The usefulness of measuring *n*-butyric acid concentration as a new indicator of blood decomposition in forensic autopsy

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Declarations of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

All data which supports these findings are supplied within the manuscript.

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Abstract

In forensic medicine, although various alcohols have been reported as indicators of decomposition in collected blood, no studies have examined short-chain fatty acids as indicators. In this study, the blood *n*-butyric acid concentration was quantified, and the association between *n*-butyric acid and decomposition was investigated to determine whether the detection of *n*-butyric acid could be a new indicator of decomposition. Among the forensic autopsies performed from 2016 to 2018 in our laboratory, the cases were divided into decomposed (n = 20) and non-decomposed (n = 20) groups based on macroscopic findings. Blood samples collected at the time of autopsy were derivatized with 3-nitrophenylhydrazine hydrochloride after solid-phase extraction. The *n*-butyric acid concentration was measured using liquid chromatography–tandem mass spectrometry. In addition, ethanol and *n*-propanol were measured using a gas chromatography-flame ionization detector. There was a significant difference ($p < 0.01$) in the concentrations of *n*-butyric acid between the decomposed and non-decomposed groups (0.343 ± 0.259 [0.030–0.973] and 0.003 ± 0.002 [0.001–0.007] mg/mL, respectively). In the decomposed group, *n*-butyric acid was detected at high concentrations, even in cases where *n*-propanol was low. These results suggest that *n*-butyric acid is more likely to be an indicator of blood decomposition than *n*-propanol.

Abbreviations: 3-NPH, 3-nitrophenylhydrazine hydrochloride; EDC, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; HS-GC-FID, headspace gas chromatography with flame ionization detection; IS, Internal standard; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PMI, postmortem interval

Keywords: Postmortem decomposition; *n*-Butyric acid; *n*-Propanol; Liquid chromatography–tandem mass spectrometry; Forensic autopsy; Postmortem production

1. Introduction

Compounds, such as *n*-propanol, isopropanol, and ethanol, are produced in the body through postmortem decomposition in the process of anaerobic decomposition by microorganisms and change from organic to inorganic matter [1,2].

When conducting a forensic autopsy, the detection of ethanol in the blood is an important information for forensic diagnosis. However, the presence of ethanol in the blood could be attributed to postmortem decomposition or the consumption of alcoholic beverages while the person was alive. Quantification of *n*-propanol has been considered an important indicator of bodily processes, such as blood decomposition and postmortem ethanol production [3,4]. However, recent reports have shown that, for some decomposed blood of rats, sufficient amounts may not be detected, suggesting that the *n*-propanol level is not always an ideal measure of blood decomposition [5].

Therefore, it is necessary to search for alternative substances that can be used as indicators of decomposition. An animal study tracked quantitative changes in short-chain fatty acids (volatile fatty acids) found in the decomposition fluid, which is formed when the body undergoes postmortem changes (decomposition) [6]. However, this metric cannot be applied to humans, as there are only reports of short-chain fatty acids being found in the soil surrounding corpses observed outside but not in the samples collected directly from the human body, such as blood samples [7].

In forensic medicine, although various alcohols, such as *n*-propanol and *n*-butanol, have been reported as indicators of decomposition in collected blood [3,4,8], no studies have examined short-chain fatty acid content. In animal experiments, *n*-butyric acid has been detected more frequently than other short-chain fatty acids [6, 7]. Moreover, we have also observed high concentrations of *n*-butyric acid in the blood of decomposed forensic autopsy cases when performing routine drug tests. Therefore, in this study, we examined whether postmortem decomposition produces *n*-butyric acid, a short-chain fatty acid, in the blood and whether it can be used as an indicator of blood decomposition.

2. Materials and Methods

2.1. Chemicals

n-Butyric acid was purchased from Tokyo Chemical Industry (Tokyo, Japan). Pyridine, 2-ethylbutyric acid, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), and 3-nitrophenylhydrazine hydrochloride (3-NPH) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Formic acid and all other solvents were of high-performance liquid chromatography (LC) grade or better and were acquired from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

2.2. Forensic samples

Among 1068 forensic autopsies performed from 2016 to 2018 in our laboratory, we selected the cases of individuals without a documented drinking habit and who had not been drinking at the time of death, based on the police investigation.

