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Liquid chromatographic–fluorimetric method for the estimation of nitric oxide biosynthesis in the central nervous system

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Abstract

Some amino acids are involved in the biosynthesis of nitric oxide (NO), which has a physiological and pathophysiological role. To study NO biosynthesis, we compared arginine and citrulline levels in the cerebrospinal fluid (CSF) from patients with infectious and/or inflammatory processes within the central nervous system (CNS), with those from patients without those disorders. Arginine concentration was not significantly different between the groups ($P = 0.115$), whereas citrulline was significantly elevated in the first group ($P = 0.020$). We propose a simple chromatographic method to estimate NO biosynthesis ex vivo within the CNS, that may be applicable for the study of neurodegenerative and psychiatric diseases such as Parkinson's disease and schizophrenia.

Keywords: Nitric oxide; Arginine; Citrulline

1. Introduction

The analysis of the amino acid composition of biological fluids is very important in several diseases. For the study of physiology and pathophysiology of the central nervous system (CNS), cerebrospinal fluid (CSF) is often used because its composition reflects the global metabolism of this system. There is a wide spectrum of pathological conditions where changes in amino acid concentrations in the CSF can be detected, including multiple sclerosis, intracraneal hypertension, hepatic coma, phenylketonuria, Parkinson's disease, epilepsy, Alzheimer's disease, Alzheimer-type dementia, major depressive disorder and schizophrenia [1–6]. The analysis of CSF is useful to determine the biochemical mechanisms underlying such pathologies.

Within the CNS, amino acids may act as substrates for metabolic processes, as neurotransmitters, as neuromodulators, or as osmolytes [5,7]. Some of them (at least asparagine, citrulline, alanine, proline, glutamate, aspartate, glycine, and γ-aminobutyric acid) do not cross the blood–brain barrier (BBB) significantly [8], so it is accepted that their concentrations in the CSF are primarily due to the CNS metabolism.

Nitric oxide (NO) is an important messenger involved in several physiological and pathophysiological processes [2,9–13]. The importance of biochemical studies about this molecule is still increasing. Most evidences about NO biosynthesis have been obtained directly, by measuring NO synthase (NOS, EC 1.14.13.39) activity [14,15], or indirectly, by the quantitation of markers such as cGMP [16,17] or nitrite and/or nitrate [17,18]; but for some experiments, samples containing the enzyme may not be available, and other markers, although very useful, may be unspecific.

The *o*-phtalaldehyde/2-mercaptoethanol (OPA/ME) reagent is commonly used for the derivatization of amino acids since it was reported by Roth [19]. A relatively simple method for reversed-phase high-performance liquid

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chromatography (HPLC) using the OPA/ME reagent was developed by Turnell and Cooper [20]. Several other methods have been described since then, but only few of them have been developed especially for the CSF. Here we describe a fast, simple and highly reproducible reversed-phase HPLC-fluorimetric method for the analysis of the main amino acids involved in NO biosynthesis, using human CSF, with precolumn OPA/ME derivatization, that is intended to be useful to understand the pathological processes occurring in some neurological and psychiatric disorders, regarding nitric oxide biosynthesis.

2. Materials and methods

2.1. Chemicals

Tetrahydrofuran (Mallinckrodt Baker, Kentucky, USA) and absolute methanol (Mallinckrodt Baker, Mexico) were both HPLC grade. *o*-Phtalaldehyde, 2-mercaptoethanol, and amino acids were obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.1.1. Solvents and solutions

All solutions were prepared in deionized distilled water (Milli-Q Water System, Millipore, Bedford, MA). Solvent A was HPLC-grade absolute methanol. The mobile phase was prepared by adding tetrahydrofuran (HPLC-grade) to a 50 mM sodium acetate buffer (pH 5.9) to a final concentration of 1.5% (v/v). This solution (solvent B) was filtered through a 0.45 μm membrane and degassed by sonication prior to use.

