ORİJİNAL ARAŞTIRMA ORIGINAL RESEARCH

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Evaluation of Formic Acid Concentrations in Postmortem Blood Samples Using HS-GC-MS System

HS-GC-MS Sistemi Kullanarak Postmortem Kan Örneklerinde Formik Asit Konsantrasyonlarının Değerlendirilmesi

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ABSTRACT Objective: In this study, we aimed to evaluate the results of formic acid concentrations in positive and control groups of postmortem blood samples obtained from autopsies conducted in the Council of Forensic Medicine, Turkey, between January 2016 and June 2018. **Material and Methods:** Formic acid concentration was measured as methylformate in the blood samples by performing headspace-gas chromatography-mass spectrometry (HS-GC-MS) system. **Results:** A wide calibration range (0.1-150 mg dL^{-1}, r^2= 0.999, internal standard: acetonitrile) was employed for determination of formic acid. LOD and LOQ values were found to be 0.003 mg dL^{-1} and 0.011 mg dL^{-1}, respectively. A positive blood sample was spiked and recovery was found to be 102%. After the analyses, the following results were obtained: In positive blood samples (N= 91), formic acid concentration range was found to be 37-141 mg dL^{-1}, while control samples (N= 50) had a range 0.3 - 5.6 mg dL^{-1}. In addition, no correlation was found between methanol and formic acid concentrations in the bloods whereas there was a very high correlation between blood and vitreous humor methanol concentrations, as expected. **Conclusion:** The most common reason for the deaths from methanol-induced metabolic acidosis is consumption of illicit alcoholic beverages produced in clandestine places and containing high concentration of methanol. In this paper, we present not only HS-GC-MS method for determination of formic acid in postmortem blood samples, but also the key points for evaluation of its results as well.

Keywords: Acidosis; blood; alcoholism; methanol; headspace gas chromatography-mass spectrometry

ÖZET Amaç: Biz bu çalışmada, Ocak 2016 ile Haziran 2018 tarihleri arasında Adli Tıp Kurumu'nda yapılan otopsilerden elde edilen pozitif ve kontrol postmortem kan örneği gruplarındaki formik asit konsantrasyonlarının sonuçlarını değerlendirmeyi hedefledik. **Gereç ve Yöntemler:** Kan örneklerinde formik asit konsantrasyonu metilformat olarak headspace-gaz kromatografi-kütle spektrometri (HS-GC-MS) sistemi ile ölçülmüştür. **Bulgular:** Formik asit tayini için geniş bir kalibrasyon aralığı (0,1-150 mg dL^{-1}, r^2= 0.999, İç standart: asetonitril) kullanıldı. LOD ve LOQ değerleri sırasıyla 0,003 mg dL^{-1} ve 0.011 mg dL^{-1} olarak bulunmuştur. Pozitif bir kan örneğine spike işlemi uygulandı ve %102 geri kazanım elde edildi. Pozitif kan örneklerinde (N= 91) formik asit konsantrasyon aralığı 37-141 mg dL^{-1} olarak bulunurken kontrol örneklerinde aralık 0,3-5,6 mg dL^{-1} (N= 50) bulunmuştur. Ayrıca, kan örneklerinde metanol ile formik asit arasında korelasyon kurulamazken kan ile göz içi sıvılarındaki metanol konsantrasyonları arasında beklendiği gibi yüksek korelasyon bulundu. **Sonuç:** Metanol kaynaklı metabolik asidozdan meydana gelen ölümlerin en yaygın nedeni gizli yerlerde üretilen yüksek konsantasyonda metanol içeren yasadışı içkilerin tüketilmesidir. Biz bu makalede, postmortem kan örneklerinde formik asit tayini için bir HS-GC-MS metodunun yanı sıra formik asit sonuçlarının değerlendirilmesindeki kilit noktaları da sunmaktayız.

