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ABOUT THE JOURNAL

Aims and Scope

Asian Archives of Pathology (AAP) is an open access, peer-reviewed journal. The journal was first published in 2002 under the Thai name "วารสารราชวิทยาลัยพยาธิแพทย์แห่งประเทศไทย" and English name "Journal of the Royal College of Pathologists of Thailand". The journal is a publication for workers in all disciplines of pathology and forensic medicine. In the first 3 years (volumes), the journal was published every 4 months. Until 2005, the journal has changed its name to be "Asian Archives of Pathology: The Official Journal of the Royal College of Pathologists of Thailand", published quarterly to expand the collaboration among people in the fields of pathology and forensic medicine in the Asia-Pacific regions and the Western countries.

The full articles of the journal are appeared in either Thai or English. However, the abstracts of all Thai articles are published in both Thai and English languages. The journal features letters to the editor, original articles, review articles, case reports, case illustrations, and technical notes. Diagnostic and research areas covered consist of (1) **Anatomical Pathology** (including cellular pathology, cytopathology, haematopathology, histopathology, immunopathology, and surgical pathology); (2) **Clinical Pathology (Laboratory Medicine)** [including blood banking and transfusion medicine, clinical chemistry (chemical pathology or clinical biochemistry), clinical immunology, clinical microbiology, clinical toxicology, cytogenetics, parasitology, and point-of-care testing]; (3) **Forensic Medicine (Legal Medicine or Medical Jurisprudence)** (including forensic science and forensic pathology); (4) **Molecular Medicine** (including molecular genetics, molecular oncology, and molecular pathology); (5) **Pathobiology**; and (6) **Pathophysiology**.

All issues of our journal have been printed in hard copy since the beginning. Around the late 2014, we developed our website (www.asianarchpath.com) in order to increase our visibility. We would like to acknowledge that our journal has been sponsored by the Royal College of Pathologists of Thailand. We have the policy to disseminate the verified scientific knowledge to the public on a non-profit basis. Hence, we have not charged the authors whose manuscripts have been submitted or accepted for publication in our journal.

On the other hand, if any authors request a printed copy of the journal issue containing the articles, each of the copied journals costs 450 bahts for Thai authors and 30 United States dollars (USD) for international authors.

Publication Frequency

Four issues per year

Disclaimer

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MANUSCRIPT REVIEWERS

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ORIGINAL ARTICLE

Pulmonary pathology in hospitalized patient with traumatic brain injury

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Abstract

Background: Traumatic brain injury (TBI) is a major cause of death encountered in forensic practice. Respiratory complications contribute to a significant portion of mortality in these victims.

Objective: To determine the prevalence and characteristics of lung pathology among hospitalized patients with TBI who underwent forensic autopsy.

Material and methods: A retrospective autopsy report review between 2022-2023 was performed at the Faculty of Medicine Siriraj Hospital to determine the prevalence, characteristic of lung pathology, and its contributing factors among hospitalized patients with TBI.

Result: A total of 198 hospitalized TBI cases were included in this study. Most common lung pathology was pneumonia (n=109, 55%), followed by pulmonary edema (n=32, 16%). The lungs were unremarkable in 50 cases (25%). For analysis, the authors divided the cases into 4 groups based on the length of hospital stay prior to death. The prevalence of pneumonia was associated with the duration of hospitalization ($P < 0.01$), which include 46% (23/50) in group 1 (≤1 day), 32% (17/53) in group 2 (2-3 days), 57% (22/39), in group 3 (4-7 days), and 82% (47/57) in group 4 (≥8 days). There is no correlation between the prevalence of pneumonia and the initial Glasgow coma scale (GCS) score.

Conclusion: Pneumonia is present in 55% of forensic autopsy population. The duration of hospital stay is an important factor contributing to the occurrence of pneumonia.

Keyword: Traumatic brain injury, Lung pathology, Pneumonia, Neurogenic pulmonary edema

Introduction

Traumatic brain injury (TBI) is a major cause of mortality and disability [(1)]. Patients with TBI may present to an emergency service with altered mental status, neurological deficits, impaired gag reflex, dysphagia, and abnormalities in the cough reflex[(2)]. In severe cases, reduced level of consciousness prompts a respiratory support, e.g., endotracheal intubation. All these factors increase the risk of respiratory complications in these patients, including pneumonia, acute respiratory distress syndrome (ARDS), and neurogenic pulmonary edema (NPE)[(2)].

Forensic pathologists play an important role in TBI autopsy. Direct causation between the act of negligence and death must be established in order to press a criminal charge or compensation. Understanding the nature of the prevalence and characteristic of lung pathology among TBI patients might enable law enforcement officers to considerate the juristic process accordingly.

The authors conducted this study at a large forensic institution in Bangkok to determine the prevalence and characteristics of lung pathology among hospitalized patients with TBI who underwent forensic autopsy.

Materials and Methods

Study design

This study was a cross-sectional retrospective review of forensic autopsy report at Siriraj Hospital. The data collection process was performed after an approval by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital (Approval number 857/2566). The authors performed a review of forensic autopsy reports during January 2022 through December 2023. The hospitalized deceased with TBI were included in this study. Case information, including age, gender, manner of injury, duration of hospitalization, underlying medical condition, initial Glasgow Coma Scale (GCS), and lung pathology, were collected. Cases would be excluded if they were dead on arrival, external examination only, or incomplete autopsy.

Statistical analysis

Statistical analysis was performed with IBM SPSS statistic software version 22 (IBM Armonk, NY, USA). Continuous variables were compared using unpaired Student's t-test. Categorical variables were compared using chi-square test. One-way ANOVA is used to assess the significant differences between 3 or more groups. The p-value of less than 0.05 was considered statistical significance.

Results

A total of 198 TBI cases were included in this study. A majority of cases were male (163, 82.3%), with the mean age of 46.47±33.13 years old. The most common manner of injury was motorcycle accident in 117 cases (59%), followed by pedestrians in 23 cases (12.6%). The median duration of hospital stay is 2.8 days.

Table 1. Manner of injury. Most common manners of injury were drivers of motorcycle accidents in 117 cases (59%), followed by pedestrians in 23 cases (12.6%).

Manner	Number of cases
Motorcycle accident(driver)	117
Pedestrian	25
Falling	23
Fall from height	11
Motorcycle accident(passenger)	10
Body assault	5
Bicycle accident	4
Car(driver)	3

The prevalence of lung findings from this study is shown in table 2. The most common lung pathology among TBI victims is pneumonia (n=109, 55%), followed and pulmonary edema (n=32, 16%). Other findings include granulomatous inflammation and organizing diffuse alveolar damage (DAD). The lung histopathology is either unremarkable or non-specific (congestion, hemorrhage) in 50 cases (25%).

Table 2. Lung pathology. Most common lung pathology is pneumonia (n=109, 55%), followed by pulmonary edema (n=32, 16%). The unremarkable or non-specific lung pathology (congestion, hemorrhage) in 50 cases (25%)

Lung pathology	Number (198)
Pneumonia	109 (55%)
- Pneumonia with diffuse alveolar damage (DAD)	9
- Pneumonia with granulomatous inflammation	2
- Resolving pneumonia	3
Pulmonary edema	32 (16%)
Granulomatous inflammation	5 (2.5%)
Organizing DAD	2 (1%)
Unremarkable	50 (25%)

The authors divided the length of hospital stay into 4 groups, namely 1 day, 2-3 days, 4-7 days and ≥8 days. The pathology of the lung and the GCS was compared between these groups.

The prevalence of pneumonia is shown in figure 1. There was significant between duration of hospitalization and the prevalence of pneumonia (P<0.01), with the highest prevalence of 82% in patients who were hospitalized more than 8 days.

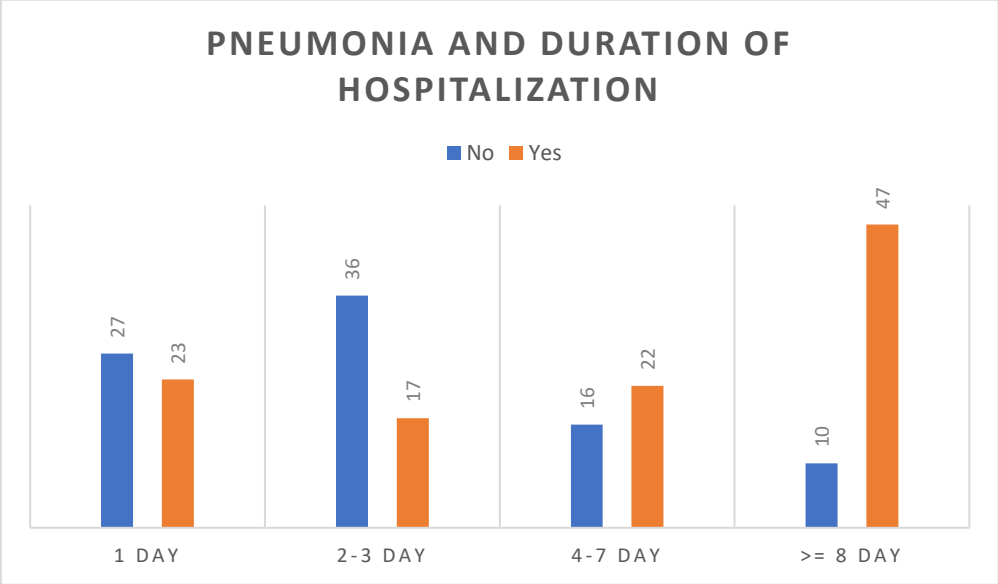

Figure 1 Pneumonia and duration of hospitalization. There was significant between duration of hospitalization and the prevalence of pneumonia (P<0.01), with the highest prevalence of 82% in patients who were hospitalized more than 8 days.

The prevalence of pulmonary edema and pneumonia show in figure 2, which there was no relation between pulmonary edema and duration of hospitalization (P=0.31)

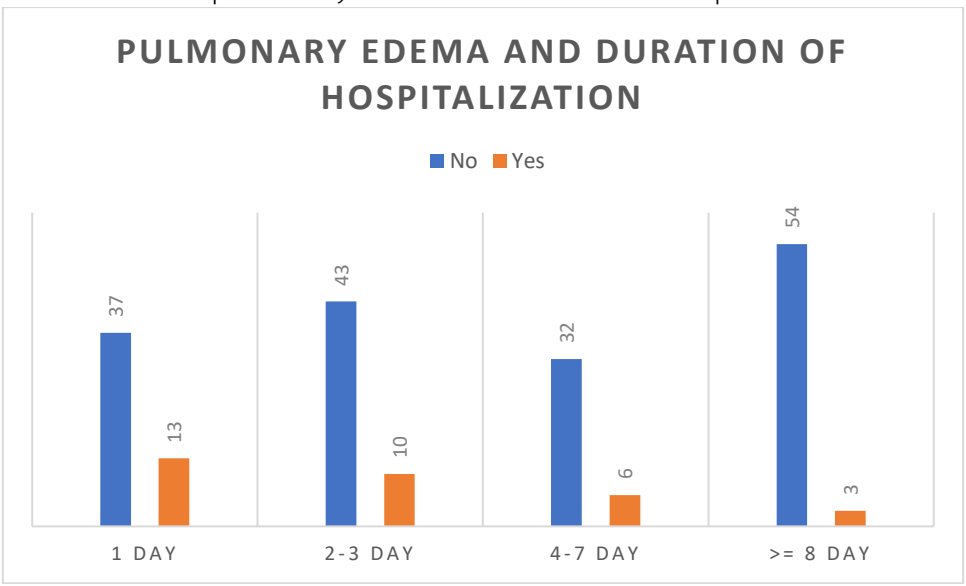

Figure 2 Pulmonary edema and duration of hospitalization. There was no statistically significant between pulmonary edema and duration of hospitalization (P=0.31)

Initial GCS and prevalence of pneumonia show in table 3, which there was no relation between initial GCS and prevalence pneumonia (P=0.57)

Table 3. Initial GCS and Prevalence of pneumonia. There was no statistically significant between initial GCS scores and prevalence of pneumonia (P = 0.57)

Initial GCS	Prevalence of pneumonia	
	Yes	no
Mild head injury	11 (44%)	14 (56%)
Moderate head injury	10 (55%)	8 (45%)
Severe head injury	60 (55%)	48 (45%)

Discussion

In this study collect cases of traumatic brain injury from 2022-2023 which have 198 cases, 90 cases in 2022 and 108 cases in 2023. The majority of cases involved males (82.3%) and the leading cause was motorcycle vehicle accidents (59%), followed by pedestrians (12.6%). These findings reflect the high-risk behaviors and environmental factors contributing to TBI, particularly among male populations and motorcycle users.