The OPA/ME reagent was prepared as described by Turnell and Cooper [20] with minor modifications: 25 mg of OPA were dissolved in 625 μl of methanol. This solution was mixed with 5.6 ml of a 0.3 M sodium borate buffer (pH 9.5) and then, 25 μl of ME were added. This OPA/ME reagent was prepared daily and maintained on ice during the analysis.

2.2. Subjects

Five patients (35.2 ± 4.43 years old) with inflammatory or infectious processes within the CNS (viral encephalitis (1), neurocysticercosis (3), or lumbar disk herniations (1)) were included in this study. In all of them, an increase in NO biosynthesis, caused by the response of the tissue, is expected. Neither of them (except the one with viral encephalitis) had severe symptoms of infection or inflammation nor were taking steroid analgesics or anti-inflammatory drugs at the time of the lumbar puncture. Six patients (39.66 ± 7.42 years old) without markers of infection or inflammation in their CSF (ophtalmologic disorders (3), controlled (inactive) neuropathies (2), and possible neurocysticercosis with negative CSF immunoreactivity (ELISA) (1)) were included in the control group. Healthy volunteers were not included because the procedure for lumbar puncture is not ethically justified for them.

2.2.1. Samples

CSF was obtained by lumbar puncture from patients attended at our Institute. Samples were obtained only when indicated by the attending clinicians. Informed consent was obtained in all cases by the patients or their relatives. CSF was stored at −80 °C until analysis. CSF was thawed and filtered through a 0.45 μm membrane; aliquots of 20 μl were diluted by the addition of 80 μl of distilled deionized water, mixed with 100 μl of the OPA/ME reagent, and allowed to react for 1 min while stirred with a vortex. Then the reaction mixture was injected into the column. The reaction time was chosen since it has been shown that 1 min is appropriate for the determination of the OPA/ME derivatives for most amino acids (such as glutamate and aspartate), except for glycine and lysine [21], none of which were included in our experiments.

2.3. Equipment

Separation of amino acid derivatives was carried out with a 200 LC Series binary pump (Perkin Elmer); signals were obtained with a fluorimetric detector (Model 157, Beckman). The peaks were integrated by an HP 3396 Series II integrator (Hewlett Packard). Injections were made with a 20 μl loop.

2.3.1. Chromatographic conditions

This method is based on that described by Jarrett et al. [22], but has some modifications. The program we used was as follows: a linear gradient step from 10 to 30% A in 12 min, a washing step with 99% A for 5 min, and a reequilibration step with 10% A for 5 min. Derivatives were separated with an Adsorbosphere OPA HS reversed phase column (5 μm particle size, 100 mm × 4.6 mm, Alltech). A flow rate of 1.5 ml/min was used. The minimum time spent between injections was 22 min.

2.4. Repeatability

A standard mixture of amino acids (aspartate, glutamate, citrulline, and arginine, 20 μM each one) and a CSF sample from a patient with communicating hydrocephalus were analyzed 10 consecutive times to determine the intra-assay variation. Both retention times and concentrations were assayed for variation.

2.5. Stability

The same standard mixture of amino acids described above was analyzed in 10 different days. To determine the effect of multiple freezing–thawing cycles in amino acids instability, one sample of CSF was thawed, analyzed, and frozen, for 10 different days. Another aliquot of the same

sample was stored at 4 °C all the time and analyzed in the same way for comparison.

2.6. Reproducibility

An aliquot of the amino acids standard mixture (prepared every 2–3 days to prevent instability) was analyzed in 10 different days to determine the inter-assay variation. Again, both retention times and concentrations were tested.

2.7. Linearity

Standard curves were prepared in the range from 0.1 to 1000 μM ($8 < n < 13$). The fluorimetric responses were considered linear when adjusted to a correlation coefficient of at least 0.9990.

