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Vitreous Humor Hypoxanthine and Potassium as Markers for Post-mortem Interval Estimation: A UPLC-Based Study

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ABSTRACT

The biochemical analysis of vitreous humor provides a valuable approach to estimating the post-mortem interval (PMI). Following death, the vitreous humor undergoes predictable biochemical changes, making it a reliable substrate for temporal estimation. This study explores the relationship between PMI and the chemical constituents of vitreous humor, with particular emphasis on potassium and hypoxanthine (Hx).
The study involved a total of 55 medico legal autopsies. Vitreous humor samples were collected from all subjects and the experiments were conducted using the Ultra-Performance Liquid Chromatography (UPLC) technique and an electrolyte analyzer.
Hypoxanthine (Hx) levels increased significantly from 78.68 to 268.97 µmol/L across PMIs determined by police records and from 87.26 to 280.09 µmol/L by physical examination. The linear regression analysis of Hx levels concerning PMI from police records and physical examinations demonstrated a statistically significant correlation, with R^2 values of 0.86 and 0.76, respectively. This suggests Hx as a more reliable PMI biomarker compared to potassium.
In contrast, potassium levels showed no statistically significant relationship with PMI based on either police records (*p=0.688*) or physical examination findings (*p=0.806*). The consistent and significant rise in Hx concentration with increasing PMI ($p < 0.001$) underscores its potential usefulness for estimating time since death. The study concludes that hypoxanthine concentration in vitreous humor serves as a more dependable indicator for PMI evaluation, whereas potassium contributes minimally to time-of-death estimation.
Keywords: Hypoxanthine; Post-mortem interval; Potassium; UPLC; Vitreous humor

INTRODUCTION

One of the key goals of forensic medicine is to determine the ‘time since death’, which refers to the duration between death and the post-mortem examination, also known as the 'post-mortem interval' (PMI). The standard techniques for estimating PMI focus on observing physical changes in the body, including temperature, muscle rigidity, and livor mortis.[1] Traditional methods for PMI estimation often rely on the measurement of body temperature and the application of various cooling models, such as the Henssge nomogram or the Forensic Pathology Temperature Model.[2] However, this approach produces accurate and reliable results only for shorter time frames (a few hours post-death). As the PMI increases, the accuracy of temperature-based PMI estimation decreases significantly.[3] Other traditional PMI estimation methods, including isotopic analysis, DNA degradation assessment, and forensic entomology, are beneficial for longer (weeks) and very extended (years) time frames, while the period between several hours and several days poses notable challenges for PMI estimation. [4-7]

The traditional methods for estimating PMI rely on the examination of three key physical changes that occur in the body after death: Rigor mortis, Algor mortis, and Livor mortis. These traditional methods have their limitations, particularly in terms of accuracy and applicability over longer PMI.[8]

To overcome these limitations, modern forensic science has explored alternative approaches, such as the use of biochemical markers, molecular techniques, and advanced imaging methods.[9] These newer methods aim to provide more accurate, objective, and comprehensive PMI estimation. One such approach involves the analysis of biochemical changes in the vitreous humor, the clear gel-like substance located in the posterior chamber of the eye. The vitreous humor undergoes predictable biochemical changes over time after death, making it a valuable source of information for PMI estimation.[10]

Two key analytes that have shown promise in vitreous humor analysis for PMI estimation are hypoxanthine (Hx) and potassium.[11] Hx, an intermediate product of purine metabolism, accumulates in the vitreous humor due to the breakdown of adenosine triphosphate (ATP) after death.[12] Potassium, an electrolyte, also exhibits a consistent increase in vitreous humor concentration over time due to the cessation of active transport mechanisms post-mortem.[13]

Numerous researchers have investigated the correlation between potassium levels in the vitreous humor and PMI. [14,15] One particular study indicated that potassium concentrations in the vitreous humor rose with an increasing time since death [13], and further confirmed a positive linear correlation between PMI and potassium levels in the vitreous humor. Rognum et al 1991 established a linear connection between the time post-mortem and the increase in Hx in the vitreous humor for up to 120 hours after death.[16]

Given the accessibility of vitreous humor samples and their resistance to decomposition, this study aimed to examine the concentration of Hx and the alterations in potassium electrolytes in the vitreous humor, as well as their implications for estimating the PMI in cases of unnatural death.

