ELSEVIER

Journal of Chromatography B, 657 (1994) 185–191

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Simple high-performance liquid chromatographic analysis of free primary amino acid concentrations in rat plasma and cisternal cerebrospinal fluid

D.J. Begley[a,*], A. Reichel[b], A. Ermisch[b]

[a] *Biomedical Sciences Division, King's College London, Strand, London WC2R 2LS, UK*
[b] *Section of Biosciences, University of Leipzig, Talstrasse 33, 04103 Leipzig, Germany*

(First received December 17th, 1993; revised manuscript received March 18th, 1994)

Abstract

The quantitation of 16 acidic, basic, small and large neutral amino acids was performed using 10-μl sample aliquots of cisternal cerebrospinal fluid (CSF) and blood plasma of rats. The analytical technique is based upon a two-buffer HPLC system with fluorimetric detection of pre-column derivatized primary amino acids with *o*-phthaldialdehyde (OPA). A modification of a well established method, the power of the present technique comes from an improved resolution and sensitivity by installing a column heater adjusted to 43°C and strictly reducing any contamination by background amino acids. The analysis is simplified by separating the amino acid derivatives with a linear buffer gradient and less time consuming by the use of a short analytical column with a higher flow-rate. Analytical precision, linearity of response and reproducibility were highly acceptable at both CSF and plasma concentrations of amino acids without changing any of the separation or detection parameters.

1. Introduction

In the brain, amino acids are involved in many processes, for example as (i) substrates of several metabolic pathways, (ii) transmitters and/or modulators of information processing [1–4], (iii) as osmolytes [5–7] and as factors in brain development [8,9]. The primary event in supplying the brain with amino acids is transport from blood to the brain extracellular fluid (ECF), the immediate fluid environment of the cells within the brain, across the capillary endothelium which forms the blood–brain barrier.

To obtain information about the amount of amino acids entering the brain and, thus, available for brain utilisation, their concentrations within the brain ECF has to be known. The direct detection of amino acids within the ECF is difficult to achieve, however, since small molecules such as amino acids have relatively unrestricted movement from CSF to ECF and *vice versa* [10–13] the assumption has been made that the composition of the ECF can be roughly estimated by analysis of CSF [14,15].

Quantitation of the amino acid levels within the CSF is difficult to carry out since (i) the volume obtainable from small laboratory animals such as rats is small, (ii) the concentration of the individual solutes is very low, and (iii) other

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00171-Z

substances may interfere with the analysis. Therefore, only limited data have been published concerning the amino acid concentrations in rat CSF (see Davson *et al.* [16] for review) with very little information on the essential amino acids.

We present a simple two-buffer reversed-phase HPLC system to analyse amino acid concentrations within CSF and, for comparison, blood plasma of the rat. The technique is based upon the fluorimetric detection of pre-column derivatized primary amino acids with *o*-phthaldialdehyde (OPA) and is a modification of a method by Jones and Gilligan [17] used for the quantitation of 16 amino acids in both fluid compartments of the rat covering 6 large neutral, 3 small neutral, 3 basic and 3 acidic amino acids including taurine.

2. Experimental

2.1. Collection of CSF and plasma

Adult Wistar rats of both sexes weighing 250–300 g were anaesthetised by i.p. injection of Sagatal (60 mg/kg, BDH Chemicals, Poole, Dorset, UK) and placed in a stereotaxic frame. The implantation of the cisternal cannula has been described in detail by Sarna *et al.* [18]. Briefly, in the exposed dorsal part of the skull a burr hole was drilled 2–3 mm lateral and approximately 5 mm caudal to lambda. Initially, a screw was drilled in the hole to give a proper connection of the dental acrylic with the skull, however, the hole alone proved to stabilise the implanted catheter.