Pneumonia emerged as the most common lung pathology, affecting 55% of the cases. Previous study on ventilator-associated pneumonia (VAP) in severe TBI case showed a prevalence of 45% (60/145 cases)[1]. In another study, which focused on aneurysmal SAH patients with mechanical ventilator, have a prevalence of VAP at 35% (16/45 cases) [8].

The prevalence of pneumonia in this study was significantly associated with the duration of hospitalization (P < 0.01). On the first day, 23 out of 50 cases (46%) had pneumonia, between 4 to 7 days, 22 out of 38 cases (57%) had pneumonia and after 8 or more days, 47 out of 57 cases (82%) had pneumonia. A prior study demonstrated patients who have severe TBI admitted to the intensive care unit (ICU) developed pneumonia 29% (86 out of 290), with an average duration of 4.62 ± 3.21 days[3]. Which relate to previous research that VAP has been associated longer duration of mechanical ventilation, longer ICU and hospital length of stay[1]. This suggests that prolong hospitalization increase risk of infection due to prolong intubation which can disturb mechanical defenses in an intubated patient, such as ciliary motion and mucus secretion, progression from colonization of the upper airway, leading to tracheal colonization and finally pneumonia[4].

There was no statistically significant relationship between initial GCS scores and prevalence of pneumonia (P = 0.57), In previous study found that brain-injured patients who developed early pneumonia were found to have a lower GCS[5] because in our study we do in autopsy case which all patient have serious complication and dead. This indicates that the most important factor in developing pneumonia is the duration of hospitalization.

Pulmonary edema or neurogenic pulmonary edema which pathophysiology is release of catecholamines by the sympathetic nervous system as a response to increased intracranial pressure, which in turn leads to increased hydrostatic pressure in the lungs and increased capillary permeability. It develops within minutes to hours after the neurological injury and spontaneously resolves within 48–72 hr.[6].

The current study revealed the prevalence of pulmonary edema in 16% of cases. While a previous study on neurogenic pulmonary edema in subarachnoid hemorrhage patient showed a prevalence of 7.2% (19/262 cases)[7].

Study limitations

This study's limitations include its retrospective nature and the lack of detail in treatment, such as antibiotic administration, which may influence the observed outcomes. Future research should explore prospective approaches to evaluate the efficacy of preventive measures and therapeutic strategies in reducing pulmonary complications in TBI patients.

Conclusion

The important factor that relates to pneumonia in TBI is duration of hospitalization. The prevalence of pneumonia in TBI fairly common, especially in those who stayed in hospital more than 8 day (82%).

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ORIGINAL ARTICLE

Simple Modified QuEChERS Extraction of Blood and Vitreous Fluid in Determination of 56 common Forensic Drugs and Narcotics by LC-MS/MS

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Abstract

Background: Whole blood is the specimen of choice and vitreous fluid is an alternative sample in forensic toxicologic investigations. Sample preparation is an essential step of forensic toxicological analysis. Conventional liquid-liquid extraction or solid phase extraction procedures require significant time and effort. QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction technique was introduced for sample preparation in pesticide analysis. Modified QuEChERS extraction has been introduced for forensic toxicological analysis of amphetamine, antidepressant, cocaine and opiates in whole blood. This study extends the modified QuEChERS method to incorporate vitreous samples and additional commonly encountered drugs and narcotics in Thailand, including ketamine, mitragynine, sibutramine, and sildenafil.

Objective: To develop and validate the modified QuEChERS extraction technique for whole blood and vitreous fluid preparation for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of common drugs and narcotics.

Materials and Methods: Whole blood and vitreous fluid were spiked with 56 drugs and narcotics. The extraction of samples via the modified QuEChERS method and subsequent analysis using LC-MS/MS were validated according to the established protocol. The method was then applied to forensic cases from autopsies and compared with the routine solid-phase extraction technique.

Results: Extraction of each sample was completed within a 15-minute timeframe. The analytes were detectable by LC-MS/MS with a lower limit of detection of 1 ng/mL. Calibration curves ranged from 1 to 100 ng/mL, exhibiting linear regression with an r2 greater than 0.99. Accuracy and precision fell within acceptable criteria. Application to forensic cases yielded consistent results compared to routine analysis.

Conclusion: The modified QuEChERS extraction method has demonstrated effectiveness in preparing whole blood and vitreous fluid samples for LC-MS/MS analysis of common drugs and narcotics. Using the same extraction technique for whole blood and vitreous fluid, characterized by its affordability, simplicity, and ease of use, has the potential to enhance the efficiency of forensic toxicology laboratories.

Keywords: Sample preparation; drugs; narcotics; QuEChERS; LC-MS/MS

Introduction

Whole blood is the specimen of choice in forensic autopsy for the determination of drugs and narcotics. However, whole blood may not be available at the autopsy or be deteriorated by decomposition and postmortem redistribution. For this reason, vitreous fluid is a good alternative matrix for forensic toxicologic investigation since it is protected in the eyeball, which delays decomposition and redistribution compared to the whole blood, and the substance concentration in vitreous fluid is quite related to the concentration in the blood[1,2].

Sample preparation is an essential step in the analysis of substances in forensic toxicology investigations[3]. Different drugs and matrices require different sample preparation methods. Common and routine sample preparation methods are liquid-liquid extraction (LLE) and solid phase extraction (SPE). LLE and SPE methods consume a processing time and chemicals. QuEChERS (quick, easy, cheap, effective, rugged, and safe) was introduced as a simple preparation to determine pesticides. It reduces chemical usage and processing time using in LLE and SPE. Numerous researchers have employed the original and modified QuEChERS techniques in forensic toxicological analysis[4,5,6,]. In this study, we propose a simple preparation method using the modified QuEChERS technique for whole blood and vitreous fluid to determine common drugs and narcotics analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Notably, this study includes the analysis of certain substances not previously examined, such as mitragynine from Kratom, ketamine, sibutramine, and sildenafil, which are prevalent in Thailand.

Materials and Methods

Materials and chemicals

High-performance liquid chromatography grade methanol and acetonitrile were purchased from Merck (Sulpeco) Germany. Ammonium formate solution and formic acid (LC-MS grade) were obtained from Sigma and Fischer Chemical US, respectively.
QuEChERS (4000 mg Mg2SO4/1000 mg NaCl/ 500 mg Na2Cit/ 1000 g Na3Cit) was from UCT Pennsylvania, US.

Alprazolam, cocaine, diazepam, estazolam, fentanyl, imipramine, lorazepam, lysergic acid diethylamide (LSD), methylenedioxy ethyl amphetamine (MDEA), methcathinone, nalorphine, nitrazepam, nordiazepam, norpseudoephedrine, phencyclidine, prazepam, pseudoephedrine, tramadol, triazolam, trimipramine, sibutramine, sildenafil, zolpidem, cocaethylene-D3, cocaine-D3, codeine-D3, diazepam-D5, estazolam-D5, heroin-D9, MDA-D5, MDEA-D5, nordiazepam-D5, imipramine-D3, oxazepam-D5 were bought from LGC Germany.
Amitriptyline, amphetamine, benzoylecgonine, cathinone, clobazam, clonazepam, cocaethylene, codeine, desipramine, dextromethorphan, ecgonine methyl ester, heroin,

ketamine, methylenedioxy amphetamine (MDA), methylenedioxy methamphetamine (MDMA), mephedrone, methadone, methamphetamine, methylphenidate, mitragynine, morphine, naloxone, naltrexone, nimetazepam, nortriptyline, oxazepam, oxycodone, pentazocine, temazepam, zaleplon, zopiclone, 3,4-methylenedioxypyrovalerone, 6-acethyl morphine were purchased from Cerilliant (Sigma Aldrich) US.

Syringe filter nylon membrane (13 mm with 0.22 µm pore size), 2 mL short tread vial (amber 9 mm tread, 11.6 x 32 mm), bonded natural PTFE/white silicone septa, 9 mm blue screw cap (6 mm center hole), and 250 µL clear insert with no spring vial were purchased from Onepuresci China.

Type I water (18.2 MΩ.cm at 25 ℃) was from MicroPure Water Purification system Thermo Scientific, US.

Whole blood was from the Thai Red Cross Society, Thailand. Vitreous fluid was from the Forensic Investigation Center, Chulalongkorn University, Thailand. Both samples were tested for drugs and narcotics by LC-MS/MS and demonstrated no drugs or narcotics.

Instrumentation

A Shidmazu Liquid Chromatography Nexera X2 LC-30AD with triple quadruple mass spectrometer LC-MS 8060 was from Kyoto, Japan. Shidmazu LabSolutions software version 5.97 was used. The parameter settings of the LC-MS/MS were presented in Table1.

Table 1. Parameter, LC condition and MS/MS condition

Parameter	
Column	Shim- pax Volex SP-C18, 1. 8 µm, 2. 1 × 100 mm column (Shimadzu, Kyoto, Japan).
Mobile Phase	10 mM Ammonium formate in water with 0.1% formic acid (mobile phase A) 10 mM Ammonium formate in methanol with 0.1% formic acid (mobile phase B).
LC Condition	
The flow rate	0.3 mL/min
Gradient of the mobile phase B	0. 00– 2. 00 min: 15% , 2. 00-10. 00 min: 50% , 10. 00– 12. 00 min: 95%, 12.00–20.00 min: 95%, 20.00–21.00 min: 5%, 21.00-26.00 min: 5%.

LC Stop Time	26 min
Column Oven	40 °C
MS/MS condition	
Mode	positive electrospray ionization (ESI) mode
Interface voltage	4 kV
Nebulizing gas (N2) flow	3 L/min
Heating gas (N2) flow	10 L/ min
Interface temperature	300 °C
Desolvation temperature	250 °C
Heat block temperature	400 °C
Drying gas (N2) flow	10 L/min
Argon	270 kPa

Preparation of the standard solutions

The mixed standard stock solutions were prepared by mixing standards of drugs and narcotics in methanol to achieve stock solution concentrations of 0.01, 0.025, 0.05, 01, 0.5, 1.0, and 2.0 µg/mL.

The internal standard of drugs and narcotics were combined with methanol to yield a mixed standard stock solution with a concentration of 200 ng/mL.

Method validation

The validation method was conducted according to the set of acceptance criteria. Calibration curves were done at concentrations of 1.0, 2.5, 5.0, 10.0, 50.0, 100.0, and 200.0 ng/mL, with accepted linearity set at r2 >0.9.

Accuracy and precision were assessed through repeated analyses of samples five times over five days, with accepted criteria being %accuracy and %CV less than 20%.

The lower limit of detection (LLOD) was calculated from the point that the signal to noise ratio (S/N) of the sample peak was greater than 3, while the lower limit of quantitation (LLOQ) was calculated as LLOD with S/N greater than 10.

Selectivity was performed with blank samples and common prescribed drugs (caffeine, acetaminophen, chlorpheniramine, and lidocaine) at a concentration of 200 ng/mL.

Recovery was determined by comparing the extraction product to standard determination.

Sample processing

The method was based on the extraction technique by Dulaurent S, et al[4]. Initially, 100 µL of both whole blood and vitreous fluid, along with 10 µL internal standard, were introduced into 1.5 mL microtubes. Mixed standard stock solution was added to get concentrations of 1.0, 2.5, 5.0, 10.0, 50.0, 100.0, and 200.0 ng/mL. Mix the solution by vortexing for 30 seconds. 200 µL of acetonitrile at -20ºC of was added and mixed by vortexing for 30 seconds. 40 mg of QuEChERS powder was added to the microtube and centrifuged at 12,000 rpm for 10 minutes. The 200 µL of supernatant was filtered by a nylon syringe filter, and the filtered supernatant was mixed with 150 mL of mobile phase A to prepare the sample for LC-MS/MS analysis.

Analysis of drugs and narcotics from forensic cases

Ten samples from autopsies conducted at the Department of Forensic Medicine, Chulalongkorn University, were subjected to testing using both routine screening methods and the proposed technique for comparison.

Results

3.1 Sample preparation

A clear supernatant was obtained following the extraction process. The entirety of this method's preparation process was accomplished within a span of 15 minutes.

3.2 Chromatogram

All analytes were effectively separated under the specified LC conditions and provided ion spectra in the multiple reaction monitoring (MRM) mode of ESI-MS analysis. Furthermore, elution of all analytes occurred within a timeframe of less than 30 minutes.

Interested drugs and narcotics were eluted from the column and identified by comparing the retention time and specific m/z as depicted in Figure 1, Table 2 and 3.

