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Determination of benzoic acid concentration in biological samples of amphetamine addicts

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Abstract

Amphetamines, a popular psychostimulant, are causing significant health issues due to their metabolism, which generates benzoic acid derivatives. This study presents an accurate analytical technique for toxicologists, chemists, and public health professionals to ensure safety for the general population. Human benzoic acid levels from amphetamine users were quantitatively analyzed using GC-MS, which is the most effective choice for confirming drug concentrations in forensic toxicology. The study collected blood and urine samples from addicted prisoners in Wasit, dividing them into four groups: those who have not used amphetamines for less than 24-48 hours, those who have not used amphetamines for 7-8 days, those who have not used amphetamines for 15-16 days, and a control group who has never used drugs. The liquid-liquid extraction method (LLE) is a method used to extract general blood and urine components. The study found that the 24-48 hours subgroup had significantly higher levels of benzoic acid in their blood and urine compared to the control group. The 24-48 hours subgroup showed the most noticeable rise in blood benzoic acid levels, while the 24-48 days subgroup showed similar levels. The study also found that the changes made in the experiment significantly impacted the amount of benzoic acid excreted in urine, with the 24-48 hours subgroup having the most significant effect, indicating that the treatment or condition being studied may have an effect on the kidneys and liver, and it provides a precise analytical technique for toxicologists, chemists, and public health professionals to accurately analyze human benzoic acid levels from amphetamine users.

Keywords: Amphetamines, metabolism of amphetamines, benzoic acid, GC-MS, and liquid-liquid extraction.

Introduction

The metabolism of amphetamines, a common psychostimulant, produces derivatives of benzoic acid, which are seriously harming people's health.. Urine drug screening can help assess illegal drug use, as it is the easiest and least invasive biological sample to collect, potentially benefiting patients with chronic illness [1, 2, 3]. Drug metabolism is a process that transforms chemical substances into metabolites, determining the levels of a drug and its metabolite in the body. Pharmacokinetics quantifies the rate of drug absorption, distribution, metabolism, and elimination, highlighting the impact on bioavailability and bioequivalence. The level of a drug and its metabolite in the bloodstream indicates

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its bioavailability and bioequivalence [4, 5, 6, 7]. Benzoic acid, an aromatic acid, is a key intermediate in bacteria's metabolic pathways and is produced endogenously in humans and animals. It is used in over-the-counter medications, personal care products, and in polyunsaturated fat diets. It is also used in dental solutions and non-oral care products [8, 9, 10]. The metabolism of benzoic acid in humans involves enzymatic and spontaneous decarboxylation pathways, primarily producing benzoyl-CoA. The relative contributions of these pathways are not fully understood, with some evidence suggesting toluene oxidation, especially in heavy users of methamphetamines, contributes to benzoic acid synthesis [8, 9, 10, 11, 13].

Benzoic acid, a compound found in many products, is absorbed from the gut and circulates through tissues. It undergoes hepatic conjugation and urinary excretion, with acetate from hepatic oxidation contributing to its excretion. Individual variability in metabolism, influenced by non-genetic factors, could lead to variations in urine levels and excretion rates. This could impact hepatic glycine concentrations and health, as glycine is involved in glutathione synthesis. Urine levels of benzoic acid may reflect underlying health issues [14, 15, 16]. This study aims to present an accurate analytical technique for toxicologists, analytical chemists, and public health professionals to ensure safety for the general population. Human benzoic acid levels from amphetamine users were quantitatively analyzed using GC-MS, which is the most effective choice for confirming drug concentrations in forensic toxicology. The study also presents a summary of excretion events from urinary data from patients with amphetamine overdoses, highlighting the importance of knowledge about esters or low molecular weight drugs for public health and illegal drug dumping [17, 18, 19, 20, 21]. Nonketotic hyperglycinemia (NKH) is a metabolic disorder causing toxic levels of glycine. Treatment involves sodium benzoate to lower glycine levels. Benzoic acid binds to glycine, forming hippurate, which can cause renal failure. Gas chromatography-mass spectrometry is used to determine benzoic acid concentrations in serum or plasma [11]. Benzoic acid, a plant and human metabolite, can increase independently due to diseases, rapid metabolizers, and drug consumption.

