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TIME OF DEATH ESTIMATION BASED ON THE ANALYSIS OF TANATHOCHEMICAL PROCESSES IN FORENSIC MEDICINE

Sonia Zięba[1], Marek Wiergowski[1,*], Marta Krzyżanowska[1], Tomasz Gos[1], Ireneusz Sołtyszewski[2,3]

[1]Medical University of Gdańsk, Faculty of Medicine, Department of Forensic Medicine, Gdańsk, [2]University of Warmia and Mazury in Olsztyn, Faculty of Law and Administration, Department of Criminology and Criminalistics, Olsztyn, [3]Medical University of Warsaw, Faculty of Medicine, Department of Forensic Medicine, Warszawa, Poland

Abstract: The aim of this study was to identify the optimal method of estimating the post-mortem interval (PMI) based on a literature analysis, especially papers on measuring the concentrations of endogenous substances. The literature review included data obtained between 1960 and 2021, and presented in the PubMed database. Identify 55 publications, and, based on these, we were able to identify the biological material and substances which had been most frequently used to assess the PMI. The most common biological materials were: vitreous humor (61%), blood (20%) and urine (7%). The most common endogenous substances were: potassium ions (42%), γ-hydroxybutyric acid - GHB (13%), hypoxanthine (11%). It has to be emphasized that the measurement of the concentration of a single endogenous substance in a biological matrix is usually not sufficient to precisely establish the PMI. The literature review indicates that it is the concentration of potassium ions that is determined most frequently in terms of the PMI. We identified factors which have an impact on the post-mortem potassium level (extraction, preliminary sample processing, mass of the corpse, age, gender, cause of death). Hypoxanthine is frequently measured along with potassium ions, and the concentration of the former increases with the PMI (24-60 hours).The literature analysis reveals that potassium and hypoxanthine demonstrate a positive correlation with the PMI. The vitreous humour still remains the preferred biological material for the PMI estimation. Some authors suggest the alternative use of synovial fluid obtained from the knee joint.

Keywords: post-mortem interval (PMI), time since death, time of death, potassium, hypoxanthine, vitreous humour.

INTRODUCTION

One of the most important aspects of forensic pathology is to accurately estimate the time that has elapsed since death, abbreviated as PMI – post-mortem interval, TSD – time since death, TOD – time of death, where the PMI abbreviation is most commonly used. In criminal cases, the correct PMI estimation may allow the identification or exclusion of the murder suspect, or the verification of defendant alibi and witnesses testimonies [1]. On the other hand, the estimation of PMI may prove helpful in civil law cases in identifying the potential heir, e.g. by the determination of the sequence of deaths in a collective accident, the sequence of deaths in a dyadic death (murder–suicide) case, or by the validation of insurance policy at the moment of death. In the early post-mortem period, which does not exceed the first 48 hours after death, the PMI estimation is related to the measurement of corpse temperature, usually taken in the rectum, and to the evaluation of post-mortem signs. The evaluation of interlethal reactions, such as mechanical or electrical muscle reactivity or pupil reaction (pupil-dilating or pupil-constricting) pharmacological substances (atropine, acetylcholine, pilocarpine), which are administered to the conjunctival sac or eyeball, may also prove useful [2].

The post-mortem interval in the later period may be determined by the examination of chemical products of tissue degradation and by means of forensic entomology [3]. The estimation of the time of death based on the measurement of chemical parameters includes usually the determination of changes in the concentrations of endogenous substances

*Correspondence to: Marek Wiergowski, Medical University of Gdańsk, Faculty of Medicine, Department of Forensic Medicine, M. Skłodowskiej-Curie 3a, 80-210 Gdańsk, Poland, E-mail: marek.wiergowski@gumed.edu.pl

which develop after death. These include potassium ions, γ-hydroxybutyric acid (GHB), hypoxanthine, chlorides, urea, uric acid, creatinine and lactates [4-8]. The changes in concentrations of the above-mentioned substances, which are mainly determined in the blood, urine and vitreous humour, depend on the decomposition stage and thus are related to the PMI [7,8]. Each of these biological matrices has its own set of advantages and disadvantages, but it is the vitreous humour that has gained significant importance in recent years. Therefore, many statistical models of PMI estimation have been developed, which are related to its analysis [8,9]. The fluid obtained from the eyeball proved to be very valuable material, as it is less prone to the autolysis when compared with other biological materials. The eyeball is filled with vitreous humour between the retina and the lens. It is a transparent fluid, which contains no blood vessels and consists of water in 98 % of its volume. The remaining components are sugars, salts, phagocytes and collagen fibres [10]. Research proved that the tanathochemical processes act more slowly in the eyeball compared to other regions because of its location and the chemical composition of vitreous humour. Experimental studies have been performed with numerous bacteria species, taking into account both gram-positive (inter alia, *Staphylococcus aureus*, *Streptococcus viridans*) and gram-negative (inter alia, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) species involved in post-mortem decomposition. These models required the introduction of corrective factors, such as the ambient temperature, the cause of death and age, as they influenced the concentrations of substances present in vitreous humour [9,11].

When the implementation of above-mentioned analytical methods is difficult or impossible, entomological methods of the quantitative and qualitative evaluation of insects found in the corpse may be applied. Forensic entomology distinguishes two observational periods of post-mortem transformations: up to 30 days for the developmental method, and over 30 days for the successive method [3].

