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The Ability of Vitreous Metabolite Concentration to Determine Time of Death

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Abstract

This paper analyzes the ability of vitreous fluid to determine time of death. The high variability in current methods used to establish post-mortem interval (PMI), or the amount of time between death and post-mortem examination make it difficult to obtain an accurate estimation. The concentration of metabolites found within vitreous fluid, such as potassium (K+), hypoxanthine (Hx), and magnesium (Mg), are the most researched alternatives to traditional methods in PMI determination because of the relative isolation of vitreous within the body and its ability to withstand degradation. There is no clear consensus on which metabolite provides the most accurate data and whether it is reliable enough to incorporate vitreous analysis into the standard method of establishing PMI. This study examines previously conducted research in order to identify the cause of discrepancies and proposes potential analysis improvement. The CRAAP [Currency, Relevancy, Authority, Accuracy, and Purpose] method of evaluation determined research validity, with each component assigned a numerical value. Studies with higher aggregate scores were considered the most useful and their results the most credible. Through this research paper, K+ was the most viable metabolite to determine PMI with the highest accuracy obtained when Hx and temperature were analyzed. Furthermore, samples of vitreous fluid should be collected in a single draw, centrifuged before testing, and subjected to the selective ion electrode method to measure K+ concentration and Hx HPLC.

Keywords

post-mortem interval, time of death, vitreous, thanatochemistry

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Introduction

In order for criminal justice to work, forensic analyses must provide accurate and reliable results. However, determining time of death—one of the most critical aspects of the investigative process—utilizes highly variable methods. Post-mortem interval (PMI), or the period of since death, is primarily determined by examining a decedent's rigor, algor, and livor mortis or, respectively, their muscle rigidity, body temperature, and blood-settling pattern. Just as rigor and livor mortis are measured subjectively, variables such as ambient temperature, body composition, or rate of decomposition influence all three factors. This variability leads to unreliable and lengthy proposed intervals, especially concerning in criminal cases, where an accurate PMI could corroborate or refute alibis.

Vitreous humor, a gel-like fluid found between the lens and retina of the eye, is relatively isolated within the body. This isolation protects the vitreous from post-mortem degradation—which blood and spinal fluid are subject to—leaving the fluid relatively unchanged by factors that often alter rigor, algor, and livor mortis, making it an ideal analyte for determining PMI. Vitreous fluid contains several metabolites, including potassium (K+), hypoxanthine (Hx), and magnesium (Mg).

Most research into vitreous analysis focuses on the change in potassium concentration after death. Due to the natural concentration gradient between the intracellular fluid and plasma during life, the concentration of K+ is significantly higher at the intracellular level. After death, the pump that transports K+ across this gradient breaks down, and potassium concentration equalizes. The linear nature of this change, described in various studies, makes K+ ideal for estimating the time of death (Rognum et al., 2016). Despite the number of studies that conclude K+ and time

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since death fit a linear model, those findings' validity has been questioned. Many suspect that this model overestimates the observed relationship (Karla et al., 2016).

Adenosine monophosphate (AMP) breaks down after death and diffuses out from the retina into the center of the vitreous, creating hypoxanthine (Hx). While evaluating the potential significance of Hx in determining PMI, Rognum et al. (2016) found a linear relationship between hypoxanthine concentration and time; their data produced an r-squared value higher than those seen in K+ analysis, suggesting that Hx has a stronger linear relationship with time and, thus, better indicates PMI. Madea and colleagues (1994) attempted to replicate these results but found K+ to have a stronger correlation to time, just as earlier studies concluded. Although the study refuted a stronger correlation between Hx and time, the relationship was still confirmed to be linear (Madea et al., 1994).

Conflicting data et al.,are prevalent within post-mortem biochemical analysis of vitreous humor (Madea, 1994; Rognum et al., 2016). However, little research has been conducted to investigate this conflict, and no consensus on the source of its discrepancies exists. Therefore, an analysis of previous research will provide insight into which metabolite should focus on post-mortem analysis and which technique should be utilized to do so.

Literature Review

Potassium and Hypoxanthine

The various analytes found within vitreous humor, including electrolytes, glucose, and ketones, are discussed by Baniak et al. (2014). Potassium (K+) is an electrolyte that moves through the vitreous fluid via a semi-selective membrane and a sodium (Na)/K+ ATPase pump. The post-mortem breakdown of these analytes causes K+ to decrease intracellularly at a rapid rate and plasma concentration to sharply increase until the two concentrations have equilibrated. Most studies, as Baniak et al. (2014) notes, describe the relationship between K+ and time since death as linear. The accuracy of this relationship has been challenged but with current studies repeatedly confirming a linear relationship, this question is likely a result of either outdated or improper analytical research.