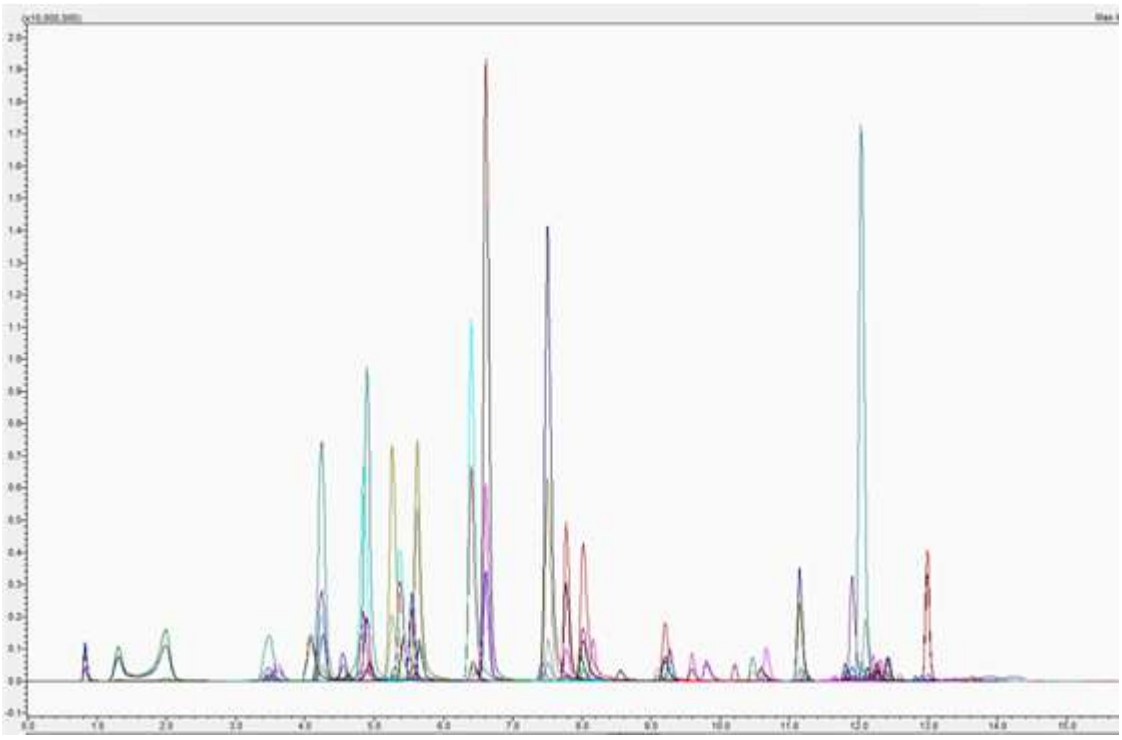

Figure 1. Ion chromatogram of the drugs, narcotics and internal standards (100 ng/mL)

Table 2. Retention time and m/z of drugs and narcotics

No	Compound	Precursor ion (m/z)	Product ions (m/z)	Retention Time	Collision energies (V)	ISTD Group	ISTD
1	3,4-Methylenedioxypyrovalerone	276.15	126.2 175.1	9.087	-24 -21	14	MDMA-D5
2	6-Acethyl Morphine	328	165 152	5.888	-37 -55	1	6-Acethyl Morphine-D3
3	Alprazolam	309.1	281.1 205.1	13.156	-26 -40	9	Diazepam-D5
4	Amitriptyline	278.1	233 117.15	12.993	-16 -22 -21	11	Imipramine-D3
5	Amphetamine	136.1	91.1 119.15	5.524	-22 -14	2	Amphetamine-D5
6	Benzoylecgonine	290.15	168.15 77	7.609	-18 -54	3	Benzoylecgonine-D3
7	Cathinone	150.1	117.15 105.15	4.326	-23 -21	14	MDMA-D5
8	Clobazam	301.05	259.05 224.1	12.909	-20 -32	7	Diazepam-D5
9	Clonazepam	316.05	270.05 214.05	12.601	-25 -36	4	Clonazepam-D4

Table 2 (Cont.). Retention time and m/z of drugs and narcotics

No	Compound	Precursor ion (m/z)	Product ions (m/z)	Retention Time	Collision energies (V)	ISTD Group	ISTD
10	Cocaethylene	318.15	196.15	9.925	-19	5	Cocaethylene-D3
			82.05		-32		
11	Cocaine	304.15	182.15	8.511	-19	6	Cocaine-D3
			82.05		-28		
12	Codeine	300.15	152.1	5.044	-63	17	Codeine-D3
			215.15		-24		
13	Desipramine	267.2	72.1	12.907	-15	11	Imipramine-D3
			208.1		-21		
14	Dextromethorphan	272.2	215.15	11.656	-23	7	Diazepam-D5
			171.1		-37		
15	Diazepam	285.1	193.05	13.452	-31	7	Diazepam-D5
			154		-26		
16	Ecgonine Methyl Ester	200.1	182.1	0.955	-18	8	EME-D3
			82.1		-25		
17	Estazolam	295.05	267.05	12.979	-24	9	Estazolam-D5
			205.05		-39		
18	Fentanyl	337.25	188.15	11.285	-23	14	MDMA-D5
			105.1		-38		
			317		-20		
19	Heroin	370.15	165.05	8.535	-47	10	Heroin-D9
			268.1		-29		
20	Imipramine	281.2	86.1	12.847	-17	11	Imipramine-D3
			58.05		-36		
21	Ketamine	238.1	125	7.901	-24	14	MDMA-D5
			179.05		-17		
22	LSD	324.2	223.15	9.936	-24	14	MDMA-D5
			207.15		-42		
23	Lorazepam	321	275	12.945	-21	7	Diazepam-D5
			229.1		-30		
24	MDA	180.1	163.15	5.936	-14	12	MDA-D5
			105.15		-21		
25	MDEA	208.15	163.1	6.912	-13	13	MDEA-D5
			105.1		-24		
26	MDMA	194.1	163.1	6.172	-13	14	MDMA-D5
			105.1		-23		
27	Mephedrone	178.1	145.15	7.002	-20	14	MDMA-D5
			144.15		-29		
			165.1		-23		
28	Methadone	310.2	265.15	12.966	-15	15	Methadone-D3
			105.05		-26		
29	Methamphetamine	150.15	91.1	5.858	-22	16	Methamphetamine-D5
			119.15		-15		
30	Methcathinone	164.1	131.1	4.666	-22	14	MDMA-D5
			130.1		-29		

Table 2 (Cont.). Retention time and m/z of drugs and narcotics

No	Compound	Precursor ion (m/z)	Product ions (m/z)	Retention Time	Collision energies (V)	ISTD Group	ISTD
31	Methylphenidate	234.15	84.1	8.736	-24	7	Diazepam-D5
			56.05		-47		
			223.1		-36		
32	Mitragynine	399.05	173.95	11.698	-32	18	Mitragynine-D3
			159		-47		
33	Morphine	286.15	152.1	2.578	-59	19	Morphine-D3
			201.1		-26		
34	Nalorphine	312.1	201	4.781	-26	20	
			181		-36		Nordiazepam-D5
			270		-23		
35	Naloxone	328.15	212.1	4.953	-38	20	Nordiazepam-D5
			268.2		-26		
36	Naltrexone	342.15	270.15	5.622	-27	20	Nordiazepam-D5
			267.15		-29		
37	Nimetazepam	296.05	250.2	12.843	-28	20	Nordiazepam-D5
			221.1		-39		
38	Nitrazepam	282.1	236.1	12.538	-23	20	Nordiazepam-D5
			180.1		-32		
39	Nordiazepam	271.05	140.05	13.309	-27	20	Nordiazepam-D5
			208.1		-27		
40	Norpseudoephedrine	151.95	134.05	4.212	-13	21	Norpseudoephedrine-D3
			115.05		-26		
			117.15		-21		
41	Nortriptyline	264.15	233.15	12.984	-14	22	Nortriptyline-D3
			105.1		-22		
42	Oxazepam	287.05	241	12.928	-23	23	Oxazepam-D5
			104.05		-35		
43	Oxycodone	316.15	241.15	5.4	-29	24	Oxycodone-D3
			256.15		-26		
44	Pentazocine	286.2	218.2	10.377	-21	20	Nordiazepam-D5
			69.1		-28		
45	Phencyclidine	244.2	91.05	10.68	-29	20	Nordiazepam-D5
			86.1		-12		

Table 2 (Cont.). Retention time and m/z of drugs and narcotics

No	Compound	Precursor ion (m/z)	Product ions (m/z)	Retention Time	Collision energies (V)	ISTD Group	ISTD
46	Prazepam	325.1	271.05	13.813	-23	23	Oxazepam-D5
			140.05		-36		
47	Pseudoephedrine	166	148	4.813	-14	25	Pseudoephedrine-D3
			133		-22		
			115.1		-26		
48	Sibutramine	280.2	125.1	13.081	-25	14	MDMA-D5
			139.1		-16		
49	Sildenafil	475.2	58.05	12.68	-42	23	Oxazepam-D5
			100.05		-29		
50	Temazepam	301.05	255.1	13.186	-25	23	Oxazepam-D5
			283.15		-13		
51	Tramadol	264.2	58.05	8.439	-24	7	Diazepam-D5
			42.1		-65		
52	Triazolam	343.05	308.2	13.098	-24	23	Oxazepam-D5
			315		-27		
53	Trimipramine	295.2	100.15	13.026	-17	11	Imipramine-D3
			58.1		-34		
54	Zolpidem	308.2	235.15	9.769	-35	26	Zaleplon-D4
			263.1		-26		
55	Zopiclone	389.1	244.95	8.165	-17	27	Zopiclone-D4
			217		-31		
56	Zaleplon	306	236	12.225	-26	26	Zaleplon-D4
			264		-21		

Table 3. Retention tine and m/z of internal Standard Data

No.	Name	Precursor ion	Product ion	RT	Collision energies (V)	ISTD Group
1	6-Acethyl Morphine-D3	331.15	165.15	5.888	-43	1
			211.1		-27	
2	Amphetamine-D5	141.15	93.1	5.524	-18	2
			124.15		-14	
3	Benzoylecgonine-D3	293.15	171.2	7.609	-20	3
			77.05		-56	
4	Clonazepam-D4	320.05	274.2	12.601	-26	4
			218.15		-39	
5	Cocaethylene-D3	321.2	199.25	9.925	-21	5
			85.2		-32	
6	Cocaine-D3	307.15	185.15	8.511	-19	6
			85.25		-31	
7	Diazepam-D5	290.1	198.15	13.452	-31	7
			154.05		-26	
8	EME-D3	204	186	0.955	-17	8
			86		-25	

Table 3 (Cont.). Retention tine and m/z of internal Standard Data

No.	Name	Precursor ion	Product ion	RT	Collision energies (V)	ISTD Group
9	Estazolam-D5	301.95	273.9	12.979	-24	9
10	Heroin-D9	379.05	212	8.03	-32	10
11	Imipramine-D3	285.2	89.2	12.847	-17	11
12	MDA-D5	185.15	168.15	5.936	-13	12
			110.15		-22	
13	MDEA-D5	213.15	163.15	6.912	-14	13
			105.2		-28	
14	MDMA-D5	199.15	165.15	6.172	-15	14
			107.15		-25	
15	Methadone- D3	313.15	268.1	12.966	-15	15
16	Methamphetamine-D5	155.15	92.2	5.859	-20	16
			121.1		-15	
17	Codeine-D3	303.2	215.25	4.95	-26	17
			181.2		-37	
18	Mitragynine-D3	402.1	177	11.698	-24	18
			238.1		177.0/-32	
19	Morphine-D3	289.15	152.1	2.578	-59	19
			201.15		-26	
20	Nordiazepam-D5	278	141.9	13.309	-27	20
21	Norpseudoephedrine-D3	155	137	4.212	-13	21
			117.1		-14	
22	Nortriptyline-D3	267.05	232.95	12.984	-15	22
			105.2		-21	
			117.15		-21	
23	Oxazepam-D5	292.1	245.9	12.928	-22	23
			109		-36	
24	Oxycodone-D3	319.15	301.1	5.4	-19	24
			259.1		-26	
25	Pseudoephedrine-D3	169	151	4.813	-14	25
			115.05		-26	
			91.15		-33	
26	Zaleplon-D4	310	239.95	12.225	-28	26
			268		-22	
27	Zopiclone-D4	393	244.85	8.165	-16	27
			216.95		-33	
			349		-10	

3.3 Method validation

No interferences from endogenous matrix and external drugs were observed. With the exception of nitrazepam (5 ng/mL), all drugs and narcotics exhibited a LLOD of 1 ng/mL or lower. The LLOQ of all tested substances was 10 ng/mL or lower.