Two further holes were drilled on the midline and joined to form a 3-mm slot right at the end of the external occipital crest in which the catheter was inserted. Catheters were formed from polyethylene tubing (0.28 mm I.D. and 0.61 mm O.D., Portex, Hythe, Kent, UK). During implantation each catheter was made rigid by the insertion of an enamelled copper wire into the lumen. A button, 6.5 mm from the catheters end, formed by gently heating the plastic, indicated the correct position within the cisterna magna when touching the brain surface after insertion. The catheter was secured in place using dental acrylic (DeTrey, Weybridge, UK). Gentle negative pressure on the catheter helped the CSF to flow. A 30- to 90-μl volume of CSF was slowly withdrawn and immediately centrifuged at 11 600 *g* for 5 min. CSF samples contaminated with visible blood were discarded. The CSF was deproteinized by adding of an equivalent volume of HPLC grade methanol (Fisons, Loughborough, UK). After centrifuging samples at 11 600 *g* for 5 min were stored frozen at −20°C until analysis the following day.

The animals were killed by withdrawing blood from the left ventricle of the heart. Blood samples were centrifuged and the plasma deproteinized by adding of a 4-fold excess of methanol. After centrifuging at 11 600 *g* for 5 min the deproteinized supernatant was stored frozen at −20°C until analysis.

2.2. Analysis of free amino acids

The present amino acid analysis was performed essentially as previously described by Jones and Gilligan [17] using a two-buffer HPLC system and fluorimetric detection by pre-column derivatization of primary amino acids with *o*-phthaldialdehyde/β-mercaptoethanol. A few parameters, detailed below, have been modified to improve resolution.

The HPLC employed (A) 0.1 *M* sodium acetate buffer containing 5% tetrahydrofuran, adjusted to pH 7.2 with glacial acetic acid, and (B) methanol containing 5% tetrahydrofuran (all HPLC chemicals were obtained from Fisons). Buffer A was ultrasonically degassed, solvent B was filtered and degassed through a 0.2-μm nitrocellulose membrane under vacuum. Separation was carried out on a 50 × 4.6 mm I.D. column of Spherisorb 5 ODS preceded by a cartridge guard column 10 × 3.0 mm I.D. 5 ODS at a flow-rate of 1.5 ml/min and a starting pressure of approximately 7 MPa at 43°C.

Free primary amino acids in CSF or plasma were detected by derivatizing to their fluorescent

isoindoles. Aliquots (10 μl) of deproteinized CSF or plasma samples and 5 μl of *o*-phthaldialdehyde (Fluoroaldehyde, Pierce Chemical) were allowed to react for 1 min at room temperature. The reaction was stopped by adding 100 μl of buffer A.

Aliquots (20 μl) of the reaction mixture were injected onto the column and separated with a linear gradient from A:B (10:90, v/v) to 100% B within 13 min. The gradient reset was delayed 4 min to extend the separation time to 17 min in order to elute all fluorescent products from the column. The column was then allowed to recycle over at least 1 min to the starting conditions. The apparatus consisted of an LDC/Milton Roy Constametric III and a Constametric I pump controlled by an LDC/Milton Roy gradient master controller. Fluorescence was detected with an LDC/Milton Roy fluoroMonitor III. Peak heights and areas were determined with an LDC Milton Roy C1-10B integrator.

Under these conditions, the following amino acid derivatives were sequentially eluted and detected: aspartic acid (Asp), glutamic acid (Glu), taurine (Tau), serine (Ser), glutamine (Glu), alanine (Ala), arginine (Arg), tyrosine (Tyr), methionine (Met), valine (Val), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), ornithine (Orn), and lysine (Lys). As a result of co-elution with other unidentified peaks: glycine (Gly), threonine (Thr), histidine (His), and asparagine (Asn) could not be quantified in plasma and CSF samples.

An aqueous standard diluted from the amino acid Standard "H" (2.5 m*M*, Pierce Chemical) consisted of the following L-amino acids at 25 μ*M*: Asp, Glu, Ser, His, Gly, Thr, Ala, Arg, Tyr, Met, Val, Phe, Ile, Leu and Lys. In addition, Tau, Gln, Asn, Trp and Orn (Sigma) standards were made also at the same concentration in a separate mixture.

Analytical precision was tested by using successive serial dilutions of the standards over the concentration range of the amino acids contained in the deproteinized CSF and plasma samples. Detection linearity was determined for each amino acid by linear regression analysis of their peak area and the dilution factor.