It can inhibit metabolism in rats and interfere with glutamate metabolism and mitochondrial oxidation [22,23,24,25,26,27,28,29,30,31,32,33,34]. Few studies have quantified benzoic acid in biological specimens, but it is found in multiple samples after chronic amphetamine use. Gas chromatography combined with mass spectrometry is suitable for qualitative and quantitative research; while sample preparation and solvent extraction are recommended for fewer complexes extracts [35, 36, 37]. GC-MS is a hybrid technology that uses gas-liquid chromatography and mass spectrometry to analyze complex compounds in organic mixtures, separating volatile compounds and identifying and quantifying analytes [38,39,40,41]. GC-MS is a highly sensitive and efficient method for separating and resolving compounds in biological samples, outperforming other methods like high-performance liquid chromatography-mass spectrometry [42, 43, 44].

Methodology

The study collected blood and urine samples from 60 addicted prisoners in Wasit Governorate, aged 18-25 years, who were free of any illnesses and were not receiving any medication during this period. They were divided into four groups:

G1. Those who had not used amphetamines for less than 24-48 hours

G2. Those who had not used amphetamines for 7-8 days

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G3. Those who had not used amphetamines for 15-16 days

4. Control group: those who had never used the drug

Table (1) Number of Urine and Blood Samples per Group (G1, G2, G3, Control)

Type of sample	G1	G2	G3	Control
Urine	15	15	15	15
Blood	15	15	15	15
Total	30	30	30	30

Table (1) the total number of urine and blood samples obtained from four different groups categorized by their history of amphetamine use. Urine samples: 15 samples from each group (total 60 samples). Almost all drug screening occurs through the detection of drug metabolites in urine, the most dynamic of which indicate substance use and how long it lingers in the system (as denoted in G1). Blood Samples: Again there are 15 blood samples from each group, leading to a total of 60 another samples. Blood tests can offer quicker information about drug levels in the body and can gauge both the presence of the drug and the biochemical changes triggered by its use or withdrawal. The 30 samples per group (15 urine and 15 bloods) indicate a balanced approach to measure the effects of amphetamines, potentially providing a clear comparative analysis.

Sample Preparation and Extraction Methods

The process of extraction involves collecting whole blood in anticoagulant tubes and centrifuging it at low speed for 10 minutes to separate plasma, buffy coat, and red blood cells. The plasma layer is pipetted from the top, avoiding disturbances to the buffy coat and red blood cells. To extract the benzoic acid, two times as much dichloromethane (DCM) is added to the separating funnel. The funnel is then shaken to increase its surface area, and the benzoic acid is left to move from the inorganic medium to the organic medium, dichloromethane. The organic layer is then rinsed and drained, and the aqueous phase can be re-extracted with fresh solvent if needed. The extracted analytes are then concentrated and reconstituted in a suitable solvent for further analysis. Acid extraction involves adding concentrated HCl to a liquid layer, making it acidic until pH reaches 2-3. Basic extraction uses concentrated ammonium hydroxide until pH reaches 10-12. We then place the extraction product in a glass container, dry it, wash it with dichloromethane, place it in a test tube, dry it again, and add methanol. The sample is then injected into a gas chromatography device (GC-MS). The process of extracting benzoic acid from urine is done in the same way after it is placed in a centrifuge and filtered from impurities; then we follow the same previous steps [45, 46, 47, 48, 49, 50, 51].

Standards and Calibrators

A. Benzoic acid control stock standard (1000 µg/ml): Add 0.25 g benzoic acid to a 250 mL volumetric flask. Then fill to volume with Methanol.

