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QUANTITATION OF FREE AMINO ACIDS IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION OF THE METHOD IN EVALUATING AMINO ACID LEVELS IN CEREBROSPINAL FLUID AND PLASMA OF PATIENTS WITH MULTIPLE SCLEROSIS

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SUMMARY

An automatic on-line high-performance liquid chromatographic method based on a precolumn derivatization with *o*-phthalaldehyde has been developed to quantitate levels of free amino acids in cerebrospinal fluid (CSF) and plasma samples from 12 patients with multiple sclerosis (MS) and 12 controls. The analytical method gave reproducible results with relative standard deviations of 0.5–3% for all amino acids. The separation of 24 amino acids was performed on a reversed-phase C_{18} column, using two solvents and a multiple-step gradient. Each chromatographic experiment was completed within 40 min. The results showed higher levels of Glu, Gln, Gly and Ala and lower levels of Met, Val, Phe and Lys in plasma of MS patients. In CSF, increased levels of Gln, Arg, Ser and Tyr and decreased levels of Asp, Glu, Met, γ-aminobutyric acid and Phe were found in MS patients, whereas the levels of other amino acids remained more or the less same in both groups.

INTRODUCTION

It is well established that among the free amino acids, glutamic acid (Glu), aspartic acid (Asp), γ-aminobutyric acid (GABA), glycine (Gly), glutamine (Gln) and taurine (Tau) are prominent in mature brain and constitute 2/3 of the free α-amino nitrogen[1]. These amino acids possibly play an important rôle as neurotransmitters and/or neuromodulators[1–3].

It is known that the pattern of free amino acid concentrations in cerebrospinal fluid (CSF) reflects a complex equilibrium of amino acid transport at the various barriers between blood, CSF and brain, as well as metabolic processes in whole body and brain tissue[4,5]. Accordingly, abnormal concentrations of one or more free amino acids in CSF are found under various neurological conditions, such as central nervous system (CNS) infections, convulsions, extrapyramidal and in various neuropsychiatric disorders[4–12].

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Since the majority of identified neurotransmitters and putative neurotransmitters are either amino acids or their derivatives, the accurate and valid measurements of CSF amino acid levels would constitute a potentially useful method for investigating the CNS function. However, aside from GABA, systematic evaluation of basic parameters has not been adequately carried out, and this is reflected in the variability in CSF amino acid levels reported in the literature[10–16].

The quantitation of free amino acids and total amino acids in biological samples has traditionally been performed by classical ion-exchange chromatography followed by post-column derivatization with ninhydrin or a fluorescence reagent, such as *o*-phthalaldehyde (OPA)[17,18]. This technique is time consuming (2–3 h), requires relatively large sample volumes (100–300 μl) and yields unreliable results for labile amino acids such as Gln and Asn. Other problems encountered, including broadening of peaks, discrepancies in results in accurate quantitation of basic amino acid, buffer contamination, baseline shift and incorrect identification of amino acids in physiological samples, have recently been reviewed[19].

In recent years, the fluorogenic reaction between OPA, thiols and primary amines has been exploited in reversed-phase high-performance liquid chromatography (RP-HPLC). The reaction is highly specific and sensitive and is completed within a few minutes at ambient temperature. Various systems are utilized for HPLC analysis after OPA derivatization to quantitate amino acids in physiological fluids[20–24]. In this study, an automatic on-line HPLC system, based on precolumn derivatization with OPA, has been developed to quantitate free amino acids in CSF and plasma samples from twelve multiple sclerosis (MS) patients. This method gave highly reproducible results with standard deviations between 0.5 and 3% for all amino acids. The results obtained for MS patients were statistically compared with results for healthy controls.

EXPERIMENTAL

Materials

Individual crystalline samples of L-amino acids (standard kit No. 20065), 30% Brij and anhydrous OPA were obtained from Pierce Eurochemie, The Netherlands. Phosphoserine, 3-methylhistidine, citrulline, carnosine, α-, β- and γ-aminobutyric acid, taurine, asparagine, ornithine and 2-mercapthoethanol (2-ME) were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade methanol was obtained from Rathburn (Walkerburn, U.K.). Anhydrous sodium dihydrogenphosphate, disodium hydrogenphosphate, 5-sulphosalicylic acid, boric acid, sodium hydroxide and hydrochloric acid were all of analytical-reagent grade (Merck, Darmstadt, F.R.G.).

