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Electrochemical detection of human brain transmitter amino acids by high-performance liquid chromatography of stable o-phthalaldehyde-sulphite derivatives

Short Communication

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Summary. A simple, sensitive, reliable and reproducible isocratic HPLC technique for the measurement of OPA/sulphite derivatives of human brain amino acid neurotransmitters is described. This employs a sample preparation that is also compatible with the concurrent determination of monoamines and their metabolites on a separate HPLC system. The method has been applied to the determination of GABA and glutamate in brain tissue taken post-mortem from patients with Huntington's disease and control subjects.

Keywords: High performance liquid chromatography, electrochemical detection, human brain, amino acid neurotransmitters.

Introduction

Several amino acids including glutamate, aspartate and γ-aminobutyric acid (GABA) are generally considered to act as neurotransmitters in the brain. Changes in these compounds in brain tissue have been demonstrated in some neurological and psychiatric disorders (e.g. Huntington's disease, Alzheimer's disease and Down's syndrome) and provide measures of the selectivity and severity of the neurodegenerative processes in these disorders (Perry et al., 1973; Rossor and Iversen, 1986; Reynolds and Warner, 1988; Reynolds and Pearson, 1987). Analysis of these neurotransmitters previously relied on non-chromatographic measurement of individual compounds (e.g. Kravitz and Potter, 1965) or application of chromatography to measure a wide range of amino acids. For a long period this latter approach employed ion-exchange chromatography

using ninhydrin post-column reaction with colorimetric detection (Spackman et al., 1958). More recently, high-performance liquid chromatography (HPLC) has been used with various detection (fluorimetric, spectrometric, electrochemical) and derivatization (e.g. dansyl chloride, ninhydrin, phenylthiocarbamyl, fluorescamine, dinitrophenyl, trinitrophenyl) techniques (DeJong et al., 1982; Rubenstein et al., 1979; Bidlingmeyer et al., 1984; Stein et al., 1973; Freeman et al., 1980; Caudill et al., 1982), frequently employing gradient column elution. These methods are often time-consuming, impractical and expensive. The most recent HPLC methods involve the reaction of o-phthalaldehyde (OPA) and an alkyl thiol to form derivatives detectable by fluorimetric methods (Lindroth and Mopper, 1979). Many of these are electroactive, making electrochemical detection a useful alternative (Joseph and Davies, 1983). Pre-column derivatization was found to be preferable to post-column reaction due to increased resolution and sensitivity (Allison et al., 1984), although the instability of these derivatives necessitates rapid preparation and reproducible timing to obtain accurate results. The most commonly used thiol was β-mercaptoethanol (Joseph and Davies, 1983), although others were tried in an effort to increase stability (e.g. ethanethiol, 3-mercaptopropionic acid, 2-mercaptopropionic acid (Allison et al., 1984). Latterly, *tert*-butylthiol was used, as this produced more stable derivatives in high yield (Allison et al., 1984). We have employed this successfully for the analysis of transmitter amino acids in human brain (Reynolds and Warner, 1988; Reynolds and Pearson, 1987), however the improved stability obtained was offset by the high volatility and noxious smell of *tert*-butylthiol which led to the method having to be abandoned in this institution. Recently the use inorganic sulphite instead of a thiol has been reported to produce stable OPA derivatives of primary amines suitable for electrochemical detection (Jacobs, 1987). We have modified this isocratic method to apply it to the determination of several neurotransmitter amino acids in human brain tissue taken at post-mortem, using sample homogenates prepared for concurrent monoamine transmitter assay on a separate HPLC system.

Material and methods

Isocratic separation, at 40 °C, of transmitter amino acid derivatives was obtained using a reverse-phase HPLC-electrochemical detection system. This comprised of a 25 cm × 4.6 mm Spherisorb ODS2 5 μm column with a BAS detector containing a glassy carbon electrode set at +0.85 V (vs. Ag/AgCl) for quantification. The mobile phase (flow rate 1 ml/min) consisted of 0.1 M phosphate buffer at pH 5.6 and containing 0.05 mM EDTA and 5% methanol. All chemicals were reagent grade, obtained from Sigma and Fisons. OPA (54 mg) was added to 1 ml ethanol, 1 ml sodium sulphite (1 M) and 18 ml disodium tetraborate (0.1 M, pH 9.5). This reagent was stable at room temperature for more than several weeks. Refrigerated stock solutions (2 mg/ml) of amino acids were made by dissolving in water with 100 μl 5 M sodium hydroxide added where necessary. Daily dilutions to 100 μg/ml were made using 0.1 M perchloric acid. β-aminobutyric acid (BABA) (2 mg/ml) was used as an internal standard, as this was found to not coelute with any compound present in tissue samples.

