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Note

Determination of free amino acids in the cerebrospinal fluid of male rats by high-performance liquid chromatography with fluorescence detection

Alterations in amino acid concentrations during sexual activity

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It is well known that glutamic and (Glu) and aspartic acid (Asp) are important excitory neurotransmitters and γ-aminobutyric acid (GABA) an equally important inhibitory neurotransmitter in the mammalian nervous system[1]. Recent studies have elucidated the anatomy of the neuronal pathways of these amino acid transmitters and their receptors and these have been found to control, *e.g.*, hypothalamic neuroendocrine functions[2–4]. Studies on the functional state of the nervous system may be facilitated by accurate and valid measurements of amino acids in the cerebrospinal fluid (CSF)[5,6]. However; apart from GABA, no systematic attempts at an accurate evaluation of basic parameters have been reported and this is reflected in the high variability of the reported data on amino acid levels in the CSF[4–8].

The quantitation of free and total amino acids in biological samples has traditionally been performed by ion-exchange chromatography and post-column derivatization with ninhydrin or a fluorescence reagent such as *o*-phthalaldehyde (OPA)[9,10]. This technique is very time consuming (2–3 h), requires large sample volumes (2–300 μl) and yields unreliable results for labile amino acids such as glutamine (Gln) and asparagine (Asn).

In recent years, the fluorogenic reactions of OPA, thiol and primary amines have been exploited in reversed-phase high-performance liquid chromatography (RP-HPLC). The reaction is highly specific and sensitive and results in rapid separations. Several systems have been utilized for HPLC analysis after OPA derivatization to quantitate amino acids in physiological fluids[11–13]. Since the reaction of amino acids and OPA is dependent on pH, temperature and time, these factors must be rigorously controlled. In our study, an automatic on-line system was used to control the reaction kinetics in order to obtain high reproducibility with a relative standard deviation (R.S.D.) between 0.2 and 2% for all amino acids. This optimized HPLC method has been applied to record alterations in the levels of amino acids in the CSF of male rats during sexual activity.

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EXPERIMENTAL

Chemicals

Individual crystalline salts of L-amino acids (AMAC standard kit No. 20065), OPA and Brij were obtained from Pierce (Rotterdam, The Netherlands), GABA, 3-methylhistidine, citrulline, phosphoserine, taurine, aspargine, ornithine and 2-mercaptoethanol from Sigma (St. Louis, MO, U.S.A.) and HPLC-grade methanol from Rathburn Chemical (Walkerburn, U.K.). All other chemical were of analytical reagent grade and were obtained from Merck (Darmstadt, F.R.G.).

Apparatus

The chromatograph consisted of two solvent delivery pumps (6000A and M45), a multiple sampler (WISP 710B), a data module, a system controller (730B), a fluorescent detector (420 nm) equipped with an excitation monochromator at 340 nm and an emission cut-off filter at 450 nm, all supplied by Waters Assoc. (Milford, MA, U.S.A.). Separation of amino acids was carried out on a 5-μm Hypersil-ODS column (150 × 4.6 mm I.D.), obtained from Shandon (London, U.K.). A pre-column (50 × 4.6 mm I.D.) packed with the same material (obtained from Waters Assoc.) was inserted before the analytical column. The column was equilibrated for 10 min between injections.

Standard solutions

Calculated amounts of each amino acid were taken to make solutions of concentration 1 μM in doubly distilled water by addition of a few drops of 0.1 M hydrochloric acid. A standard mixture containing 22 primary amino acids was prepared with concentrations of 0.01 μM of each amino acid and was stored at −20°C. This mixture was further diluted with distilled water to concentrations of 2.5, 5 and 10 nM to evaluate the relationship between the individual amino acids and the integrated peak area.

