Fluorometric Quantitation of Glutamine in Cerebrospinal Fluid

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We describe a rapid, relatively simple-procedure for fluorometry of glutamine in cerebrospinal fluid. Glutamine is de-aminated with diluted sulfuric acid at 100 °C. The ammonia liberated is then reacted with a buffered o-phthalaldehyde–mercaptoethanol solution, pH 7.4, to form a fluorochrome. Fluorescence is linearly related to the concentration of glutamine from 0.10 to 0.60 g/L (0.68 to 4.10 mmol/L). To prevent interference by endogenous ammonia, each specimen was corrected for its own background fluorescence. The mean analytical recovery was 99.8%. Within-day variation (CV) for pooled, enriched cerebrospinal fluid controls at 0.33 g/L (2.22 mmol/L) and 0.53 g/L (3.63 mmol/L) were 2.2 and 5.1%. Day-to-day variation (CV) for the same controls was 4.4 and 4.8%, respectively. Data obtained by our method correlate excellently (r = 0.980) with those by an established procedure based on the Berthelot (phenol–hypochlorite) reaction.

Additional Keyphrases: Reye’s syndrome • liver disease • encephalopathy • hypercapnea • measuring glutamine vs measuring NH₃

The clinical relevance of measuring glutamine in cerebrospinal fluid (CSF) has been well documented. CSF glutamine concentration is slightly increased in patients with chronic hypercapnea and in patients with chronic liver disease without encephalopathy (1). A moderate increase in CSF glutamine is associated with acute liver disease (2). However, as hepatic encephalopathy develops and progresses to hepatic coma, CSF glutamine markedly increases to concentrations in the range of 260–540 mg/L (3). In addition, current evidence suggests that encephalopathy of Reye’s syndrome in children is related to hepatic dysfunction and that glutamine concentrations in these patients are abnormally high (4).

In our laboratory, CSF glutamine was routinely quantitated by deaminating glutamine with diluted sulfuric acid and measuring the liberated ammonia colorimetrically, after reacting with phenol–hypochlorite reagent (5, 6). The linearity of the standard curve for this procedure extends only to 0.4 g/L. Frequently, the analysis is prolonged because of the need to make dilutions and repeat the test when specimens were abnormal. Moreover, the method involves two corrosive reagents, and a relatively large sample is required because of the low sensitivity of the procedure. To overcome these disadvantages, we developed a simple fluorometric procedure for CSF glutamine quantitation, which not only is more sensitive but requires also a smaller volume of sample.

Materials and Methods

Reagents. Alcoholic o-phthalaldehyde solution, 0.75 mol/L, was prepared by dissolving 100 mg of o-phthalaldehyde (Eastman Organic Chemicals, Rochester, NY 14650) in 10 mL of absolute ethanol. Alcoholic mercaptoethanol solution, 72 mmol/L, was prepared by dissolving 50 µL of mercaptoethanol (Sigma Chemical Co., St. Louis, MO 63178) in 10 mL of absolute ethanol. Sodium phosphate buffer, 0.2 mol/L, was prepared by dissolving 22.6 g of Na₂HPO₄ and 4.8 g of NaH₂PO₄ into 800 mL of de-ionized water. The pH was adjusted to 7.4 and the solution was diluted to 1 L with de-ionized water. The working o-phthalaldehyde–mercaptoethanol reagent was made by adding 4.5 mL of alcoholic o-phthalaldehyde solution and 4.5 mL of alcoholic mercaptoethanol solution to 91 mL of sodium phosphate buffer, pH 7.4. Sulfuric acid, 100 mL/L, was used.

Procedure. Place 0.1 mL of CSF in a disposable glass tube (10 × 75 mm) containing 0.1 mL of a 100 mL/L solution of sulfuric acid. Prepare standards and controls in the same manner. Cover all tubes with Parafilm, then transfer them to a heating block preset at 100 °C. After exactly 15 min in the heating block, remove all tubes and cool them in a water bath (21 °C). Transfer the acid hydrolysate (0.1 mL) from each tube to a test tube containing 3.0 mL of buffered working o-phthalaldehyde–mercaptoethanol reagent. Allow the fluorescent complex to develop for exactly 25 min and then measure the fluorescence (we used a Turner Spectrofluorometer, Model 430; Turner Associates, Palo Alto, CA 94303), with excitation and emission wavelengths of 412 and 490 nm, respectively. Also determine background fluorescence for each specimen in parallel by adding 0.1 mL of a mixture consisting of 0.1 mL of CSF and 0.1 mL of water to 3.0 mL of buffered working o-phthalaldehyde–mercaptoethanol solution. Measure CSF glutamine concentrations of unknowns and controls from a standard curve. (Calculation on the basis of the fluorescence of the standards may be used in an emergency, but use of a standard curve is generally preferred.)