Anahtar Kelimeler: Asidoz; kan; alkolizm; metanol; gaz kromatografi-kütle spektrometri

Acute methanol poisoning and subsequent treatment is a serious subject worldwide. Being a colorless, clear, and volatile liquid, methanol is used in paints, solvents, polishers, cleaners, perfumes, additives to fuels, anti-freezes and outlawed alcoholic drinks.[1,2] Methanol-

based poisonings generally occur by drinking it as if it were an ethanolic drink, or deliberately consuming it for commit suicide, or malpractice of several liquids.[1,3] Alcohol-addicted people find it more intriguing to buy cheap alcoholic beverages. Non-corporate markets might illegally sell alcoholic beverages much less than their market price and insensible consumers might find them appealing. However, in clandestine laboratories, cheap alcoholic beverages are produced, and technical ethanol is sometimes preferred over ethanol produced with fermentation, but generally methanol is added. Since methanol is cheaper than ethanol, the overall cost is much lowered. What is more, there are also alcohol addicted people drinking cheap cologne water as an example for methanol intake.

Methanol is first metabolized into formaldehyde with alcohol dehydrogenase (ADH), then formic acid (FA) with aldehyde dehydrogenase enzyme and these metabolites are very toxic.[1,2] Methanol creates a depression in the central nervous system, therefore is toxic, and accumulating FA has suppressive toxicity. It is therefore wise to evaluate both at the same time in a case of poisoning. Formate is strongly cytotoxic against mitochondrial cytochrome c oxidase activation, thereby inhibiting it. Accumulation of FA causes metabolic acidosis and severely impairs retina and ocular nerves.[4,5] Excessive metabolic acidosis results in death.

Although the poisoning dose of methanol varies among people, 10-20 mL of methanol intake causes visual impairment or loss of sight, whereas 30-100 mL of methanol intake causes death. In addition, literature data suggests that people poisoned from methanol and survived had methanol concentrations more than 10 mg dL^{-1}.[6] In literature, after methanol intoxication, the patients were treated with folic acid, folinic acid, ethanol, and fomepizole (4-methylpyrazole) as antidotes to block the toxicity of FA.[3-5]

A method that is used to measure FA concentration in the blood must be precise and reliable. In the literature, FA is derivatized to methylformate and measured with HS-GC-FID, derivatized to ethylformate and measured with GC-FID, derivatized to methylformate and measured with in-tube extraction GC-MS method, derivatized to isobutylformate and measured with GC-MS, derivatized to pentafluorobenzylformate and measured with GC-MS, derivatized to isopropylformate and measured with HS-GC-FID, with enzymatic test, with UV-dedection capillary electrophoresis or capillary electrophoresis with conductivity detector.[4,7-20]

In this study, we aimed to describe headspace gas chromatography-mass spectrometry (HS-GC-MS) method for the analysis of FA in postmortem blood samples and to establish FA levels not only in positive group including patients who died by metabolic acidosis after drinking methanol-containing liquids, but also in control groups of postmortem blood samples obtained from autopsies conducted in the Forensic Medicine Institute (ATK), Turkey, between January 2016 and June 2018. Furthermore, to the best of our knowledge this is the first report in Turkey to evaluate elaborately FA levels in postmortem blood samples and its correlations with blood methanol, blood ethanol, and vitreous methanol concentrations.

MATERIAL AND METHODS

This study was conducted by permission of the ATK Chairmanship, Education and Scientific Research Commission (decision number: 2018/737; date: September 18, 2018). The authors confirm that this research conducted according to the principles expressed in the Declaration of Helsinki.

REAGENTS

Formic acid 99-100%, ACS Reag. Ph. Eur, was purchased from VWR Chemicals (Fontenay-sous-Bois France). Methanol and acetonitrile (both of them 99.9%, hypergrade for LC-MS) were provided by Merck (Darmstadt, Germany). Ultrapure water of min 18.2 MΩ cm^{-1} resistivity was obtained from a New Human Power I Scholar UV system (Human Corporation, Seoul, Korea).