B. Prepare benzoic acid working calibrators using benzoic acid stock standard in 10 mL volumetric flasks according to prepare the following concentrations: (1, 2, 5, 10, 50, 100, 250 µg/ml) Using the law of dilution (M1V1=M2V2) . The standards and calibrators are stable for 6 months stored at -20 0C.

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C. Internal Standard: - Working internal standard (benzoic acid 100 µg/ml): Add10mg benzoic acid (Sigma) to a100 mL volumetric flask. Then fill to volume with Methanol.Stable 6 months stored at -20C.**3.**

Linearity

The benzoic acid calibration curve (Figure 1) showed great linearity over the concentration range of (1–250 µg/ml), as shown by a strong regression value (r^2 = 0.999). The equation y = 160525x + 501574, which describes the linear relationship, shows a high correlation between the observed signal and the benzoic acid content. This calibration curve allows for the quantitative analysis of benzoic acid within this concentration range.

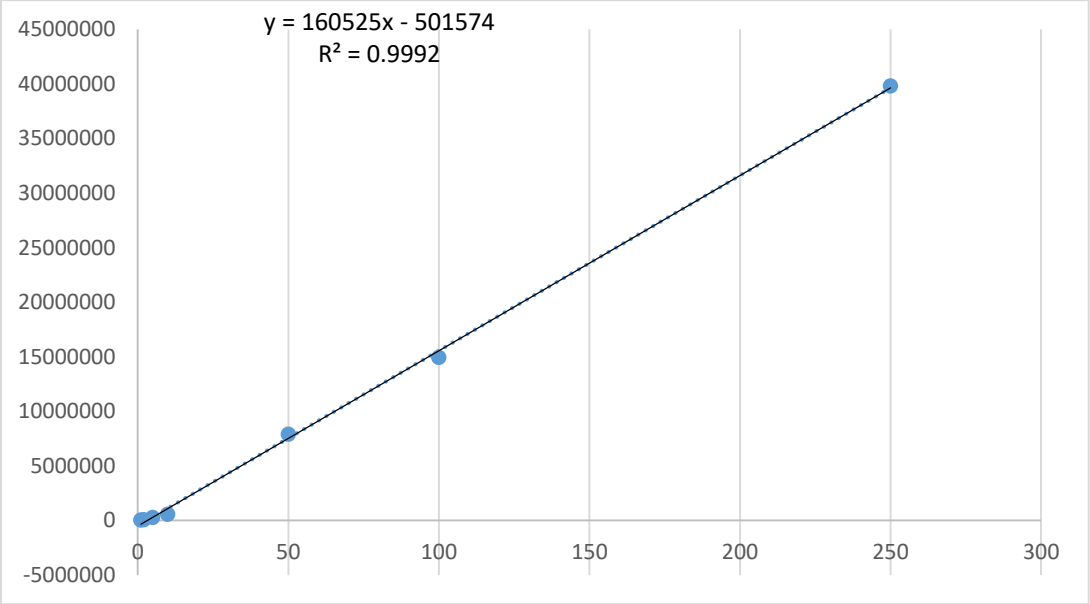

Figure (1) Standard curve for benzoic acid

Figure (1) demonstrated that the calibration curves for benzoic acid were linear across the concentration range of (1–250 µg/ml). For benzoic acid, the equation was y = 160525x +501574, and the regression coefficient (r^2) was 0.999.

Materials

This study used a Gas Chromatograph Mass Spectrometer GCMS-QP2020 NX, , and a the GC column has a maximum program temperature of 360°C and a minimum bleeding temperature of 340°C (Rxi-35Sil MS, 30 meter, 0.25 mm ID, 0.25 m df) [52].

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Figure (2). External view of Gas Chromatograph Mass Spectrometer GCMS-QP2020 NX.

