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***Original research article***

# POST-MORTEM CHANGES IN METABOLOMIC PROFILES OF HUMAN SERUM, AQUEOUS HUMOR, AND VITREOUS HUMOR

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**Abstract:** *Accurate post-mortem interval (PMI) estimation is crucial in forensic investigations, and metabolomic analysis of bodily fluids offers a promising approach. However, the suitability of ocular fluids for PMI estimation remains underexplored.* ***Objective:*** *The objective of this study was to evaluate the suitability of metabolomic analysis of ocular fluids, specifically vitreous humor (VH) and aqueous humor (AH), compared to serum for post-mortem interval (PMI) estimation and to identify the most promising metabolites for this purpose.* ***Methods:*** *A cross-sectional study was conducted at a tertiary care hospital in Karachi. Blood, AH, and VH samples were collected from human cadavers with PMIs ranging from 5 to 60 hours. Samples were prepared by removing proteins and lipids, followed by lyophilization. Metabolomic profiling was performed using an NMR Spectrometer. Pearson correlation coefficients were calculated to assess the relationship between metabolite concentrations and PMI.* ***Results:*** *The study identified 24 metabolites in blood, AH, and VH samples. Several metabolites, including glutamate, choline, glycine, and formate, exhibited significant positive correlations with PMI in all three fluids, with Pearson coefficients greater than 0.5. Notably, glutamate (blood: r = 0.585**, AH: r = 0.488*, VH: r = 0.683**), choline (blood: r = 0.435*, AH: r = 0.691**, VH: r = 0.713**), and glycine (blood: r = 0.553*, AH: r = 0.598**, VH: r = 0.690**) showed strong correlations. Conversely, glucose and pyruvate significantly negatively correlated with PMI in AH and VH.* ***Conclusion:*** *The findings indicate that VH and AH are more reliable than serum for PMI estimation due to their more stable metabolite profiles. Glutamate, choline, and glycine emerged as particularly promising biomarkers. The study supports the potential of metabolomic analysis of ocular fluids in forensic investigations to provide accurate PMI estimations.*

**Keywords:** Metabolomics, Post-Mortem, Aqueous Humor, Vitreous Humor.

## Introduction

Forensic science is one of the most essential tools in legal proceedings, which offers objective proof that may be essential to a conviction or acquittal (1). Bloodstain pattern analysis, DNA profiling, fingerprint identification, and the post-mortem interval (PMI) can all be used as forms of evidence (2). Metabolomics has recently demonstrated potential in bridging the gap in PMI estimation. The method allows tracking post-mortem metabolite changes in body fluids by analyzing an organism's system components. Studies are being conducted to determine which body fluids contain the most promising metabolites that satisfy these criteria (3). Aqueous humor (AH) and vitreous humor (VH), the fluids found in the eyes, are the most useful for assessing PMI, according to current research on animal models (4). Numerous early investigations have found metabolic alterations in a variety of body fluids. Compared to other body fluids like blood, these fluids are anatomically separated and undergo slower metabolite changes, increasing test accuracy (5, 6).
A research team at the International Tomography Center and Novosibirsk Regional Clinical Bureau of Forensic Medicine in Russia conducted a study to determine if ocular fluids show potential in human PMI estimation and identify the most promising metabolites for use as biomarkers. They utilized a Bruker Avance III NMR Spectrometer to identify metabolite biomarkers. Blood, VH, and AH samples were extracted from human cadavers at various post-mortem intervals (7). Blood plasma, AH protein-free extracts, and VH lipid-free extracts were obtained from these samples. Nuclear magnetic resonance (NMR) was used for qualitative metabolomic profiling of these samples. Forty-two metabolites were found in all three samples based on these results. The following six components of the sample set showed a high positive linear connection with PMI in all physiological fluids: glycine, glutamate, betaine, hypoxanthine, and choline.
The metabolite concentration in the AH and VH changed more gradually and smoothly than in the blood, which changed more randomly. These results determined that the AH and VH were more appropriate for PMI, which corroborates the metabolomic analysis of earlier animal research (7). The study's primary objective is to determine the associations between postmortem interval (PMI) and alterations in the fluid metabolomic composition.

