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TECHNICAL NOTE

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Free Amino Acid Concentrations in Vitreous Humor and Cerebrospinal Fluid in Relation to the Cause of Death and Postmortem Interval

ABSTRACT: We studied free amino acids in vitreous humor and cerebrospinal fluid from 58 cadavers in the course of routine medicolegal autopsies in the city of Granada. The main objective was to establish whether free amino acids contents in these fluids were related with the cause of death, postmortem interval, and severity of the classic signs of asphyxia. The amino acids (aspartic acid, glutamic acid, serine, glutamine, glycine/threonine/histidine, citruline, arginine, alanine, taurine, GABA, tirosine, valine, methionine, isoleucine, phenylalanine/tryptophan, leucine, and lysine) were quantified by high performance liquid chromatography. There were no statistically significant differences in amino acids concentrations in vitreous humor when the different causes of death were considered. Our results did not show any statistically significant relationship when asphyxial score was plotted against the vitreous content of each amino acid. A statistically significant increase with postmortem interval was observed in vitreous taurine ($r = 0.3191$, $p = 0.01461$), glutamate ($r = 0.4323$, $p = 0.0007$) and particularly in aspartate ($r = 0.4508$, $p = 0.0003$).

KEYWORDS: forensic science, vitreous humor, cerebrospinal fluid, amino acids, postmortem interval, postmortem chemistry, cause of death

Numerous methods have been used to estimate the time after death, particularly for short postmortem intervals (PMI). These methods involve most of the biochemical compounds in blood, vitreous humor, cerebrospinal fluid (CSF), and to a lesser extent in pericardial fluid. Nonetheless, many of these methods show a broad margin of error even under controlled conditions, and most of them have considered only the first day after death (1).

One exception to these criticisms is potassium concentration in the vitreous humor, which has been investigated in detail by different groups (2–5). Despite its large margin of error within the first 24 h after death, it is generally accepted as the best biochemical test available for longer PMI. In 1963, Sturner (2) was the first to report a formula to calculate PMI from vitreous potassium concentration; this approach was reformulated later using modern statistical methods (3–5), and improved by the use of potassium as the independent variable, instead of the dependent variable (5,6).

In recent dates, new methods developed in radiology, like H magnetic resonance spectroscopy (H-MRS), have been applied for the identification of metabolites emerging during decomposition of brain tissue, both in sheep brain models by Scheurer et al. (7) and in porcine brain model by Banaschak et al. (8). Their results show more precision in estimation of longer PMI.

Few publications deal with the possible role of amino acids in postmortem chemistry. Purcher and Burd (9) and Schourup (10) described a sharp rise in free amino acid nitrogen level both in blood and in CSF, probably as a result of enzymatic hydrolysis of proteins. Schleyer (11) investigated samples obtained up to 80 h postmortem, and found results in accordance with those published by Schourup. These findings suggested that the concentrations of amino acids could be used to determine PMI during the first 20 h after death: values of amino acid nitrogen not over 14 mg% probably correspond to PMI of <10 h. Vass et al. (12) studied aminoacids, neurotransmitters, and other decompositional products in various organs in relation to PMI, revealing distinct patterns useful for determining PMI up to a range of *c.* 3 weeks. The most significant biomarkers for the establishment of PMI in all tissues studied were GABA, proline, methionine, and oxalic acid.

Erdei and Vass (13) and Durham et al. (14) reported the presence of free amino acids in the vitreous humor. Later, Patrick and Logan (15) established, in sudden infant death syndrome (SIDS), that the concentrations of all vitreous amino acids rose as PMI increased. However, samples needed to be obtained within the first 24 h after death to give reliable results. These results showed no indication of a specific aminoacidopathy as a factor in SIDS.

With the exception of the publication by Patrick and Logan (15) and Vass et al. (12) most analysis of amino acid have been performed with old methods, such as amino acid nitrogen analysis with the Folin naphthoquinone method (11) or paper chromatography (13). In the present study, we tried to correlate free amino acid concentrations in CSF and vitreous humor with the PMI, and also to investigate a possible relationship with the cause of death, by the use of modern high performance liquid chromatography (HPLC).