Additionally, blood and urine samples from addicted inmates in Wasit and the Prison and Deportation Division were analyzed using a centrifuge, magnetic stirrer, oven, pH meter, vortex mixer, water bath, separator funnel, centrifuge tubes, pipettes, safety equipment, a CA syringe filter, clear glass vials

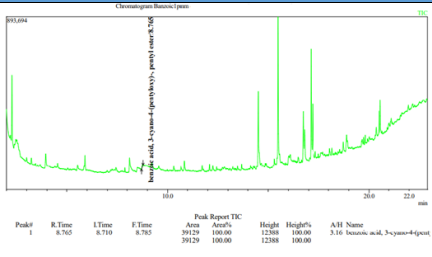
Statistical analysis

The data was analyzed using the Statistical Package for the Social Sciences version 25.0. The statistical characteristics of the subjects were examined, and the data's normality test was run, which showed that several variables differed significantly from normal distributions. Therefore, the variables were defined as medians (minimum–maximum), and the variables between the control and time-dependent subgroups were compared using Kruskal-Walli's-H with posttest (Dunn's test). Whitney, Mann Variables between sample types subgroups were compared using the U test.

Results and Discussions

The GC-MS data for known amounts of benzoic acid standards are shown in Table (2). Benzoic acid levels in blood and urine samples taken from amphetamine addicts at different times will be measured using this data to create a trustworthy calibration curve. To comprehend benzoic acid's metabolic function in this population, precise measurement is necessary.

Table (2) Concentration of benzoic acid, its area, and GC-MS chromatogram of benzoic acid

Conc. Of 4-HPA (µg/ml)	Area	chromatogram
1	**39129**	

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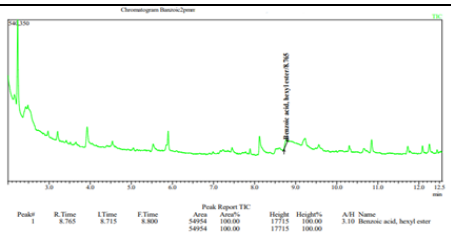
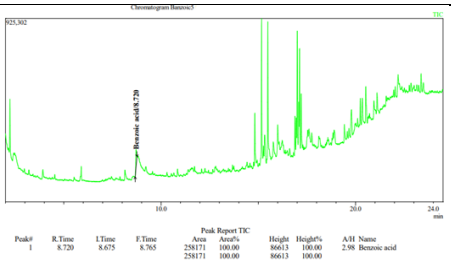
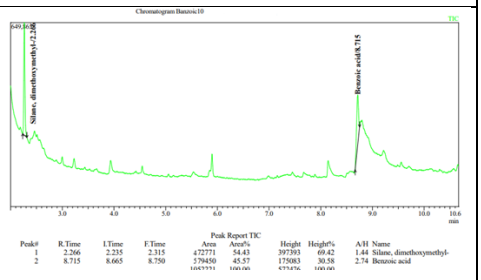
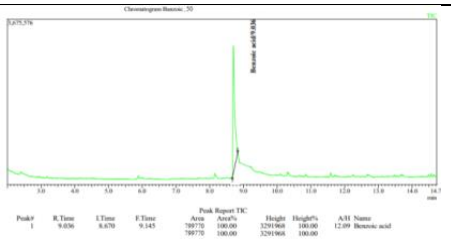
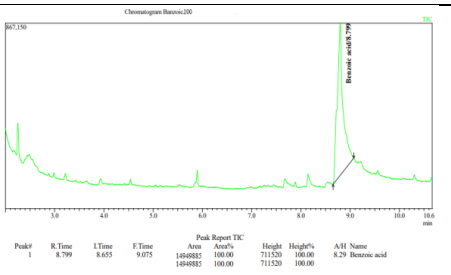
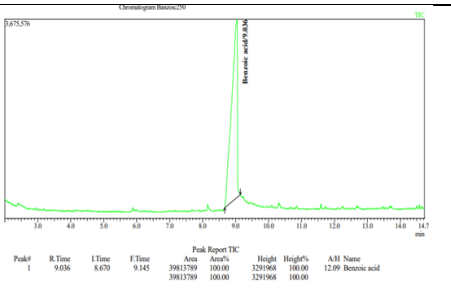
2	**54954**	
5	**258171**	
10	**572771**	
50	**7899770**	
100	**14949885**	
250	**39813789**	