Results

Fluorescence stability. Fluorescence stability was determined at four different concentrations of aqueous glutamine standards, ranging from 0.1 to 0.6 g/L (Figure 1). The intensity of fluorescence increased during incubation up to 50 min at room temperature. However, change in intensity of fluorescence was linearly related to duration of incubation for the interval 15 to 30 min for all glutamine concentrations tested. Therefore, we selected 25 min at room temperature as the optimal incubation.

Linearity study. The intensities of fluorescence were linearly related to glutamine concentration from 0.1 to 0.6 g/L.

Precision studies. Table 1 shows the reproducibility of the method, both day-to-day and within-run. The relative variability about the mean of both controls was within acceptable limits, as demonstrated by the lower CV for both controls.

Analytical-recovery studies. Ten CSF specimens were selected without conscious bias from specimens submitted to the laboratory. Each specimen was then supplemented with various concentrations of glutamine and the glutamine concentration of each specimen was determined by our method and compared with the expected value. Recovery of added glutamine averaged 99.8% (range, 94–106%).

Correlation study. Figure 2 shows the correlation (and regression analysis) between results by the proposed method.
Fig. 1. Stability of fluorescence of the reaction product
Glutamine concentrations, g/L: A, 0.6; B, 0.4; C, 0.2; and D, 0.1

Fig. 2. Correlation of CSF glutamine concentrations as measured by the present method and by the manual phenol–hypochlorite procedure
Correlation coefficient, 0.980; regression equation: \( y = 0.946 x + 0.34 \)

and an established method based on the use of phenol–hypochlorite, for 61 specimens (5, 6).

Discussion

Of the various methods for glutamine determination, one of the most common involves de-amination of glutamine with sulfuric acid. Liberated ammonia is determined with either Nessler’s reagent or phenol–hypochlorite (2, 5, 6). However, various substances interfere. For example, inorganic ions and some organic solvents interfere with the nesslerization process (7), and amines cause error in the phenol–hypochlorite method for ammonia determination (8, 9). Moreover, the sensitivities of these procedures are low.

We believe that these disadvantages can be overcome by our proposed method because: (a) our procedure extends the linearity up to 0.6 g/L of glutamine, which greatly improves the usefulness of measuring abnormal specimens, in that repetition of tests can be eliminated; (b) incorporation of mercaptoethanol in the procedure greatly enhances the specificity of the method because sulfhydryl compounds such as dithioerythritol and reduced glutathione cannot effectively replace mercaptoethanol; and (c) potential interference by endogenous substances such as ammonia can be obviated in our method, because the background fluorescence has already been taken into account in the final calculation.

CSF glutamine is increased in patients who are comatose secondary to acute hepatic necrosis or chronic liver disease (1, 2, 5). In these patients, values for CSF glutamine have generally correlated better with the clinical course than have values for blood ammonia (2, 6). Moreover, some patients with Reye’s syndrome show a normal blood ammonia concentration but an above-normal CSF glutamine concentration (4). Thus well-established evidence tends to support the idea that measuring CSF glutamine may be more relevant clinically than data on blood ammonia.

References


Table 1. Reproducibility of Glutamine Analysis

<table>
<thead>
<tr>
<th>Glutamine concentration</th>
<th>CSF Pool 1</th>
<th>CSF Pool 2</th>
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</thead>
<tbody>
<tr>
<td><strong>Within-run</strong></td>
<td></td>
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<tr>
<td>Mean, g/L</td>
<td>0.331</td>
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<td>SD, g/L</td>
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<td>CV, %</td>
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<td>n</td>
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<td>5</td>
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<td><strong>Day-to-day</strong></td>
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