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RESEARCH ARTICLE

THE STABILITY OF HEROIN METABOLITES AND ADULTERANTS IN BLOOD TO EVALUATE ROLE OF THE STABILITY ON POSTMORTEM REDISTRIBUTION

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Abstract

The present study aimed to investigate the stability of morphine, morphine-3-β-glucuronide (M3G), morphine-6-β-glucuronide (M6G), 6-acetylmorphine (6-AM) from heroin metabolites, and codeine, codeine-6-glucuronide, nor-codeine, fentanyl, norfentanyl, paracetamol, caffeine from its adulterants at room temperature for 24 hours in order to understand the effect of instability in postmortem redistribution (PMR). The heroin metabolites and adulterants were spiked into the control blood sample with the standard addition method. Then they were kept at room temperature for 4 hours and 24 hours and the blood samples were prepared by liquid-liquid extraction method. The prepared samples were analyzed using the validated analytical method with a liquid-chromatography-tandem mass spectrometry. According to the results of this study, no significant changes were observed in the analyte concentrations in the 4- and 24-hour postmortem blood, and it is thought that the other mechanisms may be more responsible than the instability of analytes for the significant concentration changes in PMR.

Introduction:-

Heroin is an opioid drug that is most commonly used and has a high mortality rate globally each year. According to CDC (Centre for Disease Control and Prevention) data, around 143,000 people died from high-dose heroin in the USA between 1999 and 2020[1]. After administration via intravenous route, it is rapidly deacetylated to 6-AM and then hydrolyzed to morphine. The half-life of heroin, which is rarely detectable in human samples, averages 2–6 minutes. The half-life of 6-AM is on average 6–25 minutes, and its detection in the blood can indicate that heroin has been administered recently[2]. 6-AM is then hydrolyzed to morphine. The accurate elucidation of the death mechanism of heroin-related deaths poses a challenge in forensic toxicology. Therewith, the changing of adulterant composition including synthetic opioids such as fentanyl and its derivatives mixed into heroin in order to increase profit by increasing the amount of substance, increase the desired effects of heroin and facilitate its administration to the body, is considered among the possible causes of the opioid epidemic in recent years [3,4].

The most common drugs in the mixture called street heroin are paracetamol and caffeine[5]. In recent years, dextromethorphan has been added to these drugs. Tittarelli et al. determined the composition of the street heroin seized by the police to update the Italian National Early Warning System launched in 2010 and the most frequently

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encountered substances were reported as caffeine, dextro/levo-methorphan (racemic mixture), and paracetamol, respectively[6].

Considering the stability of target analytes in biological matrices is vital for the correct interpretation of toxicological analysis results in heroin-related death. Forensic toxicologists and pathologists have to interpret the drug concentrations in postmortem samples in order to determine the primary or secondary effects of drugs in the drug-related death[7]. However, drug concentrations in postmortem samples change over time due to unknown mechanisms such as postmortem redistribution (PMR). Changes in drug concentrations occur after death, especially (mostly) through passive diffusion from reservoir organs to the blood or from blood to organs [8]. Therefore, drug concentrations in biological samples collected at autopsy are (mostly) high and do not reflect concentrations at the time of death. It is thought that all drugs can be affected to some degree by PMR, and the major contributing factors are periods between death and sampling, sampling location and technique, potential postmortem metabolism/production by body enzymes or bacteria, and finally the physicochemical properties of the drug[9–11]. Given the instability of morphine in blood due to bacterial action and other factors in the postmortem period, a more accurate and reliable characterization of substance concentrations at the time of death is needed. Stability studies in which potential degradation in blood is controlled by other factors such as passive diffusion are needed to understand the change in postmortem drug concentrations.

In this study, it was aimed to examine the stability of morphine, M3G, M6G, and 6-AM from heroin metabolites, and codeine, codeine-6-glucuronide (C6G), nor-codeine, fentanyl, norfentanyl, paracetamol, caffeine, dextromethorphan from its adulterants at room temperature for 4- and 24- hours in order to evaluate the impact of instability of heroin metabolites and adulterants into the PMR.