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Table (3) Participants clinical characteristics

Group / Parameter	G1	G2	G3	Control
Number	15	15	15	15
Age (Years)	22±2.5	21±1.7	21±2.2	22.8±2.2
BMI (kg/m^2)	22.5 ± 1.5	23.0 ± 1.2	23.0 ± 1.0	23±1.1

Table (4) comparison of blood Benzoic Acid between control and time dependent subgroups

Groups		Blood Benzoic Acid (ng/ml)
Control	Median(max-min)	403.17(354.06- 463.80)
G1		1783.98(1322.87 – 1943.87)
G2		757.96 (671.78 – 988.99)
G3		420.47 (479.39 – 549.58)
Kruskal-Walli's test value		36.58
P value		<0.001*

***: significant difference**

The study analyzed the effects of amphetamine abstinence on four groups of participants, each consisting of 15 individuals. The data showed that the average age and BMI values were relatively similar across the groups, indicating homogeneity in terms of age. The study also found that the differences observed in other parameters, such as physiological measures, could be attributed to the history of amphetamine use rather than age or body composition differences. This consistency helps researchers assess the effects of amphetamine abstinence while controlling for key demographic variables.

The study groups had very different median (max-min) levels of benzoic acid in their blood (Kruskal-Wallis test: 36.58, $p < 0.001$). In particular, the G1 subgroup had significantly higher amounts of benzoic acid (1783.98 ng/ml) than the control group (403.17 ng/ml). The amount of benzoic acid in the G3 subgroup was about the same as the control group's (420.47 ng/ml), but the amount in the G2 subgroup was higher (757.96 ng/ml) than in the control. These results point to a time-dependent impact on blood benzoic acid levels, with the G1 subgroup showing the most noticeable rise.

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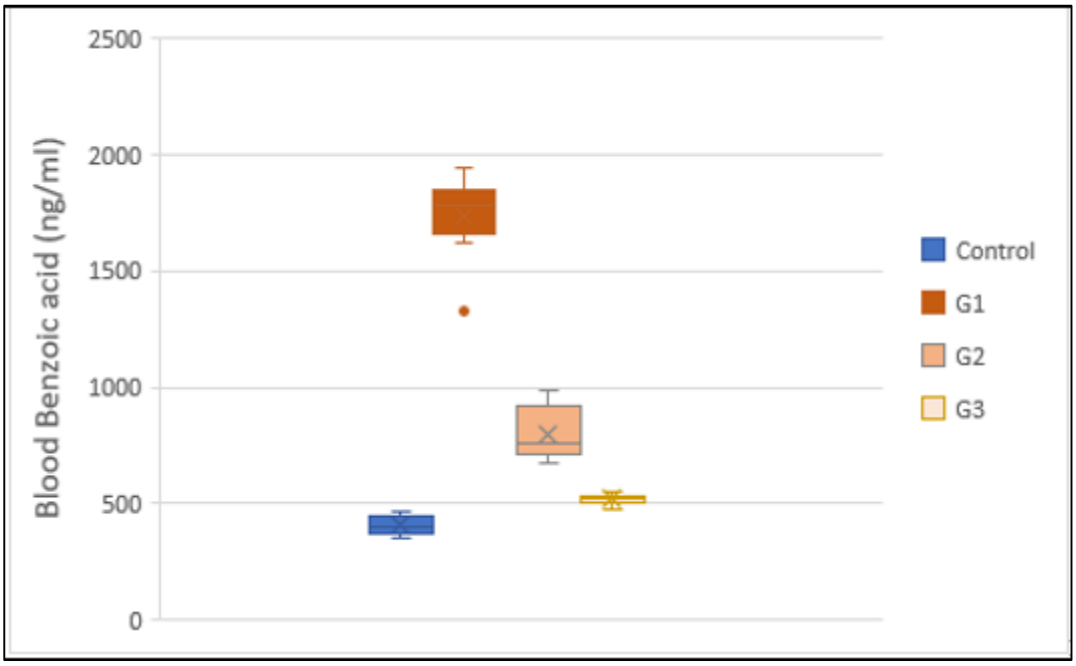