This study aimed to determine whether vitreous hypoxanthine and potassium levels correlate with the post-mortem interval (PMI) and to evaluate their reliability as biochemical markers using UPLC.

MATERIALS & METHODS

This hospital-based cross-sectional analytical study was conducted at the Department of Forensic Medicine, from September 1, 2023 to October 31, 2024. A total of 55 medico legal autopsies with known time of death were included in the study. Cases with known or suspected eye diseases and eye injury were excluded from the study.

Sample size was calculated using the formula:

$n=[(Z\alpha+Z\beta)/C]^2+3$

Where,

$C=0.5 \times \ln[(1+r)/(1-r)]$

Interpretation of Notation

$Z\alpha$ = at 95% level of confidence interval

$Z\beta$ = at 80% power of the study

R = Correlation between Hx and PMI

C = Fisher Z transformation of the minimum correlation coefficient

Estimation of Parameters from the Previous Study [17]
Zα = 1.96
Zβ = 0.84 (80% power)
R = 0.37
C = 0.38
Total Sample Size (n) = 55

METHODOLOGY

Upon receiving approval from the Institutional Research Committee (IRC) and the Institutional Ethics Committee (IEC) (Reference: IEC/HIMS/RR-367/24-11-2022), participants were enrolled in the study. The research encompassed all instances of sudden or unnatural death where a post-mortem examination was performed at the mortuary linked to the Department of Forensic Medicine in the designated area. Using a sterile 20-gauge needle, vitreous humor samples were meticulously and slowly aspirated from the posterior chamber through a puncture made 5-6 mm from the limbus (ora serrata). Each eye yielded a sample volume of 1 to 2 ml. Gentle suction was employed, and leftover fluid was preserved during the sampling to avoid detaching retinal cells and to ensure that no loose tissue within the vitreous chamber was disrupted. Any fluid that appeared cloudy or contained particulates was discarded.

The vitreous humor samples collected were quickly sent to the Central Laboratory, Department of Biochemistry, to evaluate potassium levels using an automated electrolyte analyzer (utilizing the ion-selective electrode (ISE) method with the ABBOTT Architect plus instrument). The samples were centrifuged at 2000 rpm for five minutes, and the resultant supernatant was used for potassium measurement.

Standard and sample preparation:

Hx standard (sourced from Loba Chemie Pvt. Ltd.) was dissolved in HPLC-grade water to obtain a 1 µM stock solution, which was stored at -20°C and prepared fresh. Vitreous humor samples were obtained, and each 100 microliters of the sample was diluted with HPLC-grade water before being vortexed. The diluted sample was then transferred into a Nylon 0.22 µm pore size filter unit (Shimadzu). Following that, the samples were centrifuged at 9000 rpm for 90 minutes at 4°C (using a Neuation iFUGE UC02R, India) and were kept at -20°C until Hx measurements were conducted. A total of 55 bodies (n=40, 72% male and n=15, 27% female) were analyzed, with an average age of 43.98 years, ranging from 17 to 85 years.

UPLC analysis for Hypoxanthine:

A SHIMADZU-N-series Ultra Performance Liquid Chromatography (UPLC) system was utilized for the analysis of samples. For the separation of Hx, a Shim-pack Solar-C18 (5 µm, 4.6 X 250 mm) column was employed. The mobile phase consisted of 0.05M potassium di-hydrogen ortho-phosphate (KH_2PO_4) mixed with 1% methanol (MeOH). The volume of the sample injected was fixed at 10 µL, with the column oven temperature set to 40°C. The flow rate was maintained at 1 mL/min [18], using an isocratic method.

