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In-use stability of monoamine metabolites in human cerebrospinal fluid

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Abstract

Cerebrospinal fluid (CSF) concentrations of the monoamine metabolites homovanillic acid (HVA) and 5-hydroxyindolacetic acid (5-HIAA) are commonly used to provide information about central nervous system (CNS) dopaminergic and serotonergic activity. However, little attention has been given to the effects of sample handling on the concentrations of these compounds in human CSF. Using high-performance liquid chromatography (HPLC) with electrochemical detection, we observed that, in CSF stored at −80°C, concentrations of the serotonin metabolite 5-HIAA and the dopamine metabolite HVA remained unchanged through six 1-h and six 24-h freeze–thaw cycles. Exposure to bright room light (3 h, 1230 lux) resulted in a 5-HIAA concentration that was 96.3±2.0% of the initial and an HVA concentration that was 98.8±1.03% of initial. The pH of the CSF significantly increased during both freeze–thaw series and while maintained on ice (4°C). These results demonstrate the in-use stability of 5-HIAA and HVA in human CSF under commonly-encountered laboratory conditions. Published by Elsevier Science B.V.

Keywords: Monoamines; Homovanillic acid; 5-Hydroxyindolacetic acid

1. Introduction

The monoamines serotonin (5-HT) and dopamine (DA) are widely recognized as important neurochemicals in humans as well as in all other animals that possess a nervous system [1,2]. The cerebrospinal fluid (CSF) which surrounds the brain and spinal cord of mammals not only provides the microclimate to support neuronal function but may be involved in diffusion neurotransmission [3]. In humans, CSF is a major medium in which the dynamics and metabolism of these monoaminergic systems can be readily accessed and studied. CSF concentrations of the major serotonin metabolite, 5-hydroxyindolacetic acid (5-HIAA), and the major DA metabolite, homovanillic acid (HVA), are commonly used as indices of serotonergic and dopaminergic activity, respectively, in humans and other primates. A May 2001 Medline search, using the keywords: (homovanillic acid OR HVA OR 5-hydroxyindolacetic acid OR 5-HIAA) AND (cerebrospinal fluid OR CSF) yielded 2,417 reports.

Surprisingly, little attention has been given to the way in which the handling of cerebrospinal fluid

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samples in the laboratory might affect the concentrations of serotonin and dopamine metabolites. For example, we are unable to locate any articles on the effects of repeated freezing and thawing or on the effects of light on these compounds in CSF. However, a number of studies have explored the stability of the parent compounds as well as their metabolites in other complex biological matrices and standards. Kass Simon's group, using high-performance liquid chromatography (HPLC) with electrochemical detection, observed that exposure to ''indirect sunlight'' reduces concentrations of 5-HT [4]; however, these assays were performed at supraphysiological concentrations and in standard solutions rather than in biological media. Urinary 5-HIAA remains stable for up to 3 weeks from −20°C to 20°C [5] but when stored at −18°C is no longer detectable after 6 months [6]. In plasma stored under the same conditions, 5-HIAA can no longer be detected after 9 months. 5-HIAA in CSF held at −70°C is stable for at least 1 month and in CSF held at 4°C and room temperature its concentrations remain constant for 24 h [7]. In postmortem CSF samples, the concentrations of 5-HT decrease significantly with postmortem interval while the concentration of 5-HIAA remains stable up to 1815 min [8]. HVA concentrations in urine (maintenance pH 2–5) are reportedly stable from −20°C to 20°C for up to 3 weeks [5] but begin to decrease after 3 months of storage at −18°C [6], while in plasma, at the same temperature, HVA levels remain constant for up to 9 months [6]. In canine brain homogenates, stored at −80°C, HVA is very stable; its concentration remains virtually unchanged for up to 44 months, while over the same period concentrations of DA markedly decrease [9]. In human CSF, HVA levels remain constant both at 4°C and room temperature for up to 24 h and are reportedly unaffected by freezing immediately after lumbar puncture [10]. Also CSF HVA measured at autopsy is unaffected by postmortem time, at least up to 1,815 min [8]. CSF samples can be frozen after collection then thawed once for HPLC assay without changing the concentrations of either HVA or 5-HIAA [10]. However, no assessment of refreezing or repeated freezing and thawing were mentioned in this report [10]. In short, the results from previous experiments are difficult to interpret as a result of differing methods, storage conditions, subject populations and treatment paradigms.