Materials and Methods

Material

Samples were taken from 58 cadavers (18 females and 40 males aged 13–97 years) in the course of routine medicolegal autopsies at

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the city of Granada. Average PMI was 23.9 h (range 5–60 h), although most autopsies (67.25%) were performed within the first 24 h after death. PMI was determined from witnessed death (in most cases) or death certificate and corroborated at the time of autopsy with the state of postmortem changes.

The different causes of death considered were determined after autopsy: asphyxia (27.5%), poisoning (15.5%), cardiac (24.2%), traumatic (17.3%), and miscellaneous deaths (15.5%).

Apart from ordinary autopsy reports, age, sex, estimated PMI, organ weights, and data from the clinical history related to the cause of death were recorded in every case. The "classic" signs of asphyxia were scored for severity. A score was assigned only by two of the authors in every case, on a four-point scale of 0 (absence), 1 (scarce), 2 (mild), or 3 (intense) for each of the five "classic" signs of asphyxia evaluated: petechial hemorrhages, cyanosis, congestion, pulmonary edema, and fluidity of the blood. For agreement among different observers, we reserved a score of 3 for petechial hemorrhages when there were multiple, spreading on conjunctivae, some parts of the skin, and under the thoracic serous membranes, while we reserved a score of 2 when they were evident but not present in all these zones. In relation to cyanosis, we assigned a score of 3 when this color was evident in the whole face and neck, while assigned a score of 2 when it was evident only in lips, fingers, and toes. In relation to pulmonary edema, we assigned a score of 3 when it was quite evident before lung dissection (froth exuding from mouth or nostrils or in trachea and main bronchi and lungs very increased in weight and size), while we assigned a score of 2 when edema was evident only after lung dissection (scarce froth in secondary bronchi, or only evident after tissue squeezing).

A sample of the vitreous humor was taken in each cadaver from both eyes, using a sterile syringe.

A sample of CSF was taken with a sterile syringe in each cadaver from the lateral ventricles after brain dissection according to Virchows' method.

Methods

Samples of CSF were centrifuged after autopsy and the supernatant was used. To detect possible blood contamination, the benzidine test was performed on each CSF. All samples were properly labeled and frozen at −70°C until analysis. Vitreous humor samples were frozen without additional handling. The time elapsed from collection to storage of samples was 90–140 min.

The OPA derivatives of aspartic acid, glutamic acid, serine, glutamine, glycine/threonine/histidine, citruline, arginine, alanine, taurine, GABA, tyrosine, valine, methionine, isoleucine, phenylalanine/tryptophan, leucine, and lysine were quantified by HPLC coupled to fluorimetric detection, according to the method previously described by Peinado et al. (16) with minor modifications. The samples were mixed with homo-serine as an internal standard and deproteinized with Ultrafree-MC filters (Millipore, Bedford, MA). A 40-µL sample was mixed with the OPA reagent and automatically injected into a Waters Resolve C-18 column (Waters, Milford, MA). The mobile phase consisted of sodium phosphate buffer as solvent A, plus acetonitrile/water (50:50) as solvent B. The gradient was from 90:10 (A/B) to 100 (B) in 25 min. The chromatograms were recorded and quantified with the Baseline program (Waters).

Group means were compared with one-way analysis of variance (ANOVA). Linear regression analysis was used to determine the correlations between amino acid concentrations and PMI or asphyxial score in every case.

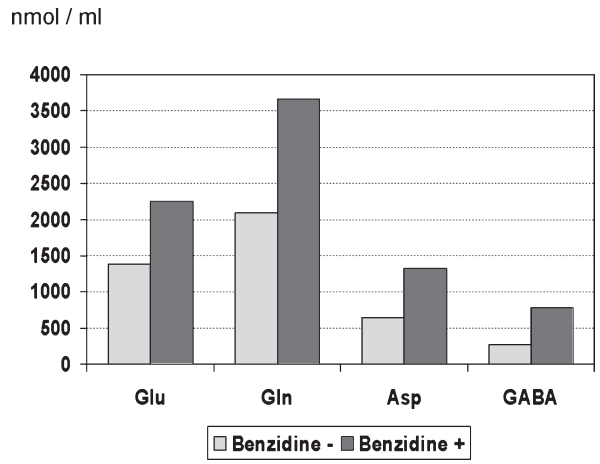

FIG. 1—*Free amino acids in benzidine (+) and benzidine (−) samples of cerebrospinal fluid.*

Results

The concentration of all amino acids in CSF was statistically higher in benzidine positive than in benzidine negative samples. The results for glutamate (Glu), glutamine (Gln), aspartate (Asp), and GABA, the most representative amino acids within the central nervous system (CNS) are shown in Fig. 1.

