Determination and Correlation of Electrolytes and Trace Elements from Vitreous Humour and Synovial Fluid by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) for Estimation of Post-Mortem Interval (PMI): Experimental Study in an Animal Model

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Abstract

The estimation of post-mortem interval (PMI) procedures seem to subjectivity in assessment and less sensitivity and specificity. Biochemical determination of trace elements from closed-system samples, such as vitreous humour (VH) and synovial fluid (SF) is an alternative laboratory investigation for PMI estimation. The inductively coupled plasma-mass spectrometry (ICP-MS) provides more rapid, precise and sensitive analysis of elements samples. However, determination of electrolytes and trace elements in VH and SF by ICP-MS, and application for PMI has not been intensively investigated. In this study, VH and SF from twenty adult domesticated pig carcasses were collected at 1, 3, 6, 12, 24, 36, and 48-hour after death. Samples were analysed for total protein concentration, Ca, Cu, Fe, K and, Na using ICP-MS. The results showed that the protein concentration in VH was significantly increased in time dependent manner, but not for SF. The level of K and Fe in both VH and SF increased in time dependent manner, with a good correlation between both sample sources. The calcium concentration of both VH and SF increased in time dependent manner, but there was no correlation between both sample sources. Sodium was significantly reduced at 36-48 h, with a correlation between both sample sources. However, the copper level in VH was increased at 36-48 h but reduced in SF. In conclusion, this study showed *for the first time* that multiple trace elements and electrolytes could be detected, using the ICP-MS, in both of VH and SF simultaneously and additionally its correlation.

Keywords: *time since death; post-mortem interval; inductively coupled plasma-mass spectrometry; vitreous humour **;** synovial fluid*

Introduction

The time since death or post-mortem interval (PMI) estimation through the analysis of post-mortem biochemistry was first investigated in the mid-twentieth century [1]. Biochemical markers are examined to predict time since death [2-4]. The majority of the PMI parameters utilize the ante-mortem and post-mortem concentration discrepancies of biochemical substances in blood, which occur rapidly and are also influenced by several factors and become limitation of interpretation [5]. A "closed compartments" such as cerebrospinal fluid, synovial fluid, and vitreous humour could be more suitable for the assessment of post-mortem changes.

The vitreous humour is one of the preferred mediums for post-mortem electrolyte concentrations [6, 7]. Several organic and inorganic analytes have been investigated [3,

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[8]. Alternatively, synovial fluid could also be a source for PMI estimation [9]. The electrolyte analytical technique that is most widely used for potassium and sodium ions in vitreous humour and synovial fluid by means of ion-selective electrodes (ISE) and flame photometry. This process utilises commercial automated or manually operated analytical instruments which are available in most hospitals and laboratories. [8, 10-14].

Inductively coupled plasma-mass spectrometry (ICP-MS) provides more rapid, precise, and sensitive analysis of very low concentrations of elements in liquid samples. However, the application of ICP-MS in the determination of electrolytes and trace elements in vitreous humour and synovial fluid has not been intensively investigated. The purpose of this study is to integrate ICP-MS into the investigation of multiple electrolytes and trace elements in vitreous humour and synovial fluid for PMI estimation.

Materials and Methods

Chemical and reagents

The internal standards for ICP-MS; Ca, Cu, Fe, K and, Na were supplied by SCP Science (Quebec, Canada). Ultrapure water was purchased from Agilent (California, United States). Other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, United States).

Materials

Twenty adult domesticated male and female pig (*Sus domesticus*) carcasses of varied ages were selected as the animal model. Each pig carcass weighed ≥ 45 kgs. The pig carcasses were purchased from a standard abattoir, Pratumthani province, Thailand. Immediately after death, the animal carcasses were immediately transported to an open field research site, where they were placed on the grass under natural shade and ambient conditions. The ambient and pig carcass temperature was recorded simultaneously in accordance with sample collection time.

Vitreous humour collection

Approximately 100 µl of vitreous humour was collected from both eyes of each pig carcass using a 21G needle with a 1 ml plastic syringe. Vitreous humour samples were collected 1, 3, 6, 12, 24, 36, and 48-hour intervals from the same animal carcasses. The same needle and syringe were used to collect vitreous humour from both eyes, stored at -80°C until further analysis.