2.8. Limits of detection (LOD) and quantitation (LOQ)

Both limits were determined according to the ACS Committee on Environmental Improvement [23]. In both cases, we considered the mean of the blank, plus three times (LOD) or 10 times (LOQ) their standard deviation.

3. Results and discussion

Figs. 1 and 2 show the chromatograms obtained from a standard mixture of amino acids (aspartate, glutamate, glutamine, glycine, citrulline, arginine, and taurine, 10 μM each one), and an aliquot of CSF (diluted 1:5) from a patient with communicating hydrocephalus, respectively. As expected, each peak that is observed in the chromatogram corresponds to only one amino acid. None of the amino acids tested co-elute at the chromatographic conditions used. With this method, repeatability (Table 1) is acceptable, being lower than 5% for both concentrations and retention times, for all of the amino acids in distilled deionized water. In the CSF, variations in concentration are considerable, especially in the case of aspartate and glutamate. For arginine and citrulline concentrations in the CSF, repeatability is below 15% (Table 1).

When prepared in distilled deionized water, amino acids have a good stability throughout 10 days of analysis (Table 2). Only glutamate presented coefficients of variation above 5%, but it is still acceptable (below 10%). Variation tends slightly to increase as a function of time. When necessary, we recommend that solutions of amino acids not be

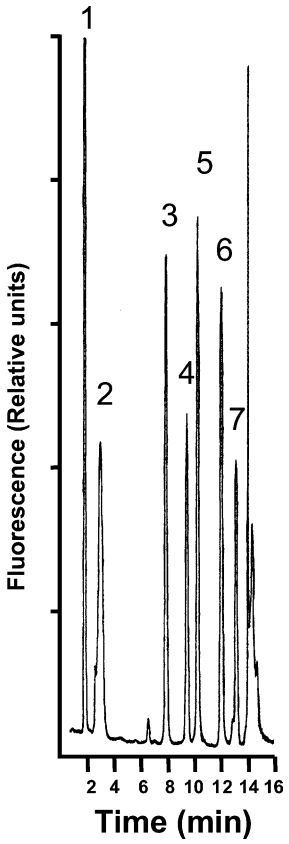

Fig. 1. Chromatogram obtained from a standard mixture of amino acids (10 μM each one). The last peak observed is due to the OPA reagent. Numbered peaks represent: aspartate (1), glutamate (2), glutamine (3), glycine (4), citrulline (5), arginine (6), and taurine (7).

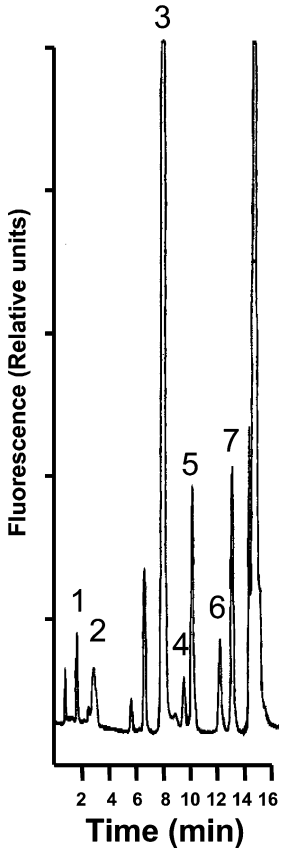

Fig. 2. Chromatogram obtained from a sample of CSF (diluted 1:5) from a patient with communicating hydrocephalus. The last peak observed is due to the OPA reagent. Numbered peaks represent: aspartate (1), glutamate (2), glutamine (3), glycine (4), citrulline (5), arginine (6), and taurine (7). Other peaks are unidentified components.