HS-GC-MS SYSTEM

The gas chromatographic analysis of FA was performed with a Perkin Elmer Clarus 680 gas chromatograph equipped with a Clarus SQ 8 T Mass

Spectrometer and HS40 headspace (HS) autosampler. Separations were achieved using a highly polar gas chromatography (GC) column (Perkin Elmer Elite-FFAP) that was appropriate for separation of acidic compounds thanks to its crossbond carbowax-PEG structure. Dimensions of the column were 30 m long, 0.25 mm i.d., and 0.5 µm df. Helium was used as the carrier gas at 1 mL min^{-1} constant flow rate with a HS pressure of 35 psi. For incubation of esterification, vials were kept in the HS oven set at 60°C for 15 min. The temperature of HS needle was 80°C. Injections were made by adjusting needle time of the loopless HS. After the incubation time finished, pressure was applied to the vial with the needle for 1.0 min and then the gas was taken from vapor phase of the vial for 0.12 min. The temperature of transfer line was set at 90°C. The GC oven temperature program was as follows: I) initially 40 °C for 7 min, II) elevated from 40°C to 220 °C at rate of 25 °C min^{-1}, III) held at 220°C for 2 min. Equilibration time of oven was 0.5 min. Gas chromatography injector temperature was 150 °C during total analysis time that was 16.20 min. Mass detection was performed at 200°C and electron energy was 70 eV of EI+ source with both full scan between 12-150 amu for identification for first 7 min, and with selected ion recording (SIR) mode for quantitative analysis. The assigned ions for SIR mode was m/z 31 and 60 between 1.5-3 min for methyl formate while m/z 40 and 41 between 4.5-5.5 min was for acetonitrile (ACN) used as internal standart (IS) with 0.04 secs of dwell time. A TurboMass version 6.1.0.1963 software was utilized for data acquisition and instrumental control for GC and MS while headspace autosampler was controlled by computer using PerkinElmer HS Driver v2.5.0.0125 software.

SAMPLE PREPARATION

Formic acid cannot be directly analyzed by GC due primarily to its low response and high reactivity behavior. Thus, it must be derivatized prior to GC analysis. FA can be esterified with methanol to yield formic acid methyl ester (methylformate). Sulfuric acid was utilized as a catalyst.[2,7,8,10,21] In our study, standard solutions and blood samples were prepared as follows: 0.5 mL of 50 mg dL^{-1} ACN was added into 22 mL headspace vial, which was in a tube holder placed on an ice box with appropriate size. Then, an aliquot of 0.2 mL blood sample and 0.1 mL pure methanol were respectively added in this solution. Finally, 0.2 mL of concentrated H_2SO_4 was gingerly added above the cold mixture. After the sample vial was immediately sealed with gas-tight polytetrafluoroethylene (PTFE)-lined rubber septum cap, it was vigorously stirred with a vortex mixer for 1 minute. This vial was placed into headspace autosampler and it was left at 60 °C for 15 min to complete the derivatization.[2,21] Derivatization reaction is demonstrated in (Figure 1).

RESULTS

VALIDATION PARAMETERS

The calibration curve was drawn by plotting the peak-area ratios of the methylformate, which was derivative of FA, to ACN (IS) versus eight different concentrations of FA. Retention times of methylformate and ACN on chromatogram were 1.93 and 5.00, respectively. All curves have exhibited good linearities in the range of 0.011 -150 mg dL^{-1} (Last one: r^2= 0.999) so far.