CSF is a remarkably robust medium although it contains less than 0.5% of the total protein found in plasma and is poorly buffered [11]. As might then be expected, despite high interindividual basal variability, CSF pH increases have been observed to occur immediately after its removal from patients with intracranial hypertension or multiple sclerosis [12]. In fact, within the first 60 s to 2 h after its withdrawal, pH increases to as high as 10 at temperatures of 4°C, 22°C, and 37°C [12]. Theoretically, high pH could affect the stability of 5-HIAA and/or HVA.

Using HPLC with electrochemical detection, we assessed changes in the CSF concentrations of HVA and 5-HIAA after both exposure to light and repeated freeze–thaw cycles. We also investigated the spontaneous changes in pH and determined the pH of 12 samples that had been stored at −80°C for periods between 72 h and 10 years.

2. Experimental

2.1. Chemicals and reagents

Standards were obtained from Research Biochemical Incorporated (Natick, MA, USA), sodium acetate from EM Science (Gibbstown, New Jersey, USA), citric acid, 1-octanesulfonic acid (OSA) and acetonitrile (ACN) from Fisher Scientific (New Jersey, NJ, USA) and ethylenediaminetetraacetic acid (EDTA) from Sigma (St. Louis, MO, USA). All reagents were HPLC grade.

2.2. Collection and storage of CSF

Subjects followed a low monoamine diet for at least 3 days prior to admission to the Veterans Affairs Medical Center, Cincinnati. A standard 666 calorie meal (20% protein, 24% fat and 56% carbohydrate) was consumed the evening before CSF collection began after which participants fasted for approximately 12 h before a catheter was placed in the lumbar subarachnoid space. Normal saline was infused at 100 ml/h during the experiment via a catheter previously inserted into an antecubital vein in the non-dominant arm. At approximately 08:00 h, after administration of intradermal lidocaine anesthesia, a 17 ga Tuohy–Schliff spinal needle was

inserted through the L3–L4 interspace as described previously [13,14]. After entry into the subarachnoid space, a 20 ga polyamide catheter was advaced cephalad 5–15 cm, capped and secured externally with tape. Later, the subarachnoid catheter was extended with 0.08 mm diameter silicone tubing which was attached to a peristaltic pump as previously described [13]. The total dead space in this system averaged <1.2 ml. At 11:00 h, approximately 3 h after subarachnoid catheter placement, CSF sampling was begun. CSF was continuously withdrawn into iced polypropylene test tubes (4°C) at a rate of 0.1 ml/min. No anti-oxidants or other additives (e.g. ascorbate) were added as previous work had shown that they are unnecessary [10,15] and potentially complicated chromatographic analysis of monoamine metabolites by increasing the size of the solvent front [15]. Every 10 min, the 1 ml of CSF was collected from the test tube and separated into four 250 μl aliquots (in polypropylene microcentrifuge tubes) which were frozen on dry ice at the bedside. The microcentrifuge tubes were then transferred from the dry ice to a freezer where they were held at −80°C until assayed; however, they were thawed once during the creation of the pool. For time-dependent pH changes during storage at −80°C, individual samples from normal volunteers and patients with posttraumatic stress disorder were used. These samples were obtained as described above but were held at −80°C, in polypropylene microcentrifuge tubes, for periods ranging from 72 h to 10 years (mean 89.6 months, median 82.9 months), during which time they had never thawed.

2.3. Light exposure

On the day of assay the CSF pool was thawed for 4–5 min at room temperature (22°C) and immediately transferred to wet ice (4°C). CSF was transferred in 80 μl aliquots to either clear or aluminum foil-wrapped 12×75 mm polystyrene culture tubes (Fisher Scientific, Pittsburgh, PA; 14-956-3D). All tubes were capped and placed horizontally on a stainless steel tray in wet ice and exposed to room light (intensity: 1230 lux). Temperature was monitored at 10-min intervals throughout the experiment and ice was replenished hourly. Light intensity was monitored with a digital light meter (Sper Scientific Limited, Scottsdale, AZ; 840020). Tubes were removed from the light treatment at 30 min, 1, 2 and 3 h; CSF was then removed and alliquoted into 300 μl vials (Bioanalytical Systems, West Lafayette, IN; MF-5270) that were capped and placed into a variable Spectra System refrigerated autosampler (ESA Incorporated Chelmsford, MA, USA; Model 540; 4°C). Injections (15 μl) were made in duplicate.