Linear regression analysis of the calibration curve was performed for each target analyte, with all demonstrating an r2 value greater than 0.99.

The intra-day precision ranged from 0.5% to 12%, while the inter-day precision ranged from 1.6% to 16%. The accuracies for both blood and vitreous fluid extraction were within the range of less than 20%.

The recovery of the analytes ranged between 80 and 120 %.

The validated data of blood and vitreous fluid have been presented in Table 4 and 5, respectively.

3.4 Forensic case application

Routine forensic toxicology analyses were conducted, alongside parallel testing of the same forensic samples using a modified QuECHerS method for sample preparation. Results obtained from both the routine method and the modified method exhibited consistency in the identification and quantification of drugs and narcotics across the tested 10 cases.

Table 4. Validate data of drugs and narcotics from whole blood

NO	Name	Range (ng/mL)	Linearity (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Intra Day (Accuracy)						Inter Day (Precision)						Matrix Effect (%)			%Recovery		
						Low		Medium		High		Low		Medium		High		Low	Medium	High	Low	Medium	High
						%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias						
1	3,4-Methylene dioxypyrovalerone	1-100	0.998	0.10	1	3.47	14.60	3.42	6.44	1.52	19.86	5.27	11.04	5.94	1.73	3.33	15.55	86.61	83.36	80.87	106.60	98.65	117.72
2	6-Acethyl Morphine	2.5-250	0.995	0.25	25	8.22	17.48	9.14	9.46	2.24	18.49	7.61	10.88	12.10	1.56	6.68	12.71	87.69	83.69	88.82	100.51	116.50	118.09
3	Alprazolam	5-500	0.999	0.50	5	12.78	-17.60	4.36	12.04	10.59	7.73	10.40	-5.77	11.42	-2.82	7.98	-2.86	119.02	88.26	84.90	103.51	83.17	87.62
4	Amitriptyline	1-100	0.999	0.10	1	10.61	-0.70	3.16	6.24	7.89	8.76	8.43	4.44	9.11	0.95	6.97	6.62	80.22	86.47	87.34	94.73	112.99	117.23
5	Amphetamine	5-500	0.999	0.50	5	3.47	-3.14	1.13	-9.35	2.54	2.34	6.44	-4.61	6.33	-11.18	5.79	8.52	95.11	90.47	90.33	98.21	80.91	101.06
6	Benzoylecgonine	2.5-250	0.998	0.25	2.5	1.23	18.78	2.99	-4.60	1.34	8.75	9.93	12.67	4.79	-5.77	5.08	11.47	114.13	96.28	87.06	94.79	98.73	119.44
7	Cathinone	5-500	0.995	0.50	5	4.31	3.68	4.29	2.02	3.85	-6.60	6.33	-1.63	5.36	1.62	3.23	-8.01	105.49	102.65	114.85	101.58	93.96	86.75
8	Clobazam	2.5-250	0.998	0.25	2.5	13.56	10.27	8.64	3.45	5.59	7.59	11.14	1.01	3.79	-1.61	2.55	7.95	84.75	86.65	88.39	84.28	94.97	110.53
9	Clonazepam	5-250	0.995	1.00	5	12.86	2.85	7.24	0.57	6.50	19.55	14.92	0.99	5.32	-5.09	5.80	12.90	87.18	88.47	86.48	85.91	98.10	118.88
10	Cocaethylene	0.5-50	0.999	0.05	0.5	2.97	-0.32	2.49	6.07	2.27	-3.10	8.69	-2.17	2.87	3.68	4.56	-0.89	82.97	81.03	80.28	82.88	106.53	106.70
11	Cocaine	5-500	0.999	0.50	5	2.17	1.73	0.73	1.18	3.75	-12.86	6.53	1.07	8.54	-3.95	14.10	-16.62	83.41	85.20	93.39	110.62	81.59	95.67
12	Codeine	1-100	0.998	0.10	1	10.71	-0.25	4.88	-4.97	3.13	3.31	8.74	-4.12	5.78	-2.91	5.30	5.54	92.52	99.86	97.29	82.17	105.27	115.27
13	Desipramine	1-100	0.997	0.10	1	9.75	0.60	7.96	0.95	7.28	9.90	9.42	2.20	10.98	-6.44	4.26	9.02	82.73	80.77	81.74	95.35	107.99	115.81
14	Dextromethorphan	1-100	0.998	0.10	1	11.94	-8.52	7.60	15.26	3.42	11.30	10.30	3.17	8.60	1.38	4.49	10.91	93.71	85.44	80.95	100.14	104.18	116.35
15	Diazepam	1-100	0.997	0.10	1	12.78	-3.57	8.22	6.88	9.48	13.98	11.56	1.75	9.96	-6.68	5.22	8.03	83.07	86.46	88.74	105.00	99.14	113.58
16	EME	5-500	0.999	0.50	5	3.39	3.18	2.09	-6.51	2.25	10.99	8.93	-7.90	5.90	-4.04	5.28	5.92	112.84	118.94	110.49	81.60	105.17	100.57
17	Estazolam	1-100	0.998	0.10	1	12.98	7.11	4.77	-0.09	4.41	11.32	11.83	-8.03	4.11	-2.72	2.90	11.59	82.83	93.04	84.43	83.24	101.27	114.82
18	Fentanyl	0.5-50	0.999	0.05	0.5	5.88	9.47	3.48	8.07	4.04	15.54	8.41	0.30	3.35	5.93	5.76	8.57	88.41	88.12	85.98	86.67	109.98	113.03
19	Heroin	5-500	0.998	0.50	5	5.18	-16.63	6.92	-4.61	4.04	-17.84	11.42	-6.84	6.88	-4.18	7.41	-11.58	84.00	92.23	86.83	105.72	88.05	80.62
20	Imipramine	0.5-50	0.999	0.05	0.5	10.51	0.98	4.01	8.87	4.41	11.35	7.43	-1.73	4.85	3.06	3.33	6.43	80.33	87.06	83.40	86.32	106.95	104.81
21	Ketamine	1-100	0.999	0.10	1	5.25	4.28	3.79	-4.60	2.62	13.58	9.14	-2.79	6.10	-2.43	4.62	11.93	103.21	84.35	86.83	81.73	106.23	115.29
22	LSD	1-100	0.998	0.10	1	5.02	17.11	3.04	-9.22	4.96	12.13	13.13	5.24	5.69	-7.64	2.00	11.64	93.35	81.33	86.58	81.53	100.60	114.56
23	Lorazepam	5-500	0.999	0.50	5	12.32	-5.99	8.46	15.87	2.02	15.59	12.75	-2.31	11.86	-1.39	3.26	11.04	95.60	98.87	97.94	114.69	89.51	106.43
24	MDA	10-500	1.000	1.00	10	3.60	3.76	2.44	-4.33	3.29	-1.76	3.49	3.12	7.90	-7.29	4.77	1.77	97.83	92.77	89.26	101.11	82.78	94.99
25	MDEA	1-100	0.996	0.10	1	3.36	12.30	3.23	7.03	3.42	17.12	6.28	12.12	4.89	-0.26	1.91	13.93	85.86	84.26	82.13	117.92	98.15	111.08

Table 4 (Cont.). Validate data of drugs and narcotics from whole blood

NO	Name	Range (ng/mL)	Linearity (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Intra Day (Accuracy)						Inter Day (Precision)						Matrix Effect (%)			%Recovery		
						Low		Medium		High		Low		Medium		High							
						%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	Low	Medium	High	Low	Medium	High
26	MDMA	2.5-250	0.996	0.25	2.5	3.61	3.32	2.10	-3.32	9.67	16.43	6.54	-4.79	5.09	-1.83	1.67	15.24	89.90	103.05	99.45	96.73	93.12	116.86
27	Mephedrone	2-100	0.995	0.10	2	3.68	-0.97	3.23	8.32	4.37	13.01	11.66	-2.47	10.30	-1.24	1.32	12.52	96.15	88.08	84.35	91.56	100.78	114.40
28	Methadone	0.5-50	0.998	0.05	0.5	15.50	7.57	8.96	13.67	3.10	16.87	12.30	-2.22	2.91	12.69	5.01	10.00	88.96	89.86	86.15	82.13	110.56	110.99
29	Methamphetamine	1-100	0.997	0.10	1	3.44	10.71	2.94	8.02	3.05	16.52	7.27	9.92	5.79	2.36	0.45	16.79	87.44	86.96	88.61	96.93	100.11	117.28
30	Methcathinone	5-500	1.000	0.50	5	2.69	3.12	1.85	-5.10	3.02	14.81	8.47	3.47	5.26	-4.49	3.51	11.08	103.47	106.91	99.01	112.82	89.16	104.45
31	Methylphenidate	1-100	0.999	0.10	1	6.99	5.84	7.31	2.33	3.77	9.38	7.51	7.32	6.64	-4.78	3.22	10.34	89.56	88.37	86.33	100.32	101.75	114.89
32	Mitragynine	1-100	0.998	0.10	1	3.88	12.40	5.01	-14.58	5.34	4.35	10.10	-0.84	8.16	-7.33	7.50	11.17	96.00	94.83	88.21	86.95	102.35	115.26
33	Morphine	5-500	0.999	0.50	5	3.89	-13.03	1.09	0.04	2.27	2.03	3.52	-12.49	4.98	-4.10	4.86	6.55	95.43	107.77	82.44	86.30	87.66	100.83
34	Nalorphine	2.5-250	0.997	0.25	2.5	11.48	-2.58	9.92	10.87	13.05	13.45	8.90	-2.47	5.87	10.10	9.75	17.78	106.77	107.95	95.46	100.47	115.55	117.48
35	Naloxone	2.5-250	0.997	0.25	2.5	16.47	-16.30	10.05	8.66	3.37	17.81	13.39	-3.47	5.23	8.51	5.77	12.70	88.90	93.60	91.55	105.09	107.50	119.09
36	Naltrexone	2.5-250	0.997	0.25	2.5	13.10	-6.04	8.68	0.51	2.38	18.04	13.05	-2.36	8.02	4.63	5.26	13.58	94.59	82.95	82.52	96.72	105.90	119.30
37	Nimetazepam	2.5-250	0.999	0.25	2.5	17.69	-1.62	9.34	2.87	16.83	8.92	12.46	-2.01	8.46	8.94	2.86	12.81	87.66	93.85	85.26	105.79	96.37	114.60
38	Nitrazepam	10-500	0.998	5.00	10	15.15	-5.38	10.72	3.95	8.81	-12.40	3.01	-7.89	7.89	-5.51	8.50	-7.48	85.86	92.17	87.23	91.49	91.09	98.53
39	Nordiazepam	2.5-250	0.998	0.25	2.5	5.63	4.39	13.25	10.48	4.24	5.22	8.16	-7.50	9.39	-4.54	7.26	5.87	83.40	87.62	83.37	92.80	91.65	117.99
40	Nor pseudoephedrine	5-500	0.999	0.50	5	6.07	-1.82	1.08	0.35	2.18	-2.66	7.39	-3.66	5.64	-2.28	4.87	-0.74	119.32	111.22	107.19	102.41	89.18	92.31
41	Nortriptyline	1-100	0.998	0.10	1	9.89	-3.91	6.26	-3.22	3.19	10.24	7.98	-7.81	6.90	-0.77	3.20	9.66	85.35	93.37	94.15	99.08	108.90	112.33
42	Oxazepam	5-500	0.999	1.00	5	16.31	-11.27	7.48	1.97	2.02	13.30	8.83	-8.07	5.52	-3.70	2.13	11.55	83.70	102.67	96.04	89.88	87.55	107.76
43	Oxycodone	5-500	0.999	0.50	5	4.52	-14.35	1.74	1.00	2.13	14.61	3.00	-13.82	6.77	-3.41	7.53	8.76	91.89	93.97	91.84	89.42	85.47	106.55
44	Pentazocine	1-100	0.998	0.10	1	8.54	4.85	9.84	9.75	4.60	11.42	15.35	3.07	4.29	11.08	2.90	11.66	91.24	80.48	87.64	81.26	112.36	112.37
45	Phencyclidine	1-100	0.995	0.10	1	9.90	1.95	9.69	6.52	8.92	5.90	13.24	-1.82	5.47	5.80	4.23	11.78	83.47	84.59	83.26	82.82	110.51	118.17
46	Prazepam	1-50	0.998	0.25	1	11.11	4.74	3.51	15.08	3.27	15.26	12.53	1.53	11.47	-0.28	5.45	9.47	86.36	106.42	105.41	86.36	106.42	105.41
47	R-Pseudoephedrine	5-500	1.000	0.50	5	1.62	3.13	1.06	-3.74	2.78	3.79	5.31	4.04	5.77	-8.70	6.10	4.99	93.76	96.22	97.29	106.44	82.54	95.92
48	Sibutramine	1-100	0.998	0.10	1	5.72	16.94	8.79	-10.56	8.10	6.23	19.15	-1.57	11.06	2.03	5.25	10.19	92.46	84.39	80.44	98.06	117.94	113.11
49	Sildenafil	5-250	0.997	0.25	5	9.64	16.73	7.66	-6.48	3.93	14.53	10.45	5.89	8.24	0.59	1.27	15.01	85.04	87.61	86.71	90.57	92.31	116.47
50	Temazepam	1-100	0.996	0.10	1	15.75	-0.71	5.73	4.51	4.77	13.18	5.05	-2.22	5.64	-1.89	9.10	11.03	101.98	87.02	80.88	92.00	103.61	126.24
51	Tramadol	1-100	0.997	0.10	1	8.36	-3.85	6.75	5.53	7.47	3.27	10.20	5.44	8.02	-0.80	7.94	5.91	88.11	87.26	84.22	109.82	109.92	119.96