Cases of severely decomposed bodies with putrefactive discoloration and gas in the whole body were included in the decomposed group; there were 20 cases, in which > 10 mL of blood could be collected from the heart (Table 1). Cases of slightly decomposed bodies with putrefactive changes localized in the abdomen or other parts of the body were excluded.

In the non-decomposed group, there were 298 cases within 3 days of death showing no putrefactive changes, drinking habits, or alcohol in the body. We randomly selected 20 cases to serve as controls for comparison with the decomposed group (Table 1). The causes of death were natural death, trauma, drowning, asphyxia, poisoning, and hypothermia in eight, four, three, two, two, and one case(s), respectively. The seasons and environments in which the cases were found were fall (11 cases) or winter (nine cases) and inside (15 cases) or outside (five cases), respectively.

The causes of death in the decomposed group were natural death, drowning, asphyxia, and poisoning in 13, three, two, and two cases, respectively. The seasons and environments where the cases were observed were spring (four cases), summer (four cases), fall (eight cases), or winter (four cases) and inside (16 cases) or outside (four cases) (individual information is available in Table 2).

The average postmortem intervals (PMIs) were 15.5 (3–60) days in the decomposed group and 1.7 (0.4–3) days in the non-decomposed group (Table 1).

2.3. Sample preparation for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis of n-butyric acid

n-Butyric acid analysis was performed according to the method package commercially available from Shimadzu Corporation (Tokyo, Japan), using the procedure developed by Han et al. [9]. The blood samples were stored at –30°C after collection, thawed at the start of the experiment and, then, treated as follows. First, 1 mL of sample was mixed with 2 mL of 2% formic acid and centrifuged at 13,000 × g at 4°C for 10 min. Next, 2 mL of the supernatant was loaded onto a solid-phase extraction column (EVOLUTE®, 50 mg, Biotage AB, Uppsala, Sweden). The solid-phase extraction column was conditioned by passing 1 mL of methanol followed by 1 mL of 0.1% formic acid aqueous solution. After adsorbing the sample solution, the column was washed with 1 mL of 5% aqueous methanol, and finally, the sample was eluted into a new vial with 2 mL of methanol. Next, reagent solutions, 50 µL of methanol eluate, 50 µL of 2 mg/L ethyl 2-ethylbutyrate ethanol solution (internal standard, IS), 50 µL of 50 mM 3-NPH solution, 50 µL of 50 mM EDC solution, and

50 μL of 7.5% pyridine solution, were prepared using a 75% aqueous methanol solution. Subsequently, samples were vortexed and placed for 30 min in a shaded area at 25°C. A fivefold dilution with 75% aqueous methanol solution containing 0.5% formic acid was analyzed using LC-MS/MS.

2.4. LC–MS/MS

A Dionex Ultimate 3000 LC system coupled with a TSQ Quantum Access Max triple stage quadrupole mass spectrometer with a heated-electrospray ionization probe (Thermo Fisher Scientific, Waltham, MA, USA) was used. The LC column used was a Mastro C18 column (2.1 × 150 mm, 3 μm; Shimadzu GLC Ltd.). The mobile phase comprised varying concentration ratios of 0.1% formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.35 mL/min. The LC was performed at 40°C with the following mobile phase gradient program: 0–6 min, 16%–25% B; 6–9 min, 25%–40% B; 9–17 min, 40%–95% B; and 17–20 min, 95% B. After measuring, the mobile phase was brought back to the initial condition (16% B) and kept for 5 min. Under these conditions, 3-NPH derivatives of *n*-butyric acid and IS were eluted at 10.3 and 12.6 min, respectively.

Mass spectrometry detection was performed by selected reaction monitoring at *m/z* 224 → 138 (*n*-butyric acid 3-NPH derivative) and *m/z* 252 → 138 (IS 3-NPH derivative) in the positive ion mode. The parameters and conditions were as follows: electrospray voltage, 3.0 kV; vaporizer temperature, 400°C; ion transfer tube (capillary) temperature, 235°C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas flow, 15 arbitrary units; tube lens offset voltage, 85 V; and collision gas (argon) pressure, 1.5 mTorr.