Salam et al. (2012) argue that vitreous humor is an ideal for establishing a time of death because of its relative isolation within the body and resistance to change. Levels of K+ and hypoxanthine (Hx), products of degradation, are significantly higher within the cells than the plasma during life. After death, the membrane that separates the cells from the plasma breaks down, and metabolite levels adjusts until equilibrium is established between cellular and plasma concentrations.

Samples were taken from approximately 70 adult autopsy cases with a known PMI to measure the return to the equilibrium described in the study conducted by Salam and colleagues (2012). Statistical and graphical analysis show a linear relationship between PMI and potassium and hypoxanthine levels, where K+ concentration within the vitreous increased with PMI at a 95% confidence level (Salam et al., 2012). However, K+ was more accurate than Hx when time since death exceeded 60 hours, making it better suited for continuing research and possible use in forensic investigations.

The period in which metabolite levels are useful is another point of contention among scientists. Salam and colleagues (2012) studied K+ levels for as long as 60 hours post-mortem while previous research found K+ levels to be valid only up to two hours since time of death. Due to this discrepancy, K+ concentration alone is inadequate to estimate a post-mortem interval (Baniak et

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al., 2014). Traditional methods of determining PMI—such as rigor mortis and corneal turbidity—were accounted for and incorporated into the equation extrapolated from their results (Salam et al., 2012). No other studies have combined traditional and biochemical analysis in this way.

Magnesium Concentration

Most research on metabolite concentration and its relationship to time of death focuses on K+. However, Mihailović et al. (2014) examined the correlation between magnesium (Mg) concentration and PMI. Their decision to examine Mg levels came from the same logic used to justify the usefulness of K+: after death, a loss in selective membrane permeability causes Mg concentration to shift both intra- and extracellularly until establishment of equilibrium (Mihailović et al., 2014). Their study contained various controls, including temperature, same eye sampling, and the decedent's manner of death, and found that Mg concentration increased with time since death. Despite K+ and Hx also sharing the same linear relationship with PMI, the correlation of Mg is too weak to be considered statistically significant and, therefore, insufficient for forensic applications. This insufficiency can be attributed to the relatively low Mg concentration within vitreous fluid both ante- and post-mortem and how quickly equilibrium is established between the cells and plasma after death.

Improving Analysis

McNeil et al. (1999) propose a way to improve vitreous fluid analysis. Their study mentions the inaccurate findings of various research and questions the cause of their differing results. To standardize experimental procedures and improve results, McNeil and colleagues (1999) suggest heating vitreous samples to 100°C for five minutes. The heat treatment, via flame photometry, reduced the samples' viscosity and improved the measurement of K^+ concentration.

Theirauf et al. (2009) examined several potential pretreatments of vitreous, including heat, centrifugation, ultrasonic bath, and enzymatic digestion. Data was gathered on 2,000 samples and showed a trend toward higher accuracy on those treated by ultrasonic bath. However, because the study reported issues in data precision, it requires further investigation into the advantages of alternative pretreatment methods.

The studies conducted investigated the effects of pretreatment on K^+ concentration yet did not include Hx (McNeil et al., 1999; Theirauf et al., 2009). Recognizing Hx's strong correlation with time since death, Camba and colleagues (2014) researched how the pretreatments used in the Theirauf et al. study impacted its concentration. The results from the 2014 study by Camba et al. agreed with the previous study conducted by Theirauf et al. in 2009, both of which found the highest accuracy when vitreous samples were treated via ultrasonic bathing before analysis. In addition, heat was the least valuable treatment for Hx determination, as it produced the highest amount of data dispersion. Though it had been proposed that heating as an acceptable pre-analytical treatment when analyzing K^+ concentration (McNeil et al., 1999), the results from later studies (Camba et al., 2014; Theirauf et al., 2009) dispute, and directly conflict with, those earlier findings, increasing the number of discrepancies seen within vitreous metabolite research.

The overall lack of biochemical analysis in death investigations is described by Tormey (2016), who reviewed several cases that requiring autopsy within a year, including a percentage of those with vitreous humor sampled and analyzed. This study did not attempt to determine the value of vitreous fluid

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in establishing a PMI but instead highlighted the absence of biochemical analysis in the autopsy process. Furthermore, Tormey (2016) argues that a biochemical module should be added to certification requirements in pathology. Despite not explicitly looking to use vitreous to answer a question, Tormey's (2016) study still highlights how vitreous analysis is underutilized in the field as a whole.

Methodology

Studies that examine the relationships between various metabolite concentrations within vitreous fluid and time since death were qualitatively analyzed; all studies referenced were either published in peer-reviewed, scholarly journals or submitted as graduate theses to accredited universities. Because similar experiments produced conflicting results, each study's validity had to be determined to establish which experimental method produced the most reliable results and utilized the most accurate experimental techniques.