Material and Methods:-

Chemicals and reagents

Ultrapure water, liquid-chromatography-grade methanol (MeOH) and acetonitrile were purchased from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Heroin.HCl was purchased from LGC standard. Certified reference standards of morphine sulphate, morphine-3-beta-glucuronide, morphine-6-beta glucuronide, 6-AM, dextromethorphan and paracetamol were purchased from Lipomed (Cambridge, MA, USA). Caffeine was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). Internal standards of morphine-d3 and diazepam-d5 were purchased from Lipomed (Cambridge, MA, USA).

Study design and sample collection

The experimental part of this study was carried out by the recommendations in the Guide for the Care and Use of Laboratory Animals[12] and was approved by the Çukurova University Health Sciences Experimental Application and Research Center Ethics Committee (the meeting number: 2, decision number: 3, date: 13 May 2020). A total of 16 rats (Wistar albino) for control group with an average weight of 220 ± 25 g were selected as experimental animals regardless of gender. Before euthanasia, heparin (1000 U/mL) was injected into the tail vein to prevent excessive coagulation. One hour after injection, rats were euthanized by CO_2 and placed in a supine position at room temperature for the desired post-mortem interval (0, 0.5, 4, and 24 hours). Cardiac blood, right and left femoral blood and subclavian blood were collected.

Sample preparation and extraction

The mixture of all analytes at a 100 ng/mL concentration except for internal standards (morphine-d3 and diazepam-d5) was spiked into 1 mL blood samples collected in tubes with K2 EDTA (ethylenediamine tetraacetic acid) and the blood samples were prepared in 5 replicates (n=5). The reason for selecting the 100 ng/mL concentration was that it was an average drug concentration detected in blood samples of postmortem cases and provides mathematical ease of percentages calculation. These samples were kept at room temperature (25±5 °C) for 4- and 24-hours periods and then analyzed by using the liquid-liquid extraction method in the present study. 100 µL samples were taken into a flat glass tube (10 mL) and 5 µL of the stock internal standard (morphine-d3 and diazepam-d5) mixture solution at a concentration of 1000 ng/mL was added. For the removal of proteins and phospholipids, 100 µL of 0.1 M Zn_2SO_4/ammonium acetate solution was added and 800 µL of MeOH kept at -20°C was added, mixed by vortex, and centrifuged at 3500 rpm for 10 minutes. After the upper phase was taken into a glass tube, it was centrifuged again at 3500 rpm for 10 minutes, then the supernatant was transferred to a new tube and evaporated at +50°C under N_2. The residue was reconstituted by adding 50 µL of mobile phase A (0.1% formic acid in ultrapure water) and mobile phase B (MeOH) (95:5) and injected into the LC-MS/MS instrument in a volume of 10 µL for analysis.

Instrumental analysis

Shimadzu 8040 liquid chromatography-tandem mass spectrometry (LC-MS/MS) device was used for chromatographic analysis (Kyoto, Japan). The separation of heroin, dextromethorphan, caffeine and paracetamol was carried out using a pentafuorophenyl propyl (PFPP) column (Allure 50×2.1 mm i.d., 5 µm, Restek, Bellefonte, PA, USA). Detection and quantification were performed by integrating the area under the specific multiple reaction monitoring (MRM) chromatograms about the integrated area of the internal standards with an electrospray ionization source operating in positive mode. Mobile phase A consisted of 0.1% formic acid in water. Mobile phase B methanol. The gradient of mobile phase B was started at 10% and was held for 10 min at a 0.4 mL/min flow rate. Then, the flow rate was increased to 1 mL/min with 90% solution B for 5 min, and the flow rate was decreased to 0.4 mL/min with 10% solution B for equilibration of the column. The other mass spectrometry LC/MS/MS analysis was performed on a Shimadzu CBM-20A Ultra Flow Liquid Chromatography UFLC equipped with a Shimadzu SIL-20A/HT autosampler system and Shimadzu 8040 LC/MS/MS Systems. The separation of the analyte was carried out using a pentafluorophenylpropyl (PFPP) column (Allure 50x2.150 mm i.d., 5 µm, Restek, Bellefonte, PA, USA), maintained at 40 °C. LC/MS-MS was operated in the Multiple Reaction Monitoring (MRM). The total run time per sample was 20 min. The LC/MS/MS method parameters was shown in Table 1.

Table 1:- Operating conditions for LC/MS/MS analysis.