Brain tissue (50–100 mg) was homogenized in a semi-micro glass/glass homogeniser in

0.5 ml 0.1 M perchloric acid containing 100 μM ascorbic acid. The homogenate was centrifuged for 3 minutes at 12 000 g. An aliquot of supernatant was derivatized and a further aliquot was used for the concurrent measurement of monoamines and their metabolites, on a different system. If required, dilution with four volumes borate buffer (0.1 M, pH 9.5) permitted storage frozen at −20 °C for later analysis. Brain tissue was obtained post-mortem from patients with Huntington's disease and from matched controls. Preparation and storage of brain tissue was as described previously (Spokes, 1979). BABA (20 μl) was added to 800 μl standard or sample supernatant and 100 μl of this mixture was reacted with 160 μl reagent for 20 minutes at room temperature. A 40 μl aliquot of the derivative was diluted with 20-fold excess of water, and 50 μl was then injected onto the HPLC column. Amino acid concentrations in samples were determined by the measurement of peak heights, calculation of a ratio of peak height to internal standard (BABA) peak height and comparison with the equivalent ratio from a standard solution.

Results

The chromatogram in Fig. 1 A shows a typical sample and in Fig. 1 B, a typical standard. Single peaks (retention times in mins) were demonstrated for aspartate (1.4), glutamate (2.2), glycine (3.8), GABA (21.0) and BABA (26.7). The peaks in the sample were assigned after comparison of retention times with those from standard solutions and by confirmation of their identical coelution by observing the expected summation of peak heights of standards added to samples. Percentage recoveries from added standards were aspartate: 99 ± 6, glutamate: 98 ± 7, GABA: 100 ± 5 (n = 5, mean ± SD). Reproducibility was tested using eight samples from two brain tissue homogenates which yielded coefficients of variation of 3% for aspartate, 6% for glutamate, 4% for glycine and 7% for GABA. Linear standard curves for concentration (0–150 μg/ml) versus response were demonstrated for all amino acid derivatives. The derivatives were shown to be stable for more than five hours when kept on ice. Optimum detector response in terms of sensitivity versus selectivity was obtained at an electrode potential of 0.85 V. Borate-diluted samples were found to be stable on storage at −20 °C; this was demonstrated in samples stored frozen for several weeks that yielded for glutamate: 98 ± 8%, aspartate: 100 ± 11%; GABA: 97 ± 10% (mean ± SD, n = 6) of the values obtained from freshly prepared homogenates of the same tissue. These results also demonstrate the low variability between different preparations from the same sample of dissected tissue. Values of frozen samples kept for several months were shown to be within their expected range. The optimal time of derivatization was tested using 4 minute increments; 20 minutes derivatization was shown to produce a maximal response. Brain amino acids were typically in the range 100–1000 μg/g tissue, although much greater sensitivity could be achieved by eliminating the dilution stages of the method. Here the limit of detection (signal to noise ratio of 3) for GABA was approximately 0.5 pmol on the column, although this sensitivity was not possible for aspartate and glutamate due to their proximity to the solvent front.

Other amino acids also giving single peaks (not shown) were serine, asparagine, threonine, glutamine and arginine. Three amino acids produced a coeluting peak: histidine, taurine and alanine. Using these conditions, no peaks

