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Note

Rapid high-performance liquid chromatographic method for the assay of glutamine in human cerebrospinal fluid

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Glutamic acid (Glu) and glutamine (Gln) are the amino acids occurring in the highest concentration in the central nervous system [1]. There is strong evidence that Glu is an excitatory transmitter in some corticostriatal neurons [2, 3]. A possible role of Glu in the pathophysiology of mental disorders such as schizophrenia and depression has been discussed [4, 5]. In the central nervous system Glu and Gln take part in the glutamine cycle [6], where Gln can be both a precursor and an end-product to the transmitter Glu. The extracellular concentration of Gln is in the same order as the total brain concentration while the extracellular concentration of Glu is low. Gln is the dominating amino acid in the cerebrospinal fluid (CSF), while the concentration of Glu is just above the detection limit [7].

Gln in CSF has previously been measured with an amino acid analyzer [7] or using an enzymatic procedure [8]. The first of these methods is time-consuming. With the enzymatic procedure it is difficult to detect interference from other components. Lindroth and Mopper [9] have recently developed a method for the analysis of amino acids using high-performance liquid chromatography (HPLC) with fluorescence detection. The method, which involves derivatization of the amino acids with *o*-phthaldialdehyde, is extremely sensitive.

On the basis of the method of Lindroth and Mopper [9] we have developed a new procedure for the analysis of Gln in CSF. The principle is similar but there are differences in the preparation of the CSF. The introduction of an internal standard (cysteic acid) and isocratic elution instead of gradient elution of the column has shortened the time of analysis considerably and made it possible to analyze as many as 50 samples per day with high accuracy and specificity.

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EXPERIMENTAL

Materials

Methanol for liquid chromatography was obtained from May and Baker (Dagenham, Great Britain). Glutamine and cysteic acid were from Sigma (St. Louis, MO, U.S.A.). Water was distilled over active carbon in a glass apparatus. Other chemicals were of analytical grade.

HPLC was performed with a Spectra-Physics System 3500 B equipped with a Valco rotary valve injector with a 10-μl loop for syringe injection. The fluorescence detector was a SFM 22 Model from Kontron (Stockholm, Sweden) equipped with a 10-μl flow-through cell. The recorder was a W + W 600 Tarkan.

A 150 mm × 4 mm reversed-phase column packed with C_{18} Nucleosil 5 μm (Macherey, Nagel & Co, Düren, G.F.R.) was used. The packing of the column was performed principally according to the procedure of Bristow et al. [10].

Buffers. The phosphate buffer was prepared from 1/15 *M* KH_2PO_4 and 1/15 *M* Na_2HPO_4 which were mixed to give pH 6.0. The borate buffer was prepared from boric acid solution (0.4 *M*) which was adjusted to pH 9.5 with 1 *M* NaOH.

Reagent. *o*-Phthaldialdehyde reagent (OPT) was prepared and handled according to the method of Lindroth and Mopper [9] with the exception that the concentration of *o*-phthaldialdehyde was doubled.

Sampling of CSF

Fresh CSF was obtained from healthy volunteers (age 21—36 years), 12 ml being collected by lumbar puncture. The samples were mixed and centrifuged for 15 min at 2000 *g* at +4°C within 1 h after sampling. The technique used for sampling is described in detail by Sedvall et al. [11]. The fresh samples were processed immediately.

A pool of frozen CSF taken for diagnostic purposes was obtained from the Department of Neurology at the Karolinska Hospital.

Sample preparation

Ethanol (300 μl) was added to a sample of CSF (100 μl). The sample was mixed, stored for 10 min in a refrigerator at +4°C and centrifuged for 10 min at 2000 *g*. A part of the supernatant (300 μl) was transferred to a plastic tube which was stored at —80°C pending analysis. On the day of analysis 50 μl of the supernatant were added to borate buffer (500 μl), pH 9.5, containing the internal standard cysteic acid (2 μg/ml). Part of the sample (20 μl) was reacted with OPT reagent (50 μl) at room temperature. After exactly 4 min 10 μl of the reaction mixture were injected into the chromatograph.

Preparation of standard samples

A stock solution of glutamine (20 mg per 100 ml) in water—methanol (1:1) was prepared every month. It was stored at —20°C. Standard concentrations in the range 100—1000 nmol/ml were prepared by dilution of the stock solution with water—ethanol (1:3). The standard solutions were handled as the supernatant of the CSF sample deproteinized with ethanol.