The CRAAP method evaluates sources based on five categories: currency, relevance, authority, accuracy, and purpose. Currency refers to how current the study is and traces any updates made to it. Relevance asks if the study adequately addresses the chosen research topic at an appropriately academic level. Authority decides whether the author has the proper credentials to collect and analyze data. Finally, accuracy ensures the study is based on proper science and factual truth, while purpose evaluates whether the author is trying to entertain or share scientific data. The source that received the highest aggregate CRAAP score was considered to have the most reliable data, and suggestions for how future research that should be conducted are based on the methods utilized by higher-scoring studies.

Studies conducted within the last five years or offered an update to previous research received the highest currency scores. These scores ensure that the information from the selected studies utilizes the most current knowledge and experimental procedures. All studies received high scores in relevance because any sources that did not accurately address the research question were disregarded before any CRAAP evaluation. Like relevance, all studies also received high scores in authority because each of their authors possessed the proper credentials and educational backgrounds to conduct and evaluate the data generated. The scores for accuracy were determined by whether the study was conducted using sound methodology and how precise of data the methodology produced. The technique itself, and the data produced, received individual scores of one through ten, with one being unacceptable to ten being reliable. All studies obtained data via scientifically proven experimental designs, earning them partially high scores inaccuracy, which made their data's precision the determining factor within this category.

While Hx concentration was always measured via HPLC, two methods of measuring K+ concentration were used in the studies analyzed: flame photometry or selective ion electrodes. Flame photometry can measure the concentration of five different elements, including K+. In this method, the vitreous humor is aerosolized into a flame, the heat from which excites the electrons. When these electrons return to their ground state, they give off energy in the form of light with a wavelength specific to the element. The wavelength and color produced allow the researcher to identify which element is present and the quantity or concentration. Flame photometry relies on the aspiration of a solution into a flame, but this grows increasingly difficult the denser the analyte solution is. Therefore, vitreous fluid, being a

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thick, gel-like substance, is challenging to completely aspirate, leading to poor sample distribution and increased error rates.

Selective ion electrodes use a sensor to convert the activity of an electron into an electric potential. This potential is directly proportional to the ion concentration. The solution does not need to be manipulated, and there are fewer errors in the selective ion electrode method. As reflected in other studies' error rates, those that implemented the selective ion electrode method over flame photometry produced results with a stronger correlation to time since death.

Lastly, these studies were criticized based upon their purpose, or whether they written for entertainment or scientific purposes. Similar to relevance, all of the studies in this category received high scores. Only studies published in scientific journals or submitted as theses were gathered, leaving out any articles that may have been published in entertainment magazines or for websites that prioritize entertainment over science.

Discussion

After determining which sources obtained the most accurate and reliable data, a pattern emerged. Studies that analyzed K+ and Hx had higher aggregate CRAAP scores and r-squared values in their research. Salam and colleagues (2012) had the highest CRAAP score—with a total of 48 out of 50 possible—and the strongest r-squared values—at 0.998 for K+ and 0.898 for Hx—when compared to time since death.

The lowest CRAAP score was given to Madea et al. (1994), whose study measured Hx concentration and no other metabolites through flame photometry. In addition to their poor experimental design, their published data lacked critical information in several areas. Despite their evaluation of a metabolite with a strong average relationship, which should produce a higher r-squared value than those who looked at weaker metabolites such as Mihailović and colleagues (2014), they reported one of the lowest r-squared values. This can likely be attributed to their usage of flame photometry rather than the selective ion electrode to measure concentration.

Baniak et al. (2014) received one of the higher CRAAP scores despite their study being roughly five years older than the others and their data producing moderate r-squared values for K+ concerning time (Baniak et al., 2014). Their higher CRAAP score can partially be attributed to this being a follow-up study to previous research. In their initial research, by looking at approximately 70 cases of known time of death and using flame photometry, they found a moderate correlation between K+ concentration and the time that passed since death. However, the selective ion electrode method was chosen for their follow-up study. By demonstrating that they have extensively studied this topic on more than one occasion, this update awarded them a higher CRAAP score, and giving credence to the hypothesis that the selective ion electrode method is the most accurate means of measuring K+ concentration.

Using the selective ion electrode method to gather data on K+ concentration within the vitreous humor is more effective than flame photometry. However, flame photometry remains the prevalent technique, likely due to the relatively low cost of its testing materials. Many labs receive funding through universities or colleges, and most coroner and medical examiner offices do not conduct in-house testing. Instead, they send samples to contracted labs. Coroner offices are affiliated with local police or sheriff departments, which are taxpayer funded and must have their budgets approved by the public. The coroner unit is typically smaller than narcotic, patrol, or sex-crime units, and is made up of

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civilian and sworn employees. This causes coroner units to be considered a low priority, leaving minimal funding to cover several expenses, including their essential ones.