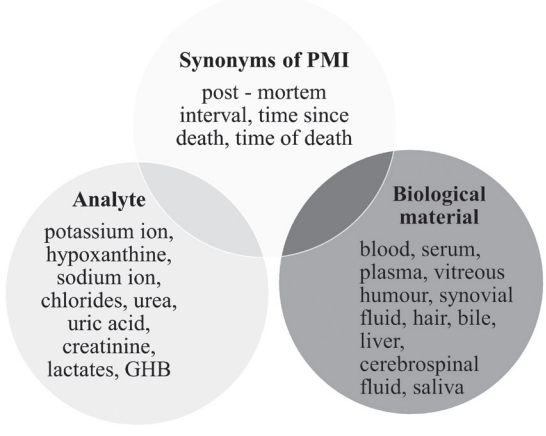

Figure 1. Keywords grouped into categories: analytes, biological materials and synonyms of PMI.

METHODOLOGY

The aim of this study was to identify the optimal method for estimating the PMI. We analysed publications presenting the autopsy results and multiparametric analysis of thanatochemical processes, which described chemical examinations aiming at the determination of the concentrations of endogenous substances. The confidence interval of such measurements has to be acceptable to meet the needs of forensic science. The literature review included data obtained between 1960 and 2021, and presented in the PubMed (https://pubmed.ncbi.nlm.nih.gov) database. 22 keywords were selected and divided into three groups:

- 9 analytes and synonyms: potassium ion (K+), hypoxanthine (Hx), sodium ion (Na+), chlorides (chloride ions, Cl-), urea (carbamide), uric acid, (2,6,8-Trihydroxypurine), creatinine, lactates, GHB.

- 10 biological materials: blood, serum, plasma, vitreous humour (vitreous body, VH), synovial fluid, hair, bile, liver, cerebrospinal fluid, saliva.

- 3 synonyms of PMI: post–mortem interval (PMI, *post mortem intervallum*), time since death (TSD), time of death (TOD).

The papers were searched through the keywords in the following categories: 'biological material' and 'synonyms of PMI' and 'analyte' and 'synonyms of PMI' (Fig. 1). Then the search area was narrowed down by selecting the time period in the years 1960-2021. This procedure significantly reduced the number of records and allowed for a better matching of the search.

In the group of keywords "analyte" and "biological material" in the years 1960-2021, most studies were found for potassium ion and hypoxanthine (Fig. 2).

The number of publications in the group of keywords "biological material" and synonym PMI was the same in the time intervals. Most results were obtained for "blood" and "vitreous humour" (Fig.3).

Table 1 presents the full results for the group of keywords: 'analyte' + 'synonyms of PMI'. The most relevant keyword turned out to be 'potassium + post-mortem interval' (n=63). The fewest results were obtained for the use of the words: TSD, TOD and post mortem intervallum. The keywords did not always

match the expected result, other hash extensions were encountered: TSD - total saponins of dioscorea, TOD - target organ damage. The word "post mortem intervallum" is most often used in the publications on forensic entomology.

Table 2 presents the full results for the groups of keywords: 'biological material + 'synonyms of PMI'. The most relevant keyword turned out to be keywords 'blood' + 'time of death' (n=137).

DISCUSSION

Biological material

The literature analysis allowed us to identify 55 publications which met the inclusion criteria. The most commonly studied biological material was the vitreous humour – in 61% of papers (Fig. 4) [1,4-9,11-58] which probably results from the fact that it is the least contaminated structure and its degradation is slower than that observed in other tissues [5]. On the other hand, researchers frequently reported problems with the small amount of vitreous humour that could be obtained for analysis, as its volume usually does not exceed 1-2 mL [45].

The second most commonly studied biological matrix was blood (20%). Its advantage lies in the possibility to collect a large volume of blood during an autopsy (up to 50 mL). On the other hand, blood is susceptible to autolysis and contamination. Of all the materials which are routinely collected during autopsy, the fewest number of studies concerned urine (7%) in comparison to blood and vitreous humour.

Table 1. Number of publications for keywords 'analytes' and 'synonyms of PMI' in the period 1960-2021

Analyte + synonyms of PMI	1960-1969	1970-1979	1980-1989	1990-1999	2000-2009	2010-2019	2020-2021	Preliminary total number	Matched total number
potassium ion + post - mortem interval	0	0	8	6	18	23	8	268	63
potassium ion + time since death	0	0	2	5	6	5	2	125	20
potassium ion + time of death	2	4	12	4	15	12	1	262	50
SUM OF PUBLICATIONS IN TIME INTERVAL	**2**	**4**	**22**	**15**	**39**	**40**	**11**	**655**	**133**
hypoxanthine + post - mortem interval	0	0	0	3	10	17	3	46	33
hypoxanthine + time since death	0	0	0	2	3	2	0	15	7
hypoxanthine + time of death	0	0	0	3	0	4	0	23	7
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**0**	**8**	**13**	**23**	**3**	**84**	**47**
sodium ion + post - mortem interval	0	0	0	2	1	6	0	58	9
sodium ion + time since death	0	0	0	0	1	3	0	21	4
sodium ion + time of death	0	0	0	1	1	0	0	67	2
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**0**	**3**	**3**	**9**	**0**	**146**	**15**
chlorides + post - mortem interval	0	0	0	0	0	2	0	35	2
chlorides + time since death	0	0	0	0	0	0	0	18	0
chlorides + time of death	0	0	0	0	0	0	1	49	1
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**0**	**0**	**0**	**2**	**1**	**102**	**3**
urea + post - mortem interval	0	0	0	1	2	4	0	24	7
urea + time since death	0	0	0	0	0	0	0	14	0
urea + time of death	0	1	0	2	1	2	1	44	7
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**1**	**0**	**3**	**3**	**6**	**1**	**82**	**14**
uric acid + post - mortem interval	0	0	0	0	1	3	0	8	4
uric acid + time since death	0	0	0	0	2	0	0	14	2
uric acid + time of death	0	0	0	1	0	0	0	27	1
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**0**	**1**	**3**	**3**	**0**	**49**	**7**
creatinine + post - mortem interval	0	0	0	0	1	2	0	41	3
creatinine + time since death	0	0	0	0	0	0	0	5	0
creatinine + time of death	0	2	3	1	2	6	0	161	14
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**2**	**3**	**1**	**3**	**8**	**0**	**207**	**17**
lactates + post - mortem interval	0	0	0	0	0	2	0	2	2
lactates + time since death	0	0	0	0	0	1	0	5	1
lactates + time of death	0	0	0	0	0	0	0	8	0
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**0**	**0**	**0**	**3**	**0**	**15**	**3**
GHB + post - mortem interval	0	0	0	0	0	6	0	6	6
GHB + time since death	0	0	0	0	0	0	0	0	0
GHB + time of death	0	0	0	0	1	0	0	1	1
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**0**	**0**	**1**	**6**	**0**	**7**	**7**