Column	Restek PFPP Allure 50x2.150 mm i.d., 5 µm
Oven temperature	40 °C
Flow	0.4 mL·min^{-1}
Mobile phase A	0.1% Formic acid in Ultrapure Water
Mobile phase B	Methanol (LC grade)
Gradient	0-0.01 min 5% solvent B
	0-10 min linear gradient to 95% solvent B
	10-15 min 95% solvent B
	15-15.01 min gradient to 5% solvent B
	20 min stop
Detector	Triple quadrupole mass spectrometer
Interface	Electrospray ionisation
Desolvation temperature	250 °C
Heat block temperature	400 °C
CID gas	17 kPa
Interface voltage	4.5 kV
Nebulizing gas flow	3.0 L/min

Method validation was fully performed for accuracy, selectivity, linearity, the limit of detection (LOD), the lowest limit of quantitation (LLOQ), dilution integrity, precision, carry-over, and matrix effect according to the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices[13]. For the development and validation of the LC-MS/MS method for analysing all analytes in whole blood, calibrators and controls at certain concentrations were prepared by using blank blood samples.

Statistical analysis

Data were maintained on the LabSolution software v5.97 (Shimadzu, Japan). All data were transferred to a single spreadsheet and mean values and percentages were calculated by Excel v16.0 (Microsoft, Redmond, Washington, D.C., USA).

Results:-

Validation of the analytical method was performed for the analysis of analytes in blood samples prepared in order to evaluate the stability with recovery selectivity, specificity, (absolute recover), linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, dilution accuracy, precision, matrix effect and carry-over parameters. The results obtained for each validation parameter were within the acceptable range specified in SWGTOX validation guidelines.

The blood stability results of heroin metabolites and adulterants in this study are shown in Table 2.

Table 2:- Stability results of analytes at 100 ng/mL concentration in the blood samples (n=5, mean %±SD).

Analyte	4-hour	24-hour
Morphine	99.9 ± 2.18	99.4 ± 0.61
Morphine-3-β-glucuronide	98.7 ± 2.00	97.4 ± 0.57
Morphine-6-β-glucuronide	100.4 ± 2.41	100.0 ± 1.24
Codeine	100.5 ± 0.94	100.2 ± 1.07
Norcodeine	99.1 ± 1.64	99.9 ± 1.00
Codein-6-β-glucuronide	99.8 ± 1.74	98.2 ± 0.91
Heroin	98.2 ± 2.55	97.3 ± 0.80
6-AM	97.1 ± 0.74	94.2 ± 0.36
Fentanyl	99.7 ± 1.63	97.8 ± 1.41
Norfentanyl	100.1 ± 2.32	99.0 ± 0.98
Dextromethorphan	98.9 ± 1.71	98.8 ± 0.91
Caffeine	100.4 ± 0.39	99.4 ± 2.12
Paracetamol	99.2 ± 1.32	99.8 ± 1.12