All reagents, standards, QCs, and samples were allowed to equilibrate to room temperature prior to assay. The working standard solutions for hypoxanthine, with concentrations ranging from 0.1 µM to 1 µM, were prepared by aliquoting different volumes of the working solution into a series of 1.5ml autosampler vials. These vials were then placed in the autosampler tray of the UPLC system, where volumes of 10 µL (in triplicate) were injected under optimal chromatographic conditions after filtering each preparation using a 0.22 µm membrane filter. Calibration curves were created by plotting the peak areas against the final concentrations, from which the corresponding regression equations were derived. It was confirmed that the calibration curve demonstrated linearity ($R^2 > 0.99$). The pre-processed samples were taken from the -20°C freezer, briefly vortexed, and centrifuged at 14,000 × g for 15 minutes at room temperature. The

resulting clear filtrates were then transferred to deactivated glass UPLC autosampler vials, with 10 µL injected into the UPLC system for analysis.
Figure 1 shows the chromatograms for hypoxanthine (RT approximately 8.6 minutes) in deionized water to indicate the retention times of the components. The technique exhibited excellent chromatographic selectivity, with no plasma interferences detected at the hypoxanthine retention times and adequate sensitivity for both of the target components, employing a PDA (Photodiode Array) detector and requiring about 13 minutes for each analytical run (this duration enables the mobile phase to equilibrate). To extend column lifetime, it was cleaned after each analytical run for 10 minutes at a flow rate of 1.0 mL/min using a mixture of acetonitrile and deionized water (90:10, v/v) to remove any retained nonpolar substances from the column.

Determination of Potassium:
The vitreous humor samples, which were pre-processed, were collected in sterile microcentrifuge tubes for the potassium estimation. Calibration standards with known potassium concentrations (such as 0, 2, 5, 10, and 20 mM) were prepared for plotting the standard graph and to calibrate the potassium ISE module. The vitreous humor samples were diluted with distilled water to ensure their concentrations were within the calibration range, and they were mixed gently to achieve homogeneity. Subsequently, the samples were placed into the ABBOTT Architect Plus instrument. The ABBOTT Architect Plus generated the results based on the calibration curve, and these results were verified against quality control samples to confirm their accuracy.

STATISTICAL ANALYSIS

Descriptive statistics:
The data was entered in MS Excel and subsequently examined with the Statistical Package for the Social Sciences (SPSS) software, version 29.0.2.0 (IBM Corp). Frequencies and proportions were used to describe categorical data, while means and standard deviations were calculated for continuous variables.

Analytical Statistics:
A one-way ANOVA was utilized to assess the differences in mean levels of Hypoxanthine and Potassium across various durations of PMI, as indicated by police reports and physical examinations. Linear regression analysis was performed to examine the relationship between hypoxanthine and potassium levels with PMI, based on police documentation and physical assessments, as well as to calculate the change in mean hypoxanthine and potassium levels for each unit change in PMI. Pearson's Correlation was computed, and a scatter plot was created to illustrate the relationship between the mean levels of hypoxanthine and potassium with PMI based on police records and clinical findings. A p-value of less than 0.05 was deemed statistically significant.

RESULT

The study involved 55 medico legal autopsies, in which the time of death was determined, and samples of vitreous humor were analyzed for Hx and potassium levels. The essential characteristics of the study group are detailed in Table 1. A total of 55 bodies (n=40, 72% males and n=15, 27% females) were examined, with an average age of 43.98 ± 15.75 years (ranging from 17 to 85 years).

Table 1: Baseline characteristics of the study population (n = 55).