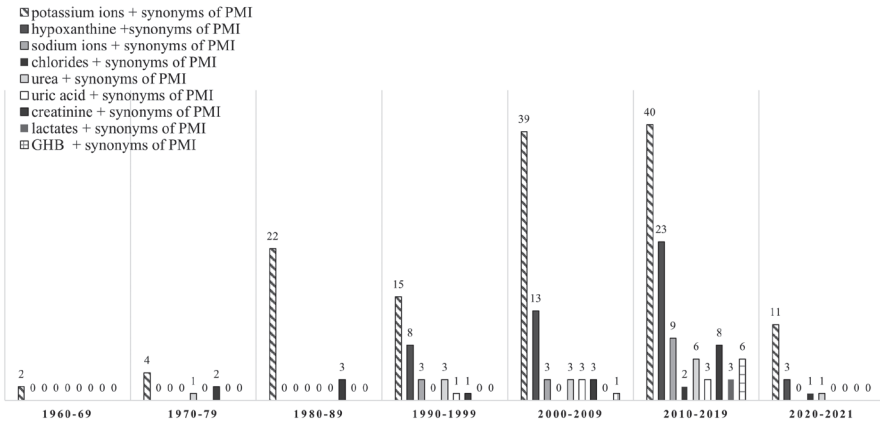

Figure 2. Number of papers in decades* with 'analytes' (potassium ion, hypoxanthine, sodium ion, chlorides, urea, urea acid, creatinine, lactates, GHB) in combination with synonyms of PMI in years 1960-2021. * - except of the years 2020-2021.

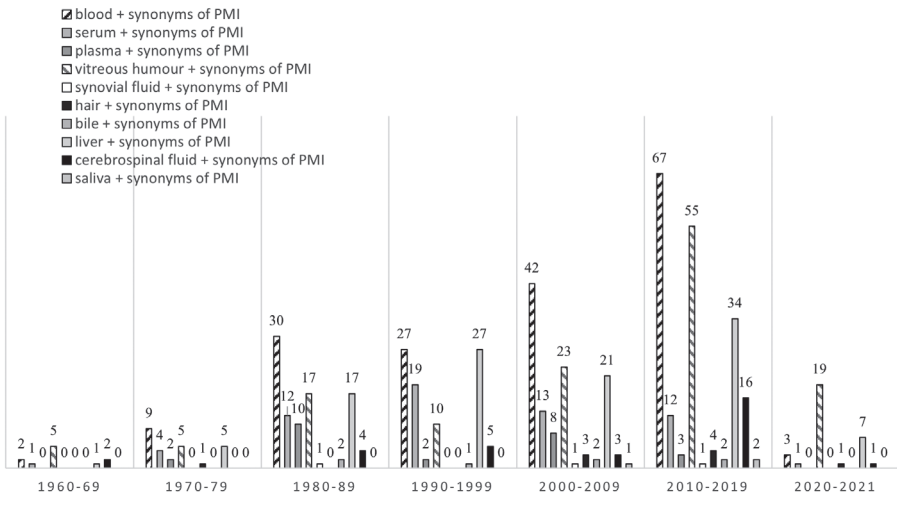

Figure 3. Number of papers in decades* with 'biological material' (blood, serum, plasma, vitreous humour, synovial fluid, hair, bile, liver, cerebrospinal fluid, saliva) in combination with synonyms of PMI in years 1960-2021. * - except of the years 2020-2021.

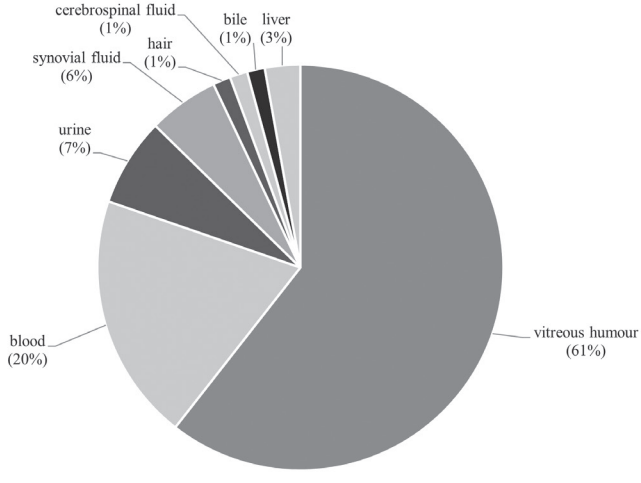

Figure 4. Distribution of biological materials used for PMI determination (in some papers two or more materials were analysed) [1,4-9,11-58].

