Automated Assay of \( \gamma \)-Aminobutyric Acid in Human Cerebrospinal Fluid

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We describe an automated amino acid analyzer with fluorescence detection (o-phthalaldehyde) which permits sensitive and rapid determinations of \( \gamma \)-aminobutyric acid in human cerebrospinal fluid. Concentrations as low as 50 nmol/liter can be accurately determined in 100-\( \mu \)l samples at the rate of one sample per hour. Concentrations in untreated cerebrospinal fluid increase rapidly after sampling by lumbar puncture. The concentration in immediately deproteinized samples from 38 patients with intervertebral disc disorders was 220 ± 81 nmol/liter (mean ± SD).

Additional Keyphrases: fluorometry with o-phthalaldehyde - tentative normal values - variation, source of - neurologic disorders - monitoring therapy

\( \gamma \)-Aminobutyric acid (GABA) has been widely implicated as a major inhibitory transmitter in the central nervous system. Certain neurological disorders may be associated with altered concentrations of GABA in the brain. In support of this hypothesis Perry et al. (1) demonstrated decreased GABA concentrations in postmortem brain tissue of patients with Huntington’s chorea. Altered GABA concentrations in brain may also occur in schizophrenia (2) and epilepsy (3). Accordingly, there is much interest in the therapeutic use of pharmacologic agents that alter brain GABA concentrations (4).

Clinical research in this area has been difficult because therapy intended to alter GABA concentrations in brain cannot be monitored by direct tissue sampling. GABA concentrations in the brain (or turnover or release) may be reflected by GABA concentrations in the cerebrospinal fluid (CSF), which can be easily sampled. Thus, a method for determining GABA in CSF could be of value both in the diagnosis of various neurological disorders involving alterations of GABA metabolism and for clinical monitoring of GABA concentrations during treatment of these conditions.

Although there are many methods for determining GABA in brain tissue, most are insufficiently sensitive or specific for detection of GABA in CSF. Recently, several investigators have reported the use of such techniques as liquid chromatography (5, 6), combined gas chromatography–mass spectrometry (7), and a radioreceptor assay (8). These methods do not appear to be entirely suitable for the clinical laboratory owing to their complexity or to limitations in reliability, speed, or simplicity.

We report a fully automatic high-performance liquid-chromatographic technique for the determination of GABA in 100-\( \mu \)l aliquots of CSF. The method is a significantly improved version of the technique used by Glaeser and Hare (5) and combines high sensitivity with simplicity. It is apparently similar to the method reported recently by Prasad and Fahn (6), details of which have not been published. We also present the results of such determinations in a group of patients with intervertebral disc disorders, who appear to be appropriate controls for comparisons with patients with various central neurological disorders. Finally, we point out an important problem concerning the handling of CSF that has not been recognized in the past and requires attention if values for GABA are to be accurate.

Materials and Methods

Materials

Chemicals. Unless otherwise stated, chemicals for the preparation of sodium and lithium citrate buffers and reagent solutions were analytical grade and were obtained from Merck, Darmstadt, Germany. Pentachlorophenol and 5-sulfosalicylic acid were from Pierce Chemical Co., Rockford, Ill. 61105, and o-phthalaldehyde from Fluka, Buchs, Switzerland.

Apparatus. Figure 1 is a schematic representation of the apparatus used. GABA is separated from other amino acids and primary amines by high-performance ion-exchange chromatography on a microbore column with a single elution buffer. Detection proceeds by flow-through fluorometry after continuous reaction of the column eluent with o-phthalaldehyde as described by Roth and Hampai (9) and Benson and Hare (10).