## Methodology

[Citation: Riaz, L., Siddiqui, A.M., Rashid, A.N., Shahid, R.A., Nasrulhuda., Butt, F., (2024). Post-mortem changes in metabolomic profiles of human serum, aqueous humor, and vitreous humor. *Biol. Clin. Sci. Res. J.*, **2024***: 966*. doi: https://doi.org/10.54112/bcsrj.v2024i1.966]

After the ethical approval from the institutional review board, this cross-sectional study was conducted at a Tertiary care hospital in Karachi from January 1, 2023, to August 30, 2023. Samples of blood, aqueous humor (AH), and vitreous humor (VH) were collected from human cadavers under the following conditions: the corpses were kept at room temperature for 1–9 hours after death, then stored at 15°C for 3–30 hours before sampling. Blood samples were taken from the subclavian artery using a 22G needle, AH samples from the anterior chamber using a 27G needle, and VH samples from the ora serrata area using an 18G needle. Blood samples were immediately centrifuged to obtain plasma, which, along with AH and VH samples, was frozen at -70°C until analysis. Initially, samples from 30 post-mortem donors were collected. Still, those who had died from cancer were excluded due to distinct metabolomic differences, and three blood samples were discarded due to contamination or coagulation. The final set included 25 blood, VH, and AH samples each, with post-mortem intervals (PMI) ranging from 5 to 60 hours. To prepare protein-free extracts from AH, methanol was added, and the mixture was shaken and centrifuged. The supernatants were lyophilized. A mixture of sample, methanol, and chloroform was used for VH and serum samples to remove proteins and lipids. The mixture was shaken, cooled, and centrifuged, separating into layers, with the upper aqueous layer collected and lyophilized. 1H NMR measurements were conducted using a 700 MHz spectrometer. Each sample underwent 96 accumulations, with a temperature of 25°C and a 90° detection pulse. The water signal was saturated, and metabolite concentrations were determined by peak area integration relative to the internal standard DSS. SPSS version 21 was used to analyze the data.

**Results**

In the post-mortem samples, several metabolites showed significant time correlations with PMI (Post-Mortem Interval) in blood, aqueous humor (AH), and vitreous humor (VH) (Table 1). Metabolites that increased with PMI and had Pearson coefficients greater than 0.5 included acetate in the blood (r = 0.547**), glutamate in the blood (r = 0.585**), AH (r = 0.488*), and VH (r = 0.683**), choline in the blood (r = 0.435*), AH (r = 0.691**), and VH (r = 0.713**) (figure 1), phosphocholine in blood (r = 0.575**) and VH (r = 0.544**), glycine in the blood (r = 0.553*), AH (r = 0.598**), and VH (r = 0.690**), and formate in blood (r = 0.562**) and VH (r = 0.587**). Isobutyrate showed a significant increase in AH (r = 0.476*) and VH (r = 0.542**), while alanine increased in AH (r = 0.488*) and VH (r = 0.639**), and succinate in AH (r = 0.540**). Lysine also increased significantly in VH (r = 0.650**). Conversely, glucose concentrations significantly decreased with PMI in AH (r = -0.537**) and VH (r = -0.567**), and pyruvate decreased in VH (r = -0.464*). Metabolites with increasing concentrations ($r > 0.5$) included acetate, glutamate, choline, phosphocholine, glycine, and formate in blood, with glutamate, choline, and glycine also increasing in AH and VH (Table 2). Significant increases were also observed for isobutyrate in AH and VH, alanine in AH and VH, succinate in AH, and lysine in VH. Metabolites with decreasing concentrations ($-0.5 < r < -0.3$) were glucose in AH and VH and pyruvate in VH. Metabolites without significant correlation ($-0.3 < r < 0.5$) included leucine, valine, isoleucine, and lactate