The downside of urine is that it may be contaminated and it is not always available. Some authors analysed more than one biological matrix, and the most commonly studied combination of biological materials used as source of obtaining the required information was blood and the vitreous humour [42,46,47]. Synovial fluid has recently also been used more frequently as biological material (6%). This biological fluid is well isolated and protected by synovial bursa, and therefore demonstrates less vulnerability to autolysis caused by bacterial growth (comparable to the vitreous humour) [31]. However, synovial fluid is rarely examined, as it is not routinely collected during an autopsy. Other matrices amounted to 6% of all biological materials and included hair, bile, liver, cerebrospinal fluid [4,33,41,46]. Some authors analysed a few other matrices [4,7,26,33,39,43,46,48].

Endogenous substances

After the initial analysis, two compounds were selected as PMI markers: potassium ions and hypoxanthines. Due to the unsatisfactory number of publications (less than 10%), the following were rejected: sodium ions, chlorides, urea, uric acid, creatinine, lactates. (Fig. 5). GHB (13%) was also rejected as the usefulness of GHB as a PMI marker is questionable. Independently of natural release after death, this psychoactive substance may be found in post-mortem analytes due to sexual abuse facilitation or recreational use [37]. Moreover, although analytical limits for postmortem GHB concentration were

established, in authors' opinion more research is needed to confirm the usefulness of GHB in PMI estimation [4,36,42,39].

Potassium ions

The method of time of death estimation based on the measurements of potassium ions concentration in the vitreous humour was developed as early as in 1960 [28]. The advantage of this method is a well-established mechanism of post-mortem release of these ions. Therefore, there are numerous publications describing the correlation between the PMI and potassium levels. The extracellular concentration of potassium ions increases as the activity of sodium-potassium pump is terminated, which consequently leads to an unconstrained leakage of K+ ions from cells [11,20] (Fig. 6).

Potassium was the most commonly used endogenous substance for the estimation of PMI, as its concentration was assayed in 37, i.e. in 42% of the analysed papers [1,4-9,11-58] (Fig. 5). However, the literature review suggests the need of simultaneous evaluation of different markers, which are potentially important for the PMI estimation. It is emphasised

Table 2. Number of publications for keywords 'biological materials' and 'synonyms of PMI' in the period 1960-2021

Biological material + synonyms of PMI	1960-1969	1970-1979	1980-1989	1990-1999	2000-2009	2010-2019	2020-2021	Prelimi-nary total number	Matched total number
blood + post - mortem interval	0	0	3	2	7	27	0	561	39
blood + time since death	0	0	0	1	1	2	0	191	4
blood + time of death	2	9	27	24	34	38	3	1216	137
SUM OF PUBLICATIONS IN TIME INTERVAL	**2**	**9**	**30**	**27**	**42**	**67**	**3**	**1968**	**180**
serum + post - mortem interval	0	0	0	0	1	8	2	169	11
serum + time since death	0	0	0	0	1	0	0	56	1
serum + time of death	1	4	12	19	13	12	1	308	62
SUM OF PUBLICATIONS IN TIME INTERVAL	**1**	**4**	**12**	**19**	**15**	**20**	**3**	**533**	**74**
plasma + post - mortem interval	0	0	0	0	0	4	0	203	4
plasma + time since death	0	0	0	1	2	1	0	52	4
plasma + time of death	0	2	10	2	8	3	0	229	25
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**2**	**10**	**3**	**10**	**8**	**0**	**484**	**33**
vitreous humor + post - mortem interval	0	0	6	3	14	26	12	169	61
vitreous humor + time since death	0	0	0	2	3	15	6	76	26
vitreous humor + time of death	5	5	11	5	6	14	1	140	47
SUM OF PUBLICATIONS IN TIME INTERVAL	**5**	**5**	**17**	**10**	**23**	**55**	**19**	**385**	**134**
synovial fluid + post - mortem interval	0	0	0	0	1	0	0	1	1
synovial fluid + time since death	0	0	0	0	0	0	0	2	0
synovial fluid + time of death	0	0	1	0	0	1	0	7	2
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**1**	**0**	**1**	**1**	**0**	**10**	**3**
hair + post - mortem interval	0	0	0	0	0	3	0	12	3
hair + time since death	0	0	0	0	0	0	0	3	0
hair + time of death	0	1	0	0	3	1	1	38	6
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**1**	**0**	**0**	**3**	**4**	**1**	**53**	**9**
bile + post - mortem interval	0	0	0	0	0	0	0	10	0
bile + time since death	0	0	0	0	0	0	0	1	0
bile + time of death	0	0	2	1	2	2	0	38	7
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**2**	**1**	**2**	**2**	**0**	**49**	**7**
liver + post - mortem interval	0	0	0	1	9	9	2	159	21
liver + time since death	0	0	0	0	0	1	0	37	1
liver + time of death	1	5	17	27	12	24	5	429	91
SUM OF PUBLICATIONS IN TIME INTERVAL	**1**	**5**	**17**	**28**	**21**	**34**	**7**	**625**	**113**
cerebrospinal fluid + post - mortem interval	0	0	0	2	0	6	0	26	8
cerebrospinal fluid + time since death	0	0	0	1	1	3	0	13	5
cerebrospinal fluid + time of death	2	0	4	2	2	7	1	64	18
SUM OF PUBLICATIONS IN TIME INTERVAL	**2**	**0**	**4**	**5**	**3**	**16**	**1**	**103**	**31**
saliva + post - mortem interval	0	0	0	0	0	1	0	2	1
saliva + time since death	0	0	0	0	0	0	0	4	0
saliva + time of death	0	0	0	0	1	1	0	12	2
SUM OF PUBLICATIONS IN TIME INTERVAL	**0**	**0**	**0**	**0**	**1**	**2**	**0**	**18**	**3**

that a range of conditions may have an impact on the methodology of PMI estimation, which are associated with the correct extraction and initial processing of the sample, ambient temperature, and the body temperature or age of the deceased [7,9].

