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Separation and quantitation of the amino acid neurotransmitters in rat brain by capillary electrophoresis

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Abstract: Capillary electrophoresis (CE) has been used to separate the CBI derivatives of the neurotransmitters γ-aminobutyric acid, glycine, glutamate, aspartate, norepinephrine and dopamine from 18 other amino acids present in the rat brain. The procedure, which requires an injection volume of <10 nl, gave detection limits of 24–40 fmol using UV detection at 420 nm. Efficiencies for the derivatized amino acids varied from 344,000 to 444,000 theoretical plates. The method was applied successfully to the quantitation of the amino acid neurotransmitters and several other amino acids in a rat brain homogenate.

Keywords: *Capillary electrophoresis; amino acid neurotransmitters; rat brain; naphthalene-2,3-dicarboxyaldehyde.*

Introduction

Four amino acids in mammalian brain have been shown to function as neurotransmitters. Glutamate and aspartate are the principal excitatory transmitters, while γ-aminobutyric acid (GABA) and glycine are the primary inhibitory transmitters [1]. These neurotransmitters have been implicated in many neurological and psychiatric disorders such as Huntington's disease, Parkinsonism, epilepsy, schizophrenia and senile dementia [2]. Thus, there is great interest in developing methods which permit quantitation of these amino acids in brain samples.

Of the various analytical methods currently in use, high-performance liquid chromatography (LC) has been routinely employed with much success and seems to be the method of choice [3]. However, the use of LC for analysis of amino acids in the brain suffers from some drawbacks. This methodology usually involves derivatization of the amino acids with orthophthalaldehyde (OPA) prior to injection to produce compounds which can be detected by light absorption, fluorescence, or electrochemical activity [4–6]. However, a disconcerting problem with the OPA derivatives is that they are unstable and the reaction must be carefully timed to allow for quantitation [5, 7].

Derivatization of primary amines with naphthalene-2,3-dicarboxyaldehyde (NDA) in the presence of cyanide produces derivatives which are more stable than those derivatized with OPA [7, 8]. The LC separation of the common amino acids derivatized with NDA has been accomplished previously [7, 9]; unfortunately, glutamate and aspartate elute early in the chromatogram and can coelute with other brain matrix components, thus interfering with accurate quantitation.

Capillary electrophoresis (CE) is an analytical counterpart to LC which offers a number of advantages, including higher column efficiency and smaller sample volume requirement. The high column efficiency allows the separation of some of the common amino acids labelled with NDA [10–12]. CE with laser-induced fluorescence detection has been employed for the determination of NDA–amino acids in neurons of the land snail *Helix aspera* [11]. However, many of the amino acid neurotransmitters, including GABA and taurine, were not identified. In this paper, the use of NDA/CN coupled to CE–UV is evaluated for the determination of the important amino acids in brain tissue samples. CE has a very low sample volume requirement, typically <10 nl; therefore, very good spatial resolution can be obtained in small brain samples. Other advantages of CE over LC are the low solvent consumption and lower equipment cost.

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In the present work, the use of CE for the separation of the NDA-derivatized amino acid neurotransmitters from other amino acids and amines which occur in the brain was investigated. Efficiencies and detection limits employing a UV–vis detector were determined.

Experimental

Electrophoretic instrumentation

Electrophoresis in the capillary was driven by a high-voltage dc (0–30 kV) power supply (Spellman High Voltage Electronics Corp., Plainview, New York) connected to platinum electrodes. The anodic high-voltage end of the capillary was isolated in a Plexiglas box fitted with an interlock for operator safety. The column used was a 115-cm × 50-μm i.d., 360-μm o.d., fused silica capillary with a detection window at 95 cm. All experiments were performed at ambient air temperature (25°C). An ISCO CV4 capillary electrophoresis detector (ISCO, Lincoln, NE) set at 420 nm using the deuterium lamp as the light source was employed. The deuterium light source was found to give less noise and better sensitivity than the tungsten light source. Sample introduction was accomplished by electrokinetic injection at 5 kV for 12 s.