Synovial fluid collection

Approximately 100 µl of synovial fluid was collected from each pig carcass using a 21G needle attached with 1 ml plastic syringe. The same needle and syringe were used to collect synovial fluid from all 4 joints at 1, 3, 6, 12, 24, 36, and 48-hour intervals from the same animal carcasses. The samples were stored at -80°C until further analysis.

Sample Preparation

An aliquot part of 50 µl of thawed vitreous humour sample was transferred into 15-ml plastic tubes. Ultrapure nitric acid 5% (v/v) was then slowly added to the sample to give a final volume of 3 ml. The samples were centrifuged at 500 x g at room temperature for 15 minutes. The supernatant was then transferred to pre-washed 15-ml plastic tubes with natural caps for multi-element analysis.

Determination of protein concentration by Bradford protein assay

Fifty microliters of the protein samples were added to 2.5 ml of Bradford reagent (BIO-RAD, USA) and incubated at room temperature. Sample absorbance was measured using a spectrophotometer at 595 nm.

Determination of electrolytes and trace elements by inductively coupled plasma-mass spectrometry (ICP-MS)

All samples were analysed three times for Ca, Cu, Fe, K and, Na using inductively coupled plasma-mass spectrometry (ICP-MS) (7500 Series, Agilent). The samples and standards were aspirated and nebulized using a quartz Scott-type spray chamber into the argon plasma through a peristaltic pump operated at a flow rate of around 0.9-1.0 ml/min. The elements were analysed with a quadruple mass analyser.

Statistical Analysis

All values were expressed as mean ± standard deviation (S.D.). All comparisons were assessed by one-

way analysis of variance (ANOVA), followed by the Tukey-Kramer test. A statistical value of less than 0.05 was considered significant. Correlation analysis was calculated using Pearson's correlation or Spearman's rank correlation.

Results

1. Alteration of carcass body temperature

The pig carcasses were placed on the grass under natural shade and ambient conditions (Figure 1A). The body temperature dropped to 28.5 °C at 12 h after death which corresponded to the ambient temperature (Figure 1B). The carcass temperature was then stable until the end of the data recording period, regardless of fluctuating ambient temperatures.

2. Determination of total protein content from vitreous humour and synovial fluid

The results showed that vitreous humour protein collected at 6 hours after death was significantly higher than at 0-3 hours. The vitreous humour protein peaked at 36 hours after death. (Figure 1C), which significantly different when compared to other periods. In contrast, the synovial fluid results showed no significant change in protein concentration.(Figure 1D).