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**Table I: Pearson coefficients for time dependences of concentrations of metabolites in post-mortem samples of serum, AH, and VH**

| Compound | Blood | aqueous humor (AH) | vitreous humor (VH) |
|---|---|---|---|
| Leucine | 0.515** | 0.281 | 0.392 |
| Isoleucine | 0.444* | 0.106 | 0.230 |
| Valine | 0.494* | 0.206 | 0.386 |
| Isobutyrate | 0.008 | 0.476* | 0.542** |
| Lactate | 0.053 | 0.316 | 0.409* |
| Alanine | 0.322 | 0.488* | 0.639** |
| Acetate | 0.547** | -0.201 | 0.607 |
| Glutamate | 0.585** | 0.488* | 0.683** |
| Pyruvate | -0.031 | -0.219 | -0.464* |
| Succinate | 0.216 | 0.540** | 0.456* |
| Asparagine | 0.281 | 0.136 | 0.480* |
| Lysine | 0.201 | 0.358 | 0.650** |
| Creatinine | 0.075 | 0.078 | 0.168 |
| Choline | 0.435* | 0.691** | 0.713** |
| Phosphocholine | 0.575** | 0.183 | 0.544** |
| Glycine | 0.553* | 0.598** | 0.690** |

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| | | | |
|---|---|---|---|
| Glycerol | 0.006 | 0.264 | 0.465* |
| Threonine | 0.532** | 0.072 | 0.219 |
| glucose | -0.115 | -0.537** | -0.567** |
| Histidine | 0.321 | 0.352 | 0.326 |
| Phenylalanine | 0.411** | 0.169 | 0.354 |
| Tryptophan | -0.186 | -0.046 | -0.052 |
| Inosine | 0.208 | 0.460* | 0.498* |
| Formate | 0.562** | -0.14 | 0.587** |

**Table II: Group-wise distribution of compounds**

| Compound | Blood | Aqueous Humor (AH) | Vitreous Humor (VH) |
|---|---|---|---|
| Group 1: Metabolite concentration increases with PMI (r > 0.5) | | | |
| Leucine | 0.515** | | |
| Acetate | 0.547** | | 0.607 |
| Glutamate | 0.585** | 0.488* | 0.683** |
| Lysine | | | 0.650** |
| Choline | 0.435* | 0.691** | 0.713** |
| Phosphocholine | 0.575** | | 0.544** |
| Glycine | 0.553* | 0.598** | 0.690** |
| Formate | 0.562** | | 0.587** |
| Isobutyrate | | 0.476* | 0.542** |
| Alanine | | 0.488* | 0.639** |
| Succinate | | 0.540** | |
| **Group 2: Metabolite concentration decreases with PMI (−0.5 < r < −0.3)** | | | |
| Glucose | | -0.537** | -0.567** |
| Pyruvate | | | -0.464* |
| **Group 3: Metabolites without significant time correlation (-0.3 < r < 0.5)** | | | |
| Isoleucine | 0.444* | 0.106 | 0.230 |
| Valine | 0.494* | 0.206 | 0.386 |
| Lactate | | 0.316 | 0.409* |
| Alanine | 0.322 | | |
| Asparagine | 0.281 | 0.136 | 0.480* |
| Creatinine | 0.075 | 0.078 | 0.168 |
| Glycerol | 0.006 | 0.264 | 0.465* |
| Threonine | 0.532** | 0.072 | |
| Histidine | 0.321 | 0.352 | 0.326 |
| Phenylalanine | 0.411** | | 0.354 |
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| Formate | | -0.14 | |

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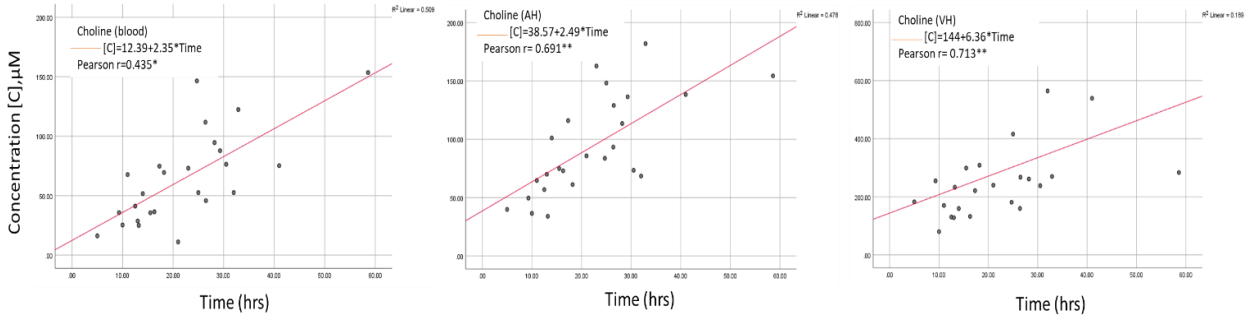