Chemicals

All amino acids and amines were purchased from Sigma (St Louis, MO) and used as received. NDA was supplied by Oread Laboratories (Lawrence, KS). Sodium cyanide and sodium borate were obtained from Fisher Scientific (Fair Lawn, NJ). All solutions were prepared in NANO pure water (Sybron-Barnstead, Boston, MA) and filtered through a 0.45-μm pore size membrane filter before use.

Sample derivatization

Samples to be electrophoresed were subjected to a procedure which converts primary amines to their 1-cyano-2-substituted-benz(f)-isoindole (CBI) derivatives [11]. Typically, 1 μl of sample or standard was mixed with 5 μl of 20 mM sodium borate buffer (pH 9.0). Next, 1.5 μl of 20 mM aqueous NaCN was added and mixed and then 1.5 μl of 20 mM NDA in acetonitrile was added and mixed thoroughly to give a total volume of 9 μl. The derivatized sample was enclosed in foil to protect it from photodegradation, allowed to stand at room temperature (25°C) and injected after 30 min. Standard solutions containing mixtures of amino acids were prepared by mixing appropriate volumes of individual stock amino acid solutions in water and then derivatizing the mixture.

Brain homogenate preparation

After a rat was sacrificed by cervical dislocation, the brain was removed and washed with water to remove extraneous matter. Subsequently, 1-2 mg of tissue obtained from punches of the frontoparietal cortex region was placed in 10 μl of 70% ethanol and sonicated in an ice bath for 5–10 s using an Artix Sonic Dismembrator, Model 150 (Artex Systems Corp., Farmingdale, NY) at a power setting of 30. The mixture was centrifuged at 16,000*g* rcf for 10 min and 1 μl of the supernatant was added to 1 μl of 4.9×10^{-4} M α-aminoadipic acid (IS) prior to derivatization and subsequent injection into the CE system.

Results and Discussion

Separation of amino acid standards

Analysis of the major amino acid neurotransmitters GABA, glycine, glutamate and aspartate in rat brain was accomplished by conversion of these amino acids to their CBI derivatives, separation by CE and detection at 420 nm. Successful analysis requires that these compounds be separated not only from each other but also from other primary amines which occur in the brain. Thus, the amino acid neurotransmitters should be separated from the other commonly occurring amino acids as well as from taurine, norepinephrine (NE), dopamine (DA), phosphoethanolamine (PEA), and 3,4-dihydroxyphenylalanine (DOPA). In addition, an internal standard (IS) is necessary for the procedure to be useful in the analysis of real samples. The amino acid α-aminoadipic acid serves the purpose well since it elutes at a position in the electropherogram at which no endogenous peaks elute.

It was quickly ascertained that in order to achieve this demanding separation a relatively long column (95 cm to the detecting window) and high voltage (maximum voltage of the power supply, 30 kV) were required. The derivatization reaction requires a pH above 8 to give reasonable yields; typically, borate buffers near pH 9 are employed [13]. This pH was also employed for the CE separation. It was found that the resolution of components

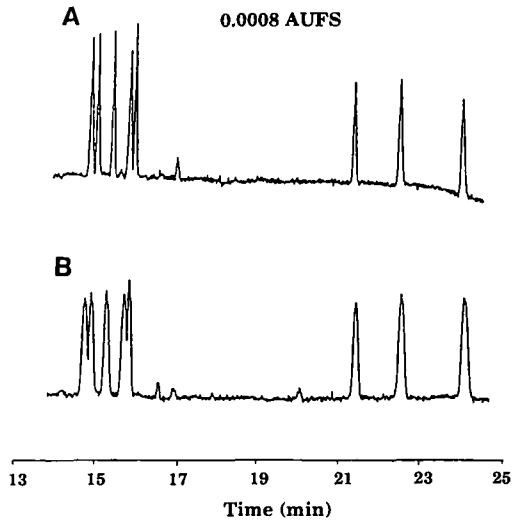

Figure 1
Electropherogram of seven NDA-derivatized amino acids. Separation conditions: 115 cm × 50 μm i.d. column in 20 mM sodium borate (pH 9.0) operated at 30 kV; 420 nm detection. Samples derivatized in: (A) 20 mM sodium borate (pH 9.0); (B) 50 mM sodium borate (pH 9.0).