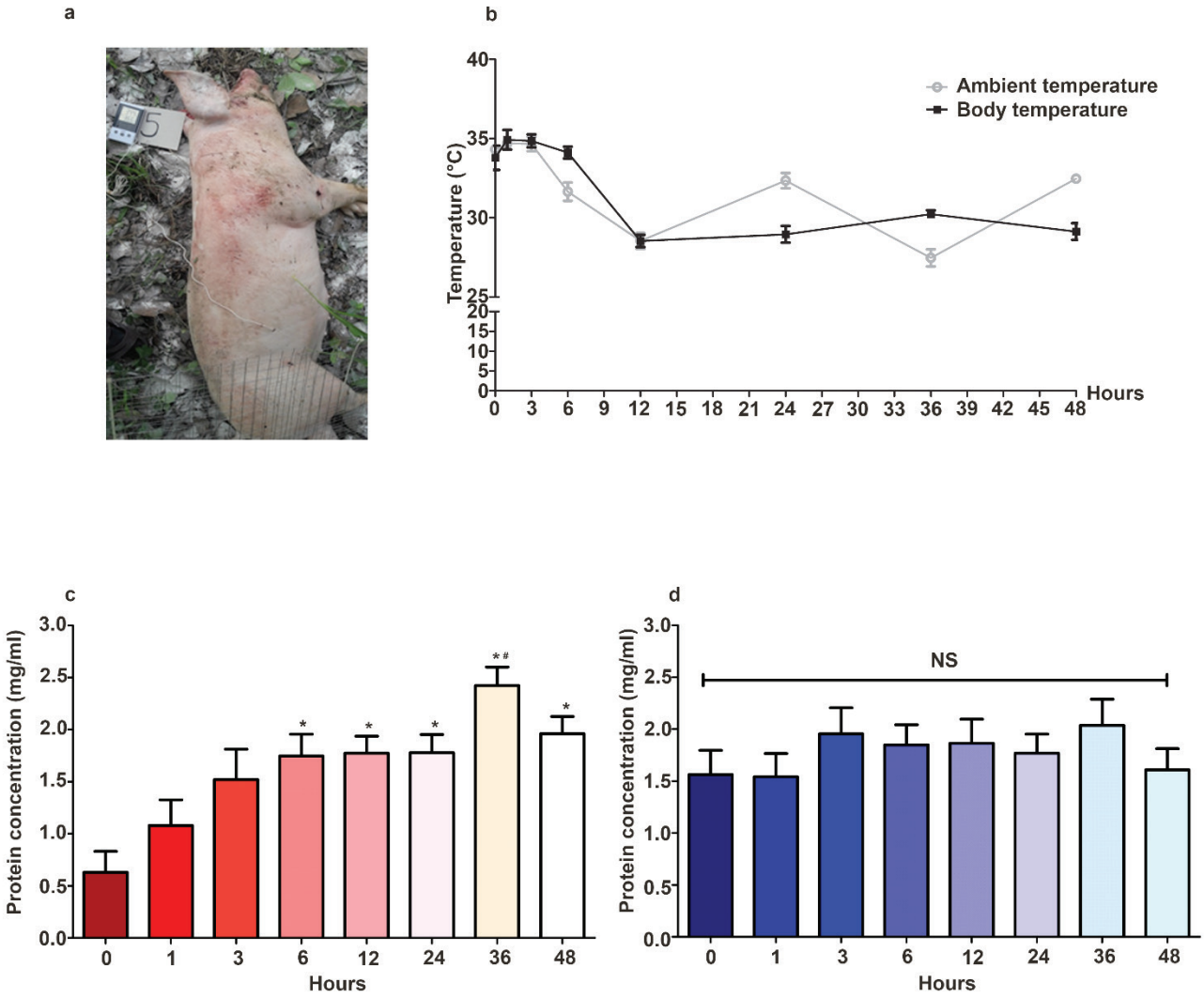

Figure 1. The animal carcasses were placed on the grass under natural shade and ambient conditions

(a). The ambient and pig carcass temperatures were recorded over 48 hours (b). The protein concentration of vitreous humour (c) and synovial fluid (d) was determined by Bradford assay.

3. Determination of sodium ion from vitreous humour and synovial fluid

The sodium ion concentration in vitreous humour and synovial fluid changed at 36 hours after death. However, the sodium ion concentration in vitreous humour at 48 hours after death was significantly lower than at earlier time checks (Figure 2A). Whereas, the sodium ion in synovial fluid at 36 hours and 48 hours after death were significantly lower than at earlier time points (Figure 2B). Interestingly, the results showed a correlation of sodium ion concentration, at 48 hours after death between vitreous humour and synovial fluid ($r = -0.529$, $p = 0.02$) (Figure 2C).