Figure (3). Comparison of blood Benzoic Acid between control and time dependent subgroups

Figure (3) presents a comparison of blood benzoic acid levels between the control and time-dependent subgroups. The concentrations of benzoic acid in the G1 subgroup were significantly higher than those in the control group. This is in line with the statistically significant difference (Kruskal-Wallis, p 0.001). The distribution of the G3 subgroup is comparable to that of the control group, even though G2 likewise seems higher in comparison to the control.

Table (5). Post Kruskal-Walli's (Dunn's test) of blood Benzoic Acid between study groups

Groups		P value
Control	G1	<0.001*
	G2	<0.001*
	G3	0.056 NS
G1	G2	0.056 NS
	G3	<0.001*
G2	G3	0.056 NS

***: significant difference, NS: Non-significant difference**

The findings of Dunn's post-hoc test, which was carried out to look into the substantial variations in blood benzoic acid levels found by the Kruskal-Wallis test, are shown in Table (5). Benzoic acid levels were considerably lower in the control group than in the G1 ($p < 0.001$) and G2 ($p < 0.001$) groups, according to the analysis. There was no discernible difference between Group G3 and the control group ($p = 0.056$). Additionally, the levels of benzoic acid in G1 were substantially higher than those in G3 ($p < 0.001$). There were no discernible differences between G1 and G2 ($p = 0.056$) or G2 and G3 ($p = 0.056$).

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Table (6). Comparison of urine Benzoic Acid between control and time dependent subgroups

Groups		Urine Benzoic Acid (ng/ml)
Control	Median(max-min)	954.76 (819.54- 1087.51)
G1		5605.75(5274.02 – 7449.89)
G2		2918.10 (2124.05 – 3217.61)
G3		1296.85 (982.76 – 1318.02)
Kruskal-Walli's test value		36.16
P value		<0.001*

***: significant difference**

The study groups had very different median (max-min) levels of benzoic acid in their urine (Kruskal-Wallis test: 36.16, $p < 0.001$). The average amount of benzoic acid in the G1 subgroup's urine was (5605.75 ng/ml), which was a lot more than the control group's (954.76 ng/ml). While subgroup G3 displayed a more moderate rise (1296.85 ng/ml) in comparison to the control group, subgroup G2 also displayed significant values (2918.10 ng/ml). The results suggest that the change made in the experiment had a big impact on the amount of benzoic acid that was excreted in the urine, with the G1 subgroup having the most significant effect.