Table 4 (Cont.). Validate data of drugs and narcotics from whole blood

NO	Name	Range (ng/mL)	Linearity (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Intra Day (Accuracy)						Inter Day (Precision)						Matrix Effect (%)			%Recovery		
						Low		Medium		High		Low		Medium		High		Low	Medium	High	Low	Medium	High
						%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias						
52	Triazolam	2.5-250	0.998	0.25	2.5	16.61	-16.93	5.26	2.83	2.89	7.86	10.27	-10.18	4.22	-1.66	3.51	6.72	86.86	86.72	81.60	81.68	92.67	108.66
53	Trimipramine	0.5-50	0.999	0.05	0.5	8.19	7.42	9.65	0.41	4.77	-7.76	3.41	4.20	4.87	5.99	7.30	-0.50	85.66	82.29	69.69	107.27	109.33	111.43
54	Zolpidem	0.5-50	0.999	0.05	0.5	3.88	-1.57	3.13	10.52	3.49	-0.84	6.75	-1.62	3.77	7.82	5.46	0.11	83.85	86.70	89.77	89.06	109.23	106.16
55	Zopiclone	2-100	0.997	0.10	2	4.53	2.38	1.53	5.51	1.37	12.97	6.21	-0.31	8.83	-0.65	3.83	16.43	90.06	84.16	81.50	98.19	93.92	113.94
56	Zaleplon	2.5-250	0.999	0.25	2.5	7.41	9.66	1.92	1.89	2.93	5.56	9.97	-1.34	2.73	-1.11	4.71	5.73	88.18	84.36	80.21	84.51	100.57	112.25

Table 5. Validate data of drugs and narcotics from vitreous fluid

No.	Name	Linearity range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Intra Day (Accuracy)						Inter Day (Precision)						% Recovery		
						Low		Medium		High		Low		Medium		High				
						%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%CV	%Bias	L	M	H
1	3,4-Methylenedioxypyrovalerone	1-100	0.999	0.10	1	15.29	1.29	12.40	0.65	11.95	3.26	9.91	13.88	13.61	1.68	12.67	4.75	115.29	112.40	111.95
2	6-Acethyl Morphine	2.5-250	0.998	0.25	2.5	-3.67	4.11	-5.50	1.54	-2.88	4.22	-0.33	12.92	7.25	7.01	5.45	7.77	96.33	94.50	97.12
3	Alprazolam	5-100	0.999	0.50	5	-13.09	4.32	-13.83	3.13	-3.61	3.87	-13.23	6.19	-5.68	11.77	2.11	4.90	86.91	86.17	96.39
4	Amitriptyline	2-100	1.000	0.10	2	-0.45	14.83	-0.85	13.53	11.77	13.05	4.35	11.79	8.17	7.20	11.80	4.22	96.98	99.15	111.77
5	Amphetamine	5-500	0.999	0.50	5	-16.37	1.85	-0.92	1.98	8.06	3.15	-12.99	3.56	4.42	6.67	10.50	7.56	83.63	99.08	108.06
6	Benzoylecgonine	2.5-250	0.999	0.25	2.5	0.03	6.60	11.96	2.59	7.90	2.73	-0.41	13.37	11.65	5.53	8.41	4.23	100.03	111.96	107.90
7	Cathinone	5-500	0.997	0.50	5	-6.47	5.55	-8.96	4.07	-8.27	5.59	-10.79	5.65	1.58	15.38	-1.83	9.08	93.53	91.04	91.73
8	Clobazam	2.5-250	1.000	0.25	2.5	-6.94	10.48	11.85	5.36	0.79	10.39	2.27	7.92	8.00	4.11	7.90	5.23	93.06	111.85	100.79
9	Clonazepam	2.5-250	0.997	0.25	2.5	8.39	6.51	7.32	8.32	3.69	11.26	5.38	12.18	7.91	6.72	9.22	4.46	108.39	107.32	103.69
10	Cocaethylene	0.5-50	0.999	0.05	0.5	-4.26	7.92	2.54	2.60	4.89	2.36	-3.12	9.01	-0.17	9.20	5.21	7.36	95.74	102.54	104.89
11	Cocaine	5-500	0.998	0.50	5	-8.34	3.57	-14.53	1.30	-13.09	3.78	-11.33	7.68	-5.06	10.94	-5.30	7.26	91.66	85.47	86.91
12	Codeine	2-100	0.996	0.25	2	-6.43	7.95	-7.27	3.39	-0.01	3.94	-1.90	4.86	-0.20	7.08	5.91	8.12	93.57	92.73	99.99
13	Desipramine	2-100	0.999	0.25	2	-2.59	10.46	6.56	9.73	8.84	9.24	1.24	5.26	12.51	4.78	13.72	2.78	97.41	106.56	108.84
14	Dextromethorphan	2-100	0.999	0.25	2	-11.23	2.63	-6.40	7.25	-2.18	8.91	3.68	13.56	5.16	9.01	10.26	6.57	88.77	93.60	97.82

Table 5 (Cont.). Validate data of drugs and narcotics from vitreous fluid

No.	Name	Linearity range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Intra Day (Accuracy)						Inter Day (Precision)						% Recovery		
						Low		Medium		High		Low		Medium		High				
						%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%CV	%Bias	L	M	H
15	Diazepam	2-100	0.999	0.25	2	-6.07	15.18	1.86	11.51	2.90	17.03	-0.01	11.22	10.26	5.61	6.68	6.31	93.93	101.86	102.90
16	EME	10-500	0.999	1.00	10	-16.53	3.72	1.97	1.89	1.98	3.08	-12.05	7.96	2.81	5.33	7.44	9.64	83.47	101.97	101.98
17	Estazolam	2-100	0.999	0.25	2	-2.20	8.31	-5.55	5.81	1.79	4.95	-2.08	6.08	1.05	9.93	6.87	7.82	97.80	94.45	101.79
18	Fentanyl	0.5-50	0.999	0.05	0.5	-7.62	6.18	3.60	3.03	5.96	4.07	-7.36	16.89	1.50	8.26	6.97	5.96	92.38	103.60	105.96
19	Heroin	5-500	0.997	0.05	5	3.97	5.25	-17.54	2.96	-9.08	3.49	-4.53	11.13	-4.59	11.27	-1.14	13.06	103.97	82.46	90.92
20	Imipramine	0.5-50	0.998	0.05	0.5	-12.34	9.21	0.94	5.10	4.16	4.74	-12.80	2.71	3.90	6.51	10.61	4.06	87.66	100.94	104.16
21	Ketamine	2-100	0.998	0.25	2	-3.08	7.35	19.94	5.50	13.47	1.42	3.13	8.36	7.65	11.00	10.62	5.77	96.92	119.94	113.47
22	LSD	1-100	0.999	0.10	1	15.25	1.79	7.62	2.44	7.63	2.63	14.61	10.22	10.75	4.62	10.54	9.15	115.25	107.62	107.63
23	Lorazepam	10-500	0.999	1.00	10	-16.84	8.38	-9.65	3.72	-5.65	5.08	-12.85	8.77	-4.05	7.22	3.45	7.97	83.16	90.35	94.35
24	MDA	10-500	1.000	1.00	10	-11.70	3.47	8.14	2.55	3.60	2.74	-12.99	4.01	8.52	5.65	5.80	5.83	88.30	108.14	103.60
25	MDEA	1-100	0.999	0.10	1	19.12	6.11	14.83	5.71	13.70	2.56	11.96	4.60	13.94	4.20	13.61	2.33	119.12	114.83	113.70
26	MDMA	2.5-250	0.999	0.25	2.5	13.02	2.92	-7.37	2.02	1.11	3.63	-1.09	10.78	-4.63	7.80	2.45	11.01	113.02	92.63	101.11
27	Mephedrone	1-100	0.998	0.10	1	7.38	3.56	18.98	2.89	13.81	2.80	13.25	4.63	16.54	3.17	12.90	6.41	107.38	118.98	113.81
28	Methadone	0.5-50	0.996	0.05	0.5	-7.84	14.11	-1.51	10.07	8.65	15.18	-10.99	8.09	-0.63	10.11	3.87	9.22	92.16	98.49	108.65
29	Methamphetamine	2-100	0.998	0.25	2	17.62	11.96	18.85	3.73	11.88	1.22	13.85	5.70	14.57	3.19	13.21	2.69	117.62	118.85	111.88
30	Methcathinone	5-500	0.999	0.50	5	-6.66	7.63	-11.33	2.08	-0.18	3.10	-16.09	9.43	-6.10	5.98	4.25	6.60	93.34	88.67	99.82
31	Methylphenidate	2-100	1.000	0.25	2	-16.13	6.44	6.52	7.08	8.07	6.16	-0.48	14.77	11.54	4.39	13.77	5.20	83.87	106.52	108.07
32	Mitragynine	2-100	0.999	0.25	2	-7.42	6.01	18.08	2.89	8.85	4.01	-1.08	8.51	12.63	4.88	10.99	7.70	92.58	118.08	108.85
33	Morphine	10-500	0.998	1.00	10	-15.30	2.87	-1.83	3.88	-4.69	6.63	-15.04	3.04	6.62	6.32	4.40	6.02	84.70	98.17	95.31
34	Nalorphine	5-250	0.999	0.50	5	-0.73	2.48	5.70	17.61	-1.60	18.06	1.45	10.30	5.41	8.56	9.65	7.67	99.27	105.70	98.40
35	Naloxone	5-250	1.000	0.50	5	-19.14	3.40	-0.65	18.65	-4.11	17.26	-3.56	6.59	4.23	11.08	1.73	13.20	80.86	99.35	95.89
36	Naltrexone	5-250	0.998	0.50	5	-19.92	11.85	12.48	18.13	1.99	15.89	0.83	9.68	4.05	11.13	7.69	5.57	80.08	112.48	101.99
37	Nimetazepam	5-250	0.999	0.50	5	-17.02	14.77	6.74	9.27	-2.72	13.71	-3.89	14.04	0.03	10.08	7.03	7.12	82.98	106.74	97.28
38	Nitrazepam	10-100	0.999	1.00	10	18.40	5.29	-0.04	10.60	-5.47	15.90	-5.37	15.73	8.34	9.18	2.63	9.13	118.40	99.96	94.53
39	Nordiazepam	5-250	0.999	0.50	5	-17.76	3.53	13.89	5.02	1.90	7.28	-4.61	14.66	8.20	10.02	6.97	8.85	82.24	113.89	101.90
40	Norpseudoephedrine	10-500	0.999	1.00	10	-8.18	7.73	-5.43	2.05	-0.49	3.13	-1.31	5.00	1.68	5.25	2.56	3.49	91.82	94.57	99.51