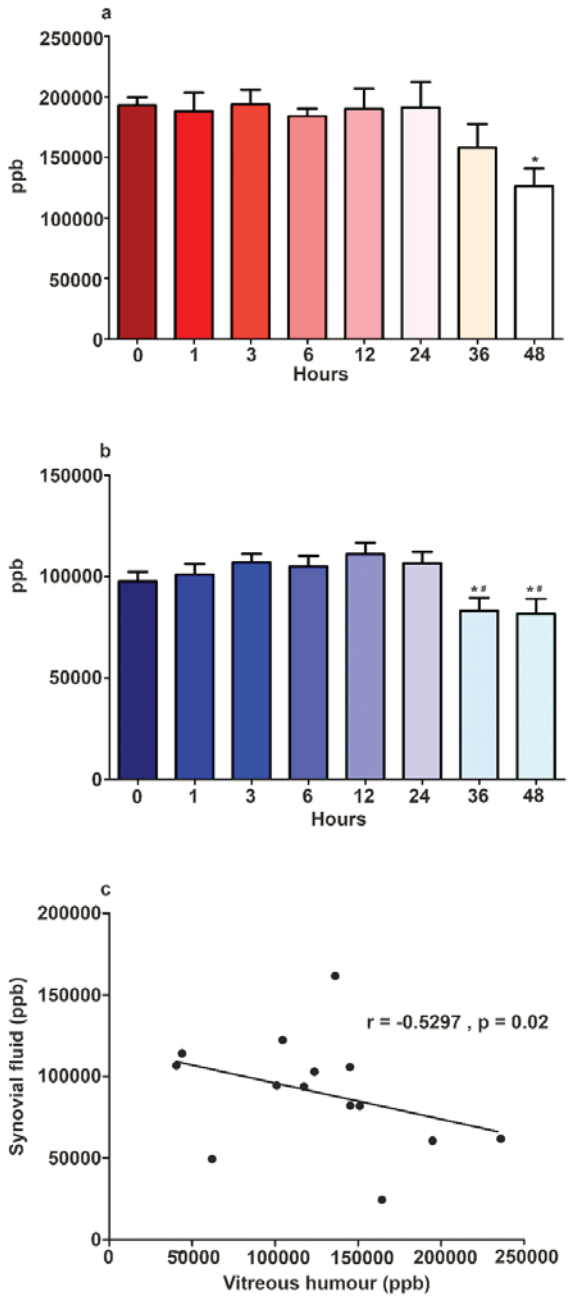

Figure 2. Determination of sodium ion from vitreous humour (a) and synovial fluid (b) and correlation of sodium ion at 48 hours after death (c)

4. Determination of potassium ion from vitreous humour and synovial fluid

The results showed that the concentration of potassium ion in both vitreous humour and synovial fluid continuously increased over the data collection period. The potassium ion concentration in vitreous humour increased significantly at 12 hours after death (Figure 3A), whereas the synovial fluid potassium ion concentration increased significantly at 6 hours after death (Figure 3B). However, there was no correlation in potassium ion concentration at 48 hours after death between vitreous humour and synovial fluid (r = -0.433, p = 0.06) (Figure 3C).

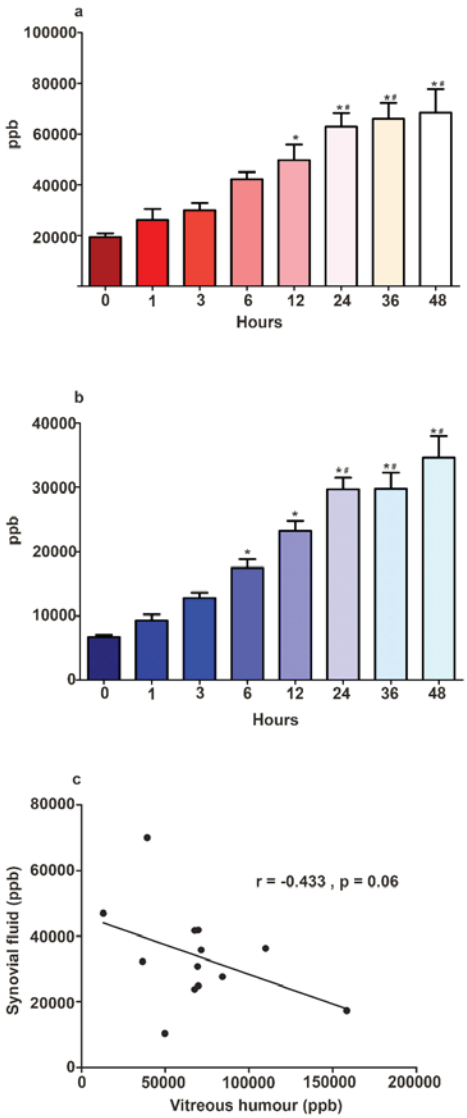

Figure 3. Determination of potassium ion from vitreous humour (a) and synovial fluid (b) and correlation of potassium ion at 48 hours after death (c)