Liquid chromatography

The chromatograph was run in an isocratic mode using methanol—phosphate buffer, pH 6.0 (45:55) as the mobile phase. The flow-rate was 1.2 ml/min; 10 μl of the sample were injected. About 3 min after the injection, the next sample was mixed with reagent. Shut-down procedures were performed according to the procedure of Lindroth and Mopper [9]. The detector settings were excitation 330 nm, emission 455 nm.

RESULTS AND DISCUSSION

A typical chromatogram of Gln in lumbar CSF from a healthy human subject is shown in Fig. 1. The retention times of cysteic acid and Gln were less than 5 min but the interval between each injection had to be 8 min due to the occurrence of some peaks after the Gln peak. The concentration of Gln in human CSF has been reported to be about 600 nmol/ml [7, 12]. We found a Gln concentration of 512 ± 109 nmol/ml (mean ± S.E.) in fresh lumbar CSF from 16 female volunteers. Naturally occurring amino acids that may interfere with the analysis are serine, asparagine and Glu. These amino acids together have a mean concentration of less than 40 nmol/ml, i.e. about 6% of the Gln level [7]. Asparagine and Glu eluted together in the small peak seen between the internal standard and the Gln peak (Fig. 1). Serine did not separate from the Gln peak, but addition of serine to CSF samples to a concentration of 30 nmol/ml resulted in only a 2% increase of the Gln level.

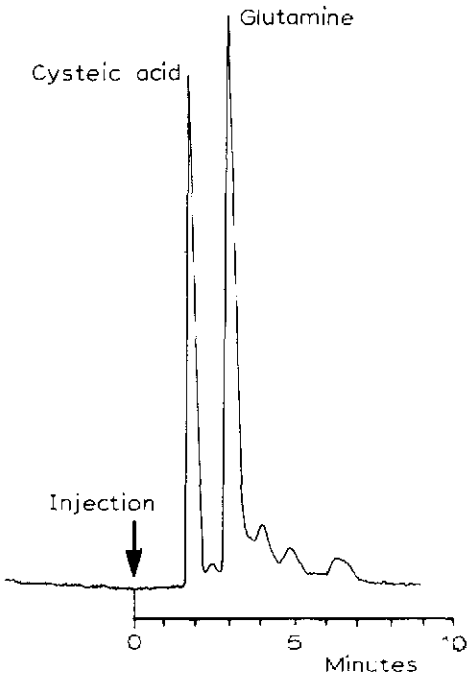

Fig. 1. Chromatogram of glutamine in human CSF. Cysteic acid was added as an internal standard. Conditions as described in the text.

TABLE I

EXPERIMENTAL ERROR AND RECOVERY OF HPLC METHOD FOR THE ANALYSIS OF GLUTAMINE

	Number of samples	Mean Gln concentration (nmol/ml)	± S.D. (%)	Recovery (%)
Frozen pool of CSF	5	568	8	—
Frozen pool of CSF*	6	98	4	—
Individual fresh CSF samples	9	516	8	—
Frozen pool of CSF + Gln 600 nmol/ml CSF	6	1198	10	98

*Frozen pool of CSF diluted 1:6 (v/v) with distilled water containing NaCl (9 mg/ml) and bovine albumin (300 μg/ml).

The standard curve was linear in the range 100—1000 nmol/ml. The recovery of Gln added to a pool of frozen CSF was 98% and the standard deviation was 8% (Table I).

The use of an internal standard was necessary as the peak heights from identical samples varied considerably during the day. This may be due to the accumulation of reagent-consuming material in the upper part of the column. Cysteic acid seems to be a suitable standard since it is apparently not present in human CSF and since its retention time interferes only with that of aspartic acid which is present in human CSF in a concentration below 1 nmol/ml [7]. The OPT derivative of cysteic acid did not reach its fluorescence maximum until after 4 min [9]. Therefore the reaction time should be at least 4 min.

The importance of rapid deproteinization of CSF samples to prevent enzymatic hydrolysis of Gln to Glu has been emphasized by several authors [7, 12]. We studied the stability of Gln in CSF by keeping samples at room temperature for different time intervals after sampling (Table II). No significant change of the Gln concentration was seen for up to 6 h without deproteinization.