Individual standard stock solutions of amino acids in concentrations of 1 μmol/ml were prepared in doubly distilled water with the addition of a few drops of 0.1 *M* hydrochloric acid. A standard mixture containing 25 amino acids was prepared with similar concentrations of amino acids. This mixture was diluted to 0.1, 0.05, 0.03 and 0.02 μmol/ml to establish a relationship between the fluorescence intensity and the concentration of the individual amino acids. Water used for the preparation of buffers and standards was deionized and sterile (Milli-Q water purification system, Millipore).

The chromatographic system consisted of two solvent delivery pumps (6000A

and M45), a multiple sampler (WISP 710B), a data module, a system controller (730B) and a fluorescence detector (420-AC) with the monochromator set at 340 nm and a 450-nm cut-off filter, all supplied by Waters Assoc. (Milford, MA, U.S.A.).

Methods

The separation of the amino acids was performed on a 5-μm LiChrosorb C_{18} column (150 mm × 4 mm I.D.) obtained from Merck. A precolumn (4 mm × 4 mm I.D.), containing similar material, was inserted between the analytical column and the injector. The column was conditioned with 50% aq. methanol and then with solvent A for 1 h before use. For pump A, a mobile phase consisting of tetrahydrofuran–methanol – 0.02 *M* phosphate buffer (pH 6.8) (1:1:98) (eluent A), and for pump B, a mobile phase consisting of phosphate buffer–methanol (35:65) (eluent B) was used. Both these eluents were filtered through 0.45-μm filter-paper and sonicated for 10 min before use. Details of the OPA reagent and buffers were given in ref. 25. The gradient used is shown in Fig. 1.

Biological samples and their treatment

Lumbar CSF and venous blood samples were collected simultaneously between 9 and 11 a.m. from 12 control subjects (7 women and 5 men aged 36–67 years, mean 49.8 ±8.8 years) and 12 patients with clinically defined MS (6 women and 6 men aged 25–65 years, mean 47 ± 11 years). None of the subjects was on any medication. The control subjects had complaints of headaches or dizziness, but no objective findings suggesting an involvement of the nervous system. All MS patients showed oligoclonal bands in their CSF and high intrathecal immunoglobulin (IgG) production (IgG index > 7). The blood samples were centrifuged at 1500 *g* for 15 min and the plasma was separated.

Prior to HPLC analysis, 100 μl of plasma or CSF samples were treated with an equal volume of cold 4% sulphosalicylic acid (SSA) and 50 μl of β-amonibutyric acid (50 nmol/ml) were added as an internal standard. The mixture was centrifuged at 1500 *g* for 15 min and the supernatant was collected and stored at −70°C if not analysed immediately. The derivatization of amino acids with OPA was carried out according to Qureshi *et al.*[21].

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram of the standard OPA-derivatized amino acids under our experimental conditions. Each peak represents 2.5 nmol of amino acid. The fluorescence intensity measured for all amino acids was linearly correlated with the amount injected over a range of 5–100 nmol giving $r^2 = 1$. By running five replicates of the standard (2.5 nmol/ml), each amino acid showed high reproducibility in terms of integrated areas and retention times, giving relative standard deviations (R.S.D.) between 0.5 and 3%. The detection limit for each amino acid was less than 1 pmol. The separation of the amino acids was completed within 40 min.

Figs. 2 and 3 show the chromatograms of plasma and CSF samples respectively from a MS patient.

Table I shows the mean plasma levels of free amino acids in controls and MS patients. Previous studies[26,27] on MS patients found increased plasma levels of Ala