Table 1
Repeatability of the concentrations and retention times from a standard mixture of amino acids (20 μM each)

Amino acid	Concentration (μM)		Retention time (min)	
	Mean ± S.E.	C.V.	Mean ± S.E.	C.V.
In water				
Aspartate	19.39 ± 0.12	2.02	1.519 ± 0.009	1.90
Glutamate	15.44 ± 0.27	4.71	2.940 ± 0.029	3.17
Citrulline	19.21 ± 0.19	3.26	10.220 ± 0.027	0.84
Arginine	19.37 ± 0.16	2.61	11.957 ± 0.022	0.60
In the CSF				
Aspartate	2.02 ± 0.11	57.70	1.608 ± 0.002	1.51
Glutamate	9.78 ± 0.17	17.77	2.875 ± 0.011	4.90
Citrulline	36.25 ± 0.49	13.75	10.801 ± 0.003	0.30
Arginine	13.06 ± 0.12	9.90	11.916 ± 0.003	0.31

S.E., standard error; C.V., coefficient of variation.

stored for more than 3 days, when variation is still at the minimum level. Glutamate solutions should be prepared daily.

When repeated analysis are to be made using the same samples, storing conditions must be considered. Under this conditions, amino acid concentrations change considerably within the samples of CSF when stored both at 4 and −80 °C (frozen–thawed samples); concentrations increase since the first days of sample storing (data not shown), except for citrulline. Protein degradation may be the main mechanism responsible for this variation. In this way, aspartate and glutamate present the greater, and citrulline (a non-proteogenic amino acid) showed the lower variation (Table 2); the formers may be present in higher proportion than arginine in the proteins of the CSF, so the concentration of this amino acid does not increased as much as the others. In contrast, citrulline concentration in the CSF tends to decrease (data not shown), maybe due to hydrolysis or other mechanisms of degradation. The conditions for sample storing would depend on which be the amino acid of interest. For arginine it is better to maintain the samples frozen, while for citrulline, storing at 4 °C is recommended (Table 2).

Table 4
Linearity of response, LOD, and LOQ for the amino acids tested

Amino acid	Linearity (μM)	C.C.	LOD (pmol)	LOQ (nM)
Aspartate	0.5–250	0.9995	3.50	416
Glutamate	0.5–1000	0.9998	1.59	155
Citrulline	0.75–500	0.9998	5.63	700
Arginine	1–1000	0.9994	6.83	901

C.C., correlation coefficient.

The results of repeatability (Table 1) using CSF are in agreement with the changes due to instability, so aspartate and glutamate show the greater variation in their concentrations. Maybe proteolysis may occur to some extent within the same day of analysis, so when aspartate and glutamate be the amino acids of interest, the use of protease inhibitors should be considered.

Reproducibility (Table 3) was also below 5% for the parameters evaluated (concentration, retention time, and the slope of the standard curves), so little changes in the chromatographic conditions between days do not significantly affect quantitation. Only glutamate showed a variation above 5%, but it is still acceptable. Reproducibility was not tested using the authentic matrix (CSF) because of the great variation in the concentration of amino acids throughout 10 days of analysis (Table 2).

Amino acids are easily detectable at the low pmol level (Table 4), and their fluorimetric responses are linear in a wide

Table 2
Variations due to the instability of the amino acids (AA) throughout 3, 5, and 10 days of analysis

AA	Deionized water			CSF (stored at 4 °C)			CSF (frozen–thawed)		
	3 days	5 days	10 days	3 days	5 days	10 days	3 days	5 days	10 days
Asp	1.26	1.57	2.00	64.43	52.67	44.02	54.72	44.85	62.94
Glu	9.66	8.21	7.57	51.69	68.31	34.80	41.60	25.04	41.53
Cit	0.31	0.85	6.26	4.99	4.54	10.92	10.13	7.93	8.61
Arg	0.47	1.34	3.54	17.15	12.31	20.20	6.75	10.85	14.15

Data shown as coefficients of variation in the corresponding period of time. Asp, aspartate; Glu, glutamate; Cit, citrulline; Arg, arginine.