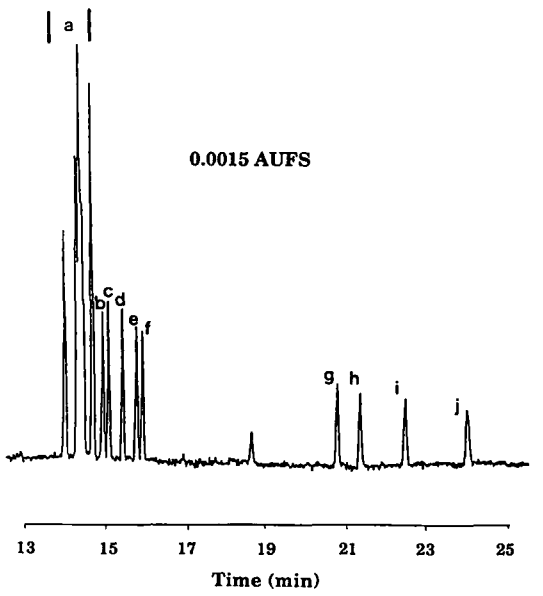

Figure 2
Electropherogram of NDA-derivatized amino acids. Separation conditions outlined in Fig. 1. Peaks (a) Trp, Phe, His, Gln, Leu, Ile, Met, Tyr, Val and Asn; (b) GABA; (c) Ser; (d) Ala; (e) taurine; (f) Gly; (g) PEA; (h) IS; (i) Glu; and (j) Asp.

did depend markedly on the concentration of the borate buffer employed in the derivatization procedure and therefore injected into the system. Demonstrated in Fig. 1 is the effect of injecting the sample in 50 mM borate versus 20 mM borate. As can be seen, all of the peaks in the electropherogram of the sample prepared in the more concentrated buffer are much broader than those in the more dilute preparation. A similar phenomenon has been observed by others where solutions more dilute than the running buffer are injected and result in narrower peaks [14]. If one attempts to use borate buffers more dilute than 20 mM, the derivatization procedure does not go to completion, especially for amino acids with acidic side chains, such as aspartate and glutamate. Thus, 20 mM sodium borate buffer was determined to be the optimum concentration for subsequent analysis.

Using the above conditions, the major amino acid neurotransmitters were separated from each other and the other common amino acids as well as from taurine and PEA, as indicated in Fig. 2. Two amino acids not included in the separation are lysine, which forms a derivatized compound and elutes as a broad peak before tryptophan, and cysteine, which oxidizes quickly to give multiple peaks. The separation of NE, DA, and their metabolic precursor DOPA from each other and the other amino acids is demonstrated in Fig. 3. Since NE, DA and DOPA can undergo oxidation when placed in the basic borate buffer, electropherograms must be run within 1 h of derivatization. Figure 3 shows the high efficiency attainable with CE as indicated by the resolution of 16 different amines commonly found in brain.

Thus, the four major amino acid neurotransmitters have been shown to be separated from each other, an internal standard, and 19 other amino acids or amines that may be found in the brain. In general, the observed order of elution seems to correlate very well with what one would expect according to capillary electrophoretic theory [15]. All derivatives except arginine are negatively charged and migrate against the electro-osmotic flow. CBI-arginine has a net charge of zero at pH 9 and elutes with the neutral marker. α-Amino acid derivatives with two negative charges, such as aspartate, glutamate, and the internal standard, have longer migration times than those with one negative charge.