Parameters	Category	*n (%)*
Age	16-30	10 (18.2)
	31-45	21 (38.2)
	46-60	15 (27.3)
	61-75	08 (14.5)
	76-90	01 (1.8)
Sex	Male	40 (72)
	Female	15 (27)

Research was conducted on the post-mortem interval (PMI) using hypoxanthine

(Hx) (144.1±64.3 µmol/L) and potassium levels (13.31±4.5 mmol/L) found in the vitreous humor, alongside police official records (13.53±4.0 hrs) and physical examinations (13.56±4.4 hrs). The validation of hypoxanthine was performed through chromatograms derived from both the standard Hx solution and the extracted Hx, as illustrated in Figures 1A and B. The calibration curve for Hx was established within a range of 10 to 100 µM.

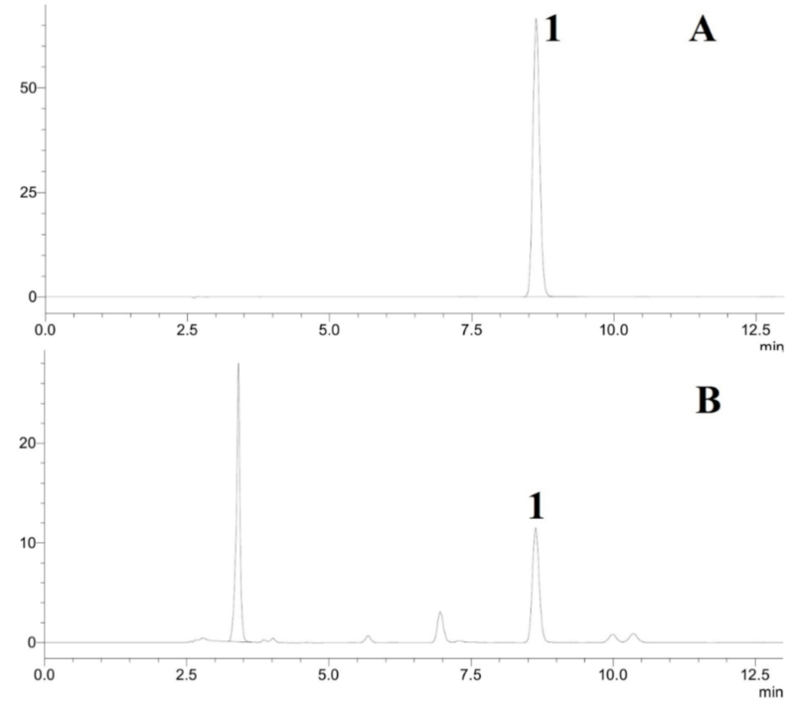

Figure 1. Chromatograms showing retention time (8.6 min) for standard (A) and vitreous sample (B) hypoxanthine using UPLC.

In Table 2, the one-way ANOVA indicated a statistically significant rise in the Mean Hypoxanthine level in the vitreous humor as the post-mortem interval increased, according to both police documentation and physical assessments (Rigor mortis-RM and Post-mortem staining-PMS). For PMI based on police records, the mean hypoxanthine level progressively increases from 78.68 ± 27.18 µmol/L for 6-10 hours to 268.97 ± 12.87 µmol/L for 21-25 hours. When PMI is estimated via physical examination, the mean hypoxanthine level also shows an increasing trend with longer PMIs, starting from 87.26 ± 25.07 µmol/L for 6-10 hours and reaching 280.09 µmol/L for 21-25 hours.

Table 2: One-way ANOVA: Association between Mean Hypoxanthine level in vitreous humor with PMI based on police record and Physical examination.

PMI (Post mortem Interval)		Mean Hypoxanthine Level in µmol/L ± SD	95% CI	F-Value	*p-value*
PMI Based on Police Records	6-10 hours	78.68 ± 27.18	62.25-95.11	79.86	*<0.001*
	11-15 hours	120.54±30.96	107.15-133.93		
	16-20 hours	203.72±24.96	189.90-217.54		
	21-25 hours	268.97±12.87	248.48-289.46		
PMI Based on Physical Examination	6-10 hours	87.26±25.07	73.90-100.63	25.45	*<0.001*
	11-15 hours	139.61±43.67	120.5-158.98		
	16-20 hours	204.96±47.29	177.65-232.7		
	21-25 hours	280.09	-		

In Table 3, there is no statistically significant difference in mean potassium levels across the different PMI intervals based on both police records and physical examination with *p-value* more than 0.05.