**Figure 1: The dependences of choline concentration in the human AH, VH, and serum on PMI**

## Discussion

The findings from this study and earlier research suggest that analyzing metabolites in anatomically separated fluids is more appropriate for estimating the postmortem interval (PMI) than analyzing serum (8, 9). Blood vessels serve as a means of connecting all areas of the body. Post-mortem metabolomic changes are influenced by various processes occurring at different rates throughout the body. These processes include biochemical reactions, anaerobic chemical cell breakdown, microbial activity, and the diffusion of metabolites from one part of the body to another. Consequently, the levels of possible PMI biomarkers in serum exhibit more unpredictability than those in the ocular fluids VH and AH (10). From a technical perspective, AH has several advantages over VH. Firstly, AH sampling is more straightforward and has a lower probability of blood contamination than VH sampling (11). Secondly, AH has significantly lower levels of lipids and proteins, eliminating the need for lipid removal during sample preparation. This simplifies the sample preparation process, decreasing the potential for experimental mistakes (12). Simultaneously, the relationship between PMI and metabolite concentrations in VH is somewhat more robust than in AH (Table 1). This discovery might be ascribed to the enhanced separation of VH from the circulatory system. The rise in metabolite concentrations in AH and VH can be due to two primary factors: the breakdown of cells in the surrounding tissues and the diffusion of metabolites from the blood to VH and AH across the hemato-ophthalmic barrier, driven by the concentration gradient (7). In an earlier investigation with experimental animals, glycerol, hypoxanthine, and choline were identified as the most favorable choices for estimating postmortem interval (PMI) (9). The quantities of these three chemicals in rabbit's ocular fluids (VH and AH) exhibit a significant and consistent increase, as observed in a study by Zelentsova et al. in 2016. The current research, including human samples, reveals that only choline fulfilled the anticipated outcomes, exhibiting a strong positive connection with PMI: only two substances, pyruvate and glucose, correlate significantly negatively with PMI (13). The decline in their levels in biological fluids after death correlates to the anaerobic glycolysis processes occurring in most human tissues. Regrettably, the concentrations of glucose and pyruvate exhibit significant fluctuations in serum, VH, and AH (14). In addition, it may be inferred that the concentrations of glucose and pyruvate decrease in an exponential rather than linear manner, which renders them inappropriate for estimating PMI. However, these levels can further confirm PMI when evaluated using other methods. If glucose concentration exceeds two mM or pyruvate exceeds 60µM in AH or VH, PMI is likely shorter than 24 hours (15).

## Conclusion

The study underscores the efficacy of metabolomic analysis of ocular fluids, specifically aqueous humor (AH) and vitreous humor (VH), for post-mortem interval (PMI) estimation. Compared to serum, these fluids exhibit more stable and predictable metabolite changes. Metabolites such as glutamate, choline, glycine, and formate showed strong positive correlations with PMI, particularly in AH and VH. Conversely, glucose and pyruvate demonstrated significant negative correlations with PMI. These findings suggest that AH and VH are more reliable for PMI determination, with hypoxanthine and choline emerging as promising biomarkers for forensic investigations.

## Declarations

**Data Availability statement**
All data generated or analyzed during the study are included in the manuscript.
**Ethics approval and consent to participate.**
Approved by the department concerned. (IRBEC-0388-92 dated 12-8-22)
**Consent for publication**
Approved
**Funding**
Not applicable

## Conflict of interest

The authors declared an absence of conflict of interest.

## Authors Contribution

***LUBNA RIAZ (Assistant Professor)***
*Data Analysis*
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*Revisiting Critically*
***MUHAMMAD NOMAN RASHID (Assistant Professor)***
*Final Approval of version*
***RIAZ AHMED SHAHID (Associate Professor)***
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*Concept & Design of Study*

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