3. Results

3.1. Verification of the amino acid analysis

Mixed standards of amino acid solutions, CSF and plasma were used to optimise the conditions of the HPLC separation. Both, high sensitivity and resolution power allowed the simultaneous detection of 16 amino acids in rat plasma and CSF. Fig. 1 shows representative chromatograms of an aqueous standard solution, plasma, and CSF sample.

CSF and plasma amino acids were identified on the basis of co-elution with and by spiking samples with single standard amino acids. Gly, His, Thr and Asn could be consistently separated in the mixed amino acid standard but not in CSF and plasma as the result of co-elution with other unidentified peaks. A few peaks remain unidentified.

To test for linearity of the detector output over the concentration range of interest, serial dilutions of the mixed amino acid standard were analysed over a 10-fold range. Slopes and correlation coefficients of the linear regressions of peak areas *vs.* dilution factor were computed for each amino acid. For all amino acids correlation coefficients were $r \geq 0.986$.

3.2. CSF and plasma amino acid concentrations

Amino acid levels in rat cisternal CSF and plasma are listed in Table 1. Mean amino acid concentrations varied between 1.59 μ*M* (Orn) and 549.47 μ*M* (Gln) in CSF and 10.21 μ*M* (Asp) and 563.27 μ*M* (Gln) in plasma, respectively. Random variability was evident, to a greater extent in plasma than in CSF, which was not attributable to analytical variability.

CSF/plasma ratios varied between 0.074 (Ile) and 0.973 (Gln) with means of 0.10 (large neutral amino acids), 0.18 (basic amino acids),

