Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 844 (2006) 278–282
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of D-serine and related neuroactive amino acids in human plasma by high-performance liquid chromatography with fluorimetric detection

Suzanne L. Grant [a,*], Yanina Shulman [b], Philip Tibbo [b], David R. Hampson [c], Glen B. Baker [a,b]

[a] *Neurochemical Research Unit, Department of Psychiatry, 12-127 Clinical Sciences Building, University of Alberta, Edmonton T6G 2G3, Canada*
[b] *Bebensee Schizophrenia Research Unit, Department of Psychiatry, University of Alberta, Edmonton, Canada*
[c] *Faculty of Pharmacy, University of Toronto, Toronto, Canada*

Received 28 April 2006; accepted 15 July 2006
Available online 4 August 2006

Abstract

A simple and versatile methodology using high-performance liquid chromatography (HPLC) with fluorimetric detection was developed to simultaneously determine D-serine along with other metabolically related neuroactive amino acids in the glutamatergic system: L-serine, L-glutamate, L-glutamine, and glycine. On-column sensitivity was in the lower picomole range. Of two chiral thiol reagents investigated, amino acid derivatives of *o*-phthaldialdehyde (OPA) in combination with *N*-isobutyryl-L-cysteine were found to have consistently higher responses than their corresponding *N*-*tert*-butyloxycarbonyl-L-cysteine derivatives. This methodology was applied to the quantitative detection of amino acids in human plasma and lays the foundation for further investigations of the role of neuroactive amino acids in the pathophysiology and treatment of neurological and psychiatric disorders.
© 2006 Elsevier B.V. All rights reserved.

Keywords: NMDA receptor; Serine racemase; Chiral thiol; Brain; Neurochemistry; Schizophrenia; Alzheimer's disease; Epilepsy

1. Introduction

Several lines of evidence suggest that changes in peripheral amino acids may be linked to central brain functions and that alterations in amino acid availability and/or metabolism may play a role in the pathogenesis of psychiatric disorders. Dysfunction in glutamatergic neurotransmission via *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in the central nervous system has been proposed to be associated with the pathophysiology of schizophrenia and other psychiatric and neurological disorders [1–4]. Accumulating evidence for the role of D-serine as an allosteric regulator of NMDA-type glutamate receptors [5,6] indicates the importance of sensitive analytical methods which can detect and quantify this compound in biological samples. Changes in plasma levels of amino acids in schizophrenia and correlation of these measures to the response to antipsychotic drug treatments have been investigated previously [7–9], but without differentiation of D- and L-serine or a targeted focus on amino acids involved with the glutamatergic system. The present study was undertaken to develop a simple and sensitive methodology to differentiate L- and D-serine isomers (L-Ser and D-Ser) and simultaneously measure the neuroactive amino acids glycine (Gly), L-glutamate (L-Glu) and L-glutamine (L-Gln) in human plasma. These amino acids are readily interconverted in vivo to modulate a number of neurological functions (Fig. 1) and Gly in particular is known to act as a coagonist at the NMDA glutamate receptor [10].

The most common and well-known method to determine amino acids, namely, reacting with *o*-phthaldialdehyde (OPA) in combination with 2-mercaptoethanol (2-ME) to produce fluorescent isoindole derivatives which are separated by high-performance liquid chromatography (HPLC) and detected fluorimetrically, does not differentiate amino acid enantiomers. By substituting a chiral thiol reagent for 2-ME, diasteromeric

* Corresponding author. Tel.: +780 492 6550.
E-mail address: suzanne.grant@ualberta.ca (S.L. Grant).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.07.022

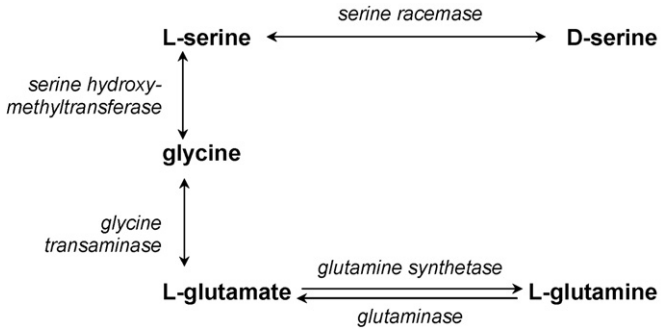

Fig. 1. Metabolic relationship of the amino acids investigated in this study.