Table 3
Reproducibility of the concentrations and retention times from a standard mixture of amino acids (20 μM each), and for the slope of a standard curve (in the 1–20 μM range)

Amino Acid	Concentration (μM)		Retention time (min)		Slope (μM^{-1})	
	Mean ± S.E.	C.V.	Mean ± S.E.	C.V.	Mean ± S.E.	C.V.
Aspartate	19.81 ± 0.07	1.17	1.523 ± 0.010	2.23	59378 ± 860	4.34
Glutamate	19.92 ± 0.49	6.97	2.820 ± 0.043	3.46	54089 ± 535	2.21
Citrulline	19.96 ± 0.05	0.70	10.191 ± 0.029	0.92	35332 ± 283	2.54
Arginine	20.30 ± 0.15	2.36	11.900 ± 0.041	1.10	31504 ± 392	3.93

S.E., standard error; C.V., coefficient of variation.

Table 5
Arginine and citrulline concentrations in the control group and patients with infectious and/or inflammatory processes in the CNS

Group	Diagnosis	Concentration (μM)	
		Arginine	Citrulline
Control	Vitreitis	20.33	27.94
	Neuropathy secondary to intestinal malabsorption	22.35	28.37
	Peripherical neuropathy	14.56	29.16
	Degeneration of the retina	15.86	22.53
	Pseudotumor cerebri	17.29	21.4
	Possible neurocysticercosis with negative CSF immunoreactivity	18.23	13.62
Inflammation	Viral encephalitis	27.87	35.67
	Neurocysticercosis	21.46	33.35
	Neurocysticercosis	17.36	26.53
	Neurocysticercosis	24.7	31.06
	Lumbar disk herniations	18.29	40.81

interval of concentrations between three and four orders of magnitude (Table 4).

With this method, we evaluated arginine and citrulline concentrations in the CSF from patients with infectious and/or inflammatory processes in the CNS (Table 5), in order to determine if the expected increase in NO biosynthesis in such cases is reflected in the levels of this amino acids. Age was not significantly different between the groups ($P = 0.269$, Student's t-test). Arginine concentration in the CSF from these patients ($22.84 \pm 1.83\,\mu M$, mean ± S.E.) was not significantly different to the control group ($18.10 \pm 1.17\,\mu M$, mean ± S.E.; $P = 0.115$, Student's t-test) (Fig. 3). Arginine transport and biosynthesis are both stimulated by cytokines such as interleukin-1 and interferon-γ [24], so its concentration is expected to increase during an immune response, but levels are also regulated by other mechanisms, such as metabolic consumption and efflux transport across the BBB [25].

Citrulline concentrations in the inflammatory group ($31.65 \pm 1.59\,\mu M$, mean ± S.E.) were significantly elevated when compared to the control group ($23.83 \pm 2.43\,\mu M$, mean ± S.E.; $P = 0.020$, Student's t-test) (Fig. 3). As expected, CSF protein concentration (measured during routine cytochemical analysis) was significantly elevated in the inflammatory group (mean ± S.E.; control: 33.66 ± 8.53 mg/dl, inflammation: 82.80 ± 17.25 mg/dl; $P = 0.024$, Student's t-test) but did not correlate with citrulline concentration ($r = 0.153$, $P = 0.804$), so the change in the concentration of this amino acid does not seem to be due to variations in BBB permeability. The same holds true for the fact that arginine concentration did not increase as well as protein concentration. The concentration of none of these amino acids correlated with age (arginine: $r = 0.133$, $P = 0.830$; citrulline: $r = 0.286$, $P = 0.639$).