There are some limitations to using data from the concentration of metabolites in vitreous humor for PMI determination. While vitreous is resistant to post-mortem degradation, the metabolites' concentration can differ in decedents that have certain diseases. For example, diabetes and its damage to the eyes, weakens blood vessels and allows for increased levels of glucose, urea, potassium, and magnesium to enter into the vitreous fluid. Similarly, heavy trauma to the eyes and orbits of the skull can damage blood vessels and hinder the ability of the vitreous to accurately establish a relationship between concentration and time since death. All of the analyzed studies acknowledged this limitation; therefore, only examine decedents without these diseases or sources of trauma.

One of the areas of disagreement throughout this research analysis is whether there is a difference between metabolite concentration in the right and left eye of a decedent. In all studies examined, this difference was mentioned, yet a reason for the apparent discrepancy was not provided. One of the studies, conducted by Kalra et al. (2016), states that "there is no significant difference in the concentration of various biochemical constituents in the same pair of eyes," but this finding contradicts many other studies (p. 1). Therefore, single versus dual-eye testing should be further investigated, and the observed variability should be explained. Without this determination, studies related to PMI will continue to face an unexplained cause of the error, which will reduce the validity of the results.

McNeil and colleagues (1999) addresses many of the repeated factors that could be generating the unpredictability of vitreous analysis. As mentioned earlier, the viscosity of vitreous is a potential limiting factor when using flame photometry to measure metabolite concentration. In that study, vitreous fluid was heated prior to analysis, creating a less dense solution. The heated samples produced greater precision than those analyzed without any heat treatment (McNeil et al., 1999). Applying heat to increase the viscosity of vitreous samples is a simple method but only improves the reliability of results when analyzing K+ concentration. As seen in a later study (Salam et al., 2012), PMI determination is most accurate when including the concentration of Hx, which is sensitive to heat. The addition of Hx concentration produced more accurate results, heat treatment should not be considered an appropriate technique to improve PMI determination. McNeil et al. (1999) and Camba et al. (2014) studied alternate pretreatment methods; both found that ultrasonic bathing of samples improved data accuracy without affecting either K+ or Hx concentrations, making it an ideal pre-analytical method.

Although the selective ion electrode method tends to be a costly procedure, money should be of no concern when finding an accurate determination of a post-mortem interval. Financial concerns limit the ability of a forensic pathologist to conduct a thorough investigation, which could greatly hinder the prosecution of criminal cases. Pretreating samples by ultrasonic bath is a relatively simple and inexpensive addition to vitreous analysis and should be considered a standard practice. Unexplained factors that may produce unreliable results need to be clearly stated and explained to support transparency and give an idea of how future research should account for these discrepancies. Finally, all methods that can reduce error, provide

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accurate data, and increase reproducibility should be further analyzed.

Conclusion

The importance of establishing an accurate time of death is often overlooked in forensic investigations, yet it is vital to the investigative process. Though the scientific community has not come to a clear consensus over the usefulness of metabolite concentration in vitreous fluid, numerous studies suggest that this concentration shares a strong enough relationship with increasing PMI and is far less influenced by the process of decomposition. Therefore, metabolite concentration ought to be included as a standard variable in all forensic autopsies.

After analyzing an array of sources and evaluating their data, the direction that continuing research should take was apparent. The methods and specific metabolites that produce the best and most meaningful results were able to be concluded. While potassium is the most well-studied metabolite that produces consistent results with the strongest relationship to time since death, hypoxanthine also exhibits a significant relationship with post-mortem interval and should not be disregarded. Studies that analyzed metabolites and traditional factors, such as ambient temperature, established the strongest linear relationship with time since death.

Just as rigor, algor, and livor mortis are assessed in the traditional method of determining the post-mortem interval, all relevant relationships should also be considered in future methods. Moreover, the traditional method is too heavily influenced by common factors, such as temperature, body composition, and animal predation, to continue being the only means of establishing a post-mortem interval. Autopsy practices have remained relatively unchanged throughout the years, but as science advances and reveals useful relationships that aid in explaining every detail of a case, so should autopsy procedures. Monetary limitations should not determine the testing methods used. Instead, using the method that offers the most accurate data should be of the utmost importance, especially in forensics.

Metabolite concentrations, particularly potassium and hypoxanthine, have an incredible potential to provide an accurate post-mortem interval. PMI is integral to forensic investigations, yet it has relied on subjective methods of determination that are easily influenced by minor changes in the environment or body composition. Each of these factors proves the importance of a reliable method for establishing time since death. The analyzed studies show that there is a way to accurately obtain a PMI from a fluid that is largely unaffected by the variability seen in the traditional method. Moving forward, analyzing potassium and hypoxanthine in conjunction would be a pivotal step toward providing an accurate determination of post-mortem interval.

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