5. Determination of calcium ion from vitreous humour and synovial fluid

The calcium ion concentration in vitreous humour increased significantly at 24 hours after death (Figure 4A), whereas the calcium ion concentration in synovial fluid was significant at 12 hours after death. (Figure 4B). There was no correlation of calcium ion concentration at 48 hours after death between vitreous humour and synovial fluid (r= 0.233, p = 0.212) (Figure 4C).

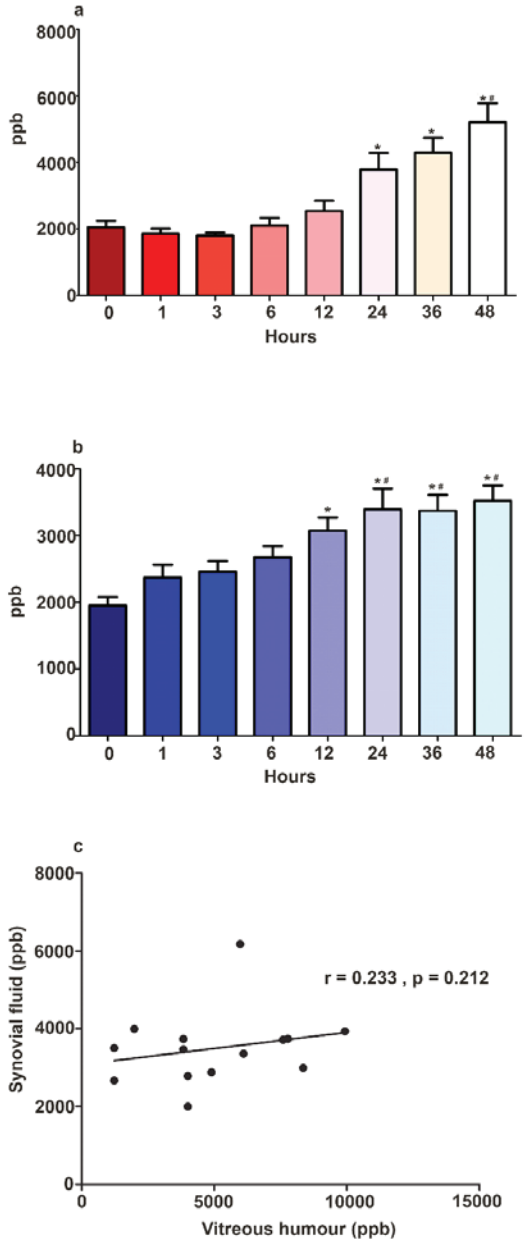

Figure 4. Determination of calcium ion from vitreous humour (a) and synovial fluid (b) and correlation of calcium ion at 48 hours after death (c)

6. Determination of iron ion from vitreous humour and synovial fluid

The results showed a continuous increase in iron concentration in vitreous humour throughout the data collection period. At 36-48 hours after death the level of iron ion concentration in vitreous humour was significantly higher than earlier time points (Figure 5A). In contrast, the iron concentration in synovial fluid increased 1 hour after death and remained unchanged thereafter. However, statistical analysis showed that at 48 hours after death the level of iron ion concentration in vitreous humour was significantly higher when compared to 0 hours (Figure 5B). Interestingly, there was a positive correlation in iron ion concentration, at 48 hours after death, between vitreous humour and synovial fluid ($r = 0.610$, $p = 0.02$) (Figure 5C).