Unless otherwise stated, all components of the instrument were purchased from Kontron, Paris, France. Reservoir bottles were connected to the buffer selection...
valve (Labotron) and to the pumps (Labotron Model HKP-1) by 0.8 mm (i.d.) Teflon tubing and Labotron fittings. To avoid stalling of the pumps by small air bubbles, we inserted air traps (Beckman, France) with an internal volume of 0.5 ml before the pump inlets. All the Teflon tubing from the exit valves of the pumps to the mixing manifold (Gilson, France) after the column was 0.35 mm (i.d.). A pressure gauge (Gilson) was connected to the column buffer line via a tee manifold. An eight-position automatic sample injector (Labotron) fitted with 80-μl sample loops served for manual and automatic sample introduction. The jacketed column (25 × 0.3 cm) and the fittings to connect the Teflon tubing were from Gilson. A Haake circulating water bath was used to maintain an appropriate column temperature. The column was filled with DC-4A cation-exchange resin (Durrum Instrument Corp., Palo Alto, Calif. 94303). A pulse dampener was included in the o-phthalaldehyde reagent flow line, to minimize pulsatile flow of the reagent. In this pulse dampener a Nupro check valve (173 kPa; Techmation, Paris) acts as a restrictor, while air in a closed glass column, 15 × 0.3 cm (Gilson), is compressed upon each pump stroke. A fluorometer (Fluoromonitac; American Instrument Co., Silver Spring, Md. 20910) equipped with a 70-μl flow cell and filters compatible with the fluorescence properties of o-phthalaldehyde (excitation, Corning 7-51; emission; Wratten 2A) was connected to the mixing manifold via a 2-m length of microbore Teflon tubing (Durrum). A 173 kPa check valve (Nupro) was inserted into the drain line to maintain system back-pressure. A Labotron PRO-1 Programmer was used for automatic operation of the instrument. Fluorometer signals were recorded on a strip-chart recorder (Heath-Schulberger, France) and digitally integrated by an Autolab System I computing integrator (Spectra-Physics, France).

Procedures

Operation of the instrument. GABA was separated from other primary amines by elution with a 67 mmol/liter sodium citrate buffer (0.2 mol of Na+ per liter), pH 5.00, at a flow rate of 12 ml/h. The column temperature was 55 °C. The o-phthalaldehyde reagent was prepared as described by Benson and Hare (10) and pumped to the column eluate at a flow rate of 12 ml/h. Under these conditions a GABA determination could be performed in 1 h, including the time for washing and regenerating of the column with 0.2 mol/liter NaOH and buffer, respectively. The total sample volume injected was 80 μl.

Sample preparation. CSF samples were obtained from 38 subjects (23 men, 15 women; mean age 45 years, range 26–66 years) hospitalized in various clinics of the Hospices Civils de Strasbourg. All were undergoing lumbar puncture for diagnostic purposes (e.g., radiography, myelography) and all were diagnosed as having intervertebral disc disorders without clinical, biochemical, or radiological evidence of central nervous system disease. Spinal fluid was always sampled as soon as the needle entered the subarachnoid space, but before the start of any other procedure. CSF was deproteinized immediately by adding one-third volume of an aqueous 200 g/liter solution of sulfosalicylic acid (11). After removal of proteins by centrifugation, the supernates were kept at 4 °C and analyzed within three days. When the effect on GABA values of untreated CSF standing at room temperature was studied, aliquots of CSF samples were deproteinized immediately and after various time intervals.

Physiological fluid amino acid analysis. Deproteinized CSF samples were also assayed by physiological fluid amino acid analysis with the same instrument after minor modifications to accommodate additional buffer reservoirs and to protect the buffers from atmospheric ammonia (sulfuric acid trap). Here, a three-buffer procedure (0.3 mol/liter Li+, 67 mmol/liter citrate, pH 2.93; 0.3 mol/liter Li+, 67 mmol/liter citrate, pH 3.83; 1.2 mol/liter Li+, 67 mmol/liter citrate, pH 4.10) was used and the temperature of the column was changed from 34 °C to 60 °C at the time of the first buffer change.