2.4. Freeze-thaw procedure

A CSF pool was prepared in a 12×75 mm culture tube. Thawing was performed at room temperature for 5 min and refreezing entailed an initial freeze in dry ice followed by placement into a −80°C freezer for 1 or 24 h. After each thaw, the tube was vortexed for 15 s and a 100 μl sample was removed and transferred to a 300 μl vial. 15 μl injections were made in duplicate.

2.5. pH measurements

CSF pH was determined with a micro pH meter which required <80 μl of CSF (B-213 Twin pH meter, Horiba, Scottsdale AZ, USA). Samples were vortexed for 15 s and 80 μl of CSF were removed and placed onto the pH sensor which was calibrated daily. pH determinations were performed in tandem with the HVA and 5-HIAA measures.

2.6. Assessment of pH after storage at −80°C

Samples (described above) were stored in 500 μl polypropylene microcentrifuge tubes (Fisher Scientific Pittsburgh, PA, USA; 0540615) and were removed from the −80°C freezer and thawed for 1–2 min. Samples were then vortexed for 15 s and 80 μl were removed and placed onto the pH sensor. Additionally, after determination of 4 samples from the pool, 4 more aliquots of the pool were held at −80°C for pH determination after 30 days.

2.7. Mobile Phase

All analyses were performed using mobile phase previously described by Artigas et al. and Schmidt et al. [16,17] with slight modifications. The mobile phase consisted of (in m*M*) 22.84 citric acid, 15.06 sodium acetate, 2.31 sodium octanesulfonic acid and 0.0067 EDTA and was adjusted with sodium hy-

droxide to pH 3.4±0.02 at 22°C. Thirteen percent acetonitrile (ACN) was added as an organic modifier. The eluate was mixed and vacuum filtered using a solvent filtration apparatus equipped with a 0.2 μm membrane filter. Mobile phase was recirculated throughout the system at a rate of 1.2 ml/min.

2.8. Chromatographic analysis

A variable Spectra System refrigerated autosampler (ESA Incorporated Chelmsford, MA; Model 540; 4°C) was programmed to inject two 15 μl samples onto a Hypersil BDS column (Alltech, Deerfield, IL, C_{18}, 5 μ, 250×4.6 mm). Thawed samples never reached room temperature. Detection was performed by an electrochemical detector as previously described [13]. Potential settings were as follows: E=300 mV, E_1=−50 mV, E_2=350 mV, R_1=1 μA, R_2=50 nA.

2.9. Calculations and statistics

The initial concentration for each sample was set to 100% and subsequent concentrations were expressed as a percentage of the initial concentration. Paired Student's *t*-tests (two-tailed) were used for comparisons between treatment groups (e.g. light and dark) as well as for comparisons between initial concentrations and the concentration after each thaw in the freeze–thaw experiments. A 1-way repeated measure ANOVA, using Greenhouse–Geisser epsilon to adjust for univariate tests, was applied to determine the significance of pH changes (SAS version 8.1, SAS Systems, Cary, NC). Means are reported±their standard errors and P values less than 0.05 are considered to be statistically significant.

3. Results

3.1. General chromatography and pH

Under our chromatographic conditions, 5-HIAA eluted at 6.5 min and HVA eluted at 8.3 min as shown in Fig. 1. The initial pH of the CSF pool was 8.22±0.01 (n=6).

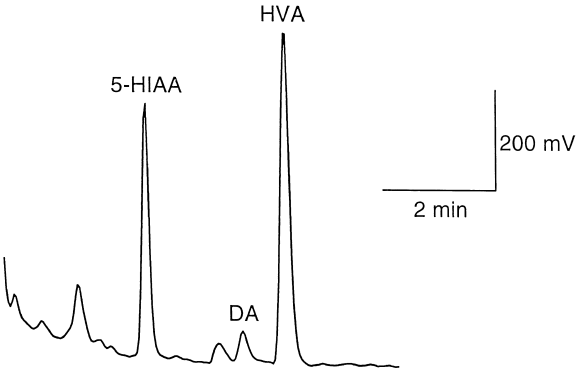

Fig. 1. A portion of a representative chromatogram illustrating the separation of 5-HIAA and HVA from human cerebrospinal fluid. Retention times for 5-HIAA and HVA were 6.5 and 8.3 min respectively.