Table 3: One-way ANOVA: Association between Mean potassium level in vitreous humor with PMI based on police record and Physical examination.

PMI (Post mortem Interval)		Mean Potassium Level in mmol/L ± SD	95% CI	F-Value	*p-value*
PMI Based on Police Record	6-10 hours	13.21±4.75	10.68-15.75	0.596	*0.621*
	11-15 hours	12.49±4.33	10.57-14.42		
	16-20 hours	14.42±4.59	11.77-17.08		
	21-25 hours	11.0	-		
PMI Based on Physical Examination	6-10 hours	13.11±3.84	10.79-15.44	0.179	*0.910*
	11-15 hours	13.03±5.45	10.67-15.38		
	16-20 hours	14.06±4.27	11.69-16.43		
	21-25 hours	12.81±3.64	7.00-18.61		

In Table 4: Each hour increases in PMI according to police records is associated with a 14.924 µmol/L rise in hypoxanthine levels, while each hour increases in PMI based on physical examination is linked to a 12.577 µmol/L increase in hypoxanthine levels, both showing statistical significance with a *p-value* of less than 0.05 and the 95% confidence interval for B does not include zero, reinforcing the significance. The standardized coefficient (β) for PMI shows a very strong positive relationship in both cases 0.931 for police records and 0.873 for physical examination.

In contrast to hypoxanthine, potassium levels do not show a statistically significant association both with PMI derived from police records and physical examination with its 95% confidence interval includes zero and the *p-value* much greater than significance level of 0.05.

Table 4: Linear Regression on Hypoxanthine, potassium level in vitreous humor with PMI based on police record and Physical examination

Sl. No	Independent Variables	Unstandardized co-efficient B	95% CI of B	Standardized co-efficient β	t-Value	*p-value*
1	Hypoxanthine level versus PMI based on Police record	14.924	13.317-16.530	0.931	18.628	*<0.001*
2	Hypoxanthine level versus PMI based Physical Examination	12.577	10.639-14.514	0.873	13.022	*<0.001*
3	Potassium level versus PMI based on Police record	0.063	- 0.252-0.378	0.055	0.403	*0.688*
4	Potassium level versus PMI based on Physical Examination	-0.035	-0.318- 0.249	-0.034	-0.246	*0.806*

The Linear Regression Model presented in Table 5 and Figure 2 indicates a strong positive correlation between the concentration of hypoxanthine in the vitreous humor and both the post-mortem interval (PMI) based on police record (R=0.931, $p < 0.05$) and the PMI determined through physical examination (R=0.873, $p < 0.05$). 86.5% of the variation in hypoxanthine levels can be explained by the PMI based on police records (Adjusted R^2 Value =0.865), while 75.7% of the variation in hypoxanthine levels is explained by the PMI derived from physical examination (Adjusted R^2 Value =0.757)

Table 5: Linear Regression analysis of hypoxanthine and potassium concentration in the vitreous humor is based on post-mortem interval information derived from police records and physical examinations with R and R^2 value.

Sl. No	Variables	R	R^2 Value	Adjusted R^2 Value	SE of the Estimate
1	Hypoxanthine Level in µmol/L Versus PMI based on Police records	0.931	0.867	0.865	23.62
2	Hypoxanthine Level in µmol/L Versus PMI based on Physical Examination	0.873	0.762	0.757	31.67
3	Potassium level in mmol/L versus PMI Based on Police record	0.055	0.003	-0.016	4.628
4	Potassium level mmol/L versus PMI based on Physical Examination	0.034	0.001	-0.018	4.633