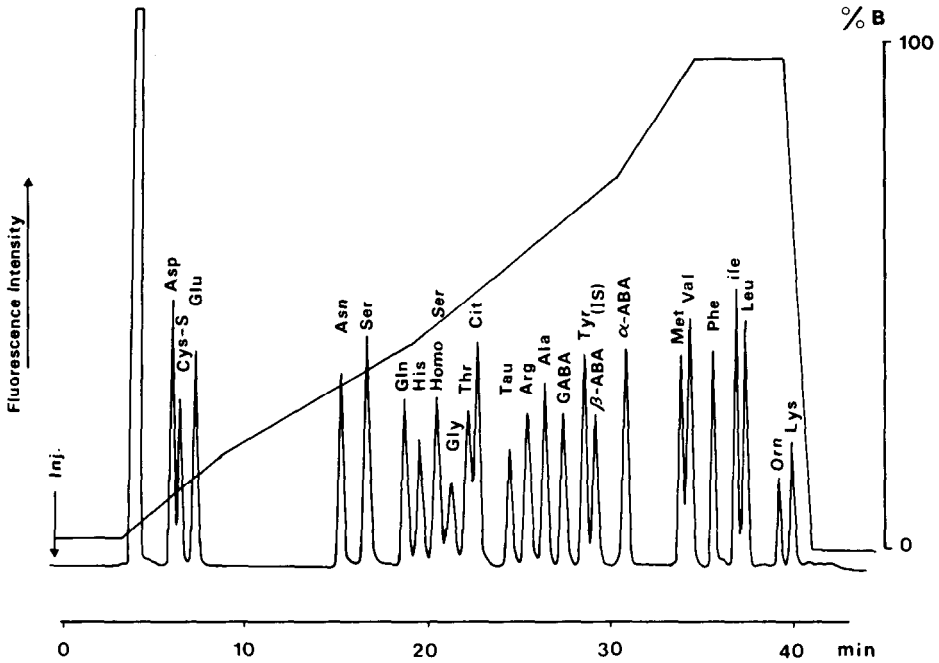

Fig. 1. Separation of standard (2.5 nmol/ml) OPA–2-ME-derivatized amino acids on 5-μm LiChrosorb C_{18} (150 mm × 4 mm I.D.) by the use of tetrahydrofuran–methanol–0.02 *M* phosphate buffer (pH 6.8) (1:1:98) (eluent A), and phosphate buffer–methanol (35:65) (eluent B). The gradient used is shown in %B. A flow-rate of 1 ml/min was maintained throughout except for the first 2 min when it was linearly increased from 0.2 to 1 ml/min. The detection was made at 340 excitation and 450 nm (emission).

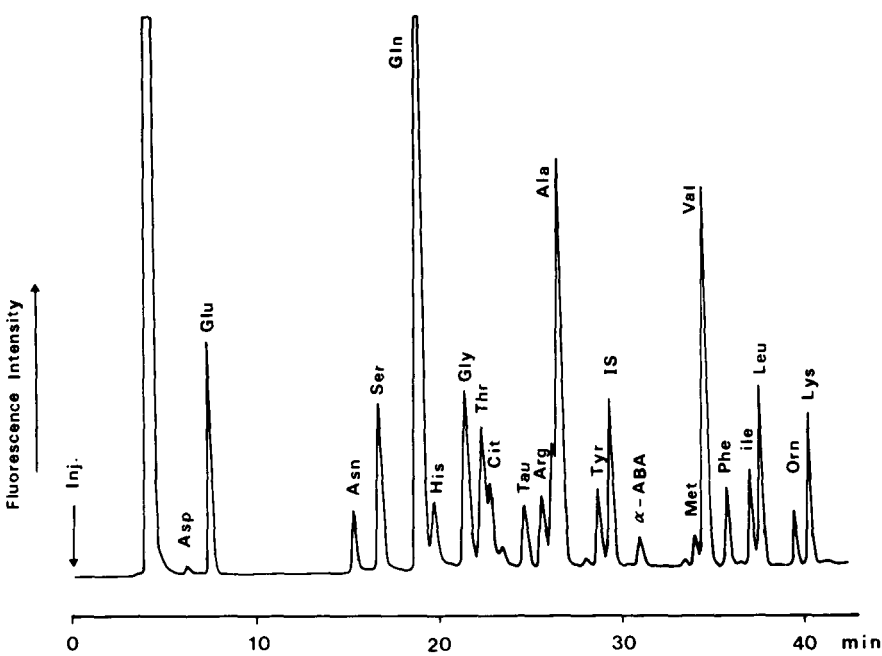

Fig. 2. Separation of OPA-amino acid derivatives in a plasma sample from an MS patient under experimental conditions identical to those in Fig. 1.