3.2. Effects of light

There were no significant differences in the time-matched light-protected and light-exposed CSF 5-HIAA over 3 h of exposure to 1230 lux room light (Table 1, n=4, P=0.42 at 3 h). Similarly, exposure of the same CSF samples to ambient light did not result in significant HVA concentration differences in comparison with dark treatments over the same 3-h period (Table 1, n=4, P=0.95 at 3 h).

3.3. Effects of repeated freeze–thaw cycles

Repeated freezing and thawing of human CSF did not result in significant changes in the concentrations of either 5-HIAA or HVA after six 1-h freeze–thaw cycles. 5-HIAA and HVA concentrations were 96.3±2.0% and 98.8±1.03% of their initial concentrations respectively (n=3, NS, duplicate injections). Likewise, six 24-h freeze–thaw cycles did not result in significant differences in the concentrations of the metabolites compared with basal levels (n=3, NS, duplicate injections). Over the course of both the 1- and 24-h freeze–thaw series, pH fluctuated but remained within 0.7 units of the initial pH (n=6). During the 24-h freeze–thaw series, pH significantly increased from 8.22 to 8.7±0.05 (n=3, P=0.001) while for the 1-h freeze–thaw cycle, pH increased to 8.89±0.11 (n=3, P=0.004).

Table 1
The effects of light on the concentrations of 5-hydroxyindolacetic acid (5-HIAA) and homovanillic acid (HVA) in human cerebrospinal fluid at 4°C

Time	5-HIAA		HVA	
	Dark (0 lux)	Light (1230 lux)	Dark (0 lux)	Light (1230 lux)
Initial	100%	100%	100%	100%
30 min	100.62±0.27%	100.37±1.54%	102.38±4.88%	96.69±5.73%
1 h	99.46±1.26%	98.4±3.01%	99.36±0.87%	93.74±4.60%
2 h	97.60±3.55%	96.78±4.67%	97.09±2.06%	99.44±7.25%
3 h	98.79±1.96%	98.80±1.42%	95.56±1.25%	97.7±4.45%

3.4. pH changes in pooled CSF at 4°C

The initial pH of the CSF pool was at 8.22±0.03 at 4°C and increased throughout the 12 h assessment to a final pH of 8.52±0.04 ($P<0.04$, $n=3$).

3.5. pH after storage at −80°C

For samples that had been stored at −80°C between 72 h and 118.3 months, there was no significant correlation between pH and storage time ($P=0.78$, $n=12$). The pHs of the four samples that had been stored for longer than 100 months were 7.21, 7.63, 7.81 and 8.26. Moreover, the pH of pooled samples did not change after 30 days at −80°C ($n=4$, $P=1.0$)

4. Discussion

The present data indicate that human CSF can be frozen and thawed at least six times without significantly changing the concentrations of 5-HIAA and HVA. Also, during the routine quantification of these metabolites by HPLC, it is not necessary to protect the CSF specimens from ambient laboratory light (<1230 lux).

The finding that exposed CSF increases in pH replicates prior observations [12]. However, the observed absence of a relationship between CSF pH and storage time is of interest. This lack of a correlation may be due to an intrinsic quality of CSF, the very cold temperatures at which the CSF was stored, or our use of bedside flash freezing of samples on dry ice. More likely, it is due to unpredictable, individual variations in CSF antioxidant status [18]. It remains to be determined if the stability of CSF monoamine concentrations across multiple in-use handling conditions persists at extreme pH and temperature.

The present data suggest high in-use stability of both CSF HVA and 5-HIAA. These findings are consistent with and extend findings of storage stability of CSF [7,10,15]. Given the high stability of monoamine metabolites in CSF and their apparently low stability in other biological media, it is possible that some quality or attribute of CSF confers protection against degradation.

These results will be of particular interest to laboratories in possession of CSF samples that might have been previously thawed and exposed to light during the course of assays for other analytes as they suggest that residual samples can be reliably assayed without compunction.

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