Buffer solutions

A 0.05 M sodium phosphate buffer of pH 7.2 was prepared from Na_2HPO_4 and NaH_2PO_4 in doubly distilled water. This buffer was diluted with water to a concentration of 0.02 M. In pump A the mobile phase consisted of 20 mM phosphate buffer–methanol–tetrahydrofuran (98:1:1) and in pump B 20 mM phosphate buffer–methanol (3:7) was used. Both solvents were filtered through a 0.45-μm filter-paper, Type HA and HV (Millipore), respectively. The flow-rate was maintained at 1 ml/min throughout except for the first 2 min, when it was increased linearly from 0.2 to 1 ml/min. The gradient used is shown in Fig. 1.

CSF samples

Male Wistar rats (320–350 g) were maintained with free access to food and water in a air-conditioned, temperature-controlled colony room. Six rats were implanted with stainless-steel guide cannulae into the cisterna magna, as described in ref. 14, and allowed a 4-day recovery period. The rats were tested with sexually receptive female rats (pre-treated with 20 μg of estradiol benzoate 48 h before testing and 0.5 mg of progesterone 6 h before testing) in circular (50 cm diameter) Plexiglas

cages. CSF samples (50–100 μl) were obtained from each male before sexual activity, immediately after ejaculation and after the end of the post-ejaculatory refractory period. One or two days elapsed between taking each CSF sample and the sampling order was random. The CSF samples were kept frozen at −70°C until assayed for amino acid concentrations.

Treatment of CSF samples

Prior to HPLC analysis, the CSF samples were treated with an equal volume of cold 4% sulphosalicylic acid (SSA), and the mixture was centrifuged at 1500 *g* for 15 min. The supernatant was collected and diluted with an equal volume of distilled water, and the solution was stored at −20°C if not analysed immediately.

Derivatization procedure

Anhydrous OPA (100 mg) was dissolved in 2 ml of methanol and 8 ml of 0.4 *M* borate buffer (pH 10.4), containing 0.6% Brij, were added. To this mixture, 200 μl of 2-mercaptoethanol were added and the solution was kept at 4°C overnight before use. The reagent is stable for 1 week.

Volumes of 30 μl of each reagent and sample (standard amino acid solution or CSF sample after its treatment) were injected into the column. The automatic system was programmed to allow these two solutions mix for 2 min in the needle before the injection. The fluorescence intensity was converted into peak area, and quantitation was made accordingly.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of the standard amino acid pool with a concentration of 5 μ*M* for each amino acid. The fluorescence response of all amino acids

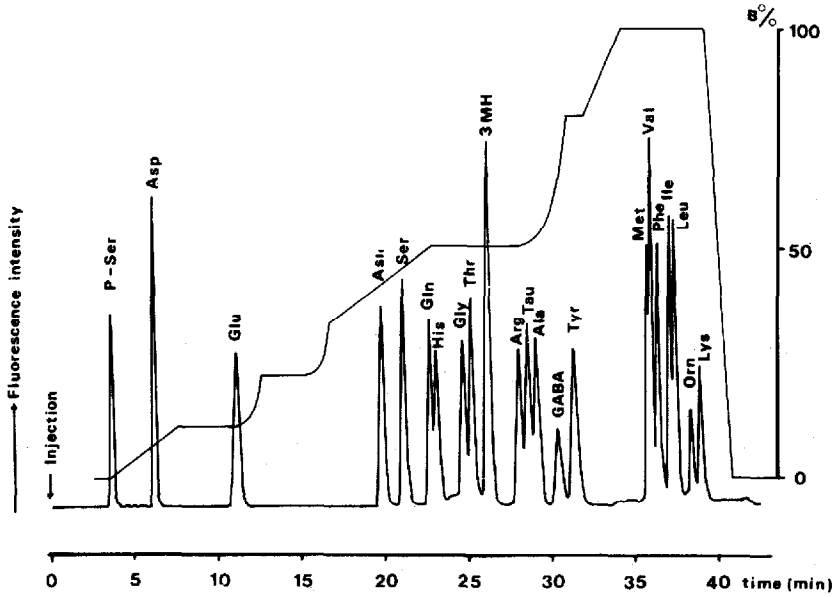

Fig. 1. Chromatogram of 23 amino acids (5 n*M*) as OPA-2ME derivatives. The gradient used is also shown.