Results

Figure 2 shows typical chromatograms of analyses of a GABA standard solution and deproteinized CSF. A standard curve for the GABA assay (Figure 3) shows good linearity over the range from 7.5 to at least 830 pmol of GABA. It can be estimated from the signal/noise ratio of the GABA peak in Figure 2 that the limit of detectability for GABA would be about 3 pmol with a signal/noise ratio of three. In terms of GABA in CSF
this would mean that the least concentration detectable would be 50 nmol/liter. Repeated injection of the same standard GABA solution (80 pmol per injection) yielded a relative standard deviation of ±2.5% (n = 13). Repeated assay of the same CSF specimen (17 pmol of GABA per injection) was reproducible within a standard deviation of ±7.9% (n = 13). Analytical recovery, determined by adding known amounts of GABA to various CSF samples, was virtually 100%.

By two criteria the new method sufficiently resolves GABA from interfering materials commonly found in physiological fluids and tissue extracts. First, a synthetic standard containing 39 amino acids and amines (Durrum physiological fluid amino acid standard) was analyzed by the new technique. The quantity of material associated with the GABA peak was, within the experimental error, identical to the quantity of GABA injected. This shows that none of the amino acids present in the synthetic standard mixture and known to be common constituents of physiological fluids and tissue extracts cochromatographed with GABA. This was further confirmed by injecting standards of amino acids and amines with chromatographic properties similar to those of GABA, such as β-alanine, α-aminoisobutyric acid, ammonia, and ethanolamine. None of these substances co-eluted with GABA. Second, CSF samples were analyzed by conventional physiological fluid amino acid analysis. The resolving power of this procedure is considered adequate for the separation of GABA from interfering substances commonly found in physiological fluids and tissue extracts (12). GABA concentration in CSF, measured in this way, was essentially identical to values obtained with the new method. This indicates that the new and more rapid procedure provides the same degree of resolution for GABA as does complete physiological fluid amino acid analysis.

With this new method, the concentration of GABA in immediately deproteinized CSF samples from 38 patients with intervertebral disc disorders was found to be 220 ± 81 nmol/liter (mean ± SD). Concentrations ranged from 59 to 413 nmol/liter and followed a normal distribution. There was no significant difference between values for men (213 ± 70 nmol/liter; n = 23) and women (232 ± 96 nmol/liter; n = 15), and the values were not age-dependent (r = 0.179).

In non-deproteinized samples of CSF kept at room temperature, GABA concentrations increased progressively. Figure 4 shows the time course of this increase in the CSF of two patients. The rate of increase of GABA concentrations of untreated CSF from different patients varied considerably. In CSF specimens from four patients GABA increased to 400–900% of its original value within 48 h of standing at room temperature. The time intervals required for doubling of the initial GABA concentrations ranged from 1 to 5 h. In non-deproteinized CSF stored at 4 °C, the GABA concentration increased more slowly (average doubling time, about 24 h). In deproteinized CSF, GABA concentrations were stable for at least three weeks at 4 °C. Regular physiological fluid amino acid analysis demonstrated that the substance formed in the CSF upon standing cochromatographed with authentic GABA.
Fig. 4. Time course of the increase in γ-aminobutyric acid concentration in the cerebrospinal fluid of two patients
CSF was obtained by lumbar puncture at time zero and kept at room temperature (22 °C). Aliquots of 100 µl were deproteinized at various time intervals after puncture. Each point represents the mean of two determinations

Discussion

Various methods have been developed for the assay of GABA in picomole quantities (13-16). Although these methods readily allow the determination of GABA in micro-dissected brain areas, they have not been applied to CSF. In contrast to the assay of GABA in brain, there are very stringent requirements for specificity in the detection of GABA in CSF. GABA is present in CSF only as a minor constituent, and great care must be taken to ensure that interfering material is completely separated. Some previous estimates of CSF GABA concentrations in humans (17) and cats (18) are relatively high and probably reflect inadequacies of the methods used. Only with the recently developed sensitive and selective methods (5-8) has it become possible to determine GABA in CSF.