derivatives are produced which can be separated by HPLC. There are a number of studies investigating the derivatization of amino acids with OPA and the chiral thiols *N*-acetylcysteine (NAC), *N*-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys), and *N*-isobutyryl-L-cysteine (IBC) for the detection of D-amino acids in a variety of biological matrices including bacteria, plants, foods, urine, and human serum [11–15]. The reaction scheme for derivatizing amino acids with OPA and chiral thiols is shown in Fig. 2. This principle has also been applied previously to simultaneously detect free amino acid enantiomers in combination with non-chiral amino acids in rat brain and serum by derivatizing with OPA and Boc-L-Cys prior to HPLC separation [16]. In a study by Soto-Otero et al. [17], free amino acids in rat brain were derivatized using a combination of OPA and NAC prior to analysis by HPLC, but enantiomers were not resolved. The methodology described in our study is an adaptation of some of the principles underlying these previous studies to specifically target amino acids in the glutamatergic system. This methodology has relevance for studying the role of peripheral and central amino acids in brain disorders as well as elucidating the neurochemical bases of drug interventions.

2. Experimental

2.1. Chemicals and reagents

Individual amino acid standards, *o*-phthaldialdehyde, and *N*-*tert*-butyloxycarbonyl-L-cysteine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). *N*-isobutyryl-L-cysteine was purchased from Novabiochem (La Jolla, CA, USA). All solvents were of HPLC grade and water was distilled and purified by reverse osmosis before use.

2.2. Apparatus

High-performance liquid chromatography was performed using a Waters Alliance 2690XE instrument equipped with an autosampler, thermally controlled sample and column compartments, and a Waters 474 programmable fluorescence detector (Waters Corporation, Milford MA). Data were acquired and processed using the Empower Pro software package from Waters. Separation was carried out on a Symmetry C_{18} column (4.6 mm × 150 mm, 3.5 μm) coupled with a guard column containing the same stationary phase (3.9 mm × 20 mm, 5 μm), both from Waters.

2.3. Chromatographic conditions

The sample and column compartments were maintained at 4 and 30 °C, respectively, for all analyses. The flow rate was constant at 1 mL/min and run time was 60 min. Solvent A comprised 850 mL 0.04 M sodium phosphate and 150 mL methanol, adjusted to pH 6.2. Solvent B comprised 670 mL 0.04 M sodium phosphate, 555 mL methanol, and 30 mL tetrahydrofuran, adjusted to pH 6.2. This particular composition of solvent B has been used in our lab previously when we were analyzing amino acids after derivatization with OPA in combination with mercaptoethanol [18]. Mobile phases were filtered through a 0.22 μm filter prior to use. The amino acids of interest were separated by a concave gradient (Curve 10 in Waters Empower Pro software) from 15% to 100% B in 35 min. The solvent mix was returned to intitial conditions by 45 min using a concave gradient (Curve 9 in Waters Empower Pro software) and maintained at that composition for 15 min prior to the next injection. Many gradient profiles were tested and this particular concave gradient was found to give the best separation of glycine from other closely eluting peaks while maintaining the resolution of the other amino acids of interest, as compared to some linear gradients investigated during method development. Amino acid derivatives were monitored at excitation and emission wavelengths of 260 and 455 nm, respectively.

2.4. Derivatization procedures

Derivatizing reagent solutions were prepared by dissolving 1 mg OPA and 2 mg IBC or Boc-L-Cys in 0.1 mL methanol followed by the addition of 0.9 mL 0.2 M sodium borate buffer (pH 10). The reagent solutions were prepared freshly every second

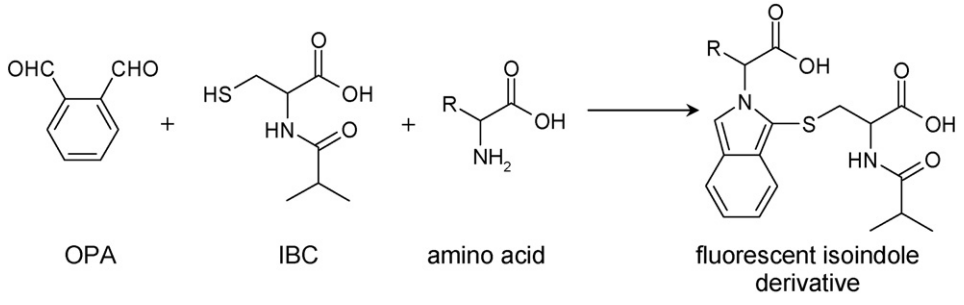

Fig. 2. Amino acid derivatization scheme.

day and stored at 4 °C when not in use. Automated pre-column derivatization was carried out by drawing up a 5 μL aliquot of sample, standard, or blank solution and 5 μL of derivatizing reagent solution, and holding in the injection loop 5 min prior to injection.