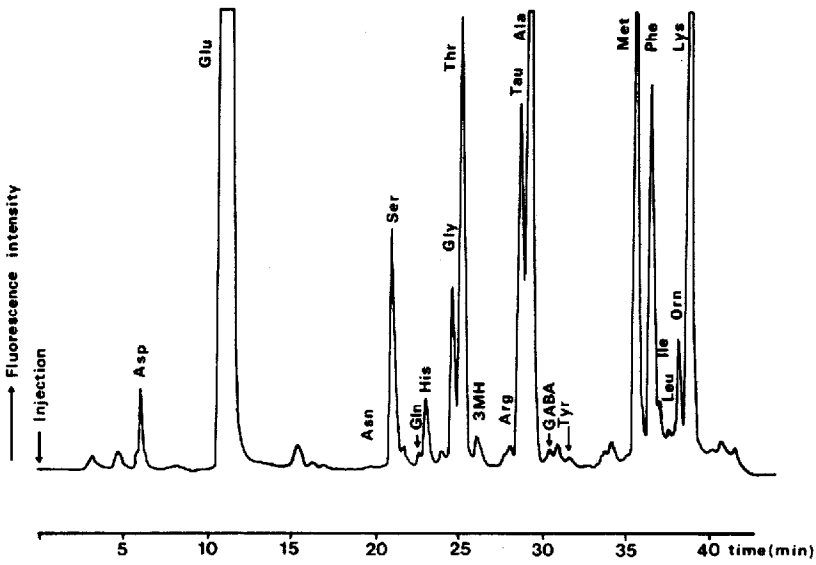

Fig. 2. Chromatogram of a CSF sample from a male rat immediately after ejaculation.

measured was linearly correlated with the amount injected over the range 20–400 pmol, giving $r = 1$.

The mean concentrations of GABA, Asp and Glu increased after ejaculation and after the end of the post-ejaculatory interval, whereas those of Ser, Arg, Ala and Leu decreased (see ref. 14). There were no alterations in any of the other amino acids measured. Fig. 2 shows a chromatogram of the CSF sample taken immediately after ejaculation.

This study has provided a method for the rapid (within 40 min) quantitation of amino acids and yielded highly reproducible results (R.S.D. = 0.2–2%) for the retention times and the integrated areas of all amino acids. The automatic on-line system avoids the variations due to the instability of the amino acid–OPA reaction. In applying this method to the determination of amino acids in CSF samples, special care was taken in the treatment of the CSF samples. Thus, on treatment with SSA at low temperatures, five replicate analyses gave R.S.D. values within 1–2% for all amino acids. These are substantially lower than the R.S.D. values in previous clinical studies on, *e.g.*, GABA[6–8].

Using the present method, we were able to record significant alterations in the concentrations of Asp, Glu and GABA in CSF samples obtained from freely moving male rats during various states of sexual excitation[14]. As GABA is a major inhibitory neurotransmitter[1], it may be of particular interest to note the increase in GABA levels after ejaculation, a physiological state during which the rat is refractory to sexual stimuli[15] and has an altered sensitivity to other sensory stimuli. The alterations in Asp and Glu levels are also of potential interest inasmuch as these amino acids are well known excitatory neurotransmitters[1]. However, as these amino acids serve as metabolic substrates for GABA formation in the brain[3,16], the alterations in their concentrations in the CSF samples measured under the present conditions may not

necessarily reflect alterations in neurotransmitter function. More work is required to delineate the physiological implications of the alterations in the CSF concentrations of Asp and Glu found in this study and also as the decreases we found in the CSF concentrations of Ser, Arg, Ala and Leu. These latter amino acids have no known relation to neurotransmission.

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