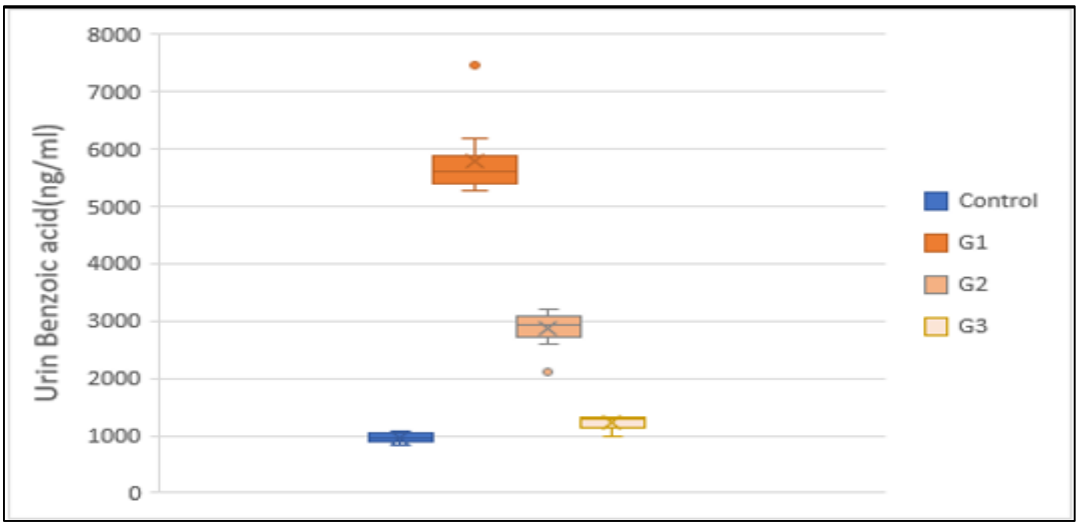

Figure (4) comparison of urine Benzoic Acid between control and time dependent subgroups

Urine benzoic acid concentrations in the control and time-dependent subgroups are graphically compared in Figure (7-4). The picture shows that the G1 subgroup, along with subgroups G2 and G3, had significantly greater quantities of urine benzoic acid than the control, indicating significant differences (Kruskal-Wallis, $p < 0.001$).

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Table (7). Post Kruskal-Walli's (Dunn's test) of urine Benzoic Acid between study groups

Groups		P value
Control	G1	<0.001*
	G2	<0.001*
	G3	0.072 NS
G1	G2	0.056 NS
	G3	<0.001*
G2	G3	0.049*

***: significant difference, NS: Non-significant difference**

Dunn's post-hoc test was used to compare urine benzoic acid levels between research groups pairwise, as shown in Table (7). There were notable variations between the control group and the G1 ($p < 0.001$) and G2 ($p < 0.001$) subgroups, suggesting that the urine benzoic acid levels in these groups were higher than in the control. G3 was substantially lower than G1 ($p < 0.001$), although it did not differ significantly from the control ($p = 0.072$). Additionally, there was a statistically significant difference between G2 and G3 ($p = 0.049$). There was no statistically significant difference between G1 and G2 ($p = 0.056$).

Table (8)Comparison between blood and urine Benzoic Acid for each time dependent group

Groups		Blood	Urine	Mann. Whitney U test	P. value
Control	Median(max-min)	403.17(354.06- 463.80)	954.76 (819.54- 1087.51)	100.00	<0.001*
G1		1783.98(1322.87 – 1943.87)	5605.75(5274.02 – 7449.89)	100.00	<0.001*
G2		757.96 (671.78 – 988.99)	2918.10 (2124.05 – 3217.61)	100.00	<0.001*
G3		420.47 (479.39 – 549.58)	1296.85 (982.76 – 1318.02)	100.00	<0.001*

***: significant difference**

The Mann-Whitney U test is used in Table (8) to compare the levels of benzoic acid in the blood and urine of each research group. Urine benzoic acid levels were considerably greater than blood benzoic acid levels within each group (Control, G1, G2, and G3) ($p < 0.001$). For example, the median (max-min) values for blood and urine were "for the G1 group, 1783.98 (1322.87–1943.87) and 5605.75 (5274.02–7449.89) ng/ml, respectively" and "for the Control group, 403.17 (354.06–463.80) and 954.76 (819.54–1087.51) ng/ml, respectively." All of the groups that looked at it showed that there was a big difference in the amounts of benzoic acid in the two types of samples (U statistic = 100; $p < 0.001$). Therefore, the G1 group had significantly higher levels of benzoic acid than the control group. This shows that the treatment or condition being studied may have an effect. More research may reveal underlying mechanisms that are causing these high concentrations, which means that the metabolic pathways involved need more study.