The concentrations of all amino acids in the vitreous humor were lower than in the CSF.

There were no statistically significant differences in vitreous amino acid concentrations when the different causes of death were considered (Figs. 2 and 3). We found no statistically significant relationship when we plotted asphyxial score against the vitreous content of each amino acid.

As expected, the asphyxial score was higher in asphyxia (mean = 7.7) and poisoning deaths, most of the latter due to pulmonary failure from drug abuse (mean = 8.3), than in traumatic (mean = 2.5) and miscellaneous deaths (mean = 3.4).

In relation to PMI, a statistically significant increase with time was observed in vitreous concentrations of taurine ($r = 0.3191$, $p = 0.01461$), glutamate ($r = 0.4323$, $p = 0.0007$), and particularly in aspartate ($r = 0.4508$, $p = 0.0003$) (Fig. 4).

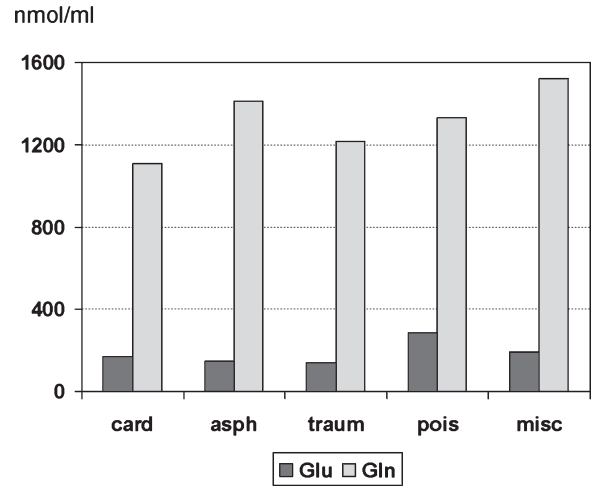

FIG. 2—*Vitreous glutamate and glutamine in different causes of death.*

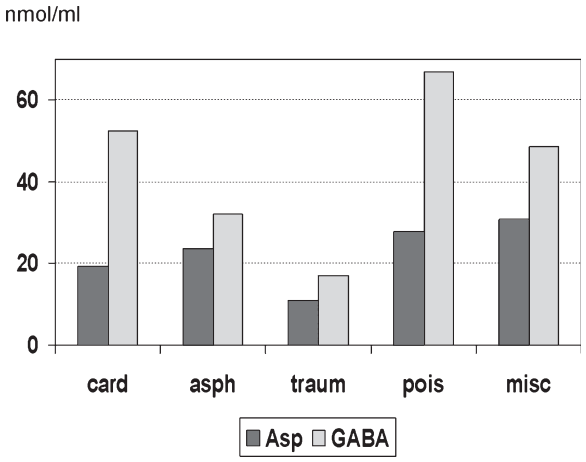

FIG. 3—*Vitreous aspartate and GABA in different causes of death.*

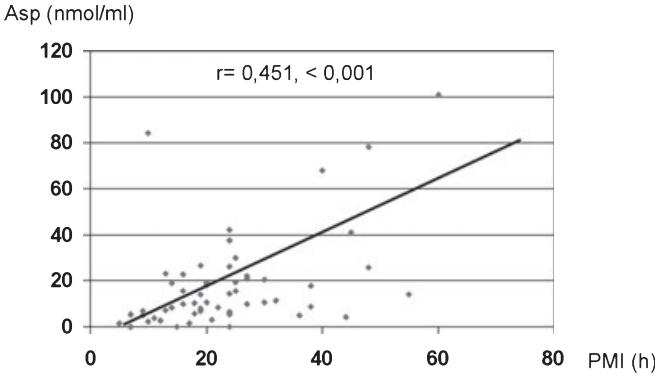

FIG. 4—*Linear increase in aspartate with postmortem interval.*

Discussion

We analyzed the concentrations of amino acids in CSF and vitreous humor from cadavers to establish the possible relationships between these values and the cause of death, severity of signs of asphyxia, and PMI.