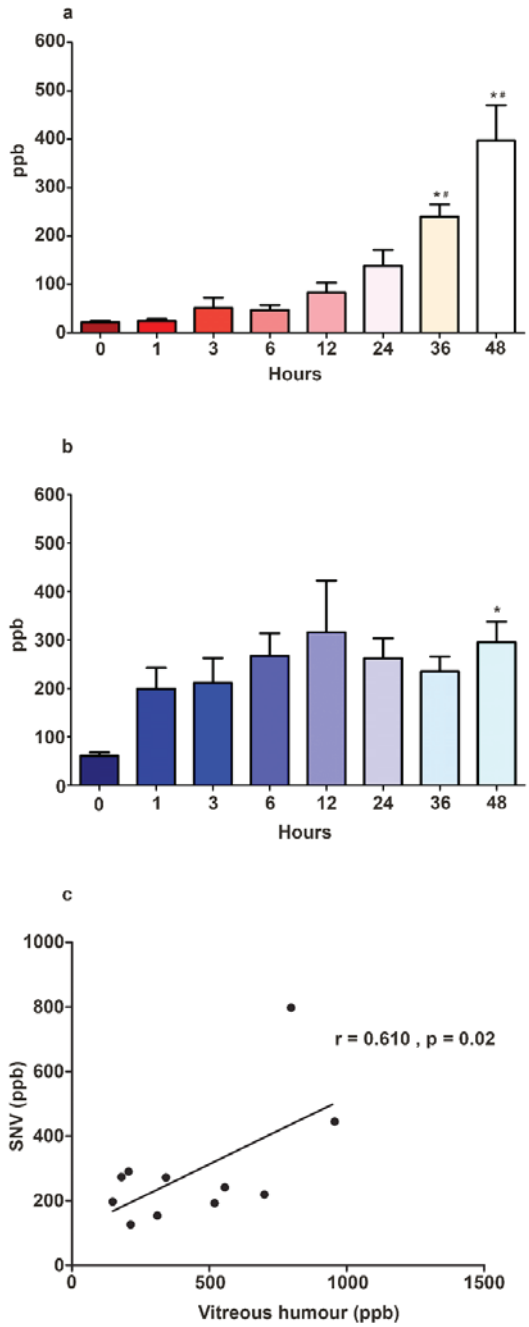

Figure 5. Determination of iron ion from vitreous humour (a) and synovial fluid (b) and correlation of iron ion at 48 hours after death (c)

7. Determination of copper ion from vitreous humour and synovial fluid

At 24-48 hours after death the level of copper ion concentration in vitreous humour was significantly higher from other time points (Figure 6A). In contrast, the copper ion concentration in synovial fluid was unchanged until 36-48 hours after death. The level of copper ion concentration in synovial fluid was significantly lower from 36 hours onwards when compared to the previous time points (Figure 6B). Interestingly, there was a significant correlation of the copper ion concentration, at 48 hours after death, between vitreous humour and synovial fluid ($r = 0.517$, $p = 0.02$) (Figure 6C).

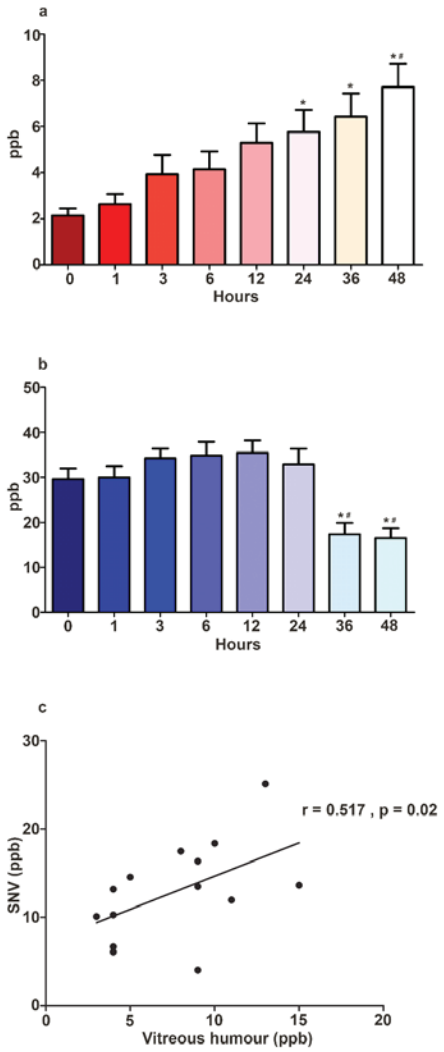

Figure 6. Determination of copper ion from vitreous humour (a) and synovial fluid (b) and correlation of copper ion at 48 hours after death (c)

Discussion

The majority of post-mortem interval (PMI) studies concentrate on ^{12} in vitreous humour and there is insufficient information available on other electrolytes and minerals beyond K, Na, Cl and Ca. Moreover, studies on the correlation of various electrolyte and mineral concentrations between different body fluid compartments are limited [15].