Table 5 (Cont.). Validate data of drugs and narcotics from vitreous fluid

No.	Name	Linearity range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Intra Day (Accuracy)						Inter Day (Precision)						% Recovery		
						Low		Medium		High		Low		Medium		High				
						%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%CV	%Bias	L	M	H
41	Nortriptyline	2-100	0.997	0.25	2	-10.78	12.02	-1.39	4.45	5.93	4.06	-5.03	10.19	4.54	8.23	6.63	4.87	86.99	98.61	105.93
42	Oxazepam	10-500	0.999	1.00	10	-17.43	6.70	10.15	9.47	12.76	7.48	-11.27	11.27	8.58	2.87	8.16	6.33	82.57	110.15	112.76
43	Oxycodone	10-500	0.999	1.00	10	-19.77	2.80	-4.32	1.15	-0.12	1.59	-15.41	5.20	0.79	3.42	2.28	5.62	80.23	95.68	99.88
44	Pentazocine	2-100	0.996	0.25	2	-8.65	8.72	14.86	17.32	2.61	16.26	5.27	11.79	11.97	5.89	11.40	5.08	91.35	114.86	102.61
45	Phencyclidine	2-100	0.996	0.25	2	-8.43	6.24	10.79	18.40	2.02	15.98	0.90	10.91	6.76	12.98	7.41	6.34	91.57	110.79	102.02
46	Prazepam	1-50	0.999	0.25	1	18.30	37.13	1.20	8.63	11.27	17.25	-1.00	14.01	1.45	11.99	4.13	13.34	118.30	101.20	111.27
47	Pseudoephedrine	10-500	0.999	1.00	10	-16.98	2.54	-5.32	1.15	3.33	1.87	-15.99	5.50	0.03	9.83	7.88	5.29	83.02	94.68	103.33
48	Sibutramine	1-100	0.999	0.10	1	-7.29	14.04	7.87	10.94	7.14	2.07	-7.33	11.16	12.87	3.47	11.92	3.28	92.71	107.87	107.14
49	Sildenafil	5-250	0.995	0.25	5	-4.73	10.40	-11.29	16.79	6.38	12.71	-5.94	9.07	1.25	13.57	5.21	9.44	95.27	88.71	106.38
50	Temazepam	2-100	1.000	0.10	2	-13.99	12.87	-10.43	12.01	4.34	9.92	-7.05	8.52	4.32	11.46	6.07	8.34	86.01	89.57	104.34
51	Tramadol	2-100	1.000	0.25	2	-17.54	6.57	-13.78	8.26	-12.85	7.17	-0.86	12.89	2.30	13.56	5.53	10.53	82.46	86.22	87.15
52	Triazolam	2.5-250	0.999	0.25	2.5	-2.61	12.70	-13.85	13.62	6.61	12.70	-7.45	11.21	0.42	10.55	5.31	8.51	97.39	86.15	106.61
53	Trimipramine	0.5-50	0.999	0.05	0.5	1.30	15.32	13.27	6.51	10.02	9.84	-7.41	6.60	7.56	13.06	6.80	10.17	101.30	113.27	110.02
54	Zolpidem	0.5-50	0.998	0.05	0.5	-7.20	18.07	11.73	5.27	10.64	7.62	-9.19	7.56	4.75	6.81	8.19	6.99	92.80	111.73	110.64
55	Zopiclone	1-100	0.999	0.10	1	12.36	7.54	19.48	0.61	16.75	2.58	3.12	13.04	17.60	1.61	15.19	3.90	112.36	119.48	116.75
56	Zaleplon	2.5-250	0.998	0.25	2.5	-11.27	10.35	-3.75	1.93	1.92	4.96	-5.11	5.47	-0.35	3.40	5.22	5.74	88.73	96.25	101.92

Discussion

Routine solid phase extraction (SPE) typically necessitates approximately 60 minutes for sample preparation, and various analytes and sample matrices often require distinct SPE techniques. However, the modified QuEChERS method requires only about 15 minutes for sample preparation, encompassing 56 common drugs and narcotics. In this experiment, it demonstrated reliability in facilitating the simultaneous extraction of various drugs, narcotics, and metabolites of forensic interest from both whole blood and vitreous fluid specimens. Moreover, it offers cost-effectiveness compared to SPE, which demands expensive equipment such as vacuum manifolds, pumps, and nitrogen evaporators. Additionally, the use of disposable devices eliminates the potential for cross-contamination of samples.

This method could serve as a valuable sample preparation technique for untargeted determination in forensic domains, given its coverage of up to 56 common drugs and narcotics including ketamine, mitragynine, sibutramine and sidelnafil. However, this study excluded cannabinoids due to findings from the pilot study, indicating that the modified QuEChERS method was unsuitable for extracting cannabinoids from either whole blood or vitreous fluid.

Conclusion

The preparation of whole blood and vitreous humor using the modified QuEChERS technique proves suitable for application in forensic toxicological investigations of common drugs and narcotics. Method validation confirms that this approach satisfies the acceptable criteria and results obtaining from real cases are consistent with the routine method of the laboratory. Adopting the same extraction technique for whole blood and vitreous fluid, characterized by its affordability, simplicity, and ease of use, has the potential to enhance the efficiency of forensic toxicology laboratories.

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ORIGINAL ARTICLE

Correlation between immunohistochemical stain in skeletal muscle and different postmortem intervals using CD56 and C5b-9

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Abstract

Background: Estimating the postmortem interval (PMI) is a key challenge in forensic investigations. Various methods have been proposed to improve the accuracy of PMI estimation. Given the postmortem cell lysis process, immunohistochemical staining has been suggested as a potential tool for PMI estimation.

Objective: To evaluate the correlation between immunohistochemical staining in psoas muscle and postmortem intervals using CD56 and C5b-9 in a humid tropical climate.

Materials and Methods: A prospective study was conducted using skeletal muscle tissues from 25 medicolegal cases. Each sample was divided into four pieces and fixed in formalin at different time points (Day 0, 1, 2, 3). Day 0 was the day of the autopsy. Day 1, 2, and 3 were 24 hours, 48 hours, and 72 hours later. PMI was calculated from the known time of death to the fixation date. Samples were stored at room temperature in a closed container before fixation. A total of 100 formalin-fixed specimens were processed into paraffin blocks and stained with H&E, CD56, and C5b-9. Digital images of each slide were analyzed using Adobe Photoshop 2024 to measure mean colorimetric values (mean CV).

Results: As PMI increased, the mean CV in C5b-9-stained samples increased, whereas the mean CV in CD56-stained samples decreased, though neither relationship was statistically significant. The Spearman correlation coefficient showed weak associations for both markers.

Conclusions: CD56 and C5b-9 staining may serve as valuable tools for PMI estimation. However, additional studies with a larger sample size, increased sampling frequency, and consideration of other influencing factors are recommended.

Keywords: C5b-9, CD56, skeletal muscle, PMI, IHC

Introduction

One of the primary objectives in forensic investigations is determining the time since death. A tool commonly used for such investigation is physical examination (e.g., livor mortis, rigor mortis, algor mortis, signs of decomposition). Since many factors can affect physical postmortem changes (e.g., humidity, temperature, clothing)[1-3], there is still no best tool or method to accurately determine the time since death, especially when time has passed and decomposition has developed. There is a lot of ongoing research on techniques that are best for determining the time since death. Immunohistochemical staining is also one of the methods studied due to the tissue's ability to stain positively, which varies by the degradation process after death.

During death, cells across the body also go through the cell death process, whether necrosis or apoptosis[4]. The membrane attack complex (MAC or C5b-9 complex) is formed during complement system activation, which leads to cell death[5]. C5b-9 might be a good candidate in helping estimate time since death since it is thought to be formed during the death process and altered by the time passed since death. According to Abd Elazeem et al.[6], C5b-9 can be found positive in heart tissue up to 48 hours after death and is also a reliable marker for predicting time since death.

CD56 is a marker for neuroendocrine differentiation and natural killer cells. It is also a marker for damaged or regenerating muscle fibers (7). Positive staining for CD56 can also persist for several days postmortem, even though cells underwent the postmortem process and cell architecture had been altered [8, 9].

While research has examined PMI estimation through IHC staining, few studies have been conducted in tropical climates like Thailand, where higher temperatures and humidity accelerate decomposition. This study aims to analyze the correlation between CD56 and C5b-9 staining and PMI in such an environment.

Nowadays, a scoring system using a scale such as grading is commonly used to grade the positivity intensity of immunohistochemistry staining. This method is sometimes questionable due to its possible effect of subjective bias. Many methods have been proposed to find a way to describe such positivity more objectively and reproducibly [10]. A study by Nhu-An Pham et al. [11] found that using CMYK with the Yellow channel method to detect the positivity of immunohistochemistry staining. In the CMYK color model, every shade in an image consists of four different colors: Cyan (C), Magenta (M), Yellow (Y), and Black (K). This method was also used in Mondello's work [12] to detect immunohistochemistry positivity via color intensity. This study will use this method to reduce bias from different pathologists and get the quantitative value of immunohistochemistry positivity.

Materials and Methods

Sample collection

This study included 25 medicolegal cases that underwent full autopsy (external and internal examination, histological examination of vital organs, and necessary lab tests) with exact times of death recorded from June to September 2024. Cases with abnormal gross findings in the abdominopelvic area (e.g., traumatic lesion, hemorrhage) or known diseases associated with musculoskeletal or immunological systems were excluded. Twenty-five cases were included in this study with informed consent from the deceased's legal proxy according to the Human Research Ethics Committee, Faculty of Medicine Ramathibodi Hospital Regulation (MURA 2024/226). Demographic data such as sex, age, and underlying disease were recorded. Cause of death, time of death, and autopsy time were also recorded after the full autopsy was done. During the autopsy, muscle samples from the right psoas muscle were collected, divided into four pieces, and labeled as Day 0, Day 1, Day 2, and Day 3 for each case. Samples labeled as Day 0 were immediately fixed with 10% buffered formalin. The remaining samples were stored in a box lined with mesh to prevent insect activity in a room environment (ventilated airflow with daily recorded temperature and humidity). After 24 hours, 48 hours, and 72 hours, the samples labeled as Day 1, Day 2, and Day 3 were fixed in 10% buffered formalin accordingly. The Postmortem Interval (PMI) for the Day0 sample was calculated by measuring the time between the documented time of death and autopsy time in hours. The others were calculated by summing up the time they were kept before fixation and the PMI of each case.

Slide preparation

After the samples were fixed, the tissues were embedded in paraffin wax, then cut to a thickness of 3 micrometers and mounted on slides. Three slides were obtained from each paraffin block. For each sample, one slide was stained with hematoxylin-eosin to study morphology under a light microscope by a qualified muscle pathologist to ensure no abnormal pathological findings in every case. The second slide from each block was stained with antibody against CD56 via an automated machine. The last slide from each block underwent immunohistochemistry staining using the heat-mediated antigen retrieval method with citrate/EDTA buffer, followed by overnight incubation with primary antibodies (dilution 1:50) at room temperature. Antibody against C5b-9 was used via an automated machine.

Image analysis

Stained slides were examined using an Olympus BX53F microscope, using 4x field magnification. The images were captured using an Olympus DP21 microscope digital camera with identical settings for all slides and saved as JPEG format files. Using Adobe Photoshop 2024, the images were processed in CYMK color mode. Using only the yellow channel, all images were converted into greyscale images and then inverted in color. The mean colorimetric values (mean CV) were measured using the whole image. The mean CV ranges from 0 to 255 (from no yellow signal to the most intense yellow signal).

Statistical analysis

Categorical data were expressed as frequencies and percentages. Numerical data were presented as means, ranges, and standard deviations (SDs). Data were analyzed using STATA version 18 software. The relationship between PMI and mean CV was analyzed. Since the data were not normally distributed, correlation analysis was done using Spearman's correlation coefficient. P-values less than 0.05 were considered statistically significant.

Results

The decedents included in this study were predominantly male (84%), with the majority aged between 20 and 60 years (88%). Cardiovascular disease was identified as the most common cause of death, accounting for 40% of cases. Due to limitations in the availability of background information, data regarding underlying medical conditions were accessible for only 60% of the individuals (as presented in Table 1). The temperature in the storage rooms where specimens were kept ranged from 21.9°C to 28.1°C, with relative humidity levels varying between 47% and 69%.

Table 1. shows demographic data of all included medicolegal cases collected between June and September 2024

		Cases (percentage)
Sex	Female	4 (16%)
	Male	21 (84%)
Age	Below 20 years	0
	20 – 40 years	12 (48%)
	40 – 60 years	10 (40%)
	Above 60 years	3 (12%)
Underlying disease	Cardiovascular disease	2 (8%)
	Asthma	1 (4%)
	COPD	1 (4%)
	Diabetes mellitus	3 (12%)
	Hypertension	4 (16%)
	Dyslipidemia	1 (4%)
	Gastric ulcer	1 (4%)
	Viral hepatitis	1 (4%)
	Depression	1 (4%)
	None	5 (20%)
	N/A	10 (40%)
Cause of death	Exsanguination	3 (12%)
	Severe head injury	4 (16%)
	Severe neck injury	1 (4%)
	Asphyxia	3 (12%)
	Infection	2 (8%)
	Cardiovascular disease	10 (40%)
	Brain disease	1 (4%)
	Intoxication	1 (4%)

A total of 100 specimens were obtained from 25 medicolegal cases, with four samples collected per case. The postmortem interval across all specimens ranged from 2.50 to 99.38 hours. The mean CV for CD56-stained samples ranged from 150.18 to 199.85, whereas the mean CV for C5b-9-stained samples ranged from 153.98 to 209.63 (as shown in Table 2).