There is some controversy about postmortem changes in amino acids within the CNS. Perry et al. (17) found that glutamate, glutamine, and taurine remained unchanged in rat and human brains under simulated mortuary conditions. GABA increased very rapidly during the first 3 h, while aspartate rose slowly after the first 4 h. Vass et al. (12) found linear increases of GABA, methionine, aspartate, isoleucine, phenylalanine, tyrosine, histidine, and proline, useful for the establishment of PMI, particularly in early time ranges. Kärkelä (18) found a linear increase after death in most CSF amino acids during the first 24 h. Particularly, evident was the increase in GABA and glutamate during the first 2 h postmortem, suggesting that these amino acids were potentially useful in forensic medicine for the determination of PMI.

In our hands, the levels of all the amino acids studied in CSF from cadavers were higher than in CSF from live people (19); the difference was particularly evident in benzidine positive samples. This suggests that CSF samples were contaminated with blood and therefore yielded highly variable amino acid contents. Because of this possibility, the CSF data were not evaluated further. Thus, collection of CSF from the lateral ventricles after Virchows' brain dissection does not seem a suitable technique, being probably a better way to obtain CSF from suboccipital cisternal puncture.

Most amino acid concentrations in the vitrous humor tended to increase with lengthening PMI. This relationship was statistically significant for taurine, glutamate, and particularly aspartate, which increased linearly after death.

Very few publications deal with amino acids in vitreous humor. Patrick and Logan (15) found that all amino acid concentrations were directly related to the PMI in a logarithmic linear regression, although at different rates.

It is known that the lower levels of amino acids in the vitreous humor, in comparison to plasma under steady state conditions, is due to their constant removal from vitreous humor into blood by an active transport mechanism located in the pigment epithelium of the retina (20). Thus, the increase in amino acid concentrations in the vitreous humor after death is probably caused by breakdown of the blood-vitreous barrier as a result of increased permeability of the pigment epithelium, as well as to a failure of the transport mechanism. Proteolysis may also contribute to this increase; this factor would account for the generalized rise in most amino acids. We found a positive correlation between vitreous aspartate content and PMI, suggesting that this amino acid may serve as additional data, particularly in difficult cases, to establish the time since death.

We found no statistically significant differences in vitreous amino acid content in relation to the different causes of death. Moreover, there was no evidence that vitreous amino acid concentrations were related to asphyxial score.

One of the main objectives of this study was to assess whether any of the amino acids, particularly glutamine, could be used as a marker of asphyxial deaths. Glutamine is not only one of the most abundant amino acids in the brain, where it is a precursor of transmitters such as glutamate and GABA, but also plays an important role in the ammonia-detoxifying mechanism. In fact, Coe (1) found high concentrations of glutamine in CSF associated with hepatic failure. Glutamine increases in the brain gradually as a result of increasing CO_2 pressure, whereas glutamate decreases (21,22), and the same effect is observed in plasma (23). Because the ammonia content is increased under hypercapnic conditions, stimulated synthesis of glutamine from glutamate has been suggested to remove excess amounts of ammonia in the brain (21). Nevertheless, we found no significant differences in vitreous glutamine content between the different causes of death considered here. Nor did we find variations in vitreous amino acid concentrations in relation to the asphyxial score.

In conclusion, these preliminary results show that vitreous aspartate concentration may be a useful tool for additional data to establish PMI in difficult cases. Further studies are needed with larger sample sizes, CSF obtained by suboccipital cisternal puncture, and an improved classification of the cause of death and asphyxial score system.

Acknowledgments

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References

1. Coe JI. Postmortem chemistry update. Emphasis on forensic application. Am J Forensic Med Pathol 1993;14(2):91–117.
2. Sturner WQ. The vitreous humour: post-mortem potassium changes. Lancet 1963;1:807–8.
3. Villanueva E. Progresos técnicos de la autopsia médico-legal y sus exámenes complementarios. In: Gisbert-Calabuig JA, editor. Técnicas bioquímicas, Actas de las II Jornadas Mediterráneas de Medicina Legal. Valencia: Universidad de Valencia, 1977;45–70.