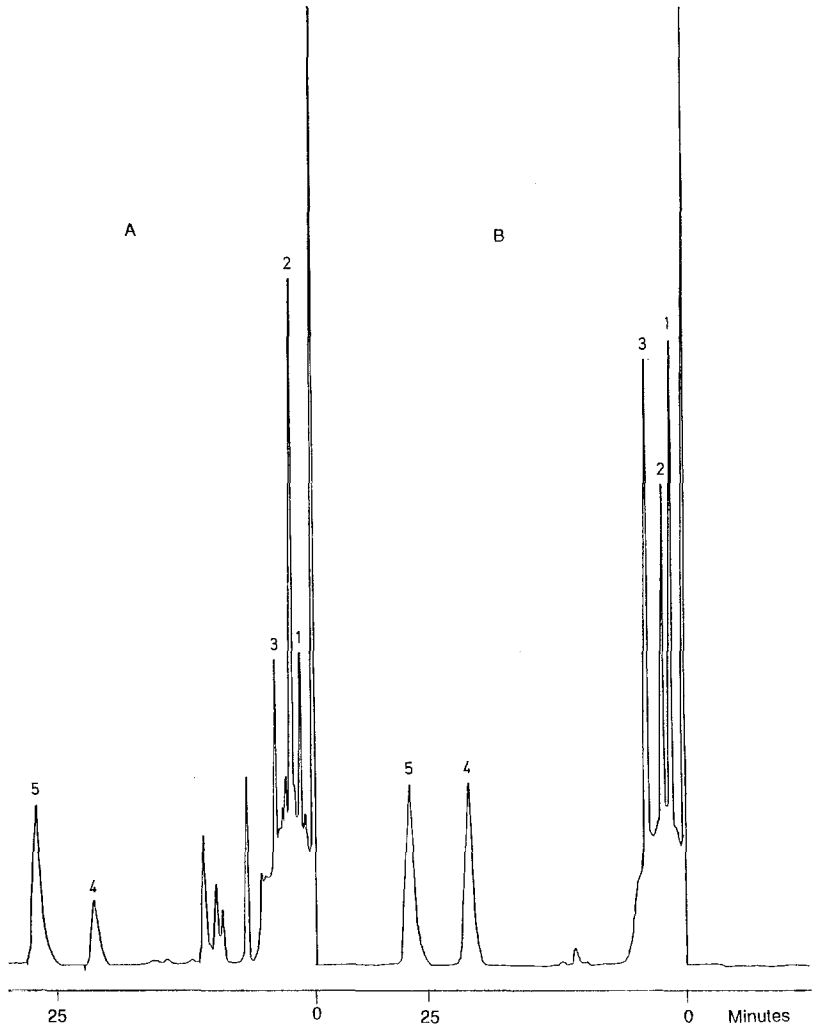

Fig. 1. Chromatogram of OPA-sulphite derivatives (f.s.d. = 500 nA). **A** shows a typical brain sample; **B** shows a typical standard (100 µg/ml). Peaks are in order: aspartate, glutamate, glycine, GABA and BABA

were identified for tyrosine, lysine, tryptophan, methionine, phenylalanine, cystine, valine, isoleucine, cysteine, leucine or homocysteine within a 30 min elution time. However the three aromatic acids of neurochemical importance as neurotransmitter precursors could be measured using a modified buffer to increase the elution rate; for optimal separation of tryptophan, phenylalanine and tyrosine, a mobile phase of pH 5.7 and containing 22% methanol was used, yielding retention times (relative to BABA: 100) of 664, 555 and 127 respectively. These late running derivatives were too low in concentration to interfere with the chromatography of subsequent samples, that normally could be injected directly after the elution of the BABA peak.

Table 1 demonstrates the application of this technique to the measurement

Table 1. GABA and glutamate in brain tissue

	Controls (n = 27)	Huntington's disease (n = 46)
Frontal cortex		
GABA	196 ± 37	140 ± 37**
Glutamate	1507 ± 272	1366 ± 407*
Substantia nigra		
GABA	405 ± 177	209 ± 138**
Glutamate	1001 ± 146	1275 ± 319**
Pallidum (medial)		
GABA	783 ± 67	318 ± 105**
Glutamate	749 ± 139	1114 ± 541**

Values are means ± s.d. in μg/g tissue.
*$p < 0.05$, **$p < 0.001$ by t-test after log transformation of data.
Cortical data from Reynolds et al. (1990)

of concentrations of GABA and glutamate in brain tissue taken post-mortem from patients with Huntington's disease and control subjects.

Discussion

Glutamate, GABA and (occasionally) aspartate were measured routinely in investigations of brain tissue. The resolution of aspartate and glutamate derivatives could be improved by increasing retention time (with, for example, less methanol in the mobile phase) and adjusting the pH, although this would reduce sample throughput by increasing the retention times for GABA and BABA. However such a modification could be of value if GABA measurement was not needed, whereupon a more appropriate internal standard could be used. Similarly, if GABA alone were required, sample throughput and sensitivity could be improved by diminishing its retention time. This would make the method more applicable to the determinations of GABA in dialysates from the recently-developed in vivo brain dialysis techniques (Marsden, 1984). Cross (personal communication) has indeed applied the method in this way. An alternative approach would be to employ gradient elution which would also permit the determination of a wider range of amino acids; however this would be at the expense of the simplicity and reproducibility of the present isocratic system.

Values obtained from the application of this technique are consistent with previous control results (Perry et al., 1971; Ellison et al., 1987), and confirm the well-established loss of GABA in the brain in Huntington's disease (Perry et al., 1973; Reynolds and Pearson, 1987). Our results also confirm our previous observation of a cortical deficit of glutamate in this disorder, which also occurs in the striatum and limbic areas (Reynolds and Pearson, 1987; Reynolds et al.,

1990) but here does not appear to extend to the pallidum and substantia nigra, in which regions glutamate is significantly increased above control values.

Thus we feel that this method provides a simple, sensitive, reliable, and reproducible isocratic HPLC technique for the measurement of tissue concentrations of several amino acids considered to be important in brain function.

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