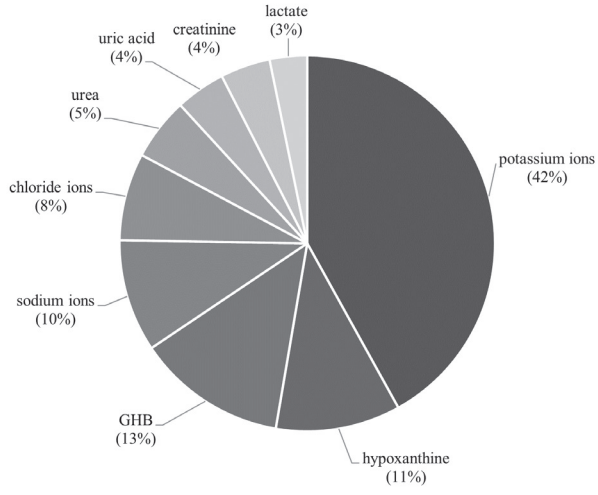

Figure 5. Distribution of examined substances in the analysed publications (in some papers two or more substances were analysed) [1,4-9,11-58].

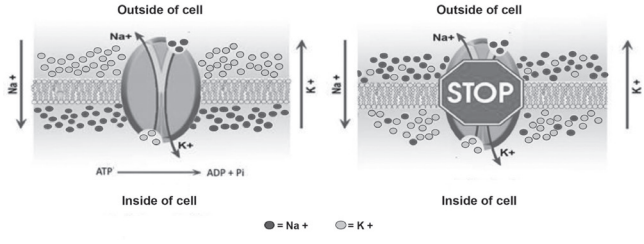

Figure 6. Migration of potassium ions in active (a) and inactive (b) sodium-potassium pump [11, 20].

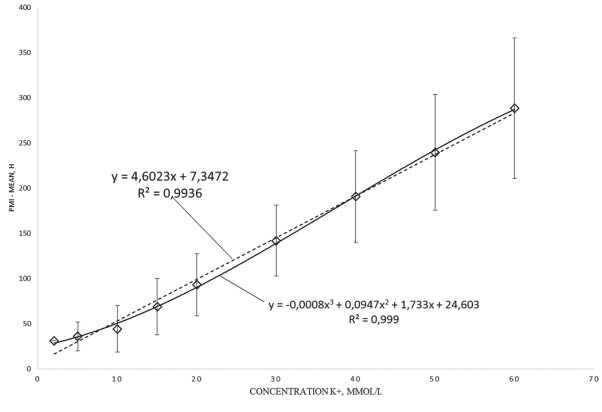

Figure 7. Averaged relationship between the potassium ion concentration and the PMI (based on the data presented in Table 2) with the calculated standard deviations and linear (dashed line) and polynomial (continuous line) correlation coefficients [17- 23, 26,27,32,33,35].

Lateralisation of deceased

Rathinam *et al.* [27] analysed the influence of intrinsic and extrinsic factors on the post-mortem concentration of potassium ions in vitreous humour obtained from both eyeballs. The study group consisted of 55 deceased, excluding subjects with head trauma and ophthalmic diseases, and whose vitreous humour was not suitable for the analysis (turbid or contaminated with blood). The mean potassium ion concentrations in the right and left eye were very similar (8.8 ± 3.9 mmol/L and 8.9 ± 3.9 mmol/L, respectively). Therefore, no statistically significant lateralisation effect was found.

Gender of deceased

The same conclusion was drawn by Chandrakanth according to the impact of gender on this marker [5]. However, whereas differences between females and males were statistically insignificant, a higher ratio of sodium to potassium was found in vitreous humour in males [5].

Age of deceased

There is a series of studies, which evaluated the concentration of potassium in vitreous humour in relation to this variable. Cordeiro *et al.* [9] stated that age had no impact on the PMI in subjects 18-97 years old but emphasised that the measurements were performed on the first day after death. Similarly, Jashnani [21] revealed that age did not influence the concentration of potassium ions in individuals whose age ranged from 15 to 88 years. The studies by Foster [19] and Rathinam *et al.* [27] corresponded with those reports, although the study by Foster did not include individuals under 18 years. On the contrary, Zilg *et al.* [11] reported an accentuated impact of age on the concentration of potassium ions in vitreous humour of younger individuals and found up to a 16% variation between different age groups.

Cause of death

Cause of death can importantly influence potassium concentration in vitreous humour. Zilg *et al.* [11] studied the impact of following causes of death: poisoning, drowning, immediate and delayed traumatic death, kidney failure, and diabetic coma. However, only three cases of diabetic coma were present in his study and potassium levels were higher in all of them. Apart from that, Zilg *et al.* [11] did not note any differences due to disease. According to Foster [19], no differences were observed due to medication or disease. Munoz

Table 3. Methods of PMI biomarkers evaluation in different biological matrices (abbreviations: VH - vitreous humour, SF - synovial fluid) [5,6,9,11,12,14,17,19,21,24-26,28,30,31,33,45,57]