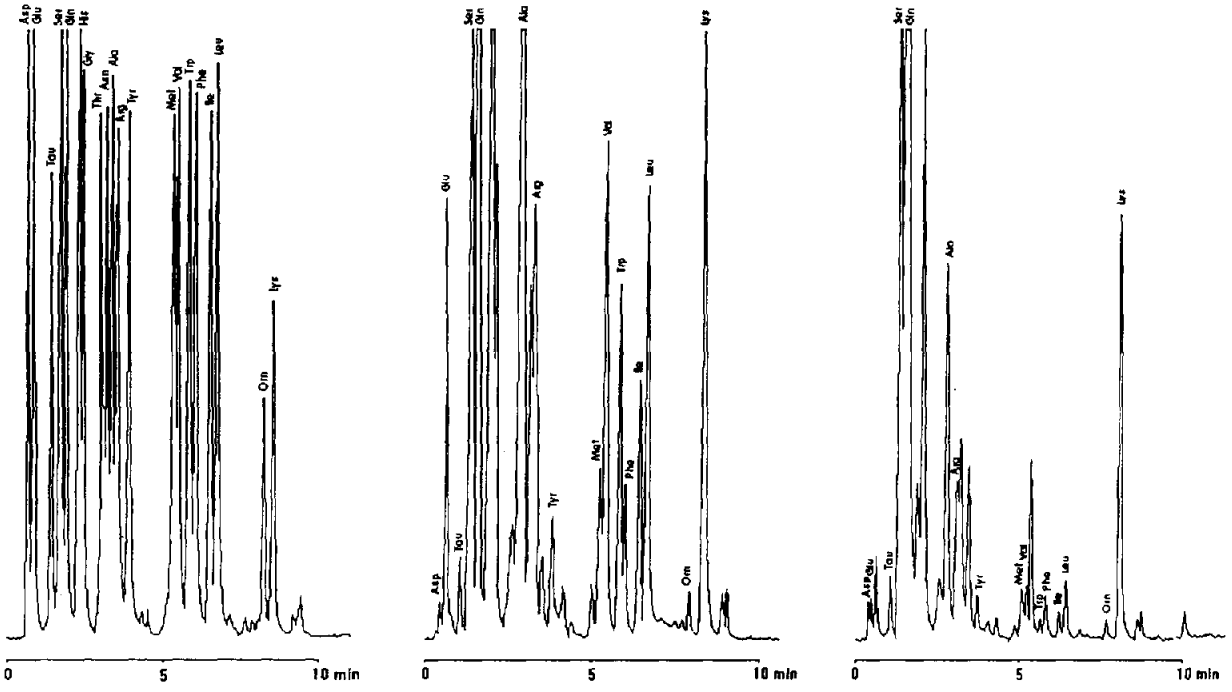

Fig. 1. Representative HPLC elution profiles of an aqueous standard solution (left), a CSF (middle) and a plasma sample (right). Each peak of the standard solutions represents 50 pmol. In the plasma and CSF samples Gly, Thr and Asn could not be resolved due to co-elution.

0.36 (acidic amino acids) and 0.55 for small neutral amino acids, respectively.

4. Discussion

The present method allows the simultaneous detection of more than a dozen amino acids in 10-μl sample aliquots in spite of a 10^3 range of amino acid levels and the presence of a variety of different background compounds in biological fluids, such as CSF and blood plasma. The method fulfils the requirements essential for a reliable chromatographic procedure such as a high (1) specificity, (2) resolution, (3) sensitivity, (4) reproducibility, (5) accuracy, and a (6) quick and simple detection to allow routine analysis.

The specificity is dependent upon the derivatization reaction. The derivatization of free amino acids with *o*-phthaldialdehyde is a quantitative reaction with a high specificity of the formed isoindole derivatives. Since only amino acids with free primary amino groups are reactive with OPA, secondary amino acids such as proline are not detectable by this procedure [19].

In CSF and plasma samples and in aqueous solutions 16 amino acids were accurately resolved without altering the separation parameters. Thr, Gly and His were only reliably resolved in the aqueous standard, whereas in the CSF and plasma samples these amino acids tended to co-elute. Asn and GABA (γ-aminobutyric acid) co-eluted with other unidentified peaks. Neither slower elution (decreased flow-rate or a less steep gradient) or changes of the buffer mixture (various pH values [6.5–7.4] and ionic strength [0.05–0.2 *M* sodium acetate] of buffer A or tetrahydrofuran content [2.5–7.5%] of both A and B) could solve the problem. A column heater adjusted to exactly 43°C was found to optimise the number of resolved peaks [Met and Val co-eluted at a somewhat lower temperature (*ca.* 40°C), whereas Asp and Glu

Table 1
Amino acid concentrations (μM) in rat CSF and plasma and corresponding CSF/plasma ratios

Amino acid	CSF	Plasma	CSF/plasma ratio
Phe	3.57 ± 1.70	37.24 ± 12.69	0.096 ± 0.051
Leu	8.75 ± 4.44	104.59 ± 28.06	0.084 ± 0.041
Tyr	4.24 ± 2.17	26.94 ± 10.41	0.148 ± 0.098
Trp	2.20 ± 1.66	92.94 ± 27.00	0.026 ± 0.018
Met	7.60 ± 6.32	41.37 ± 12.19	0.183 ± 0.109
Ile	3.45 ± 1.48	65.34 ± 22.16	0.074 ± 0.088
Val	10.81 ± 4.16	111.77 ± 35.19	0.096 ± 0.059
Ser	75.39 ± 32.60	144.61 ± 77.51	0.496 ± 0.135
Ala	65.54 ± 22.70	350.91 ± 91.92	0.189 ± 0.052
Gln	549.47 ± 182.71	563.27 ± 173.30	0.973 ± 0.313
Asp	4.24 ± 2.12	10.21 ± 5.40	0.411 ± 0.111
Glu	16.88 ± 9.98	49.67 ± 13.19	0.304 ± 0.089
Lys	74.52 ± 35.13	430.24 ± 136.71	0.193 ± 0.088
Arg	38.76 ± 16.99	185.82 ± 73.32	0.247 ± 0.122
Orn	1.59 ± 0.75	15.58 ± 6.49	0.098 ± 0.048
Tau	3.91 ± 1.59	18.82 ± 9.15	0.205 ± 0.095

Values are given as $x \pm$ S.D. ($n = 9$–31).

gave a common peak at *ca.* 46°C]. Some of the co-eluting peaks, *e.g.* Gly and GABA, could be separated by manipulating the conditions but only at a considerable sacrifice of the total number of peaks resolved.