Vitreous humour, potassium ion ^{12} is the most extensively investigated biochemical marker as its concentration increases after death [15]. Vitreous humour is one of the most suitable mediums for investigation because, firstly, electrolytes are secure and isolated in the eyeball. The extreme density and toughness of the sclera offers significant protection and stabilization of internal content. In which the electrolyte concentrations in vitreous humour can be stable for up to 120 hours post-mortem [3]. Therefore, vitreous humour is considered ideal for the study of PMI, which firstly reported in 1959 [16]. However, there was some discrepancy in conclusions due to the estimated intercepts and gradients or slopes of regression line(s), the reliability and confidence interval and the standard error or deviation [12, 17].

Alternatively, synovial fluid is also considered as medium for PMI estimation. The protein and large biochemical constituents in synovial fluid are well established in rheumatology [18]. The joint capsule (stratum fibrosum) and the inner layer comprises a unique cellular lining of synoviocytes creating the synovial membrane [19]. A study by Ropes *et al*. [20] found the presumptive absence of fibrinogen in human synovial fluid in the absence of significant pathology, which leads to a probable advantage of synovial fluid over other mediums for PMI.

The results from the current study demonstrated that all measurable elements displayed significant changes over the data collection period. Interestingly, three of the eight elements; sodium, iron and copper ions revealed significant correlations between vitreous humour and synovial fluid. The study found that there was no single element that could serve as a marker for universal PMI estimation. The pattern of alteration did not clearly indicate a process of diffusion of some intracellular elements within vitreous body or synovial fluid.

A study by Tumram et. al. [14] also exhibited significant changes in potassium and glucose concentration in both vitreous humour and synovial fluid in cadavers.

It was apparent that different elements displayed different relationships with PMI. Thus, it was feasible to obtain a high degree of accuracy of PMI estimation using multiple vitreous elements as predictor variables. To obtain multi-elemental analysis simultaneously, the ICP-MS appeared to be the most appropriate technique and its potential has not been sufficiently explored .

Conclusions

This study is *the first* to quantitatively determine multiple elements in vitreous humour and synovial fluid simultaneously and additionally its correlation using the ICP-MS. Moreover, utilizing alterations and correlations of multiple elements in the vitreous humour and synovial fluid tends to increase the accuracy of post-mortem interval estimation. The latter has a potential use as an alternative medium for the estimation of time since death.

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Conflicts of Interest: The authors declare that they have no competing interests

Ethical Clearance: The ethical Clearance was proved by National Research Council of Thailand.