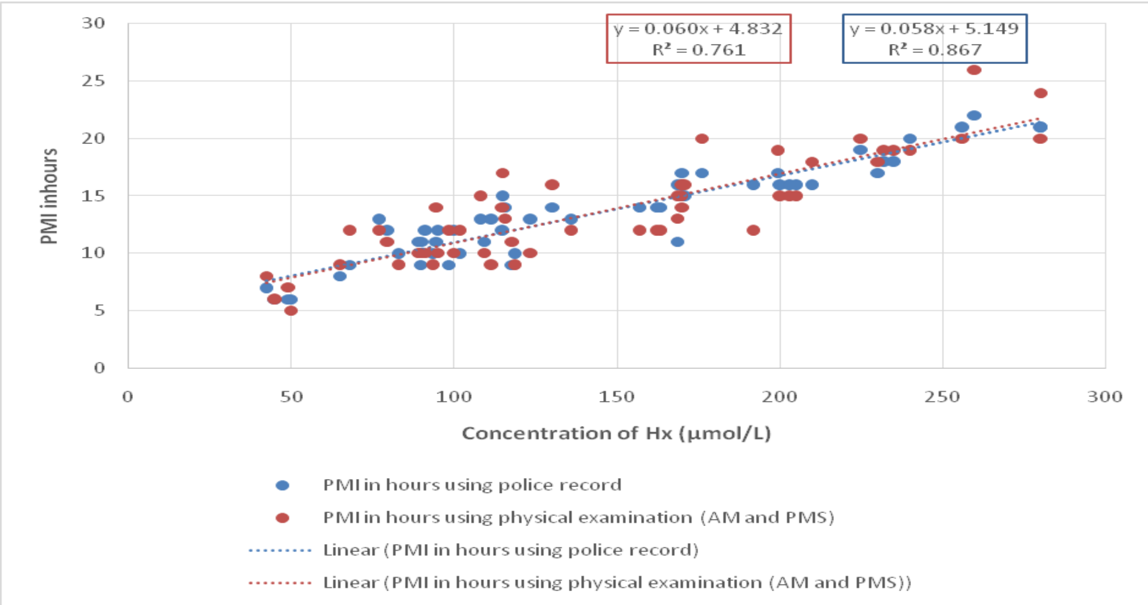

Figure 2: Scatter Diagram showing a correlation between Hypoxanthine level and PMI based on police record and physical examination.

The Linear Regression Model illustrated in Figure 3 indicated a very weak relationship between potassium levels and PMI, according to both police records (R=0.055) and physical examination (R=0.034).

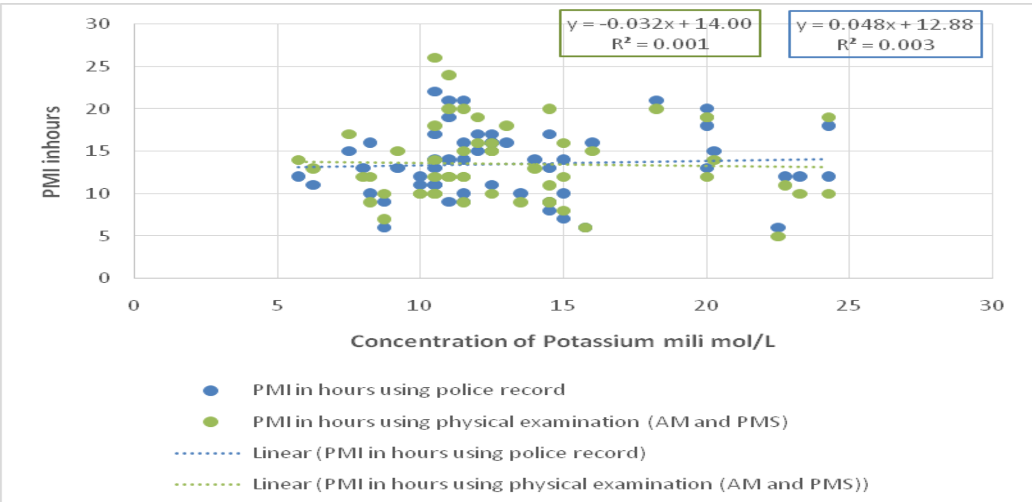

Figure 3: Scatter diagram showing a correlation between potassium level and PMI based on police records and physical examination.