The sensitivity of the method was high enough to estimate amino acid levels injected onto the column over at least 3 orders of magnitude, with a lower limit of 10^{-8} mol/l or 10^{-13} mol. The breakdown of unstable derivatives, *e.g.* of lysine [20,21], was minimised by the very short duration of the HPLC separation (only 13 min) permitting amino acid quantitations at a high sensitivity. The *o*-phthaldialdehyde/β-mercaptoethanol derivatizing reagent obtained from Pierce (Fluoroaldehyde) proved to give a higher sensitivity than a laboratory-made solution [19]. Displacing mercaptoethanol by ethanethiol as suggested by Hill *et al.* [22] did not further improve the sensitivity. The commercial reagent also contains the surfactant Brij 35, which increases the fluorescent response of the rapidly degenerating isoindole derivatives of Lys and Gly [23]. Although there is a rapid decay in the reagent reactivity due to evaporation of the thiol, the addition of further mercaptoethanol as proposed by Jones *et al.* [19] did not restore the original reagent activity. Thus, we divided the reagent by aliquoting in numerous glass vials (2.5 ml) and kept them sealed in a refrigerator. Using a freshly opened glass tube every third day was found to give a constant day-to-day reproducibility.

Because of the large amount of proteins and peptides in plasma, the samples were deproteinized. In contrast to Roettger and Goldfinger [15], CSF samples were also deproteinized in this study. This helped to guarantee a reproducible separation of some problem peaks (*e.g.* Met-Val) by avoiding excessive flow changes within the analytical column as the result of possible accumulation of macromolecules within the column. Using acid for deptoteinization Grossman *et al.* [24] found artificially increased amino acid concentrations in CSF by the addition of only one-third by volume of 20% aqueous sulfosalicylic acid, an often used deproteinizing agent, presumably due to acid hydrolysis of small

peptides (*e.g.* homocarnosine). Therefore, in this study methanol instead of acid was used for the deproteinization to avoid peptide degradation and thus contamination with additional amino acids by acidic hydrolysis of peptides.

The reproducibility of the method was assessed as analytic precision. Analytic precision, *i.e.* peak area variability [(S.D./mean)·100] for each amino acid ranged from 1.5% to 9% depending upon the instability of the fluorescent adduct [17] but this may be further improved by applying an autosampler or an internal standard which minimises pipetting errors that occur during the derivatization procedure. Enhancing the accuracy of a method may decrease the minimum concentration changes detectable above/below endogenous amino acid levels which was estimated to be at *ca.* 15% under similar conditions by Roettger and Goldfinger [15].

The accuracy, *i.e.* the linearity of response, was examined over a 10-fold range, which was within the concentration range of the deproteinized CSF and plasma samples. For each amino acid, correlation coefficients of the linear regressions of peak area *vs.* dilution factor were $r \geq 0.986$, showing very acceptable detection characteristics. To guarantee the linearity of response in the lower picomole range and to improve sensitivity, a reduction of the background presence of amino acids is essential. Therefore, all collecting, storage and reaction vessels and all pipette tips were methanol washed prior to usage. Where possible, plastic tubes were replaced by glass tubes, and glass tubes were used exclusively for CSF samples. Furthermore, all chemicals must be of HPLC grade and plastic surgical gloves were worn to prevent amino acid contaminations from hands, since single "fingerprints" contain amino acids in the nanomolar range [25].

The present method is suitable for routine analysis because of its simplicity and short analysis time. The length of the column is reported not to effect the resolution of amino acid derivatives [17]. Using a short analytical column (50 × 4.6 mm I.D. instead of 250 × 4.6 mm I.D.) and applying a flow-rate of 1.5 ml/min reduced the analysis time dramatically (~13 min). A linear gradient allowed a simplification of the analysis improved the reproducibility and helped to avoid delays due to long post-run periods required to bring the column to the starting equilibrium. Most importantly the separation parameters did not need to be changed between measurements of CSF, plasma or aqueous standard samples providing a similar precision of analysis for all samples.