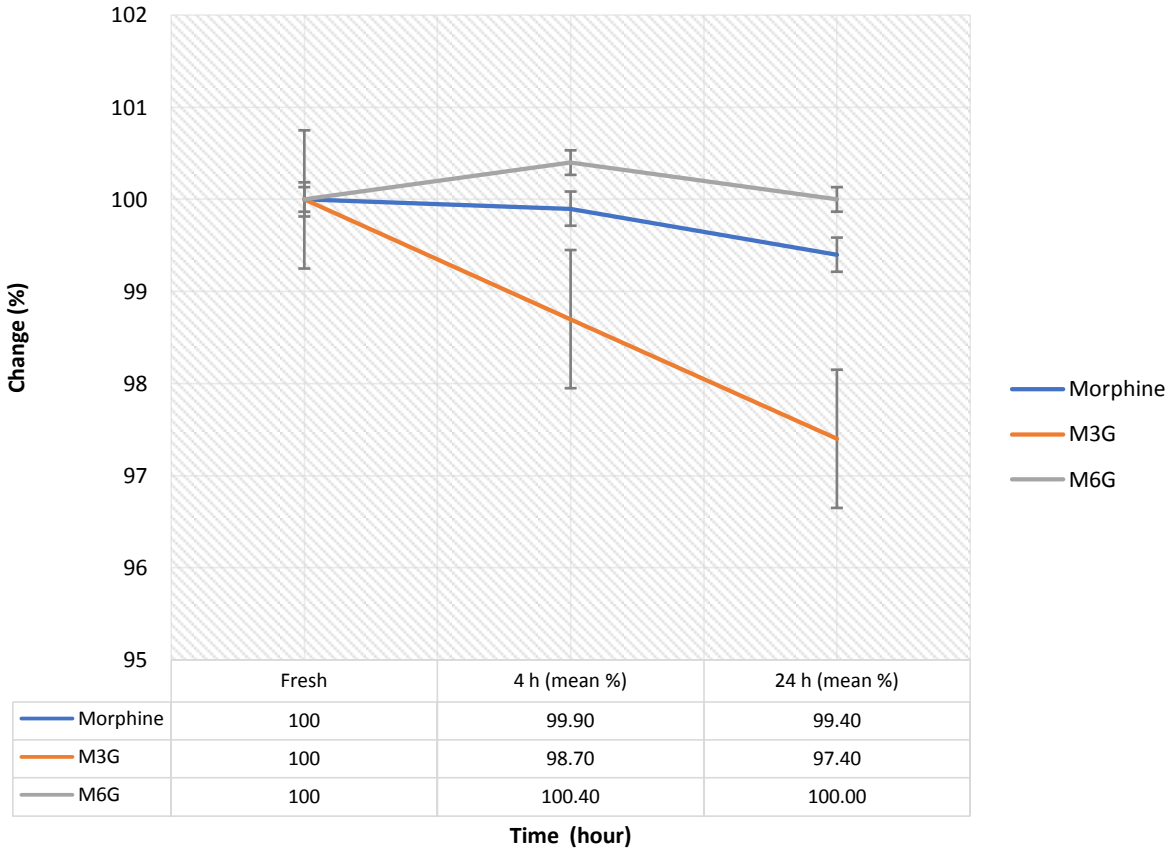

Figure 1:-The concentration changes of morphine, M3G and M6G in fresh, 4- and 24-hour blood samples.

Figure 1 shows the variation of the stability of morphine, M3G and M6G in blood across time (hours). Considering the percentage change rates, although it was not statistically significant, the most stability change was observed in M3G. Also, there was insignificant changes in the concentrations of all adulterants and their metabolites including codeine.

According to the results of the present study, no significant changes were observed in the analyte concentrations in the 4- and 24-hour postmortem blood, and it is thought that the other mechanisms may be more responsible than the instability of analytes for the significant concentration changes in PMR.

Discussion:-

The stability of drugs in biological samples is a major problem, especially in postmortem toxicological investigations. One of the impacts that change drug concentrations in tissues and body fluids of postmortem cases is drug instability [14]. The assessing the instability of drugs may enable to understand other factors causing the drug concentration change especially in PMR. When the stability results were evaluated, it was observed that there was no significant change in the 4th and 24th hour samples. In the study conducted by Al-Asmari and Anderson, in which the 24-hour stability of all opioid drugs and their metabolites evaluated in this study at room temperature, no significant change was observed in all substances except 6-MAM, and less than 15% degradation in blood was observed in 6-MAM [15].