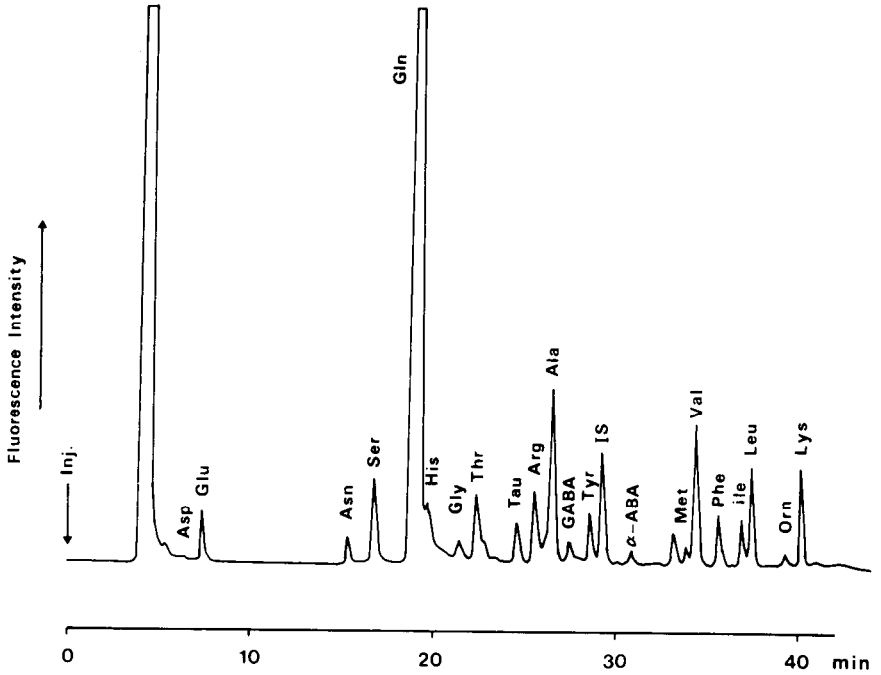

Fig. 3. Separation of OPA-amino acid derivatives in a CSF sample from an MS patient under experimental conditions identical to those in Fig. 1.

TABLE I

LEVELS OF FREE AMINO ACIDS IN PLASMA FROM 12 CONTROLS AND 12 PATIENTS WITH MULTIPLE SCLEROSIS

Amino acid	*Plasma levels, mean ± S.D. (n = 12) (μmol/l)*	
	Controls	*MS patients*
Asp	14.5 ± 4.3	11.9 ± 3.7*
Glu	29.2 ± 3.7	40.3 ± 4.3
Asn	53.3 ± 9.2	57.6 ± 12.7
Ser	102.3 ± 14.7	106.7 ± 16.7
Gln	490.2 ± 49.1	544.5 ± 48.0
His	81.3 ± 16.8	83.2 ± 13.7
Gly	210.2 ± 21.7	251.3 ± 32.1
Thr	112.3 ± 20.3	121.3 ± 23.7
Cit	32.9 ± 8.3	37.3 ± 6.8
Tau	66.1 ± 12.3	68.2 ± 10.2
Arg	110.4 ± 25.7	136.3 ± 32.1
Ala	230.7 ± 32.6	301.3 ± 47.2**
Tyr	55.3 ± 10.4	49.8 ± 4.6
Met	28.1 ± 5.8	23.3 ± 7.3*
Val	206.1 ± 27.6	179.8 ± 19.3*
Phe	67.6 ± 8.3	53.7 ± 11.8
Ile	69.1 ± 7.6	66.8 ± 10.7
Leu	137.2 ± 17.4	125.7 ± 25.2
Orn	63.1 ± 14.7	58.9 ± 5.6
Lys	196.3 ± 23.4	142.1 ± 17.6**

* $p < 0.05$.
** $p < 0.01$.

and Arg, and decreased levels of Leu, Ile, Val, Tyr and Phe, but these results were not confirmed by a third group[28]. However, elevated levels of Glu were shown in MS patients during relapses.

In our study, the levels of Glu, Asn, Gly, Arg and Ala were increased while the levels of Asp, Met, Val, Phe and Lys were decreased in MS patients. Among the amino acids Ala is known to function as a carrier for an amino base outside of brain tissue and this amino base may be overproduced in the course of an infection[29,30]. Under similar conditions, the increase as well as the decrease in certain amino acids may also be due to the selective disruption of the amino acid transport systems since the plasma or serum concentration of an amino acid is generally of the same order of magnitude as the binding constant for its transport into the brain.

Little information is available on CSF levels of amino acids in MS patients and few attempts[6,12,31,32] have been made to study the rôle of amino acids in this disorder. Table II shows the mean levels of amino acids for both groups. The levels of Ser, Gln, Tyr and Orn showed consistent increases, whereas Asp, Glu, GABA and Phe showed a tendency to decrease in MS patients. The levels of other amino acids remained more or less similar to those of the control group. Among the amino acids, the increase in Tyr in CSF in MS patients may be important as Tyr is involved in