No.	Analyte	Matrix (volume)	Sample preparation	Instrumental analysis	LOQ/LOD	R^2	Ref.
1.	K^+	VH (0.1 mL)	Samples were stored at -20°C. Centrifuged at 16.000 r/min for 10 min, only the supernatant was decanted.	photometric, potentiometric, turbidimetric (ARCHITECT 8000)	n/a	0.851	23
2.	K^+	VH (0.1 mL)	Samples were stored at -20°C. Diluted 1:20 with a 40 mg/mL aqueous solution of barium chloride (internal standard).	capillary electropherograph with a UV absorbance detector (A PACE MDQ; capillary column - 75 mm ID × 50 cm, 60 cm)	LOD 9 µmol/L	0,999	16
3.	K^+	VH (n/a)	Samples were centrifuged.	ion selective electrode (ADVIA 2400 chemistry system)	n/a	n/a	18
4.	K^+	VH (0.2 mL)	Not pre-treated (without dilution, centrifugation, or sonication).	ion selective electrode (ABL 625 radiometer with UniCel DxC 800)	LOQ 100 µmol/L	n/a	11
5.	K^+	VH, blood (1,5-2 mL)	Blood in test tubes with EDTA. VH in sterile plain vials. Samples centrifuged at 3500 r/min for 10 min.	ion selective electrode (Analyser AU680)	n/a	n/a	25
6.	K^+	VH (0.1 mL)	Samples were stored at -70 °C.	turbidimetric (Humalyzer Junior)	n/a	n/a	29
7.	K^+	VH (2 mL)	Centrifuged at 4500 r/min and supernatant was transferred to another container.	ion selective electrode, flow-through, liquid membrane electrode (Roche 9180 Electrolyte Analyzer)	n/a	n/a	5
8.	K^+	VH (0.15 mL)	n/a	indirect potentiometry (Advia 2400).	n/a	0,99	9
9.	K^+	VH (3-4 mL)	Samples were stored at 4°C, centrifuged at 2000 r/min for 5 min.	flame photometry (FLM3, Biolyte 2000)	n/a	n/a	20
10.	K^+	VH (2.5 mL)	Centrifuged at 2050 r/min for 10 min.	ion selective electrode (LX20 Automated Analyzer)	n/a	n/a	44
11.	K^+	SF (1-1.5 mL)	Samples were stored at -80°C, centrifuged at 3500 r/min for 10 min.	ion selective electrode (AVL 9181)	n/a	0.756	30
12.	K^+	VH (1.5–2 mL)	Centrifuged at 3500 r/min for 10 min.	ion selective electrode (indirect potentiometry method)	n/a	n/a	13
13.	K^+	VH (n/a)	Samples were stored at 4°C, centrifuged at 3000 r/min for 10 min	indirect potentiometry (BM/747)	n/a	n/a	24
14.	K^+	VH (n/a) SF	Centrifuged at 13,000 g for 10 min. Supernatant solutions were stored at -80 °C. Vortexed for 10 sec. Viscous SF samples were diluted with deionized water.	photometric, potentiometric, turbidimetric (ARCHITECT c16000)	n/a n/a	n/a	11
15.	K^+	CSF (5 mL) VH (2 mL)	Samples were stored at -18 °C and -70 °C. Centrifuged at 3000 r/min for 10 min.	ion selective electrode (Beckman auto-analyser)	n/a	n/a	32
16.	K^+	VH (2mL)	Samples were stored at -80 °C. Vortexed for 30 s using the highest level. Centrifuged, 1650 × g for 8 min. The supernatant was divided into four aliquots	potentiometric method (VLYTE1 Integrated Multisensor K800A),	n/a	0,73	57
17.	Hx	VH (n/a)	Prepared in deionized water and filtered through a filter unit with a 0.45 mm pore size. Vortexed and pipetted to filter unit. Centrifuged at 9000 r/min for 90 min at 4 °C. Samples were stored at -20°C and -75°C.	capillary electrophoresis (BioFocus 3000, column: 50 mm id. × 30 cm)	n/a	0.921	27
18.	Hx	VH (0.1 mL)	Samples were stored at -70°C.	colorimetric (Amplex Red Xanthine/ Xanthine Oxidase Assay Kit.)	n/a	n/a	29
19.	Hx	VH (0.15 mL)	Centrifuged at 14.500 r/min for 10 min. Samples added 2 mL of 2 mM ammonium hydroxide and 25 µL of internal standards. SPE (OASIS MAX) employed for sample clean-up. Cartridges were conditioned with 2 mL of methanol and 2 mL of water before loading the samples into the SPE column. Washed 2 mL 5% ammonium hydroxide in water and 2 mL 5% ammonium hydroxide in methanol. Dried the cartridges for 10 min under vacuum, elution two steps: first with 2 mL of 2% formic acid in water and second with 2.5 mL of formic acid in methanol. The eluates were collected in the same tube and evaporated with nitrogen at 40 °C. Samples were reconstituted in 100 µL of 10 mM ammonium acetate (pH = 4.5).	high performance liquid chromatography with tandem mass spectrometry HPLC-ESI-MS/MS-MRM (Waters Alliance 2795 HPLC Separation Module, column - Atlantis T3 2.1 mm x 100 mm, 3 µm)	LOQ 2.5 µmol/L	0,99	9
20.	Hx	VH (n/a)	Samples were stored at 4°C. Centrifuged at 3000 r/min for 10 min.	high performance liquid chromatography (Waters 996 PDA)	n/a	n/a	24
21.	Hx	VH (1.5 mL)	Sample 1.5 mL and 10 µL of ISTD (5 µM) were added into 2 mL of 2 mM ammonium hydroxide. Vortexed for 1 min and centrifuged at 1177 g for 10 min. A total of 2 mL of the supernatant was loaded into the preconditioned cartridge. Oasis MAX® cartridge was conditioned with 2 mL of MeOH and 2 mL of DI water. Cartridge was washed with 2 mL of 5% ammonium hydroxide (aq) and 2 mL of 5% ammonium hydroxide in MeOH and dried for 10 min. The analytes were eluted with 2 mL of 2% formic acid (aq) and 2.5 mL of 2% formic acid in MeOH. The elute was evaporated under nitrogen gas at 40 °C. The residue was reconstituted with 1 mL of a solution of MeOH and 0.1% formic acid (1:1, v/v).	high performance liquid chromatography with tandem mass spectrometry HPLC-ESI-MS/MS-MRM (HPLC - Agilent 1260, MS/MS - Sciex 3200 QTrap, column - poroshell 120 EC-C18 2.7 µm, 4.6×50 mm, guard column 5×2 mm)	LOD 10 µmol/L LOQ 50 µmol/L	0.9977	6

