analyzing a rat brain tissue homogenate sample. As can be seen, the four amino acid enantiomers, i.e. D-/L-Asp and D-/L-Ser, were well separated and could be simultaneously quantified. Thanks to the enantiomeric selectivity of chiral HPLC in combination with the peak identification capability of MS/MS detection, these analytical results were more reliable and more likely free from interference from endogenous components in tissue sample matrix as compared with the results obtained by using HPLC with fluorescence and electrochemical detection.

In this study, high levels of D-Asp and D-Ser were detected in rat brain. However, in all of the samples analyzed, D-Glu was not detectable (< 110 ng / g tissue). Brain samples taken from rats at different developmental stages were analyzed. The results of D-Asp contents are summarized in Fig. 3. As can be seen, D-Asp level in rat brain maintained at a relatively high level in prenatal stages. The enantiomeric ratio of Asp in rat brain homogenates prepared from 5-day prenatal pups was found to be $9.42 \pm 0.76\%$ D-Asp (mean $\pm$ SD, $n = 5$). After birth, D-Asp level gradually decreased and became undetectable (< 150 ng /g tissue) in the brain of 90-day old rats. These results are in accordance with the previously reported results by other researchers. For example, using an HPLC method, Dunlop et al. [20] found the content of D-Asp was 100 nmol /g tissue (i.e. 13.3 μ g /g tissue) in the cerebrum of newborn rats and it decreased rapidly with increasing age. In another study on D-Asp regional distribution in the brain of 6-week old rats, no D-Asp was detected in regions including cerebrum, hippocampus, pyrothalamus, and cerebellum by using an HPLC method with fluorescence detection [21]. These findings support that D-Asp is involved in prenatal development of the nervous system [13].

D-Ser was detected in all of the brain tissue homogenate samples analyzed. This study revealed that D-Ser occurred in the brain of rats through out their developmental stages and maintained at a high level when rats became adult. As shown in Fig. 4, D-Ser percent of total Ser increased steadily from $4.94 \pm 0.41\%$ D-Ser in 5-day pre-natal rats to $13.7 \pm 2.8\%$ D-Ser in 90-day old rats. It suggests that rats utilize D-Ser in their whole lifetime. D-Ser has been identified as a neurotransmitter /modulator [4,7]. To study the anatomical distribution of D-Ser in rat brain, five brain regions of 90-day old female SD rats, i.e. thalamus, cerebellum, cortex, hippocampus and striatum were analyzed to determine D- Ser. The results are summarized in table 1. As can be seen, D-Ser is not homogeneously distributed in rat brain. The regional levels of D-Ser percent (D-Ser / (D-Ser+L-Ser)) were found to be $20.73 \pm 1.2\%$ (mean $\pm$ SEM, $n = 4$) in cortex, $17.44 \pm 1.15\%$ in striatum, $16.67 \pm 0.88\%$ in hippocampus, and $8.27 \pm 1.40\%$ in thalamus. Using the method of Student-Newman-Keuls, D-Ser percent in thalamus was significantly different from those in the other brain regions ($p < 0.001$). T-test results were $p < 0.001$ for thalamus vs. hippocampus, striatum, and cortex and $p < 0.05$ for hippocampus vs. cortex.

Further, D-Ser level was below the limit of the detection of this method in cerebellum (<172 ng /g tissue).

In summary, high levels of D-Asp and D-Ser were detected by using a highly sensitive and selective HPLC-MS/MS analytical method. In prenatal rats, D-Asp level in rat brain was found relatively high. After birth, it gradually decreased and became undetectable (< 150 ng /g tissue) in the brain of 90-day old rats. These results suggested that D-Asp played an important role in the prenatal development of the nervous system. Interestingly enough, this study found that the level of D-Ser in rat brain increased through the developmental stages and remained at a high level when rats become adult. Study of the regional distribution of D-ser revealed that D-Ser was localized in the frontal brain such as hippocampus, striatum, cortex and thalamus. No D-Ser was detected in the cerebellum of rat brain.

ACKNOWLEDGEMENTS

Financial support from NIH grants (S06GM08047 and partially 12RR13459) is gratefully acknowledged.