4. Madea B, Henssge C, Hönig W, Gerbracht A. References for determining the time of death by potassium in vitreous humor. Forensic Sci Int 1989;8:231–43.
5. Munoz JI, Suárez-Penaranda JM, Otero XL, Rodríguez-Calvo MS, Costas E, Miguéns X, et al. A new perspective in the estimation of postmortem interval (PMI) based on Vitreous [K+]. J Forensic Sci 2001;46(2):209–14.
6. Munoz Barús JI, Suárez-Penaranda JM, Otero XL, Rodríguez-Calvo MS, Costas E, Miguéns X, et al. Improved estimation of postmortem interval (PMI) based on differential behaviour of vitreous potassium and hypoxantine in death by hanging. Forensic Sci Int 2002;125:67–74.
7. Scheurer E, Ith M, Dietrich D, Kreis R, Husler J, Dirnhofer R, et al. Statistical evaluation of time-dependent metabolite concentrations: estimation of post-mortem intervals based of in situ 1H-MRS of the brain. NMR Biomed 2005;18(3):163–72.
8. Banaschak S, Rzanny R, Reichenbach JR, Kaiser WA, Klein A. Estimation of post-mortem metabolic changes in porcine brain tissue using 1H-MR spectroscopy – preliminary results. Int J Legal Med 2005;119:77–9.
9. Purcher G, Burd L. A preliminary study of the chemistry of post-mortem blood and spinal fluid. Bull Buffalo Gen Hosp 1925;3:11–3.
10. Schourup K. 1950 (quoted by Coe JI). Postmortem chemistry update Emphasis on forensic application. Am J Forensic Med Path 1993;14:91–117.
11. Schleyer F. Determination of the time of death in the early postmortem interval. In: Lundquist F, editor. Methods of forensic science, Vol. II. London: Wiley Interscience, 1963;253–93.
12. Vass AA, Barshick SA, Sega G, Caton J, Skeen JT, Love JC, et al. Decomposition chemistry of human remains: a new methodology for determining the post-mortem interval. J Forensic Sci 2002;47(3):542–53.
13. Erdei Z, Vass Z. Chromatographic investigations of free amino acids of the vitreous body. Acta Ophthalmol 1967;45:22–4.
14. Durham DC, Dickinson JC, Hamilton PB. Ion-exchange chromatography of free amino acids in human intra ocular fluids. Clin Chem 1971;17:285–9.
15. Patrick WJA, Logan RW. Free amino acid content of the vitreous humor in cot deaths. Arch Dis Child 1988;63:660–2.
16. Peinado JM, Mc Manus KT, Myers RD. Rapid method for microanalysis of endogenous amino acid neurotransmitters in brain perfusates in the rat by isocratic HPLC-EC. J Neurosci Methods 1986;8:269–76.
17. Perry TL, Hansen S, Gandham SS. Post-mortem changes of amino compounds in human and rat brain. J Neurochem 1981;36(2):406–12.
18. Kärkela JT. Critical evaluation of post-mortem changes in human autopsy cisternal fluid. Enzymes, electrolytes, acid-base balance, glucose and glycolysis, free amino acids and ammonia. Correlation to total brain ischemia. J Forensic Sci 1993;38(3):603–16.
19. Iribar MC, Montes J, González-Maldonado R, Peinado JM. Alanyl-aminopeptidase activity decrease in cerebrospinal fluid of Alzheimer patients. Dement Geriatr Cogn Disord 1998;9:44–9.
20. Reddy VN, Chakrapani B, Lim CP. Blood-vitreous barrier to amino acids. Exp Eye Res 1977;25:543–54.
21. Weyne J, Van Leuven F, Leusen I. Glutamate and glutamine in the brain: influence of acute PCO_2 changes in normal rats and in rats under sustained hypercapnia or hypocapnia. Life Sci 1973;12:211–8.
22. Siesjö BK, editor. Brain energy metabolism. New York: Wiley Interscience, 1978.
23. Kvamme E. Glutamine. In: Lajtha A, editor. Handbook of neurochemistry. Vol 3. Metabolism in the nervous system. New York: Plenum Press, 1983;405–22.

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