In contrast, Linear regression model in Table 5 and Figure 3 potassium levels show no statistically significant correlation with PMI in this analysis, making them limited value for PMI estimation based on these findings.

DISCUSSION

Hypoxanthine

The concentrations of chemicals in the vitreous humor of the eye change over time post-mortem, which aids in investigating and estimating the time since death. [18,19] This study examined the correlation between the levels of Hx and potassium with the post-mortem interval (PMI) using one-way ANOVA, linear regression, and Pearson's Correlation test. Hx is a metabolite formed from the breakdown of adenosine triphosphate (ATP) during purine metabolism, and its concentration in the vitreous humor increases after death due to the lack of oxygen. Furthermore, our results showed that Hx levels escalated as the PMI lengthened. For the statistical analysis, we utilized the average Hx concentrations from both eyes. Hx levels rise linearly with PMI (Figure 2), aligning with previous research. [15,16,18] This increase in Hx concentration after death is consistent with recent studies. [14,19-21]

The levels of Hx in the 55 samples varied from 42.5 to 280.1 µmol/L, with a correlation to PMI calculated from both police records and physical examination showing R=0.931 and R=0.873, respectively (Table 5). This result is consistent with findings from previous studies that reported values of 0.51, 0.531, and 0.68, as documented by Henssge and Madea; Rognum et al.; and Munoz et al., respectively.[1,16,18] The typical Hx level in the vitreous humor of living humans is 7.6 µmol/L.[16] The principal conclusion of this study is the demonstration of a linear association between PMI and the increase in Hx levels in the vitreous humor. Hx concentration markedly rises with an increase in PMI, according to both police records and physical examinations, across four defined intervals (6-10h, 11-15h, 16-20h, and 21-25h), showing ranges of 78.68 – 268.97 µmol/L and 87.26 – 280.09 µmol/L respectively, which were statistically significant with a *p-value < 0.001* (Table 2). Consequently, this research effectively establishes a linear relationship between Hx levels and PMI over various timeframes, corroborating earlier research findings [11,14-16] and reinforcing the reliability of the results. An increase of one hour in PMI as indicated by police records correlates with a 14.9 µmol/L rise in Hx levels, while a one-hour increase in PMI based on physical examination correlates with a 12.577 µmol/L rise in Hx levels, both of which were statistically significant with a *p-value < 0.001* (Table 4). The established correlation between PMI and vitreous humor Hx level is well-documented [11,14-16], which enhances the reliability of the findings and indicates that the observed trends in Hx levels are strongly supported by the existing literature.

Potassium

Previous studies have suggested that it's feasible to estimate the time of death based on the electrolyte concentration changes in the vitreous humor.[22,23] The potassium levels have been observed to increase in a linear manner, as recorded by various researchers.[12,24] In our current investigation, the potassium concentration in the vitreous humor does not demonstrate any linear relationship with the post-mortem interval (PMI) based on police records (R=0.055) or on physical examinations (R=0.034) (Table 5). This is further illustrated by only -1.6% of the variation in potassium levels being linked to PMI according to police records (Adjusted R^2 Value = -0.016), while -1.8% of the variation in potassium levels corresponds to the PMI as assessed during physical examinations (Adjusted R^2 Value = -0.018) (Table 5). The scatter plot indicates no correlation between potassium concentration and PMI based on police records with an R^2=0.0031 (Table 5 and

Figure 3), as well as with the PMI derived from physical examinations, R^2=0.0011 (Table 5 and Figure 3). In light of these findings, the fluctuations in vitreous potassium concentrations reported in this study were not statistically significant, aligning with previous research.[25,26] Chandrakanth et al. noted no significant correlation between vitreous potassium levels and time since death, particularly within the first 36 hours after death.[25] The present research did not reveal any statistically significant rise in potassium levels with increasing PMI, according to both police records (*p=0.6*) and physical examinations (*p=0.9*), across four groups (6-10h, 11-15h, 16-20h, 21-25h), which is deemed statistically insignificant (Table 3).