LOD was 0.003 mg dL^{-1} and LOQ was 0.011 mg dL^{-1} which were calculated by the standard de-

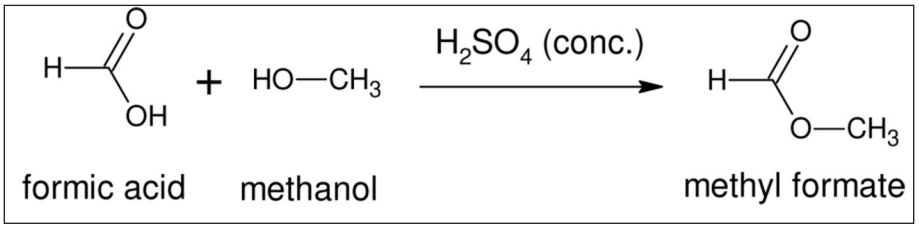

FIGURE 1: Derivatization reaction of formic acid to methyl formate.

viation (s'_0), obtained from ten replicate measurements of low concentration of FA in a post-mortem blood sample, multiplied by k_Q factors which were 3 and 10 (the IUPAC default values), respectively.[22]

To evaluate the accuracy of the method, a positive postmortem blood sample was investigated. For this reason, a sample with 76.1 mg dL^{-1} (RSD %= 0.13) FA was spiked with two different levels that were 40 mg dL^{-1} and 75 mg dL^{-1}. Final concentrations were 106.7 mg dL^{-1} (CV= 1.7, N= 3) and 149.7 mg dL^{-1} (CV= 1.4, N= 3), respectively. Consequently, recoveries of which average value was 102% were calculated as 96% and 108%, respectively. Moreover, under the optimum chromatography conditions no interference peak has been observed so far, most likely due to that similar components were also derivatized and, therefore, their retention times were increased.

In order to compare the signal-to-noise (S/N) ratios of the analyte peaks obtained from the total ion chromatogram (TIC) and SIR mode, a 60 mg dL^{-1} aqueous FA solution was injected and 10-150 m/z ions were scanned for the TIC and at the same time 31 m/z was scanned for the SIR mode. Methylformate's peak S/N ratios were: TIC: 777; 31 m/z (TIC): 3353; 31 m/z (SIR): 4963. Consequently, for qualitative analysis, 10-150 m/z ions were scanned and a NIST mass spectral database (Version 2.0 g 2011) was used for identifying the peak while 31 m/z at SIR mode was assigned for quantification.

ANALYSIS RESULTS AND THEIR DISTRIBUTIONS

Our control group consisted of 50 postmortem cases and the maximum FA concentration was found to be 5.6 mg dL^{-1}. On the other hand, FA concentration range was between 37 and 141 mg dL^{-1} in the group of the deaths (N= 91) attributed to methanol poisoning. In Table 1, a statistical summary was presented for blood FA samples of people who did not have methanol intoxication (control group) and those died without receiving any medical treatment (positive group). In addition, for both groups the whisker plots were drawn and exhibited in Figure 2 for clarity. It is clear from Table 1 and Figure 2 that there is approximately 50-fold difference between the average values of the control and positive groups.

CORRELATION OF BLOOD FORMIC ACID VERSUS BLOOD/VITREOUS METHANOL

Noteworthy is that to reach 91 number of positive FA results, we took all FA values regardless of whether methanol and ethanol had been measured or not. Besides, the results of methanol and ethanol measured samples were inserted in (Table 2). Methanol and ethanol concentration results were obtained from the accredited Alcoholmetry Laboratory where alcohol analyses were carried out by operating HS-GC-FID system ($r^2 \geq 0.999$ for both compounds). The first 36 lines of Table 2 show the FA, methanol, and ethanol concentrations of postmortem blood samples and vitreous humor samples (where available) which were not subjected to medical treatment. Since analysis of FA in vitreous humor is not examined in our laboratory, the respective results could not be presented. These 36 cases were sorted in the increasing order of FA concentration. No ethanol was detected in these samples. There was not a correlation between FA and methanol concentrations as seen in Figure 3 (A), consistently with the literature (r^2= 0.093).[11] However, blood and vitreous humor methanol concentrations had a high correlation (r^2= 0.963). Figure 3 (B) shows the graph for methanol found in the blood and the vitreous humor.