The present method offers distinct advantages over other procedures in terms of sensitivity, simplicity, and speed. Its very high sensitivity permits analysis of small CSF samples and is sufficient for determinations on the CSF of small laboratory animals. It will permit investigation of possible correlations between GABA concentrations in CSF and brain under a variety of experimental conditions, including the administration of drugs known to affect brain GABA metabolism. The present procedure is also relatively simple. A single buffer is sufficient to elute GABA with good separation from interfering substances. The use of a single buffer simplifies the design and the operation of the instrument, and contamination of the buffer with air-borne ammonia and other primary amines becomes inconsequential. In contrast, in the two-buffer procedure of Glaeser and Hare (5) great care must be taken to exclude ammonia from the buffers during preparation and instrument operation, because ammonia in the buffers may interfere with the GABA analysis, particularly at high sensitivity. The single-buffer procedure also greatly increases the assay speed. A GABA analysis can be performed in 1 h, whereas the system of Glaeser and Hare requires 2 h per sample. Excellent stability of the reagent was observed. A solution prepared according to the procedure of Benson and Hare (10) was usable for at least two weeks at room temperature without any protection from the atmosphere.

Perry and Hansen (19) have recently questioned the validity of the values for GABA in CSF reported by Glaeser and Hare (5), because in their experiments they found unknown substances that, on cation-exchange chromatography, co-eluted with GABA. Consequently, the suggestion that the actual concentrations are at least one order of magnitude lower than those reported by Glaeser and Hare, i.e., less than 50 nmol/liter, and thus not detectable in individual CSF specimens by existing methodology.

We have established that the present method is as effective in resolving GABA from other CSF constituents as regular physiological fluid amino acid analysis. We recognize, however, that chromatographic procedures alone, even when as powerful as physiological fluid amino acid analysis, cannot give final proof of the homogeneity of any peak eluted. Nevertheless, we feel confident about the GABA values provided by the present method, because other investigators using independent techniques have recently reported very similar values for patients with various neurological disorders not suspected to be associated with altered GABA concentrations. Enna et al. (8), using a radioreceptor assay, found concentrations of 225 ± 39 nmol/liter (mean ± SEM); Huizinga et al. (7) used a gas chromatographic/mass fragmentography method and reported values between 115-815 nmol/liter.

Welch et al. (20, 21) could not measure GABA in the CSF of control subjects, although their modification of an enzymatic fluorometric method (16) was claimed to be as sensitive as our chromatographic fluorometric method. This discrepancy may be due to inadequacies in terms of sensitivity or specificity (or both) of the enzymatic fluorometric method after its modification for CSF analysis. To our knowledge, these authors have not published any methodological details that permit the validity of their procedure to be evaluated.

The finding that GABA increases rapidly in untreated CSF after CSF removal from the subarachnoid space suggests enzymatic generation of new GABA under these conditions. We now are investigating the mechanism responsible for the formation of new GABA. Clearly, CSF samples for GABA determinations must be deproteinized immediately after lumbar puncture. Freezing and storing untreated CSF at −20 °C may result in the formation of a significant amount of new GABA during freezing and thawing. We believe that this potential source of error has not been recognized previously and that some reported values for GABA in CSF may be spuriously high for this reason.

Ethical considerations make it difficult to define the range of GABA concentrations in the CSF of normal subjects. In previous publications, control groups have usually consisted of ill-defined patients with a variety of neurological disorders. We have attempted to establish the range of values in a homogeneous group of
patients who had no evident central neurological disorder. This group appears suitable as control for studies dealing with values for patients with central nervous system disease.

Low GABA concentrations have been found in the CSF of patients with Huntington’s chorea (22), a condition associated with lowered brain GABA concentrations. We find that values for GABA in the CSF of cats increase after intraperitoneal injection of γ-vinylimidazole, GABA, a drug that increases brain GABA concentrations by selective and irreversible inhibition of the GABA-metabolizing enzyme, aminobutyrate aminotransferase (EC 2.6.1.19) (23). These findings strongly support the suggestion that GABA concentrations in CSF reflect brain GABA concentrations. Thus, measurements in CSF may be helpful for diagnosis and for guidance in therapy with drugs that influence brain GABA metabolism.

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References