TABLE II

LEVELS OF FREE AMINO ACIDS IN CSF FROM 12 CONTROLS AND 12 PATIENTS WITH MS

Amino acid	*CSF levels, mean ± S.D. (n = 12) (μmol/l)*	
	Controls	*MS Patients*
Asp	1.72 ± 0.24	1.11 ± 0.19*
Glu	26.13 ± 4.81	18.96 ± 3.85*
Asn	8.34 ± 1.76	10.14 ± 0.93
Ser	20.65 ± 3.37	23.84 ± 3.46
Gln	450.00 ± 38.18	561.17 ± 84.72**
His	19.31 ± 4.28	20.56 ± 6.34
Gly	11.76 ± 2.44	9.83 ± 1.78*
Thr	44.26 ± 11.63	48.80 ± 9.11
Cit	4.33 ± 1.63	4.18 ± 1.14
Tau	5.85 ± 1.91	4.52 ± 1.24
Arg	15.73 ± 5.21	20.78 ± 6.53*
Ala	26.26 ± 6.31	28.70 ± 4.71
GABA	0.12 ± 0.01	0.07 ± 0.01***
Tyr	14.17 ± 2.63	26.73 ± 4.76**
Met	6.08 ± 1.44	2.98 ± 0.54***
Val	23.63 ± 5.04	21.88 ± 4.45
Phe	15.86 ± 31.16	7.89 ± 2.94**
Ile	9.16 ± 2.34	8.04 ± 1.96
Leu	19.91 ± 3.46	20.94 ± 4.16
Orn	8.57 ± 1.99	9.63 ± 2.37
Lys	42.45 ± 4.30	42.81 ± 6.85

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

many metabolic pathways resulting in neurotransmitters such as catecholamines and their metabolites.

It is known that the amino acid concentrations of CSF are for the most part lower than in plasma or serum and moreover the ratio between CSF and plasma amino acids differs from one amino acid to another. Hence, it is postulated that either an active transport mechanism or a dynamic exchange exists within the blood–brain barrier[33]. Furthermore, it is also argued that various factors do participate in this transport and that the transport mechanisms differ according to various groups of amino acids. Hence, comparing the ratios of amino acids in CSF and plasma might provide a key to understanding either metabolic changes in blood and CNS or abnormalities in the transport mechanism to and from CSF.

Table III shows the ratios of amino acids in CSF and plasma in controls and MS patients. Apart from Val, Tyr, Orn and Lys, most of the ratios are lower or unchanged in MS patients. Comparing Tables I, II and III it is evident that it is not only the blood concentration of an amino acid which is of importance in influencing its concentration in CSF, but an additional local factor in the brain related to the MS condition may play a rôle. It may be the transport function of the blood–CSF barrier in MS patients which leads to the marked difference as compared to the control group.

TABLE III

RATIOS OF FREE AMINO ACIDS BETWEEN CSF AND PLASMA FROM 12 CONTROLS AND 12 PATIENTS WITH MS

Amino acid	*CSF/Plasma ratios, mean ± S.D. (n = 12)*	
	Controls	*MS Patients*
Asp	0.14 ± 0.02	0.10 ± 0.02*
Glu	0.89 ± 0.10	0.47 ± 0.05*
Asn	0.21 ± 0.02	0.18 ± 0.01
Ser	0.35 ± 0.02	0.31 ± 0.04
Gln	1.10 ± 0.11	1.00 ± 0.14
His	0.24 ± 0.04	0.26 ± 0.03
Gly	0.05 ± 0.01	0.04 ± 0.01
Thr	0.37 ± 0.09	0.36 ± 0.06
Cit	0.09 ± 0.01	0.09 ± 0.02
Tau	0.10 ± 0.01	0.07 ± 0.00*
Arg	0.18 ± 0.02	0.15 ± 0.01
Ala	0.12 ± 0.02	0.08 ± 0.01**
Tyr	0.23 ± 0.04	0.41 ± 0.08***
Met	0.17 ± 0.02	0.11 ± 0.01**
Val	0.10 ± 0.02	0.15 ± 0.01*
Phe	0.23 ± 0.04	0.16 ± 0.03
Ile	0.09 ± 0.01	0.10 ± 0.01
Leu	0.28 ± 0.04	0.21 ± 0.05
Orn	0.16 ± 0 03	0.18 ± 0.04
Lys	0.19 ± 0.03	0.24 ± 0.05*

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

The use of an automatic HPLC method giving rapid quantitation of amino acids has been effective in our study of the complex phenomena involved in the transport mechanisms of amino acids between blood and brain. In addition the method is programmed to make OPA react with amino acids automatically, thereby controlling the reaction kinetics and avoiding variations in results due to the instability encountered with the OPA derivatives. Special care was taken in the treatment of the biological samples including the use of milder conditions (4% SSA) resulting in complete deproteinization and giving highly reproducible results with R.S.D. $< 3\%$ for all amino acids.