Table 2. shows PMI from 100 specimens in the CD56-stained and C5b-9-stained groups and CV values detected using the Adobe Photoshop 2024 program for each group.

	Mean (Min. – Max.)	Standard deviation
PMI (hours)	52.14 (2.50 - 99.38)	27.86
Mean CV – CD56	183.27 (150.18 - 199.85)	12.14
Mean CV -- C5b-9	185.80 (153.98 – 209.63)	12.08

Spearman's correlation analysis revealed a positive but weak and statistically non-significant association between the mean CV values of C5b-9-stained samples and PMI (Rho = 0.0600). In contrast, the mean CV values of CD56-stained samples exhibited a negative correlation with PMI, which was likewise weak and not statistically significant (Rho = 0.0251) (as presented in Table 3).

Table 3. shows statistical results from Spearman's correlation coefficient in the CD56-stained and the C5b-9-stained groups. The relationships in both groups show statistical insignificance with the P-value above 0.05.

Relationship	rho	P-value
PMI & mean CV -- CD56	-0.0251	0.8042
PMI & mean CV -- C5b-9	0.0600	0.5533

Discussion

The C5b-9 membrane attack complex is formed as part of the terminal pathway of the complement system during cell lysis. This process initiates within minutes to hours after death and progresses over time. The findings of the present study demonstrate the detectability of this complex via immunohistochemical staining. These results are consistent with the work of Thomsen and Held [13], who observed persistent C5b-9 positivity in cardiac muscle tissue up to 11 days following experimentally induced putrefaction and autolysis. Similarly, Elazeem et al. [6] reported increased immunoreactivity of C5b-9 with increased postmortem intervals in both injured and uninjured cardiac muscle.

CD56 staining, a marker for muscle stem cells, is typically used to identify viable muscle tissue. These stem cells are present in vital muscles and progressively degrade postmortem. Consistent with prior studies [7, 9], CD56 positivity was observed during the postmortem period in the current investigation. Furthermore, a negative correlation between CD56 staining intensity and PMI was identified, corroborating previous findings that demonstrate the progressive decline in detectable muscle stem cells as time elapses after death.

It is important to note that this study employed an artificial decomposition model, which may not fully replicate the conditions of natural in situ decomposition. Several external variables that significantly influence the decomposition process—such as ambient temperature, humidity, and prior resuscitative efforts—were not controlled in this study. Temperature is a well-known determinant of decomposition rate, with warmer conditions accelerating the process [2, 3, 14], whereas colder or freezing environments tend to preserve tissue. High humidity similarly facilitates faster decomposition, potentially due to increased enzymatic activity, microbial proliferation, and chemical degradation [15]. In cases involving attempted resuscitation, the interval between somatic and cellular death may be prolonged due to artificial circulation and respiration. This could induce more inflammatory responses and cellular damage, thereby affecting the accuracy of protein detection by immunohistochemistry in determining postmortem interval. Furthermore, decomposition within the body may be influenced by intrinsic factors such as enzymatic degradation from adjacent organs and endogenous flora, as well as by the relatively higher moisture and temperature compared to externally decomposed muscle tissue. These considerations may contribute to variability or limitations in the study's results.

Tissue sampling in this study was conducted at 24-hour intervals over a 72-hour period. This sampling frequency may have been insufficient to capture critical windows of histological change, particularly given the accelerated decomposition associated with tropical climates such as that of Thailand. Future studies should consider more frequent sampling intervals to improve temporal resolution.

In this study, image analysis was conducted using JPEG-format images. JPEG compression involves data reduction that may result in loss of image detail, potentially affecting the accuracy of the calculated mean CV and thereby introducing bias into the results.

In recent years, various image analysis methodologies have been developed to enable reproducible, objective quantification of immunohistochemical staining positivity. Artificial Intelligence (AI)-based tools have been demonstrated to yield results comparable in accuracy to those of experienced pathologists [16, 17], while also mitigating inter-observer variability, grading inconsistencies, and errors arising from visual perception. Although commercial AI-assisted platforms are widely available, their high cost often limits accessibility in resource-constrained settings. In such contexts, conventional image editing software may offer a practical, albeit less precise, alternative for digital image analysis, despite its limitations in differentiating cellular compartments (e.g., nucleus, cytoplasm).

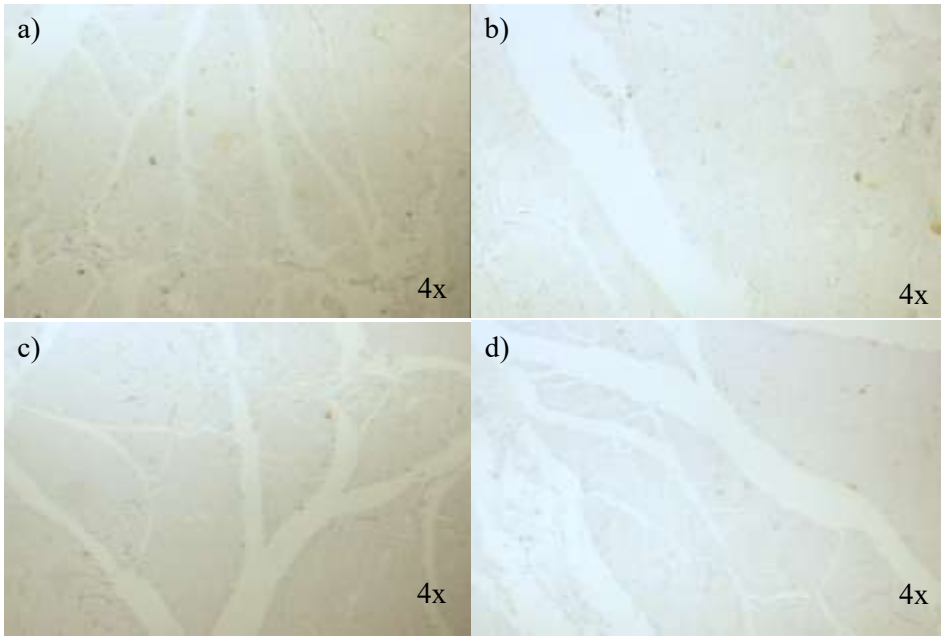

Figure 1: Example images taken by an Olympus DP21 microscope digital camera via an Olympus BX53F microscope, using 4x field magnification. The images were stained using CD56 at fixation day of Day0 in a); Day1 in b); Day2 in c) and Day3 in d)

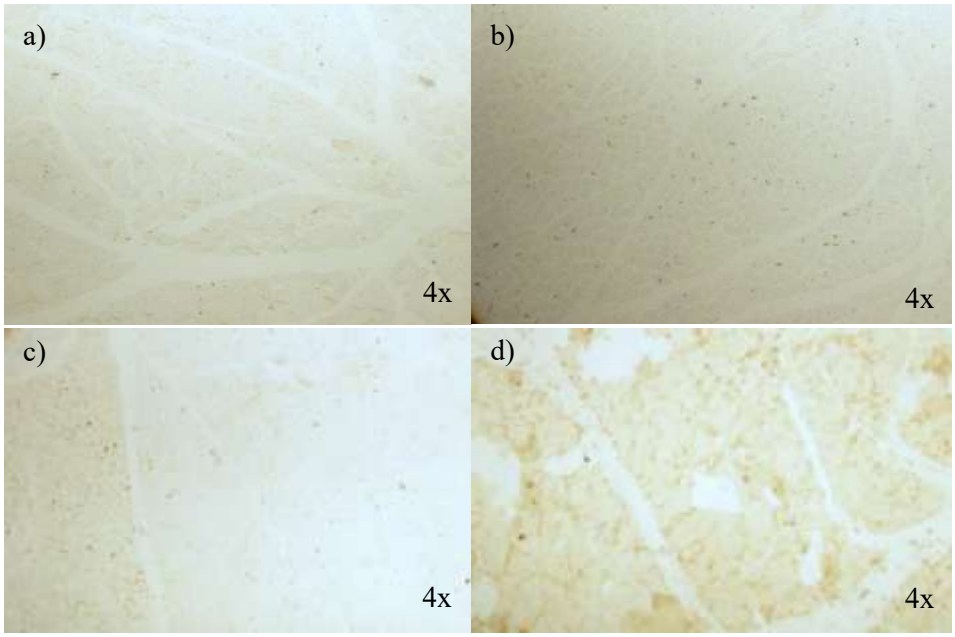

Figure 2: Example images taken by an Olympus DP21 microscope digital camera via an Olympus BX53F microscope, using 4x field magnification. The images were stained using C5b-9 at fixation day of Day0 in a); Day1 in b); Day2 in c) and Day3 in d)

Conclusion

The findings in this study support the possible usefulness of C5b-9 and CD56 immunohistochemical staining in estimating PMI. Since postmortem and decomposition processes can be affected by many factors, including climate, more studies in humid tropical areas could be helpful in a precise method of PMI estimation. Future studies with frequent sampling and more factors could help detect a stronger relationship.

Acknowledgements

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

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APPENDIX 1
INFORMATION FOR AUTHORS

All authors listed in a paper submitted to Asian Archives of Pathology (AAP) must have contributed substantially to the work. It is the corresponding author who takes responsibility for obtaining permission from all co-authors for the submission. When submitting the paper, the corresponding author is encouraged to indicate the specific contributions of all authors (the author statement, with signatures from all authors and percentage of each contribution can be accepted). Examples of contributions include: designed research, performed research, contributed vital new reagents or analytical tools, analysed data, and wrote the paper. An author may list more than one type of contribution, and more than one author may have contributed to the same aspect of the work.

Authors should take care to exclude overlap and duplication in papers dealing with related materials. See also paragraph on Redundant or Duplicate Publication in "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" at http://www.icmje.org/index.html.

The submitted manuscripts will be reviewed by the members of the Editorial Board or the expert reviewers. At the discretion of the Editorial Board, the manuscripts may be returned immediately without full review, if deemed not competitive or outside the realm of interests of the majority of the readership of the Journal. The decision (reject, invite revision, and accept) letter will be coming from the Editorial Board who has assumed responsibility for the manuscript's review. The editor's decision is based not just on technical merit of the work, but also on other factors such as the priority for publication and the relevance to the Journal's general readership. All papers are judged in relation to other submissions currently under consideration.

Categories of Manuscripts

1. Letters to the Editor

The letters to the editor are the reactions to any papers published in AAP. These letters will be reviewed by the Editorial Board and sent to the authors of the original paper with an invitation to respond. Letters and eventual responses will be published together, when appropriate.

- *Word Count: 300 – 500 words (excluding references and figure or table legends)*
- *Abstract: Not required*
- *References: Maximum of 10*
- *Figure or Table: Maximum of 1 (if needed)*

2. Original Articles

The original articles are the researches describing the novel understanding of anatomical pathology, clinical pathology (laboratory medicine), forensic medicine (legal medicine or medical jurisprudence), molecular medicine or pathobiology. Systematic reviews, meta-analyses and clinical trials are classified as articles. The articles should be clearly and concisely written in the well-organised form (see ***Organisation of Manuscripts***): abstract; introduction; materials and methods; results; discussion; and conclusions. The manuscripts that have passed an initial screening by the Editorial Board will be reviewed by two or more experts in the field.

- *Word Count: 3,000 – 5,000 words (excluding abstract, references, and figure or table legends)*
- *Structured Abstract (see* ***Organisation of Manuscripts****): 150 – 200 words*
- *References: Maximum of 150*
- *Figures or Tables: Maximum of 6*

3. Review Articles

The review articles are generally invited by the Editor-in-Chief. They should focus on a topic of broad scientific interest and on recent advances. These articles are peer-reviewed before the final decision to accept or reject the manuscript for publication. Therefore, revisions may be required.

- *Word Count: 3,000 – 5,000 words (excluding abstract, references, and figure or table legends)*
- *Unstructured Abstract: 150 – 200 words*
- *References: Maximum of 150*
- *Figures or Tables: Maximum of 4*

4. Case Reports

AAP limits publication of case reports to those that are truly novel, unexpected or unusual, provide new information about anatomical pathology, clinical pathology (laboratory medicine) or forensic medicine (legal medicine or medical jurisprudence). In addition, they must have educational value for the aforementioned fields. The journal will not consider case reports describing preventive or therapeutic interventions, as these generally require stronger evidence. Case reports that involve a substantial literature review should be submitted as a review article. The submitted case reports will undergo the usual peer-reviewed process.