The stability of Gln in CSF during storage for up to two months was also studied (Fig. 2). CSF samples from two healthy volunteers were pooled within 1 h after sampling. One part of the sample (control) was deproteinized within 1 h of sampling. It was analysed on the same day as it was sampled. The rest of the sample was divided into four fractions which were processed as described in Fig. 2. After two weeks there was no difference between the samples. After storage for one or two months the Gln concentration had decreased about 15% in the fraction which was stored at −20°C without deproteinization. The low Gln concentration found for all four fractions after two weeks' storage as compared to the control illustrates that the coefficient of variation between assays may be larger than within assays. Values of 20—30% were reported for the coefficient of variation for between-assay analysis of amino acids with HPLC in another investigation [13]. The deproteinization was made with ethanol, as perchloric acid (3%) caused a minor hydrolysis (< 1%) of Gln. Deproteinization with ethanol will be of importance if Glu, too, is analysed in the samples as the

TABLE II

GLUTAMINE CONCENTRATION IN CSF STORED AT ROOM TEMPERATURE

The samples were deproteinized at 0, 3 or 6 h after lumbar puncture. Otherwise they were handled as described in the Experimental section. Data respresent mean of duplicate samples.

Time of storage (h)	Gln concentration (nmol/ml)
0	470
3	493
6	503

Glu concentration in CSF is only about 0.2% of the Gln level [7] (Alfredsson, unpublished observation).

The present method has been used to determine Gln in CSF from patients with an acute psychosis of schizophrenic type. Samples were taken before and after treatment with chlorpromazine. Before treatment there was a tendency to a positive correlation between Gln concentrations in CSF and the severity of the illness. During treatment the reduction in psychotic symptoms was significantly correlated to a decrease in Gln levels in CSF [14].

The advantages of the present procedure for measuring Gln in CSF are its

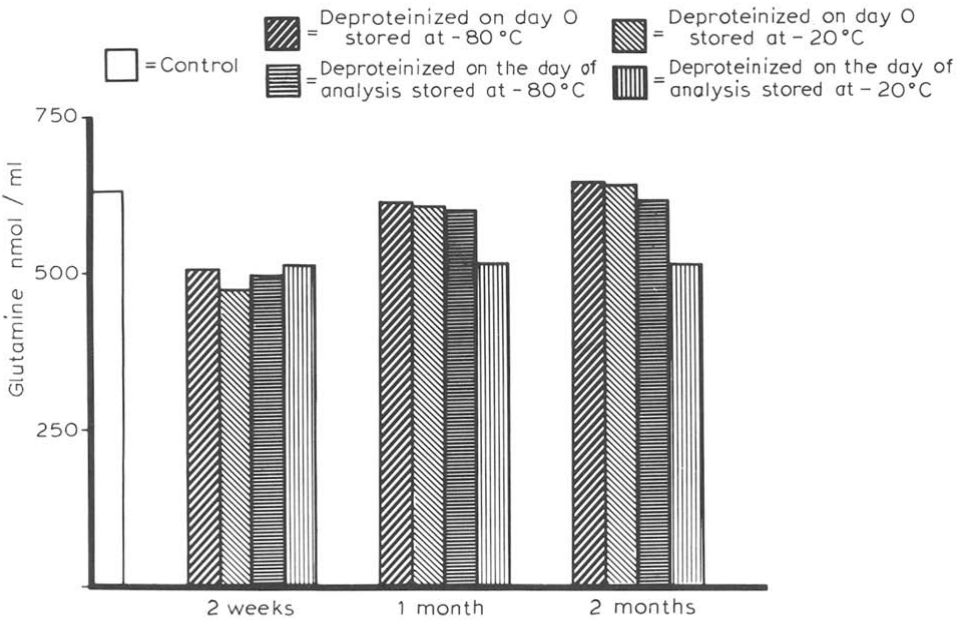

Fig. 2. CSF samples from two healthy volunteers were pooled within 1 h after sampling and deproteinized and analysed on the day of sampling (control). The rest of the sample was divided into four fractions. Two of the fractions were immediately deproteinized and thereafter stored at −20°C or −80°C. The other two fractions were stored at −20°C or −80°C without deproteinization pending analysis. All samples were run in duplicate.

accuracy, rapidity and simplicity, allowing the analysis of up to 50 samples per day. With an amino acid analyzer each sample takes several hours. The method is suitable for the analysis of Gln levels in CSF from patients with neuropsychiatric disorders in order to elucidate the role of Gln in the pathophysiology of such conditions.

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