Citrulline concentration does not depend exclusively on NOS activity, although this amino acid has been detected in

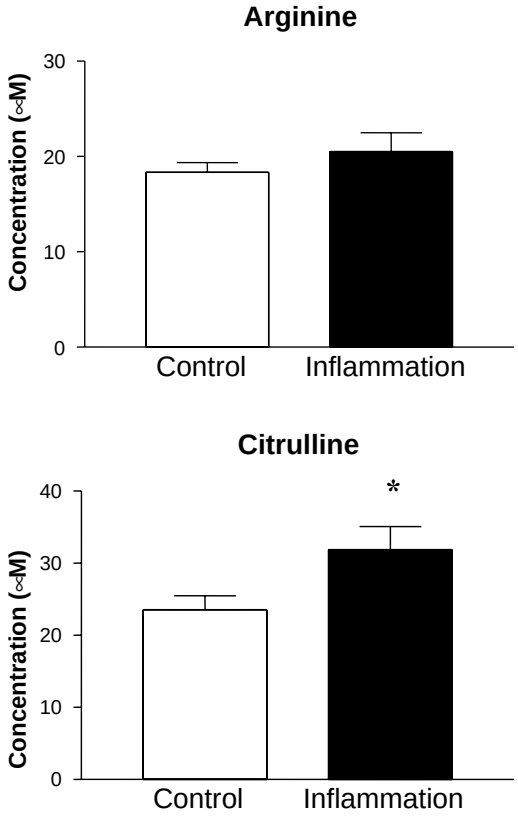

Fig. 3. Arginine and citrulline concentrations in the CSF from the control group and from patients with inflammatory and/or infectious processes in the CNS. Arginine was not significantly different between groups. $^{*}P < 0.05$ (Student's t-test).

the CNS by immunohistochemistry primarily in the neurons that express NOS [26]. Also, citrulline immunoreactivity in the brain is lacking in nNOS knockout mice [26], suggesting a direct relationship between citrulline formation and NOS activity. Ornithine carbamoyltransferase (EC 2.1.3.3) which synthesizes citrulline is absent from the CNS [25]. Dimethylargininase (dimethylarginine dimethylaminohydrolase, EC 3.5.3.18), which converts methylated arginines (NOS competitive inhibitors) into citrulline is expressed in the nervous system [25] and co-localizes with NOS [27]. Brain levels of the substrates for this enzyme (monomethyl-L-arginine and asymmetrical dimethyl-L-arginine) are at least 10 times lower than arginine content in this organ [25], and CSF concentration of asymmetrical dimethylarginine has been estimated to be below 50 nM in controls [2] so the activity of dimethylargininase does not seem to be the most likely explanation for the 7 μM increase in CSF citrulline concentration that we have observed in this work. Indeed, it has been reported that the levels of methylated arginines do not change significantly when endothelial cells are stimulated with a calcium ionophore to synthesize NO [28], and dimethylargininase has been shown to be inhibited by S-nitrosylation during cytokine-induced NOS II activity [29].

There is no other known citrulline-synthesizing enzyme in the CNS [25]. Argininosuccinate synthetase (ASS, EC 6.3.4.5) needs citrulline, as well as aspartate, for the rate-limiting step in arginine biosynthesis [25], so decreased ASS activity would result in an increase in citrulline concentration. This enzyme is co-induced with NOS II during an immune response [25,30,31] because citrulline recycling to arginine is important to maintain up to 80% of NO biosynthesis, at least in endothelial cells under bradykinin stimulation [28]. Also, the fact that citrulline and NOS co-localize in the CNS [26], but NOS and ASS do not in most neurons [32] suggests that the presence of this amino acid is related to NOS activity more than to ASS. In this way, decreased ASS activity is not expected to occur during an immune response and does not seem to be the most likely explanation to our results. However, as ASS activity was not evaluated in this report, the possible influence of this enzyme in our results cannot be completely ruled out.