LOD – limit of detection, LOQ – limit of quantification, R^2- coefficient of determination.

Barus *et al.* [25] divided investigated individuals in two groups, i.e. the former of hanging cases and the latter of remaining causes of death. There were accentuated differences between them in potassium and hypoxanthine concentrations. The discrepancies could be explained by different terminal venous blood pressure in both groups, which may influence vascular leakage. Therefore, Zilg [11] separated cases of sudden death (e.g. by shooting or hanging) from others. The former cases had a 1% higher concentration of potassium compared to the latter, which was an insignificant difference. In sudden death cases Sturner [35] found electrolyte disturbances, which influenced potassium concentration in vitreous humour.

Ambient temperature

The relation between ambient temperature and post-mortem potassium concentration was also investigated. Zilg *et al.* [11] applied a mathematical formula with a temperature factor to evaluate its impact on potassium level and found that it was responsible for 5% of parameter variation. Rognum [28] revealed a positive correlation between both potassium and hypoxanthine concentrations and the ambient temperature.

Other factors

Other factors which may impact PMI estimation by K+ measurements in vitreous humour are: analytical technique, method of sampling and storage, and the stage of corpse decomposition [33].

Vitreous humour is collected from both eyes with a needle and a 5-10 mL syringe using an appropriate technique, which allows approx. 2-3 mL of fluid to be obtained from each eye. This fluid needs to be transferred into a clean tube and centrifuged at 3000 g for 15 minutes. The supernatant is then transferred into another clean tube and frozen at -20 °C [28]. The vitreous humour collected from both eyes may be transferred to and stored in a single container as the difference in the level of potassium ions between eyes is small and statistically insignificant [14]. The concentrations of potassium, sodium and chloride ions are most commonly measured using an ion-selective electrode (indirect potentiometry). The literature analysis, in terms of the methodology of preparing the sample of biological material (mainly the vitreous humour) for analysis (Table 3), revealed that centrifugation was required to remove the biological matrix (2000-3500 RPM for at least 5 minutes). The instrumental analysis most commonly included a potentiometric method utilising ion-selective electrodes. Biological samples were stored cooled or frozen for further analysis (4°C, 20°C, -70°C, -80°C) [12,21,22,24,30,57].

The baseline potassium ion concentration in the vitreous humour is in physiological range before death, i.e. 3.5 to 5.0 mmol/L [31]. The research confirms that the increase in post-mortem concentration of potassium ions corresponds with PMI [9,26]. There are, however, contradicting reports concerning the impact of age and ambient temperature on this parameter. Zilg *et al.* [11] carried out studies which proved that the increase in potassium ion level is not linear. The effect is related to the influence of two mentioned variables (ambient temperature and age). The authors developed mathematical formula which enables the calculation of estimated PMI and takes into account the potassium ion level, ambient temperature, and age of the deceased (https://slbd.shinyapps.io/pmiPredictor/). Furthermore, Zilg *et al.* [11] stated that after approx. 7 days the potassium level reaches an equilibrium of 35 mmol/L. It is still under debate which method of PMI estimation based on potassium and hypoxanthine measurements is the most precise as the hypoxanthine level also increases after death.

Table 4 presents linear regression formulas (n=16) for the PMI calculation concerning the content of potassium ions in vitreous humour, which were found in the literature [17- 23, 26,27,32,33,35,57] . They allow also of the estimation of minimal and maximal potassium ion levels for PMI=0 and the maximal PMI range, respectively.

The calculations with first nine formulas present in Table 4 allowed us to find the mean K+ ion concentrations and respective standard deviations, which subsequently enabled us to determine the average linear relationship (dashed line, R^2=0,9936) and polynomial curvilinear dependence (continuous line, R^2=0.999) (Fig. 7). The number of investigated individuals was in range of 32-210.

Cordeiro *et al.* [9] suggested formulas which were intended to estimate the PMI based on the value of a few parameters: the concentration of potassium ions, hypoxanthine, urea, rectal temperature, ambient temperature, and body mass. The authors used generalized additive models (GAMs) applied in criminalistics to develop these formulas. These models were obtained from the website http://cran.es.rproject.org/web/packages. According to Cordeiro *et al.* [9], Model 1 is most convenient. They also found that body mass has an accentuated impact on the corpse cooling,

and thus on the estimated PMI. Rathinam *et al.* [27] studied samples of vitreous humour up to 40 hours after death. The highest concentration increase was observed between 24 and 40 hours after death. After 40 hours the concentration of potassium ions began to drop. The authors decided to use only samples collected from the right eye, as there is no statistical difference between the right and the left eye. Jashnani *et al.* [21] studied potassium ion levels in vitreous humour up to 50 hours after death. The post-mortem K+ level was in range of 4.80 - 45.60 mmol/L and showed a positive linear correlation with PMI, especially in the first 20 hours after death.