References

1. Fisher GH. Appearance of D-amino acids during aging: D-amino acids in tumor proteins. *EXS* 1998;85:109–118. [PubMed: 9949871]
2. Hamase K, Morikawa A, Zaitzu K. D-Amino acids in mammals and their diagnostic value. *J. Chromatogr. B* 2002;781:73–91.
3. Fuchs SA, Berger R, Klomp LW, de Koning TJ. D-amino acids in the central nervous system in health and disease. *Mol Genet Metab* 2005;85:168–180. [PubMed: 15979028]
4. Snyder SH, Kim PM. D-Amino acids as putative neurotransmitters: focus on D-serine. *Neurochem. Res* 2000;25:553–560. [PubMed: 10905615]
5. Wang H, Wolosker H, Morris JF, Pevsner J, Snyder SH, Selkoe DJ. Naturally occurring free D-aspartate is a nuclear component of cells in the mammalian hypothalamo- neurohypophyseal system. *Neurosci* 2002;109:1–4.
6. Boehning D, Snyder SH. Novel neural modulators. *Annu. Rev. Neurosci* 2003;26:105–131. [PubMed: 14527267]
7. Miller RF. D-serine as a glial modulator of nerve cells. *Glia* 2004;47:275–283. [PubMed: 15252817]
8. Bauer D, Hamacher K, Broeher S, Pauleit DC, Zilles K, Coenen HH, Langen K-J. Preferred stereoselective brain uptake of - serine - a modulator of glutamatergic neurotransmission. *Nuclear Med. Biol* 2005;32:793–797.
9. Schell MJ, Cooper OB, Snyder SH. D-Aspartate localizations imply neuronal and neuroendocrine roles. *Proc. Natl. Acad. Sci. USA* 1997;94:2113–2018.
10. Wolosker H, D'Aniello A, Snyder SH. D-Aspartate disposition in neuronal and endocrine tissues: ontogeny, biosynthesis and release. *Neurosci. (Oxford)* 2000;100:183–189.
11. D'Aniello G, Tolino A, D'Aniello A, Errico F, Fisher GH, Fiore MM. The role of D-aspartic acid and N-methyl-D-aspartic acid in the regulation of prolactin release. *Endocrinol* 2000;141:3862–3870.
12. D'Aniello A, Fiore MM, Fisher GH, Milone A, Seleni A, D'Aniello S, Perna AF, Ingrosso D. Occurrence of D-aspartic acid and N-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release. *FASEB J* 2000;14:699–714. [PubMed: 10744627]
13. D'Aniello A. D -Aspartic acid: an endogenous amino acid with an important neuroendocrine role. *Brain Res. Rev* 2007;53:215–234. [PubMed: 17118457]
14. Miao H, Rubakhin SS, Sweedler JV. Confirmation of peak assignments in capillary electrophoresis using immunoprecipitation. *J.Chromatogr. A* 2006;1106:56–60. [PubMed: 16199049]
15. Hamase K. Sensitive two-dimensional determination of small amounts of D-amino acids in mammals and the study on their functions. *Chem. Pharm. Bull* 2007;55:503–510. [PubMed: 17409537]

16. Wilm M, Mann M. Analytical properties of the nanoelectrospray ion source. *Anal. Chem* 1996;68:1–8. [PubMed: 8779426]
17. Desai MJ, Armstrong DW. Analysis of native amino acid and peptide enantiomers by high-performance liquid chromatography /atmospheric pressure chemical ionization mass spectrometry. *J. Mass Spectrom* 2004;39:177–187. [PubMed: 14991687]
18. Song Y, Quan Z, Liu YM. Assay of histamine by nano-liquid chromatography /tandem mass spectrometry with a packed nanoelectrospray emitter. *Rapid Commun. Mass Spectrom* 2004;18:2818–2823. [PubMed: 15508140]
19. Song Y, Shenwu M, Zhao S, Hou D, Liu YM. Enantiomeric separation of amino acids derivatized with 7-fluoro-4-nitrobenzoxadiazole by capillary liquid chromatography/tandem mass spectrometry. *J. Chromatogr. A* 2005;1091:102–109. [PubMed: 16395798]
20. Dunlop DS, Neidle A, McHale D, Dunlop DM, Lajtha A. The presence of free D-aspartic acid in rodents and man. *Biochem. Biophys. Res. Commun* 1986;26:27–32. [PubMed: 3801000]
21. Hamase K, Homma H, Takigawa Y, Fukushima T, Santa T, Imai K. Regional distribution and postnatal changes of D-amino acids in rat brain. *Biochim. Biophys. Acta* 1997;1334:214–222. [PubMed: 9101716]

Fig. 1.