NOS II expression and activity occurs in the CNS as a response of glial cells to the immunological stimulation [18,25,30], and it is responsible for a high output release of NO [12,33]. This effect causes that the levels of some markers of NO biosynthesis, such as nitrite and/or nitrate, increase. It has been shown that nitrate plus nitrate content in brain tissue correlates with NOS activity in rats [34]. This anions, as well as cGMP, have been useful for the study of NO biosynthesis in different studies [13,14,35,36]. It is well accepted that NO neurotoxicity secondary to an increase in NO biosynthesis is associated with the pathogenesis of neurodegenerative disorders such as Parkinson's disease [11,37] and Alzheimer's disease [37], but some studies have found nitrate concentration to be decreased in the CSF from patients with both disorders [38], and nitrite and nitrate concentration to be decreased in the plasma from patients with Alzheimer's disease [39], and not significantly different in the CSF from patients with Parkinson's disease [36]. Also, CSF levels of cGMP have been reported to be not significantly different in patients with Parkinson's disease compared to controls, although it tended to be higher [40]. This contradictory results may be explained by the fact that cGMP is a good marker of NO biosynthesis that unfortunately it is unspecific. On the other hand, nitrite and/or nitrate concentration should be expected to be decreased if the over-activity of NOS would lead NO to form peroxynitrite instead of the other anions. This seems to be the case in general, as nitrotyrosine, the biological marker of peroxynitrite formation, has been detected in brain tissue from people who died with Alzheimer's disease [41]. Also, we have observed a trend towards an increase in citrulline concentration in the CSF from patients with Parkinson's disease, in a preliminar study (Pérez-Neri et al., unpublished results). Nitrotyrosine concentration in the same samples remains to be determined. Citrulline concentration could be taken as complementary to the detection of other markers of NO biosynthesis, and a reduction in nitrite and/or nitrate levels combined with an increase in citrulline concentration might be considered suggestive of peroxynitrite formation.

The analysis of nitrite and/or nitrate has a disadvantage, as also cGMP, as they are not produced directly by NOS. In contrast, citrulline is produced directly by NOS activity in equimolar amounts to NO [14,18], and (to our knowledge) almost none of the reports about NO evaluates its concentration, except for the NOS activity assay [14]. In this report, we found that the concentration of citrulline in the CSF is elevated in some pathologies that involve an increase in NOS activity, so this amino acid concentration can be taken as a marker of NO biosynthesis. As citrulline does not cross the BBB significantly [8], its concentration in the CSF depends mainly on the CNS metabolism, almost without interference from peripheral tissues. NOS seems to be the main enzyme that synthesizes this amino acid in the brain [32], so the variations in citrulline concentration in the CSF may reflect changes in NOS activity.

It should be considered, also, that the main result of our work is the application of this method to the analysis of NO biosynthesis, and not the clinical finding. An increase in CSF nitrite and nitrate concentrations has been reported in other infectious disorders [42]. The increase in citrulline concentration is an expected result. In this way, our results can be reasonably questioned about important factors such as sample size and heterogeneity in patients diagnosis, but we suggest that our work should be considered as the validation and application of a chromatographic method instead of a clinical protocol.

A chromatographic method for the assay of NOS has been previously reported by Carlberg [43], and consists in the isolation of the enzyme from tissue homogenates, and after an incubation period with L-arginine, citrulline is quantitated fluorimetrically with an HPLC method. NOS does not seem to be present in the CSF [44]. Here we report an indirect assay for NOS activity in samples that do not contain the enzyme, based on the analysis of citrulline concentration, endogenously synthesized within the human CNS. This is important because the in vitro assay for the enzyme may not reflect NOS activity in vivo, as it is also regulated by some mechanisms that are not reproduced in vitro, such as arginine transport across the neuronal plasma membrane [24] and cofactors availability [33].

As reported here, glutamate and aspartate concentrations can also be measured in relationship with NOS, as the enzyme (NOS I) is dependent on glutamatergic receptor stimulation [33].

In this paper we describe a reproducible and highly sensitive method for the analysis of some amino acids in the human CSF, with possible applications for other biological fluids, and a wide group of neurological disorders, and psychiatric diseases such as schizophrenia, that are related to NO biosynthesis. This could help us to explore the pathophysiological processes involved in such diseases.

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