References

1. Luna A. Is postmortem biochemistry really useful? Why is it not widely used in forensic pathology? Legal medicine. 2009;11 Suppl 1:S27-30. doi: 10.1016/j.legalmed.2009.02.040. PubMed PMID: 19342268.
2. Li C, Wang Q, Zhang Y, Lin H, Zhang J, Huang P, et al. Research progress in the estimation of the postmortem interval by Chinese forensic scholars. Forensic sciences research. 2016;1(1):3-13. doi: 10.1080/20961790.2016.1229377. PubMed PMID: 30483604; PubMed Central PMCID: PMC6197124.
3. Madea B. Is there recent progress in the estimation of the postmortem interval by means of thanatochemistry? Forensic science international. 2005;151(2-3):139-49. doi: 10.1016/j.forsciint.2005.01.013. PubMed PMID: 15939145.
4. Coe JI. Postmortem chemistry update. Emphasis on forensic application. The American journal of forensic medicine and pathology. 1993;14(2):91-117. doi: 10.1097/00000433-199306000-00001. PubMed PMID: 8328447.
5. Madea B, Henssge C. Eye changes after death. In: Henssge C, Knight B, Krompecher T, Madea B, Nokes L, editors. The estimation of the time since death in the early postmortem period, second ed. London: Edward Arnold; 2002. p. pp. 103-33.
6. Thierauf A, Musshoff F, Madea B. Post-mortem biochemical investigations of vitreous humor. Forensic science international. 2009;192(1-3):78-82. doi: 10.1016/j.forsciint.2009.08.001. PubMed PMID: 19729257.
7. Madea B, Musshoff F. Postmortem biochemistry. Forensic science international. 2007;165(2-3):165-71. doi: 10.1016/j.forsciint.2006.05.023. PubMed PMID: 16781101.
8. Coe JI. Postmortem chemistries on human vitreous humor. American journal of clinical pathology. 1969;51(6):741-50. doi: 10.1093/ajcp/51.6.741. PubMed PMID: 5770672.
9. Madea B, Kreuser C, Banaschak S. Postmortem biochemical examination of synovial fluid--a preliminary study. Forensic science international. 2001;118(1):29-35. doi: 10.1016/s0379-0738(00)00372-8. PubMed PMID: 11343852.
10. Sturner WQ. The vitreous humour: postmortem potassium changes. Lancet. 1963;1(7285):807-8. doi: 10.1016/s0140-6736(63)91509-5. PubMed PMID: 13978991.
11. Jashnani KD, Kale SA, Rupani AB. Vitreous humor: biochemical constituents in estimation of postmortem interval. Journal of forensic sciences. 2010;55(6):1523-7. doi: 10.1111/j.1556-4029.2010.01501.x. PubMed PMID: 20666922.
12. Munoz Barus JI, Suarez-Penaranda J, Otero XL, Rodriguez-Calvo MS, Costas E, Miguens X, et al. Improved estimation of postmortem interval based

on differential behaviour of vitreous potassium and hypoxantine in death by hanging. Forensic science international. 2002;125(1):67-74. doi: 10.1016/s0379-0738(01)00616-8. PubMed PMID: 11852204.

13. Stephens RJ, Richards RG. Vitreous humor chemistry: the use of potassium concentration for the prediction of the postmortem interval. Journal of forensic sciences. 1987;32(2):503-9. PubMed PMID: 3572343.
14. Tumram NK, Bardale RV, Dongre AP. Postmortem analysis of synovial fluid and vitreous humour for determination of death interval: A comparative study. Forensic science international. 2011;204(1-3):186-90. doi: 10.1016/j.forsciint.2010.06.007. PubMed PMID: 20638804.
15. Coe JI. Vitreous potassium as a measure of the postmortem interval: an historical review and critical evaluation. Forensic science international. 1989;42(3):201-13. doi: 10.1016/0379-0738(89)90087-x. PubMed PMID: 2676789.
16. Naumann HN. Postmortem chemistry of the vitreous body in man. Archives of ophthalmology. 1959;62:356-63. doi: 10.1001/archopht.1959.04220030012003. PubMed PMID: 14426195.
17. Adelson L, Sunshine I, Rushforth NB, Mankoff M. Vitreous Potassium Concentration as an Indicator of the Postmortem Interval. Journal of forensic sciences. 1963;8(4):503-14. PubMed PMID: 14070464.
18. H.R. S. Synovial fluid analysis and synovial biopsy. In: W.N. K, editor. Textbook of Rheymatology. 3 ed. Philadelphia: WB Saunders; 1989.
19. Iwanaga T, Shikichi M, Kitamura H, Yanase H, Nozawa-Inoue K. Morphology and functional roles of synoviocytes in the joint. Archives of histology and cytology. 2000;63(1):17-31. doi: 10.1679/aohc.63.17. PubMed PMID: 10770586.
20. Ralphs JR, Benjamin M. The joint capsule: structure, composition, ageing and disease. Journal of anatomy. 1994;184 (Pt 3):503-9. PubMed PMID: 7928639; PubMed Central PMCID: PMC1259958.