A key factor for the inconsistent reports regarding potassium concentration variations at the post-mortem interval (PMI) could be differences in study methodologies and potential sample handling prior to analysis. A significant difference may arise from the aspiration techniques used by certain researchers. Bito L Z reported that the concentrations of various solutes in the vitreous humor differ between the anterior and posterior vitreous chambers.[27] It has been suggested that the variations in the same pair of eyes may be due to sample dilutions prior to analysis, which is why measuring the samples without dilution has been recommended.[26] In the current study, suitable dilutions were conducted; previous studies that did not find significant differences between the eyes concerning vitreous components have also implemented the necessary dilutions.[28] The extended time between the collection of vitreous-humor samples and their biochemical analysis could be another factor that accounts for the discrepancies. In some research, samples were stored at -70°C before being analyzed biochemically. The varying storage conditions may have affected the results to some extent, and we believe that after long-term storage at low temperatures, the findings may not reliably reflect the biochemical concentrations present in the vitreous humor.[29] Henssge and Madea proposed that numerous internal and external factors, both antemortem and post-mortem, impact the characteristics of the curve and its starting point.[30] Therefore, these influencing factors need to be quantitatively assessed to enhance the accuracy of estimating the time since death.

Our findings echo earlier research where the association between vitreous potassium levels and PMI was not strictly linear.[31] Adjutantis and Coutselinis proposed that potassium level measurements in the vitreous humor might allow for an accurate estimation of the time of death within a two-hour margin.[32] However, Leahy and Farber uncovered no mathematical relationship between vitreous potassium concentrations and PMI in sudden death occurrences.[33]

CONCLUSION

The current research presents strong evidence that Hx concentrations in the vitreous humor can serve as a dependable biomarker for assessing the post-mortem interval (PMI). The notable linear relationship identified between Hx levels and PMI highlights its potential as a significant asset in forensic studies. As Hx concentrations consistently increase with time after death, the results reinforce the case for utilizing Hx as a quantitative benchmark for PMI assessment. The robust correlation between Hx levels and PMI (R=0.931 and R=0.873) points to a consistent relationship, supporting Hx's role as a predictive marker for estimating time since death.

Conversely, the research did not identify a meaningful correlation between potassium levels and PMI, which corresponds with prior studies that cast doubt on the reliability of potassium as a marker in the initial post-mortem timeframe. These findings underscore the complexities involved in utilizing various biochemical markers and suggest that Hx may offer greater accuracy for PMI estimation.

In summary, vitreous hypoxanthine concentration demonstrated a strong linear relationship with PMI and may serve as a reliable biochemical indicator of time since death. Potassium, however, did not show significant correlation, suggesting it may have limited standalone utility. Future multicentre studies with broader post-mortem intervals and controlled environmental variables are recommended.

Limitations of the study

The current study has certain limitations, primarily due to the small sample size. There was a lack of data regarding whether the deceased experienced any electrolyte issues prior to death. The only details we have concerning the health, medications, and drug history of the deceased were provided by the relatives present. The absence of a significant link between potassium levels and post-mortem interval (PMI) raises doubts about potassium's effectiveness as a measurement. The findings of this study align with previous research [25], indicating that potassium may not serve as a dependable indicator of PMI. While notable results were found for Hx, the lack of significant outcomes for potassium prompts questions about the strength and dependability of using potassium as a predictor for PMI. Future research with larger sample sizes will be undertaken to confirm the correlation equation for Hx levels as an indicator of PMI.

Declaration by Authors

Ethical Approval: Approved from the Institutional Research Committee (IRC) and the Institutional Ethics Committee (IEC) (Reference: IEC/HIMS/RR-367/24-11-2022)

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Conflict of Interest: The authors declare no conflict of interest.

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