The last 11 results in Table 2 show that postmortem blood of whose received medical care had a FA concentration range of 1.5-74 mg dL^{-1}. The interrelation of these results showed that increasing

TABLE 1: Distributions of FA concentrations in postmortem blood samples of control and positive groups.

Analysis group	Number of cases	Range (mg dL^{-1})	Mean (mg dL^{-1})	Median (mg dL^{-1})	Standard Deviation
Control	50	0.3 – 5.6	1.7	1.2	1.4
Positive	91	37-141	69	66	18

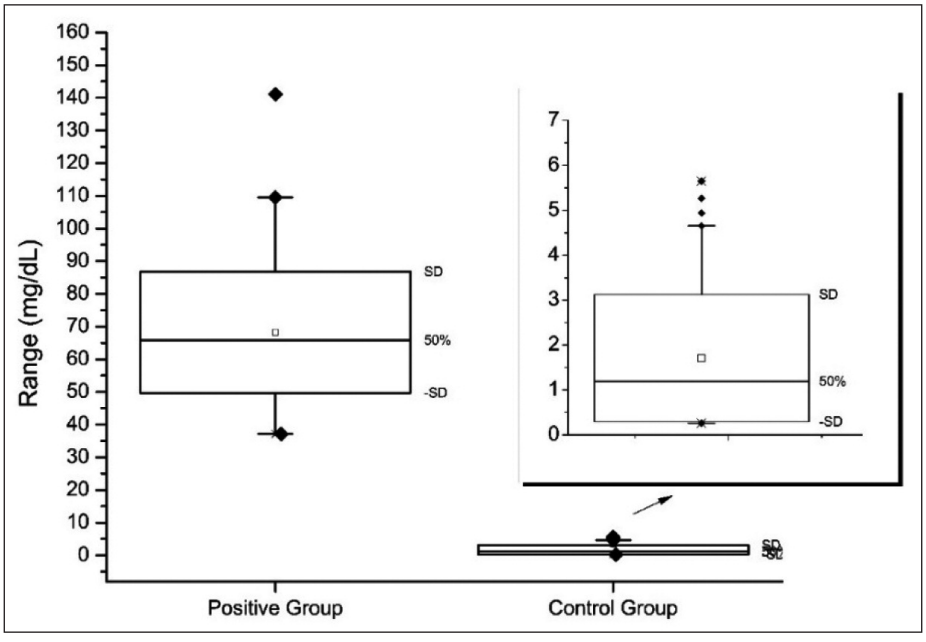

FIGURE 2: Whisker plots of FA positive group and control group.

FA concentration value could not be correlated with methanol (Figure 4 (A)) and ethanol (Figure 4 (B)) concentrations (r^2= 0.217 and r^2= 0.133, respectively). We, however, could find a high correlation between blood and vitreous humor methanol concentrations (Figure 4 (C)), r^2= 0.996).

DISCUSSION

The literature has some postmortem blood FA concentration ranges and these were compared with our values and summarized in (Table 3). Though our average value (69 mg dL^{-1}) seems to be smaller than the other averages values, there is a good agreement between the distributions of our laboratory-originated results and the others'.

It should be noted that the patients received treatment with ethanol before death, postmortem blood FA concentrations did decrease significantly, as shown by other studies in the literature.[4]

In this study, no experiment was carried out for analysis of FA in urine samples and vitreous humor. Viinamäki et al. demonstrated that the mean formic acid concentrations were 6 mg dL^{-1} and 25 mg dL^{-1} in normal urine samples and putrified urine samples, respectively while it was found 4 mg dL^{-1} and 24 mg dL^{-1} in normal and putrified blood samples, respectively. Formic acid concentration can reach to 25 mg dL^{-1}, which is a high enough value like the values obtained from methanol intoxication cases, during the postmortem period owing to decomposition of lipids, carbohydrates, and proteins or by bacterial action. On the other hand, they also reported that ten putrefied samples were transferred into the tubes containing sodium fluoride as a preservative, and then they were reanalyzed after 3-4 months. After storage, they obtained similar results for FA concentrations.[11] Therefore, it is very important that a postmortem blood sample must be sent to the laboratory with the tube containing sodium fluoride for FA analysis.