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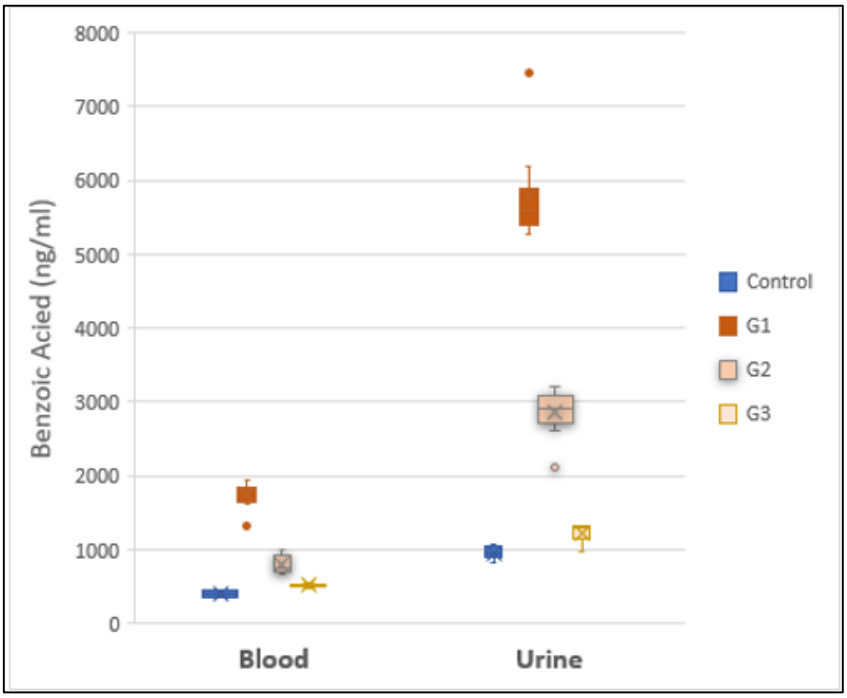

Figure (5) comparison between blood and urine Benzoic Acid for each time dependent group

Figure (5) shows the relationship between blood and urine benzoic acid levels. This is in line with how the kidneys are thought to get rid of benzoic acid. All of the groups had higher amounts of benzoic acid in their urine, which means that the body is filtering and getting rid of it effectively.

Conclusion

This study presents an accurate analytical technique for toxicologists, chemists, and public health professionals to ensure safety for the general population. Human benzoic acid levels from amphetamine users were quantitatively analyzed using GC-MS, the most effective choice for confirming drug concentrations in forensic toxicology. The study collected blood and urine samples from addicted prisoners in Wasit, dividing them into four groups: those who have not used amphetamines for less than 24-48 hours, those who have not used amphetamines for 7-8 days, those who have not used amphetamines for 15-16 days, and a control group who has never used drugs. The study found that the G1 subgroup had significantly higher levels of benzoic acid than the control group, indicating that the treatment or condition being studied may have an effect on the kidneys and liver. Further research is needed to understand the underlying mechanisms causing these high concentrations. A study aimed to optimize a method for detecting benzoic acid in Blood, and urine samples using gas chromatography-mass spectrometry technique. This could contribute to forensic science, particularly in drugs of abuse, and help toxicologists and law enforcement authorities evaluate preventive policies. The results could also provide more information and structure for other scientists, potentially leading to clinical treatments and policy evaluations. The study is relevant in forensic drug analysis and analytical toxicology, impacting public health, particularly among the baby boomer and millennial generations.

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Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of science]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Science], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

Hassan H. Khalifea: conducted some experiments, collection a part of literature review and conducted some characteristics of the products and drafted the initial manuscript.

Noor M. Ali: Contributed to the conception and design of the study, conducted some experiments, data rearrangement.

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