- *Word Count: 1,200 – 2,000 words (excluding abstract, references, and figure or table legends)*
- *Unstructured Abstract: 150 – 200 words*

- *References: Maximum of 20*
- *Figures or Tables: Maximum of 4*

5. Case Illustrations

Case illustrations are aimed to provide education to readers through multidisciplinary clinicopathological discussions of interesting cases. The manuscript consists of a clinical presentation or description, laboratory investigations, discussion, final diagnosis, and up to 5 take-home messages (learning points). Regarding continuous learning through self-assessment, each of the case illustrations will contain 3 – 5 multiple choice questions (MCQs) with 4 – 5 suggested answers for each question. These MCQs are placed after the final diagnosis and the correct answers should be revealed after the references. The questions and take-home messages (learning points) are included in the total word count. The manuscripts that have passed an initial screening by the Editorial Board will be reviewed by two experts in the field.

- *Word Count: 1,000 – 2,000 words (excluding references and figure or table legends)*
- *Abstract: Not required*
- *References: Maximum of 10*
- *Figures: Maximum of 2*
- *Tables: Maximum of 5*

6. Technical Notes

The technical notes are brief descriptions of scientific techniques used in the anatomical pathology, clinical pathology (laboratory medicine), forensic medicine (legal medicine or medical jurisprudence), molecular medicine or pathobiology. The submitted manuscripts are usually peer-reviewed.

- *Word Count: Maximum of 1,000 words (excluding references and figure or table legends)*
- *Abstract: Not required*
- *References: Maximum of 5*
- *Figures or Tables: Maximum of 2*

Organisation of Manuscripts

1. General Format

The manuscripts written in English language are preferable. However, Thai papers are also acceptable, but their title pages, abstracts, and keywords must contain both Thai and English. These English and Thai manuscripts are prepared in A4-sized Microsoft Word documents with leaving 2.54-cm (1-inch) margins on all sides. All documents are required to be aligned left and double-spaced throughout the entire manuscript. The text should be typed in 12-point regular Times New Roman font for English manuscript and 16-point regular TH SarabunPSK font for Thai manuscript.

The running titles of English and Thai manuscripts are placed in the top left-hand corner of each page. They cannot exceed 50 characters, including spaces between words and punctuation. For the header of English paper, the running title will be typed in all capital letters. The page number goes on the top right-hand corner.

Footnotes are not used in the manuscripts, but parenthetical statements within text are applied instead and sparingly. Abbreviations should be defined at first mention and thereafter used consistently throughout the article. The standard abbreviations for units of measure must be used in conjunction with numbers.

All studies that involve human subjects should not mention subjects' identifying information (e.g. initials) unless the information is essential for scientific purposes and the patients (or parents or guardians) give written informed consent for publication.

2. Title Page

The title page is the first page of the manuscripts and must contain the following:

- The title of the paper (not more than 150 characters, including spaces between words)
- The full names, institutional addresses, and email addresses for all authors (If authors regard it as essential to indicate that two or more co-authors are equal in status, they may be identified by an asterisk symbol with the caption "These authors contributed equally to this work" immediately under the address list.)
- The name, surname, full postal address, telephone number, facsimile number, and email address of the corresponding author who will take primary responsibility for communication with AAP.
- Conflict of interest statement (If there are no conflicts of interest for any author, the following statement should be inserted: "The authors declare that they have no conflicts of interest with the contents of this article.")

3. Abstract

A structured form of abstract is used in all Original Article manuscripts and must include the following separate sections:

- *Background: The main context of the study*
- *Objective: The main purpose of the study*
- *Materials and Methods: How the study was performed*
- *Results: The main findings*
- *Conclusions: Brief summary and potential implications*
- *Keywords: 3 – 5 words or phrases (listed in alphabetical order) representing the main content of the article*

4. Introduction

The Introduction section should clearly explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

5. Materials and Methods

The Materials and Methods section must be described in sufficient detail to allow the experiments or data collection to be reproduced by others. Common routine methods that have been published in detail elsewhere should not be described in detail. They need only be described in outline with an appropriate reference to a full description. Authors should provide the names of the manufacturers and their locations for any specifically named medical equipment and instruments, and all chemicals and drugs should be identified by their systematic and pharmaceutical names, and by their trivial and trade names if relevant, respectively. Calculations and the statistical methods employed must be described in this section.

All studies involving animal or human subjects must abide by the rules of the appropriate Internal Review Board and the tenets of the recently revised Helsinki protocol. Hence, the manuscripts must include the name of the ethics committee that approved the study and the committee's reference number if appropriate.

6. Results

The Results section should concisely describe the findings of the study including, if appropriate, results of statistical analysis which must be presented either in the text or as tables and figures. It should follow a logical sequence. However, the description of results should not simply repeat the data that appear in tables and figures and, likewise, the same data should not be displayed in both tables and figures. Any chemical equations, structural

formulas or mathematical equations should be placed between successive lines of text. The authors do not discuss the results or draw any conclusions in this section.

7. Discussion

The Discussion section should focus on the interpretation and the significance of the findings against the background of existing knowledge. The discussion should not repeat information in the results. The authors will clearly identify any aspects that are novel. In addition, there is the relation between the results and other work in the area.

8. Conclusions

The Conclusions section should state clearly the main summaries and provide an explanation of the importance and relevance of the study reported. The author will also describe some indication of the direction future research should take.

9. Acknowledgements

The Acknowledgements section should be any brief notes of thanks to the following:

- *Funding sources*
- *A person who provided purely technical help or writing assistance*
- *A department chair who provided only general support*
- *Sources of material (e.g. novel drugs) not available commercially*

Thanks to anonymous reviewers are not allowed. If you do not have anyone to acknowledge, please write "Not applicable" in this section.

10. References

The Vancouver system of referencing should be used in the manuscripts. References should be cited numerically in the order they appear in the text. The authors should identify references in text, tables, and legends by Arabic numerals in parentheses or as superscripts. Please give names of all authors and editors. The references should be numbered and listed in order of appearance in the text. The names of all authors are cited when there are six or fewer. When there are seven or more, only the first three followed by "et al." should be given. The names of journals should be abbreviated in the style used in Index Medicus (see examples below). Reference to unpublished data and personal communications should not appear in the list but should be cited in the text only (e.g. A Smith, unpubl. Data, 2000).

- *Journal article*

  1. Sibai BM. Magnesium sulfate is the ideal anticonvulsant in preeclampsia – eclampsia. Am J Obstet Gynecol 1990; 162: 1141 – 5.

- *Books*

2. Remington JS, Swartz MN. Current Topics in Infectious Diseases, Vol 21. Boston: Blackwell Science Publication, 2001.

- *Chapter in a book*

3. Cunningham FG, Hauth JC, Leveno KJ, Gilstrap L III, Bloom SL, Wenstrom KD. Hypertensive disorders in pregnancy. In: Cunningham FG, Hauth JC, Leveno KJ, Gilstrap L III, Brom SL, Wenstrom KD, eds. Williams Obstetrics, 22nd ed. New York: McGraw-Hill, 2005: 761 – 808.

11. Tables

The tables should be self-contained and complement, but without duplication, information contained in the text. They should be numbered consecutively in Arabic numerals (Table 1, Table 2, etc.). Each table should be presented on a separate page with a comprehensive but concise legend above the table. The tables should be double-spaced and vertical lines should not be used to separate the columns. The column headings should be brief, with units of measurement in parentheses. All abbreviations should be defined in footnotes. The tables and their legends and footnotes should be understandable without reference to the text. The authors should ensure that the data in the tables are consistent with those cited in the relevant places in the text, totals add up correctly, and percentages have been calculated correctly.

12. Figure Legends

The legends should be self-explanatory and typed on a separate page titled “Figure Legends”. They should incorporate definitions of any symbols used and all abbreviations and units of measurement should be explained so that the figures and their legends are understandable without reference to the text.

If the tables or figures have been published before, the authors must obtain written permission to reproduce the materials in both print and electronic formats from the copyright owner and submit them with the manuscripts. These also follow for quotes, illustrations, and other materials taken from previously published works not in the public domain. The original resources should be cited in the figure captions or table footnotes.

13. Figures

All illustrations (line drawings and photographs) are classified as figures. The figures should be numbered consecutively in Arabic numerals (Figure 1, Figure 2, etc.). They are submitted electronically along with the manuscripts. These figures should be referred to specifically in the text of the papers but should not be embedded within the text. The following information must be stated to each microscopic image: staining method, magnification (especially for electron micrograph), and numerical aperture of the objective

lens. The authors are encouraged to use digital images (at least 300 d.p.i.) in .jpg or .tif formats. The use of three-dimensional histograms is strongly discouraged when the addition of these histograms gives no extra information.

14. Components

14.1. Letters to the Editor

The Letter to the Editor manuscripts consist of the following order:

- *Title Page*
- *Main Text*
- *References*
- *Table (if needed)*
- *Figure Legend (if needed)*
- *Figure (if needed)*

14.2. Original Articles

The Original Article manuscripts consist of the following order:

- *Title Page*
- *Structured Abstract*
- *Introduction*
- *Materials and Methods*
- *Results*
- *Discussion*
- *Conclusions*
- *Acknowledgements*
- *References*
- *Table (s)*
- *Figure Legend (s)*
- *Figure (s)*

14.3. Review Articles

The Review Article manuscripts consist of the following order:

- *Title Page*
- *Unstructured Abstract*
- *Introduction*
- *Main Text*
- *Conclusions*
- *Acknowledgements*
- *References*

- *Table (s)*
- *Figure Legend (s)*
- *Figure (s)*

14.4. Case Reports

The Case Report manuscripts consist of the following order:

- *Title Page*
- *Unstructured Abstract*
- *Introduction*
- *Case Description*
- *Discussion*
- *Conclusions*
- *Acknowledgements*
- *References*
- *Table (s)*
- *Figure Legend (s)*
- *Figure (s)*

14.5. Case Illustrations

The Case Illustration manuscripts consist of the following order:

- *Title Page*
- *Clinical Presentation or Description*
- *Laboratory Investigations*
- *Discussion*
- *Final Diagnosis*
- *Multiple Choice Questions (MCQs)*
- *Take-Home Messages (Learning Points)*
- *Acknowledgements*
- *References*
- *Correct Answers to MCQs*
- *Table (s)*
- *Figure Legend (s)*
- *Figure (s)*

14.6. Technical Notes

The Technical Note manuscripts consist of the following order:

- *Title Page*
- *Introduction*
- *Main text*
- *Conclusions*

- *Acknowledgements*
- *References*
- *Table (s)*
- *Figure Legend (s)*
- *Figure (s)*

Proofreading

The authors of the accepted manuscripts will receive proofs and are responsible for proofreading and checking the entire article, including tables, figures, and references. These authors should correct only typesetting errors at this stage and may be charged for extensive alterations. Page proofs must be returned within 48 hours to avoid delays in publication.

Revised Manuscripts

In many cases, the authors will be invited to make revisions to their manuscripts. The revised manuscripts must generally be received by the Editorial Board within 3 months of the date on the decision letter or they will be considered a new submission. An extension can sometimes be negotiated with the Editorial Board.

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Asian Archives of Pathology (AAP) is an open access journal. Open Access makes your works freely available to everyone in the world. It provides a significant boost to the readership of your articles, and has been shown to have an increase in positive influence on citations and reuse. Hence, open-access leads to more recognition for our esteemed authors.

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Since AAP is also a peer-reviewed journal, the submitted manuscripts will be reviewed by the members of the Editorial Board or the expert reviewers. The decision on these manuscripts is processed very fast without any delay and in shortest possible time. The processing period is 1 – 2 weeks. These decisions of the reviewers are unbiased and the decision (reject, invite revision, and accept) letter coming from the Editorial Board is always conveyed to the authors.

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Step 5: Proceed to fill up the Submission Form online and follow the directions given therein.

Step 6: Upload your manuscript file (s).

Step 7: Re-check the content of your manuscript (s) and the uploaded file (s) more carefully prior to the submission. If you have submitted your manuscript file (s) incorrectly, you must contact Editor-in-Chief of Asian Archives of Pathology immediately. The Editor-in-Chief can clear the incorrect attempt and allow you another submission.

Step 8: Click the "Submit Manuscript" button under Important Notice.

If you have any further enquiries, please do not hesitate to contact the Journal.

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