Hypoxanthine

Besides potassium ions and GHB, hypoxanthine is the third most commonly assayed analyte in terms of PMI estimation. It is a product of purine degradation and after death it diffuses with a concentration gradient from the retina into vitreous humour, where its level is commonly measured along with the concentration of potassium ions [6,8,28,30,38]. Samples are prepared by centrifugation or by isolation and enrichment with solid-phase extraction techniques (SPE) and instrumental methods using high-performance liquid chromatography with tandem mass spectrometry (HPLC/MSMS) [6,8,28].

The post-mortem hypoxanthine level increases with the PMI. This increase is dependent on the temperature, i.e. the higher the ambient temperature, the higher the hypoxanthine level [6]. Cordeiro *et al.* [9] developed five models to estimate the PMI (presented in Table 5) and observed that the concentrations of potassium and hypoxanthine increase with time, but the correlation becomes weaker. The increase in hypoxanthine level may be observed up to 120 hours after death [29]. Age and gender do not have any impact on the hypoxanthine level [30]. Salam *et al.* [30] carried out a study analysing the correlation of potassium ions and the hypoxanthine level with the PMI (Table 5).

Table 4. Estimation of the PMI based on the determination of the potassium concentration and a combination of methods [17- 23, 26,27,32,33,35,57]

	Reference	Number of cases	PMI formula	PMI range, [h]	Concentration of $[K^+]_{min}$, mmol/L (for PMI=0)	Concentration of $[K^+]_{max}$, mmol/L (for PMI=maximum)
1.	Adelson *et al.* (1963)	209	PMI = 5.88 [K+] - 31.53*	21	5.362	8.934
2.	Sturner (1964)	125	PMI = 7.14[K+] - 39.10*	104	5.476	20.042
3.	Hanson *et al.* (1966)	203	PMI = 5.88 [K+] - 47.10*	310	8.010	60.731
4.	Stephens and Richards (1987)	1427	PMI = 4.20 [K+] - 26.65*	35	6.345	14.679
5.	Madea (1989)	107	PMI = 5.26[K+] - 30.90	34 - 120	12.338	26.688
6.	Coe (1969)	145	PMI = 6.15[K+] - 38.10*	100	6.195	22.455
7.	James *et al.* (1997)	100	PMI = 4.32[K+] - 18.35	n/a	4.248	n/a
8.	Munoz *et al.* (2002)	176	PMI = 3.63[K+] - 17.33	1 - 29	5.049	12.763
9.	Zhou *et al.* (2007)	62	PMI = 5.88[K+]- 32.71	1 - 27	5.732	10.155
10.	Jashnani *et al.* (2010)	120	PMI = 1.08[K+] - 2.82	50	2.611	48.907
11.	Bortolotti *et al.* (2011)	164	PMI = 5.77 [K+] - 13.28	2 - 110	2.648	21.366
12.	Mihailovic *et al.* (2012)	32	PMI = 2.75[K+]-11.98	3 - 30	5,447	15.271
13.	Siddhamsetty *et al.* (2014)	210	PMI = 4.75[K+]- 27.9	72	5.872	21.031
14.	Bohra *et al.* (2014)	200	PMI = 3.75[K+]- 16.22	n/a	4.325	n/a
15.	Foster (2016)	78	PMI = 6.42[K+]- 40.94	6 – 162	7.311	31.610
16.	Murthy (2019)	100	PMI = 5.26 [K+] - 30.9	3 - 52	6.444	15.671
17.	Focardi (2020)	120	PMI = 6,16[K+]- 32,49	20–72	5.274	n/a

$[K^+]$ concentration of potassium ions, *- original unit (meq/L) , n/a- not applicable.

Table 5. Estimation of the PMI based on the concentration of potassium ions (K+), hypoxanthine (Hx), rigidity, hypostasis, corneal turbidity [30]

	PMI prediction formula [h]	R^2
1.	$PMI=1.377K^+ + 9.050$	0.370
2.	$PMI=0.027Hx + 15.401$	0.207
3.	$PMI=0.978K^+ + 0.014\ Hx + 9.178$	0.285
4.	PMI=2.02 rigidity + 6.31 hypostasis + 6.94 corneal turbidity - 24.94	0.717
5.	$PMI=0.667K^+ + 0.003Hx$ - 0.191 rigidity + 4.907 hypostasis + 4.68 corneal turbidity - 13.624	0,780

$[K^+]$- concentration of potassium ions, Hx-concentration of hypoxanthine, R^2- coefficient of determination.

They discovered that the highest concentration may be observed in the period between 24 and 60 hours after death, and the lowest values are in the first few hours after death. The most important conclusion of this study was that up to 60 hours after death the evaluation of post-mortem changes is more reliable than tanathochemical examination in estimating the PMI.

In conclusion, despite the fact that for many years the time of death has been estimated based on the concentration of potassium ions, researchers are attempting to concomitantly measure other endogenous substances. The literature analysis reveals that potassium and hypoxanthine demonstrate a positive correlation with the PMI. The vitreous humour still remains the preferred biological material for the PMI estimation. Some authors suggest the alternative use of synovial fluid obtained from the knee joint.

The usefulness of current methods of endogenous substances analysis applied to the PMI estimation should be emphasized. It is also worth noting that current methods reveal a narrower uncertainty margin than methods applied previously. The improved accuracy of mathematical models is related to simultaneous application of different variables, which may influence the PMI estimation, such as ambient temperature, and age of the deceased.

Conflict of interest

The authors declare that they have no conflict of interest.

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