In conclusion, we present a simple HPLC analysis of more than a dozen amino acids with a resolution and sensitivity high enough to measure the very low levels of the acidic amino acids in CSF and a detection linearity up to the range of plasma amino acids, thus employing the same HPLC conditions for both biological fluids.

Acknowledgement

This work was supported by a research grant from the Biomedical Sciences Division, King's College. Andreas Reichel was holding a fellowship grant by the German Academic Research Council (Grant No. 332 4 00 075). David Begley was holding a British-German Academic Research Collaboration Grant for travel from the British Council.

References

[1] W. Zieglgänsberger, *Prog. Brain Res.*, 55 (1982) 297.
[2] H. Kettenmann and M. Schachner, *J. Neurosci.*, 5 (1985) 3295.
[3] J.W. Johnson and P. Aschner, *Nature*, 325 (1987) 529.
[4] N.W. Kleckner and R. Dingledine, *Science*, 241 (1988) 835.
[5] H.P. Morales and A. Schousboe, *J. Neurosci. Res.*, 20 (1988) 505.
[6] J.E. Olson and M.D. Goldfinger, *J. Neurosci. Res.*, 27 (1990) 241.
[7] R.O. Law, *Comp. Biochem. Physiol.*, 99A (1991) 263.
[8] S.A. Lipton and S.B. Kater, *Trends Neurosci.*, 12 (1989) 265.
[9] J.W. McDonald and M.V. Johnston, *Brain Res. Rev.*, 15 (1990) 41.
[10] J.D. Fenstermacher and C.S. Patlak, in H.F. Cserr, J.D. Fenstermacher and V. Fencl (Editors), *Fluid Environment of the Brain*, Academic Press, New York, 1975, p. 201.

[11] T.H. Milhorat and N.K. Hammok, in J.H. Wood (Editor), *Neurobiology of Cerebrospinal Fluid*, Plenum Press, New York, 1983, p. 1.
[12] M.L. Rennels, T.F. Gregory, O.R. Blaumanis, K. Fujimoto and P.A. Grady, *Brain Res.*, 326 (1985) 47.
[13] B. Hutto, L. Brady and M. Herkenham, *Neurosci. Abstr.*, 12 (1987) 710.
[14] M.W.B. Bradbury, *The Concept of a Blood-Brain Barrier*, Wiley, Chichester, UK, 1979, p. 32.
[15] V.R. Roettger and M.D. Goldfinger, *J. Neurosci. Methods*, 39 (1991) 263.
[16] H. Davson, K. Welch and M.B. Segal, *The Physiology and Pathophysiology of the Cerebrospinal Fluid*, Churchill Livingstone, New York, 1987, p. 271.
[17] B.N. Jones and J.P. Gilligan, in J.C. MacDonald (Editor), *HPLC Instrumentation and Applications*, International Scientific Communications, Fairfield, CT, 1986, p. 434.
[18] G.S. Sarna, P.H. Hutson, M.D. Tricklebank and G. Curzon, *J. Neurochem.*, 40 (1983) 383.
[19] B.N. Jones, S. Pääbo and S. Stein, *J. Liq. Chromatorgr.*, 4 (1981) 565.
[20] R.F. Chen, C. Scott and E. Trepman, *Biochim. Biophys. Acta*, 576 (1979) 440.
[21] L.A. Allison, G.S. Mayer and R.E. Shoup, *Anal. Chem.*, 56 (1984) 1089.
[22] D.W. Hill, F.H. Walters, T.D. Wilson and J.D. Stuart, *Anal. Chem.*, 51 (1979) 1338.
[23] W.S. Gardner and W.M. Miller, *Anal. Biochem.*, 101 (1980) 61.
[24] M.H. Grossman, T.A. Hare, N.V. Bala Manyam, B.S. Glaeser and J.H. Wood, *Brain Res.*, 182 (1980) 99.
[25] P.B. Hamilton, *Nature*, 205 (1965) 284.