Figure 4 shows a plot of migration time versus molecular weight (MW) for the amino acids containing neutral side chains. Electrophoretic mobilities were calculated for all the amino acid derivatives and the relationship between electrophoretic mobility and molecular weight was explored based on the work

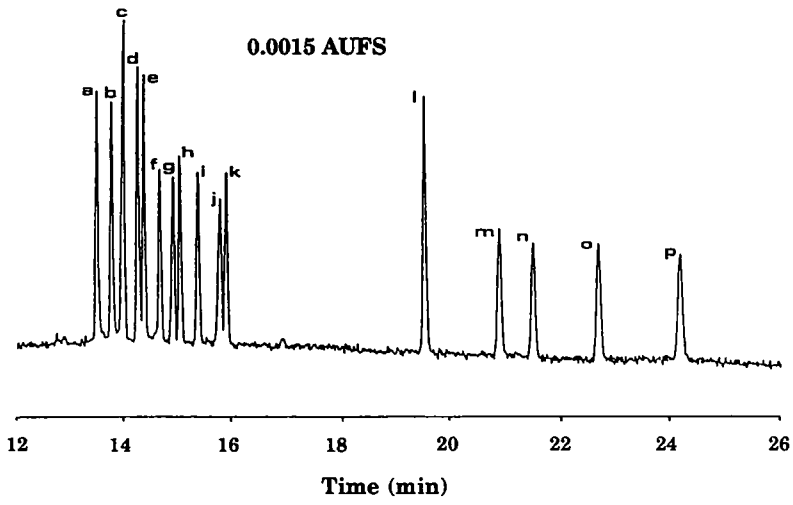

Figure 3
Electropherogram of NDA-derivatized amino acids and amines. Separation conditions outlined in Fig. 1. Peaks (a) NE, (b) DA, (c) Trp, (d) Phe, (e) Tyr, (f) Asn, (g) GABA, (h) Ser, (i) Ala, (j) taurine, (k) Gly, (l) DOPA, (m) PEA, (n) IS, (o) Glu and (p) Asp.

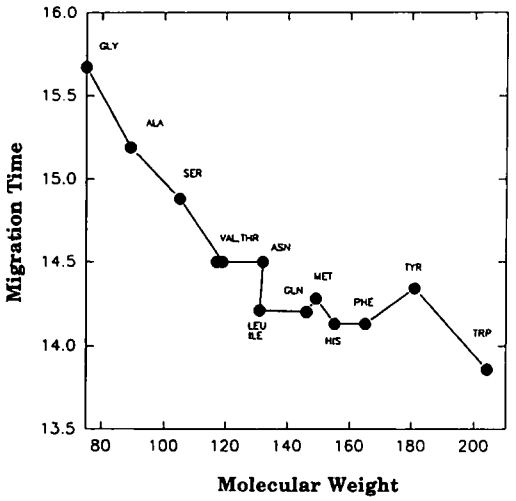

Figure 4
Plot of migration versus molecular weight for amino acids with neutral side chains.

of Rickard *et al.* for biopolymers [16]. Three possible relationships were proposed. In our case the best fit was obtained by plotting the mobility versus cube root of the molecular weight (correlation coefficient 0.998). This relationship is predicted by Stokes' Law, which says that the frictional drag is proportional to the radius (r) of a spherical molecule. (Since MW is proportional to $4/3\pi r^3$ (volume), a plot of electrophoretic mobility versus $q/MW^{1/3}$ should give a straight line.) Electrophoretic mobility was also plotted versus q/MW (volume) and $q/MW^{2/3}$ (surface area relationship). However, the correlation coefficients were not as good as for the Stokes' Law relationship (0.987 and 0.964). Further investigation of the relationship between MW and migration time of amino acids is underway [17].

Efficiencies and detection limits

The efficiency of separation was determined by calculating the number of theoretical plates from retention times and peak half-widths for the neurotransmitters and some other amino acids. The detection limits for these compounds were also calculated based on a S/N = 2 and were in the low (3–10) micromolar range. The results of the efficiency and detection limit determinations are given in Table 1. Efficiencies were generally around 400,000 theoretical plates and detection limits were at the femtomole (micromolar) level.

Response factors

Response factors based on peak height were determined for the amino acid neurotransmitters GABA, glycine, glutamate and aspartate, as well as the four amino acids which elute near them, serine, alanine, taurine and PEA. These eight amino acids were mixed with the internal standard to give concentrations of 2, 3, 4 and 5 $\times$ 10^{-5} M for each amino acid. The result of eight runs is summarized in Table 2.