2.5. Standards and sample preparation

2.5.1. Standard solutions

Stock solutions of L-Glu, L-Gln, L-Ser, D-Ser, and Gly were prepared in 20% (v/v) methanol at a concentration of 1.0 mg/mL. An intermediate standard mixture was prepared by combining 20 μL each of L-Glu and L-Gln stock solutions and 5 μL each of L-Ser, D-Ser, and Gly stock solutions with 945 μL 20% (v/v) methanol. For calibration using a 7-point standard curve, this intermediate standard was diluted to 0.75, 0.5, 0.25, 0.1, 0.025, and 0.01 times its original strength.

2.5.2. Human plasma samples

To determine reference values for amino acids, blood samples from 28 normal healthy volunteers (male, mean age = 22 ± 3 years) were collected after an overnight fast. Plasma was separated immediately by centrifugation and stored at −80 °C until use. The protocol was conducted with the approval of the University of Alberta Health Research Ethics Board and with written informed consent from all participants. On the day of analysis, plasma samples were thawed and deproteinized by adding of 3 volumes of methanol, vortexing thoroughly, and leaving the sample on ice for 10 min. Following centrifugation at 4 °C, 5 μL of supernatant was analyzed for neuroactive amino acids as described above.

3. Results and discussion

The OPA-IBC derivatives of D- and L-Ser, L-Glu, L-Gln, and Gly were separated within 35 min with excellent resolution. Typical chromatographs are shown in Fig. 3. These amino acids were resolved from L-aspartate, L-serine-*o*-phosphate, L-alanine, γ-aminobutyric acid, and L-tryptophan, which were also added to the standard mixture to test for potential interference from these common components in biological samples. The ability to resolve these additional amino acids in a standard mixture suggests that they could also be determined in biological samples using this methodology, provided they can be adequately separated from sample matrix interference. In plasma samples, there was no interference from other unidentified plasma components co-eluting with the five amino acids of interest.

The fluorescent responses of the amino acid derivatives were linear in the range 2.5–100 ng per injection for L-Glu and L-Gln and 0.63–25 ng per injection for D-Ser, L-Ser, and Gly ($R^2 > 0.99$ for all). The on-column detection limits for each of the amino acids were in the lower picomole range (2.3 pmol for D- and L-Ser, 3.3 pmol for Gly, and 6.8 pmol for L-Glu and L-Gln), which translated under the described sample preparation conditions to 5.5 μmol/L for L-Glu and L-Gln, 2.7 μmol/L for Gly, and 1.9 μmol/L for D- and L-Ser on a plasma basis.