Enantioseparation of an authentic D-/L-amino acid mixture containing NBD-L-Asp-d₃ (internal standard), NBD-D-/L-Asp, NBD-D-/L-Ser, and NBD-D-/L-Glu; (A) TIC of m/z 300, m/z 297, m/z 269 and m/z 311; (B) extracted mass chromatogram of m/z 300 $\rightarrow$ 282 for NBD-L-Asp-d₃ from A and the full scan MS/MS spectrum of m/z 300, respectively; (C) extracted mass chromatogram of m/z 297 $\rightarrow$ 279 for NBD-Asp from A and the full scan MS/MS spectrum of m/z 297, respectively; (D) extracted mass chromatogram of m/z 269 $\rightarrow$ 223 for NBD-Ser from A and the full scan MS/MS spectrum of m/z 269, respectively; (E) extracted mass chromatogram of m/z 311 $\rightarrow$ 293 for NBD-Glu from A and the full scan MS/MS spectrum of m/z 311, respectively. HPLC conditions: teicoplanin aglycone CSP (17.00 cm $\times$ 0.25 mm ID) column, and isocratic elution with methanol: water: TFA (60: 40: 0.012; v: v: v) at 2.34 μ L/min. Final concentrations of amino acids were 350 ng/mL for L-amino acids and 500 ng/mL for D-amino acids.

Fig. 2.

Detection of D-amino acids in brain tissue homogenates prepared from SD rats at different life stages: A, E and H, TICs of m/z 311, m/z 300, m/z 297, and m/z 269; B, F and I, extracted mass chromatogram of m/z 297 $\rightarrow$ 279 for Asp enantiomeric quantitation; C, G and J, extracted mass chromatogram of m/z 269 $\rightarrow$ 223 for Ser enantiomeric quantitation; No D-Glu was detected in these samples (data not shown). HPLC conditions were as in Figure 1.

Fig. 3. Occurrence of D-Asp in the brain of rats at different life stages. Results are shown in D-Asp percent, i.e. $\text{D-Asp} / (\text{D-Asp} + \text{L-Asp}) \times 100$.

Fig. 4.

Occurrence of D-Ser in the brain of rats at different life stages. Results are shown in D-Ser percent, i.e. $\text{D-Ser} / (\text{D-Ser} + \text{L-Ser}) \times 100$.

Table 1

Distribution of D-Ser in rat brain

Rat number	Brain region	D-Ser content ($\mu\text{g/g tissue}$, n= 3)	D-Ser/(DSer +L-Ser) (%)
#1	Thalamus	31.46 ± 0.71	6.55 ± 0.057
	Cerebellum	ND	ND
	Cortex	101.51 ± 4.19	21.03 ± 1.79
	Hippocampus	52.56 ± 1.81	14.25 ± 0.62
	Striatum	50.95 ± 5.18	15.23 ± 1.29
#2	Thalamus	62.26 ± 5.13	8.76 ± 0.63
	Cerebellum	ND	ND
	Cortex	480.11 ± 8.74	23.94 ± 0.19
	Hippocampus	218.48 ± 8.04	16.51 ± 0.73
	Striatum	279.15 ± 7.29	20.67 ± 0.66
#3	Thalamus	140.26 ± 0.26	12.02 ± 0.55
	Cerebellum	ND	ND
	Cortex	455.93 ± 29.57	18.38 ± 0.22
	Hippocampus	132.70 ± 1.40	17.76 ± 0.65
	Striatum	250.50 ± 7.01	16.78 ± 0.85
#4	Thalamus	65.36 ± 3.39	5.74 ± 0.15
	Cerebellum	ND	ND
	Cortex	308.59 ± 14.31	19.56 ± 0.71
	Hippocampus	215.33 ± 9.77	18.16 ± 0.38
	Striatum	218.22 ± 4.95	17.08 ± 1.71

ND: not detected.