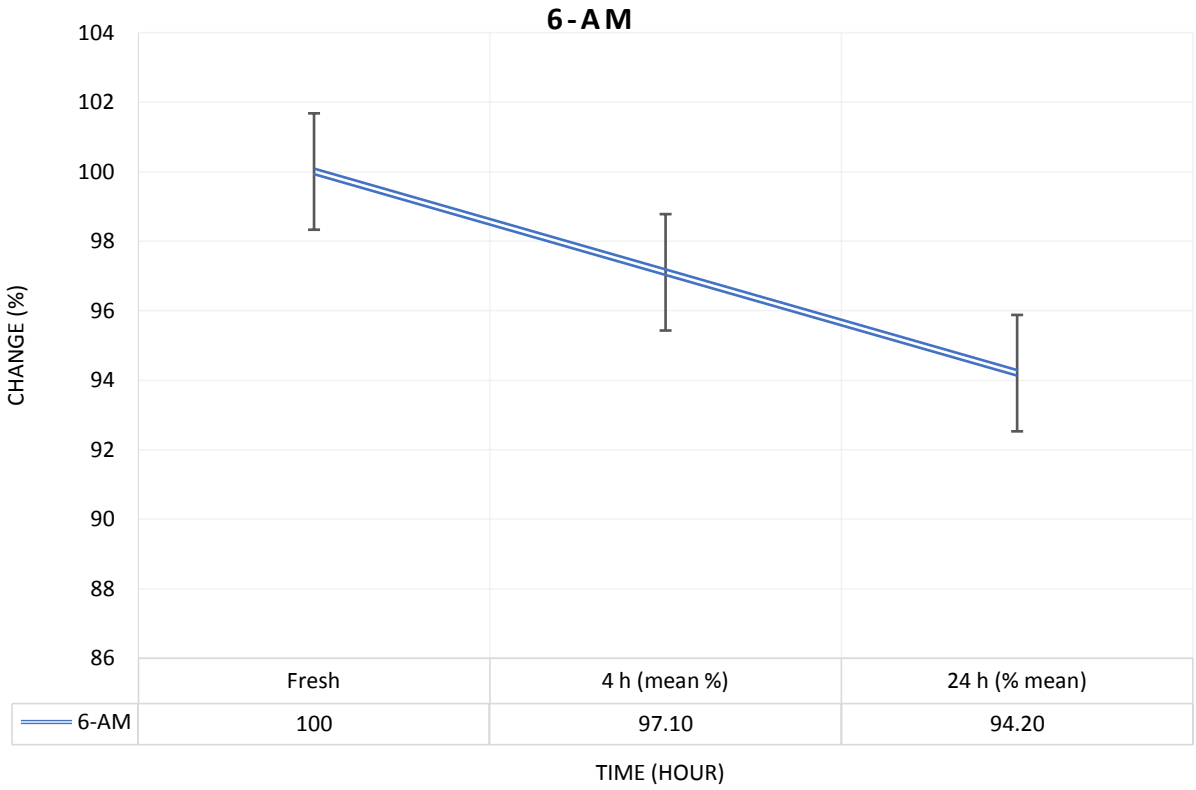

Figure 2:- The concentration change (ng/mL) of 6-AM in fresh, 4- and 24-hour blood samples.

The present study showed that the 24-hour degradation percentage of 6-AM at room temperature was 5.8±0.36 % (mean±SD) (Figure 2). The concentration decrease of 6-AM was linear. It is known that 6-AM is hydrolyzed in vivo by carboxylesterase and acetylcholine esterase in blood[16,17]. However, chemical acidic hydrolysis outcoming from the change in blood pH is another degradation mechanism of 6-AM[18]. In the study conducted by Papoutsis et al. in which evaluated the stability of morphine, codeine and 6-AM under different storage conditions, no significant change was observed in the tubes in which only NaF was used as a preservative of reference standard materials added to blood samples[19]. In this and similar studies, different storage conditions of opioid analytes in blood samples collected into tubes with anticoagulant in daily and monthly periods were evaluated, while ignoring short-term changes[19,20].

Conclusions:-

In this study, the stability of heroin metabolites and adulterants in vitro was determined in order to evaluate its effect on PMR. The present study demonstrated that no significant changes were observed in the analyte concentrations in the 4- and 24-hour postmortem blood. The findings of our study proposedthat the other mechanisms related-PMR such as bacterial metabolism, passive diffusion, postmortem environment and etc. may be more responsible than the instability of analytes for the significant concentration changes in PMR of heroin.

These data can contribute to the understanding of the mechanism of PMR in order to exclude or include stability during the different postmortem intervals in PMR investigations. However, the limitations of our study can be listed as working with repetitive samples in additional concentrations in the presence of preservatives such as EDTA and not evaluating biochemical parameters affecting degradation such as blood gases and pH. Future studies will design in vivo studies controlling for the factors listed above on the PMR investigations for other psychoactive drugs including opioids.

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Conflict of interest
We declare that there is no conflict of interest for the article: "The stability of heroin metabolites and adulterants in blood to evaluate role of the stability on postmortem redistribution".

Ethical approval
The experiment section of the present study was conducted at the Experimental Research and Application Center of Medical Sciences at Cukurova University, Adana, Turkey. Çukurova University Animal Experiments Local Ethics Committee (AELEC) in Adana, Turkey approved the study. All applicable international, national, and/or institutional guidelines were followed for the use of the animals.

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