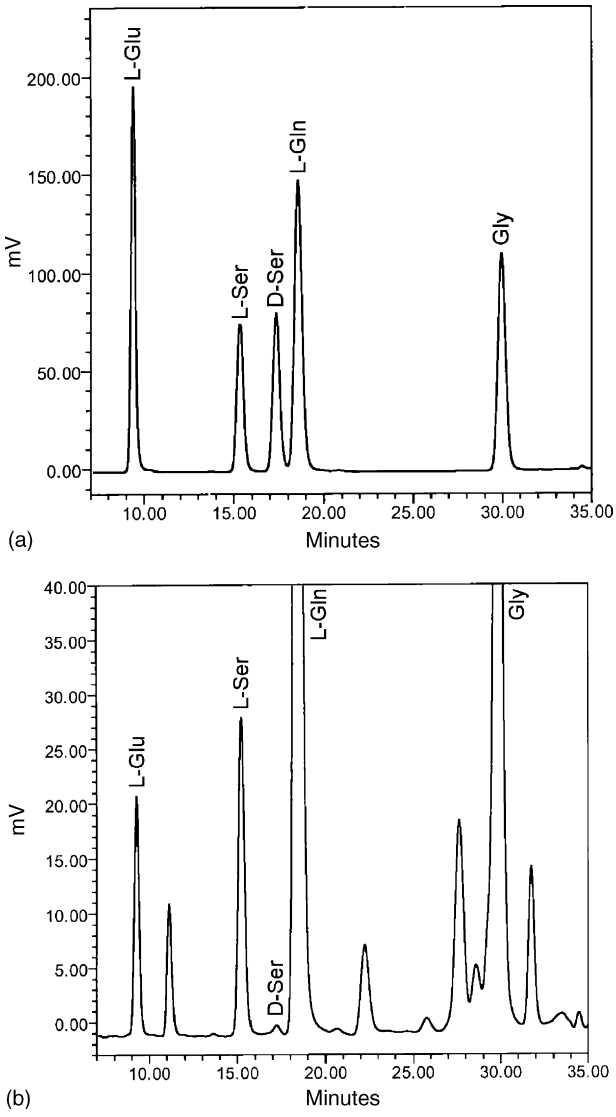

Fig. 3. HPLC chromatographs of (a) a mixture of amino acid standards with concentrations of 15, 3.75, 3.75, 15, 3.75 μg/mL for L-glutamate (L-Glu), L-serine (L-Ser), D-serine (D-Ser), L-glutamine (L-Gln), and glycine (Gly), respectively, and (b) human plasma.

Interassay variability was evaluated by injecting on three non-consecutive days a standard mixture containing L-Glu and L-Gln at concentrations of 15 μg/mL each, and L-Ser, D-Ser, and Gly at concentrations of 3.75 μg/mL each. To evaluate the interassay variability in plasma samples, two different samples were each injected on three non-consecutive days. Results of the interassay variability study are reported in Table 1. Measurements in the plasma samples were slightly more variable than in the standard mixture, but were still within acceptable limits. No breakdown of glutamine to glutamate was observed as a result of the derivatization conditions or during storage of the standard mixtures for several weeks at 4 °C.

Since previous work has shown that Boc-L-Cys is a useful reagent to determine amino acid enantiomers in biological samples [16], we were interested in comparing the responses of amino acids derivatized with either OPA/Boc-L-Cys or OPA/IBC. To adequately separate the more hydrophobic Boc-L-

Table 1
Interassay variability (%) of amino acid standards and amino acids in two different human plasma samples over three non-consecutive days

	Standard	Plasma 1	Plasma 2
L-Glutamine	4.3	2.5	4.5
Glycine	3.1	7.4	10.2
L-Serine	3.6	2.3	6.8
L-Glutamate	4.3	2.5	4.5
D-Serine	3.5	11.4	8.2
Average	3.8	5.2	6.8

Cys derivatives, the initial conditions of the gradient described above were changed to 45% B instead of 15%. All amino acid derivatives of IBC had greater signal intensity than the corresponding Boc-L-Cys derivatives over the studied concentration range. The response per unit mass was greater by 58%, 39%, 38%, 29%, and 19% for IBC derivatives of Gly, L-Ser, L-Ser, L-Glu and L-Gln, respectively. Thus, the thiol component of the derivatizing reagent affects the sensitivity of the assay and the change in response does not necessarily apply uniformly to all analytes. For this reason, the peak profile in a mixture may not be consistent among analyses using different thiol reagents. Using L-Ser as a reference peak, we found that the relative response of the IBC derivative of Gly was significantly higher than its Boc-L-Cys derivative ($p < 0.05$), and the relative responses of IBC derivatives of L-Glu and L-Gln were significantly lower than their Boc-L-Cys counterparts ($p < 0.05$). There was no relative difference in D-Ser response, which suggests that D- and L-Ser react and respond similarly. Since the signal response was consistently higher for IBC derivatives, this reagent was chosen over Boc-L-Cys for subsequent analysis of the plasma samples.

Having established suitable analytical conditions, amino acids in plasma from 28 healthy volunteers were determined and these values were similar to those reported previously in other studies (Table 2). D-Serine was detectable in most of the plasma samples, but levels were just above the limit of detection. Our findings are in agreement with previous studies which have shown that blood concentrations of D-Ser tend to be very low in comparison to brain levels [3], possibly due to the high activity of D-amino acid oxidase in liver and kidney. Because of the extensive metabolism of D-Ser in peripheral tissues, it is thought that D-Ser in the blood originates from the brain [1].

Table 2
Comparison of amino acid concentrations in plasma of healthy volunteers (mean ± standard deviation, $n = 28$) to literature values

Amino acid	Present study (μmol/L plasma)	Literature values		
		μmol/L plasma	n	Reference
L-Glutamine	490.6 ± 84.3	468 ± 76	32	[20]
Glycine	212.4 ± 57.4	226.5 ± 114.8	11	[9]
L-Serine	70.3 ± 19.6	175.0 ± 30.6	42	[9]
L-Glutamate	26.0 ± 14.3	38.8 ± 18.6	11	[1]
D-Serine	3.6 ± 2.2	2.3 ± 0.6	42	[1]

Thus, peripheral measurement of D-Ser may reflect central levels. Furthermore, intraperitoneal injection of either L- or D-serine has been shown to elevate brain levels of both L- and D-serine in rats [19], suggesting a link between the two compartments. Interestingly, the same study found that glycine administered intraperitoneally also resulted in elevated brain levels of both L- and D-serine. These findings highlight the importance of analytical methods which can simultaneously determine metabolically related compounds.