A poor correlation was found between blood formic acid concentrations and blood methanol concentrations (r^2= 0.093) like the earlier studies (r^2= 0.003, r^2= 0.1463).[9,11] In addition, a high correlation was observed between blood and vitreous humor methanol concentrations not only in our study (r^2= 0.963), but also in the earlier study (r^2= 0.9859).[9] It is clear from the correlations and the concentration ranges that this study and the earlier studies in the literature are consistent. However, in methanol positive cases, some additional correlation

TABLE 2: Results of formic acid in postmortem blood samples and also ethanol and methanol concentrations and related vitreous ethanol and methanol concentrations.

		Blood	Blood		Vitreous humor	
Case	Medical Care	FA (mg dL^{-1})	Methanol (mg dL^{-1})	Ethanol (mg dL^{-1})	Methanol (mg dL^{-1})	Ethanol (mg dL^{-1})
1	No	37	344	ND	441	ND
2	No	38	324	ND	NA	NA
3	No	44	130	ND	139	ND
4	No	45	281	ND	322	ND
5	No	46	523	ND	403	ND
6	No	50	54	ND	62	ND
7	No	51	147	ND	200	ND
8	No	51	210	ND	232	ND
9	No	53	109	ND	128	ND
10	No	54	270	ND	392	ND
11	No	54	305	ND	NA	NA
12	No	56	183	ND	NA	NA
13	No	59	86	ND	90	ND
14	No	60	58	ND	NA	NA
15	No	61	178	ND	226	ND
16	No	63	57	ND	81	ND
17	No	63	81	ND	92	ND
18	No	64	150	ND	193	ND
19	No	64	122	ND	145	ND
20	No	67	77	ND	NA	NA
21	No	68	331	ND	336	ND
22	No	77	62	ND	72	ND
23	No	77	181	ND	201	ND
24	No	79	183	ND	NA	NA
25	No	80	325	ND	420	ND
26	No	81	389	ND	396	ND
27	No	84	662	ND	719	ND
28	No	85	282	ND	326	ND
29	No	85	266	ND	NA	NA
30	No	85	192	ND	216	ND
31	No	88	117	ND	123	ND
32	No	95	348	ND	406	ND
33	No	95	355	ND	366	ND
34	No	107	212	ND	221	ND
35	No	110	472	ND	464	ND
36	No	141	69	ND	NA	NA
37	Yes	1.5	48	25	54	27
38	Yes	7.6	223	18	241	28
39	Yes	9	25	6	33	ND
40	Yes	10	56	138	75	0
41	Yes	29	114	386	122	416
42	Yes	34	61	35	70	ND
43	Yes	43	302	73	362	91
44	Yes	48	74	18	80	5
45	Yes	59	46	19	57	32
46	Yes	71	244	128	264	144
47	Yes	74	71	138	86	142

*NA: Not available; ND: Not detected.