Separation and quantitation of amino acids in rat brain

Capillary electrophoresis was performed on

Table 1
Efficiencies and limits of detection for various amino acids

Amino acid	Efficiency*	Detection limit (μM)	Detection limit (fmol)†
GABA	344,000	3.8	24.0
Serine	425,000	3.5	21.9
Alanine	445,000	3.7	22.7
Taurine	360,000	4.4	26.4
Glycine	444,000	4.3	25.6
Internal standard	386,000	7.4	32.8
Glutamate	414,000	8.7	36.6
Aspartate	385,000	10.2	40.4

* Number of theoretical plates.
† Calculated by multiplying micromolar values by the apparent volume injected.

Table 2
Response factors for various amino acids

Amino acid	Mean*	% RSD
GABA	0.758	5.67
Serine	0.593	3.32
Alanine	0.611	4.10
Taurine	0.813	4.29
Glycine	0.643	3.73
PEA	1.034	3.88
Glutamate	1.135	3.92
Aspartate	1.180	4.81

* Average of five runs.

an NDA-derivatized sample of a rat brain punch using the protocol described in the Experimental section. The resulting electropherogram is given in Fig. 5. All peaks were identified by spiking the sample with standards. Use of the internal standard and the response factors listed in Table 2 enabled quantitation of these amino acids. Given in Table 3 are the concentrations of the amino acids in the homogenate and the corresponding concentrations in the brain. The values for the

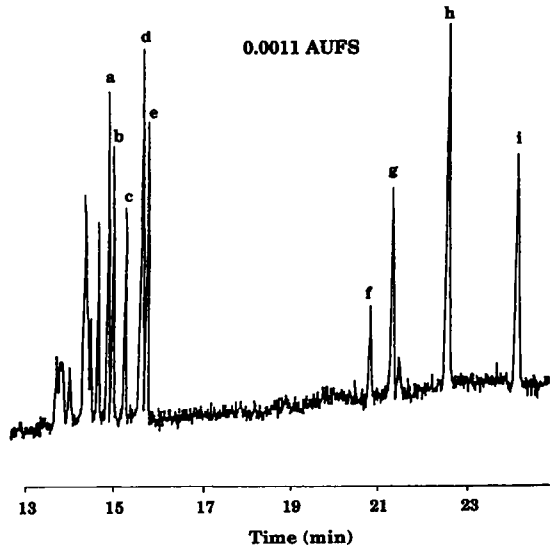

Figure 5
Electropherogram of NDA-derivatized rat brain homogenate. Separation conditions outlined in Fig. 1. Peaks (a) GABA, (b) Ser, (c) Ala, (d) taurine, (e) Gly, (f) PEA, (g) IS, (h) Glu, (i) Asp.

Table 3
Analysis of rat brain homogenate

Amino acid	Concentration: In homogenate (μM) Mean	% RSD	In brain (μmol g^{-1} rat brain)
GABA	0.808	3.9	5.05
Serine	0.533	2.2	3.33
Alanine	0.425	3.5	2.65
Taurine	0.979	2.1	6.12
Glycine	0.625	2.7	3.91
Glutamate	1.367	3.2	8.54
Aspartate	0.897	2.9	5.60
PEA	0.316	3.3	1.98

* In 1.6 mg sample of brain tissue (n = 3).

amino acids are similar to those reported by Tossman *et al.* [3] except for glycine and alanine, which were much higher in our samples.

It has been shown that CE of NDA-derivatized standards results in the efficient separation of the amino acid neurotransmitters from other amino acids, NE and DA. UV detection is sensitive enough for the determination of amino acids in rat brain homogenates. Increased sensitivity is, of course, attainable through the use of fluorescence [11] or electrochemical detection [18]. However, even with this relatively inexpensive instrumentation, it was possible to quantitate the amino acid neurotransmitters and several other amino acids in a very small mass (1 mg) of rat brain.

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