4. Conclusion

The method described in our study is sensitive, sample preparation is rapid and simple, and derivatization is automated. The method has the advantage of simultaneously measuring several amino acids which are readily interconverted in vivo and have unique neurochemical activites of their own. This methodology is useful for studying the role of amino acids in the glutamatergic system in the pathophysiology and treatment of neurological and psychiatric disorders. It is readily adaptable to other biological tissues and fluids, as well as to the simultaneous analysis of other relevant amino acids and their enantiomers.

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada, the Canadian Institutes of Health Research and the Canada Research Chairs and Canada Foundation for Innovation programs. The technical assistance of Ms. Gail Rauw is gratefully acknowledged.

References

[1] K. Hashimoto, T. Fukushima, E. Shimizu, N. Komatsu, H. Watanabe, N. Shinoda, M. Nakazato, C. Kumakiri, S. Okada, H. Hasegawa, K. Imai, Arch. Gen. Psychiatry 60 (2003) 572.
[2] K. Hashimoto, T. Fukushima, E. Shimizu, S. Okada, N. Komatsu, N. Okamura, K. Koike, H. Koizumi, C. Kumakiri, K. Imai, M. Iyo, Prog. Neuropsychopharmacol. Biol. Psychiatry 28 (2004) 385.
[3] A. Hashimoto, T. Oka, Prog. Neurobiol. 52 (1997) 325.
[4] M. Steffens, H.J. Huppertz, J. Zentner, E. Chauzit, T.J. Feuerstein, Neurochem. Int. 47 (2005) 379.
[5] H. Wolosker, R. Panizzutti, J. De Miranda Neurochem. Int. 41 (2002) 327.
[6] M. Shleper, E. Kartvelishvily, H. Wolosker, J. Neurosci. 25 (2005) 9413.
[7] T. Sumiyoshi, H.Y. Meltzer, Int. J. Neuropsychopharmacol. 7 (2004) S72.
[8] F.M.A.A. van der Heijden, D. Fekkes, S. Tuinier, A.E.S. Sijben, R.S. Kahn, W.M.A. Verhoeven, J. Neural Transm. 112 (2005) 577.
[9] A. Tortorella, P. Monteleone, M. Fabrazzo, A. Viggiano, L. De Luca, M. Maj, Neuropsychobiology 44 (2001) 167.
[10] B. Hassel, R. Dingledine, Glutamate, in: G.J. Siegel, R.W. Abbers, S.T. Brady, D.L. Price (Eds.), Basic Neurochemistry: Molecular, Cellular, and Medical Aspects, seventh ed., Elsevier, Amsterdam, 2006.
[11] H. Bruckner, T. Westhauser, Amino Acids 24 (2003) 43.
[12] H. Bruckner, M. Langer, M. Lupke, T. Westhauser, H. Godel, J. Chromatogr. A 697 (1995) 229.
[13] H. Bruckner, S. Haasmann, A. Friedrich, Amino Acids 6 (1994) 205.
[14] H. Bruckner, S. Haasmann, M. Langer, T. Westhauser, R. Wittner, J. Chromatogr. A 666 (1994) 259.
[15] H. Bruckner, R. Wittner, H. Godel, J. Chromatogr. 476 (1989) 73.

[16] A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, T. Hayashi, J. Chromatogr. 582 (1992) 41.
[17] R. Soto-Otero, E. Mendez-Alvarez, J. Galan-Valiente, E. Aguilar-Veiga, G. Sierra-Marcuno, Biomed. Chromatogr. 8 (1994) 114.
[18] M. Parent, D. Bush, G. Rauw, S. Master, F. Vaccarino, G. Baker, Methods 23 (2001) 11.
[19] K. Takahashi, F. Hayashi, T. Nishikawa, J. Neurochem. 69 (1997) 1286.
[20] F.J. Jimenez-Jimenez, J.A. Molina, P. Gomez, C. Vargas, F. de Bustos, J. Benito-Leon, A. Tallon-Barranco, M. Orti-Pareja, T. Gasalla, J. Arenas, J. Neural Transm. 105 (1998) 269.