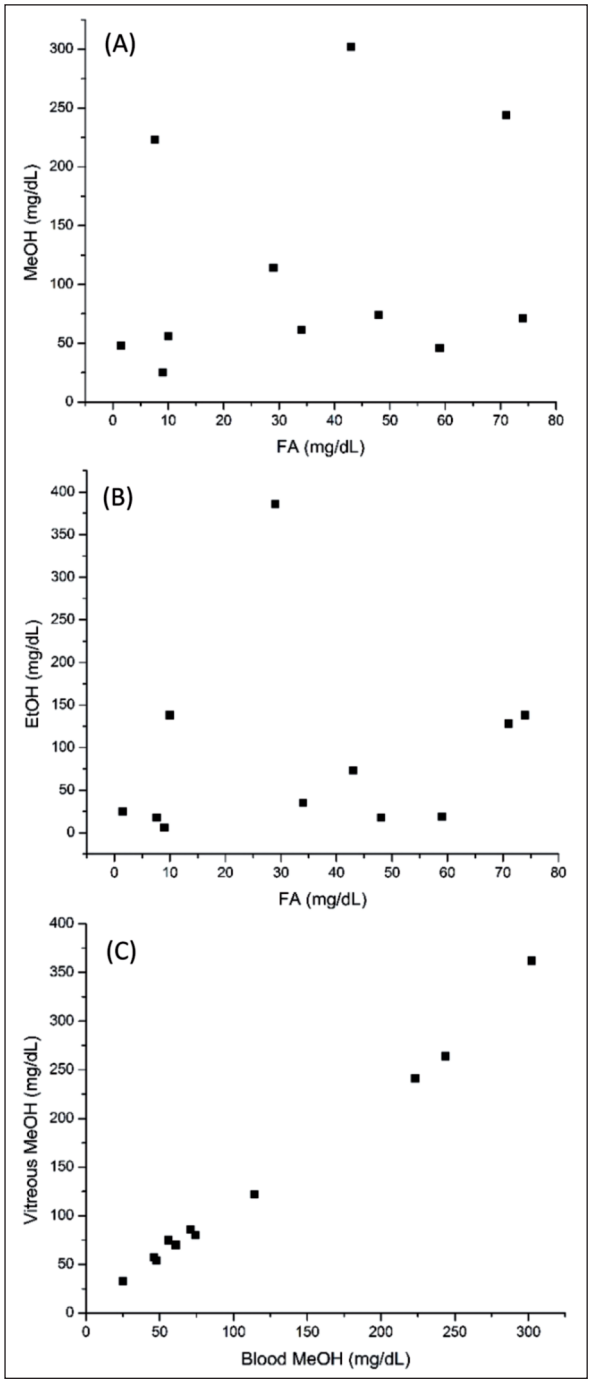

FIGURE 3: Correlation plots of (A) postmortem blood FA versus blood methanol (r^2= 0.093) and (B) postmortem blood methanol versus vitreous humor methanol (r^2= 0.963) concentrations in 36 methanol fatalities.

experiments were conducted in the literature unlike our study. For instance, correlations between: vitreous humor FA concentration and vitreous humor methanol concentration (r^2= 0.0632), vitreous humor FA concentration and blood FA concentration (r^2= 0.2646), urine methanol concentration and blood methanol concentration (r^2= 0.977), urine FA concentration and blood FA concentration (r^2= 0.067), urine FA concentration and urine methanol concentration (r^2= 0.037).[9,11]