REFERENCES

1 J. W. Wood, in A. Lajtha (Editor), *Handbook for Neurochemistry*, Vol. 3, Plenum, New York, 1982, pp. 415–487.
2 A. T. B. Moir, G. W. Asheroft and T. B. B. Crawford, *Brain*, 93 (1970) 357.
3 F. E. Bloom, *Neurosci. Res. Program Bull.*, 10 (1972) 127.
4 J. Piek, A. Boldt-Göth, D. Wieczorek and W. J. Bock, *Neurochirurgia*, 78 (1985) 33.
5 D. I. Heiblim, H. E. Evans, L. Glass and M. M. Agbayani, *Arch. Neurol.*, 35 (1978) 765.
6 K. Iijima, S. Takase, K. Tsumyraya, M. Enda and K. Itahara, *Tohoku J. Exp. Med.*, 126 (1978) 133.
7 A. W. Teelkenand and J. P. Lakye, in J. P. Lake (Editor), *Parkinson Disease*, Excerpta Medica, Amsterdam, 1977, pp. 85–112.
8 R. N. Corston, E. H. F. McGale, C. Stonier, E. C. Hutchinson and G. M. Aber, *J. Neurol., Neurosurg. Psychiatry*, 44 (1981) 86.
9 H. Briem, E. H. Hultman, M. E. Kalin and P. R. Lundberg, *J. Neurol., Neurosurg. Psychiatry*, 42 (1979) 881.
10 E. H. F. McGale, I. F. Pye, C. Stonier, E. Hutchinson and G. M. Aber, *J. Neurochem.*, 24 (1975) 587.
11 T. Kruse, H. Reiber and V. Neuhoff, *J. Neurol. Sci.*, 70 (1985) 129.
12 K. J. Scholt and D. Meier, *Acta Neurol. Scand.*, 75 (1978) 304.
13 T. N. Ferraro and T. A. Hare, *Brain Res.*, 338 (1985) 53.
14 P. J. Goodnick, H. E. Evans, D. L. Dunnar and R. R. Fiere, *Biol. Psychiat.*, 15 (1980) 557.
15 J. C. Dickinson and P. B. Hamilton, *J. Neurochem.*, 13 (1966) 1179.
16 T. L. Perry, S. Hansen and J. Kennedy, *J. Neurochem.*, 24 (1975) 587.
17 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
18 R. J. Benson and P. E. Hare, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 619.
19 A. P. Williams, *J. Chromatogr.*, 373 (1986) 175.
20 J. D. Cooper and D. C. Turnell, *Clin. Chem.*, 28 (1982) 527.
21 G. A. Qureshi, L. Fohlin and J. Bergström, *J. Chromatogr.*, 297 (1984) 91.
22 H. Godel, T. Graser, P. Földi, P. Pfaender and P. Fürst, *J. Chromatogr.*, 297 (1984) 49.
23 R. J. Smith and K. A. Panico, *J. Liq. Chromatogr.*, 8 (1985) 1783.
24 H. R. Zielke, *J. Chromatogr.*, 347 (1985) 320.
25 G. A. Qureshi and P. Södersten, *J. Chromatogr.*, 400 (1987) 247.
26 F. Monaco, S. Fumero, A. Mondino and R. Mutani, *J. Neurol., Neurosurg. Psychiat.*, 42 (1979) 640.
27 F. C. Westall, A. Hawkin, G. M. Ellison and L. W. Myers, *J. Neurol. Sci.*, 47 (1980) 353.
28 A. Mori, *Amino Acids and Brain*, Chygai-Igakusha, Tokyo, 1976, p. 259.
29 W. H. Oldendorf and J. Szabo, *Am. J. Physiol.*, 230 (1976) 94.
30 W. M. Pardridge and W. H. Oldendorf, *Biochem. Biophys. Acta*, 401 (1975) 128.
31 J. J. Martin and W. Schole, *J. Neurol. Sci.*, 15 (1972) 49.
32 D. E. Lumsden and D. McAlpine, in D. E. Lumsden and E. D. Achesson (Editors), *Amino Acids in Multiple Sclerosis*, Livingstone, Edinburgh, 1972, pp. 433–441.
33 F. L. Hymoller, O.J. Mahler and M. M. Parker, *Int. J. Neuropsych.*, 2 (1966) 293.