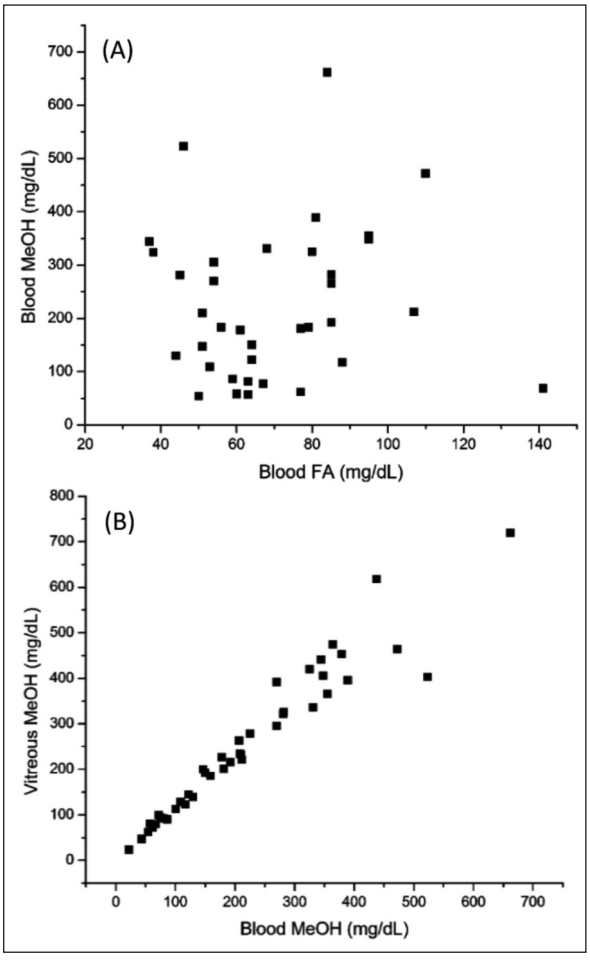

FIGURE 4: Correlation plots of (A) postmortem blood FA versus blood methanol (r^2= 0.217); (B) blood FA versus vitreous blood ethanol (r^2= 0.133) and (C) postmortem blood methanol versus vitreous humor methanol (r^2= 0.996) concentrations in 11 methanol fatalities in which ethanol was also detected.

TABLE 3: Statistical summary of the results of positive FA concentrations in postmortem blood samples of our study and the literature studies.

		Literature works		
	This Study	**Jones et al.**	**Wallage et al.**	**Viinamäki et al.**
Range (mg dL^{-1})	37-141	12-140	64-110	19-103
Mean (mg dL^{-1})	69	84	88	80
Median (mg dL^{-1})	66	83	93.5	87.5
N	91	74	6	24
Std deviation	18	19	17	24

Even though a case history is unknown, measured high concentrations of methanol and FA in the blood of the victim provide important clues for the solution of the file. However, before conveying the final report, a broad investigation with caution must be exercised. Another crucial point is that whether formalin or a similar protective solution was applied to a cadaver after autopsy because formaldehyde might be oxidized to FA on contact with aerial oxygen.[23] In addition, formalin solutions contain methanol to prevent polymerization of formaldehyde. Therefore, formaldehyde and FA might be present together in the same matrix. If these are not considered, serious errors will occur in the post-analysis evaluations.

CONCLUSION

This is the fist paper to demonstrate the results of FA in postmortem blood samples in detail. Metabolic acidosis resulting from elevated formic acid concentration seriously threatens the living health and is even fatal. Therefore, it is very important to provide fast and reliable results for FA concentration in the blood samples. This work investigated the determination of FA in postmortem blood samples with a reliable and sensitive HS-GC-MS method. It is clear that our results coherent with the previously published results in the literature. For the control group, maximum FA concentration was 5.6 mg dL^{-1} whereas the minimum concentration of the positive group was 37 mg dL^{-1}. This means that there is at least a six-fold difference between these groups while there is approximately fifty-fold difference between the mean values of two groups. According to our results and the results of the early studies, > 50 mg dL^{-1} of FA in blood is definitely fatal. Another key point for evaluation of FA in postmortem blood samples is that the samples must be immediately transferred into the tube containing sodium fluoride as a preservative after autopsies, because FA is formed and even its concentration can reach to 25 mg dL^{-1} during postmortem period. Unless the postmortem blood sample has been putrefied much and external formalin was involved, high concentrations of FA and methanol proves the metabolic acidosis-related death due to methanol intoxication. Although the concentration of FA in urine and vitreous humor samples were not measured in this study, their results may provide supporting information as to the cases.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Orhan Destanoğlu, İsmail Ateş;* ***Design:*** *Orhan Destanoğlu;* ***Control/Supervision:*** *Orhan Destanoğlu, İsmail Ateş;* ***Data Collection and/or Processing:*** *Orhan Destanoğlu;* ***Analysis and/or Interpretation:*** *Orhan Destanoğlu, İsmail Ateş;* ***Literature Review:*** *Orhan Destanoğlu;* ***Writing the Article:*** *Orhan Destanoğlu;* ***Critical Review:*** *Orhan Destanoğlu, İsmail Ateş;* ***References and Fundings:*** *İsmail Ateş;* ***Materials:*** *Orhan Destanoğlu.*

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