Under these conditions, *n*-butyric acid was measured with a detection range of 0.001–1.000 mg/mL

2.5. Analysis of alcohols

Quantitative analysis of ethanol and *n*-propanol in blood was performed using headspace gas chromatography with flame ionization detection (HS-GC-FID). Analytical samples were prepared by mixing 0.5 mL of blood with 0.5 mL pure water containing an internal standard (acetonitrile, 1 g/L). Samples were analyzed using a TRIPLUS300 headspace system coupled with a TRACE1319 gas chromatography system (Thermo Fisher Scientific). The gas chromatography column was a TG-BOND U column (30 m × 0.53 mm × 20.0 μm; Thermo Fisher Scientific). The conditions of HS-GC-FID were as follows: headspace oven, 55°C; needle temperature, 70°C; transfer tube temperature, 70°C; headspace gas pressure, 2.0 bar; carrier gas (helium), 1.5 mL/min, constant flow; gas chromatograph oven, from 80°C to 160°C (12 min) at 10°C/min; injection, 190°C; and detector, flame ionization detection, 250°C (H_2 flow, 35 mL/min; airflow, 350 mL/min; constant column + makeup flow, 20 mL/min). Under these conditions, ethanol and *n*-propanol were measured with detection ranges of 0.001–4.000 mg/mL and 0.001–0.200 mg/mL, respectively.

2.6. Statistical analyses

Student's *t*-test ($p < 0.01$) was performed to compare the measured values for the decomposed and non-decomposed groups. In addition, a simple regression analysis was performed to determine whether there was a correlation between *n*-butyric acid and ethanol and between *n*-butyric acid and PMI. Statistical analyses were performed using JMP (version 11.0.0; SAS Institute Inc., Cary, NC, USA).

2.7. Ethics

These data analyses were performed within the framework of routine medicolegal casework following the autopsy guidelines (2009) and ethics guidelines (2003) of the

Japanese Society of Legal Medicine and approved by our institutional ethics committee (No. 16-015).

3. Results

Table 1 shows various alcohols and *n*-butyric acid measurement results from the non-decomposed and decomposed groups. Ethanol was detected in high concentrations in the decomposed group and in low concentrations in the non-decomposed group. The average concentration ratio between the decomposed and non-decomposed groups was approximately 58:1. The concentration of *n*-propanol was significantly different between the samples from the decomposed and non-decomposed groups ($p < 0.01$). Additionally, the average concentration ratio of *n*-butyric acid between the decomposed and non-decomposed groups was 114:1, which was also significant ($p < 0.01$). The concentrations of blood alcohols and *n*-butyric acid for all cases in the decomposed group are presented in Table 2. The ratio of ethanol to *n*-propanol in the blood of the decomposed group ranged from 4:1 to 244:1 (Table 2), while the ethanol to *n*-butyric acid ratio ranged from 0.3:1 to 15.6:1 (Table 2).

The correlations between *n*-butyric acid and ethanol and between *n*-butyric acid and PMI were analyzed using simple regression analysis. However, the correlation coefficients were 0.03 and –0.04, respectively, indicating no strong correlation.

4. Discussion

When conducting a forensic autopsy, it is necessary to measure the concentrations of certain compounds, such as ethanol and acetone, to determine the cause and manner of death and aid in criminal investigations. In places, such as our facility, reference values (cutoff values), which are ≥ 0.100 mg/mL for ethanol and ≥ 0.010 mg/mL for acetone, isopropanol, and *n*-propanol, are used to aid in reporting abnormal values. When interpreting measurements of these substances, the effects of postmortem changes due to factors, such as autolysis and decomposition, must always be considered. For a cadaver, the femoral or subclavian veins are the optimal sites to collect blood samples because they are far from the organs of the gastrointestinal tract, such as the stomach, and are the least susceptible to the diffusion of ethanol into the blood [8]. However, even if the PMI is short and no decomposition is observed, a sufficient blood sample often cannot be collected from the femoral vein because of the effects of postmortem hypostasis and blood coagulation reactions. Additionally, it is difficult to collect blood from peripheral blood vessels as decomposition progresses; therefore, in this study, we used blood collected from the heart.

Since postmortem ethanol is often produced together with *n*-propanol, the detection of *n*-propanol is used to indicate the postmortem production of ethanol. The ethanol concentration produced by decomposition is < 20 times the *n*-propanol concentration produced after death [3,4,10]. Accordingly, if ethanol is detected at a concentration level > 20 times greater than the detected *n*-propanol concentration levels, there is a high possibility that alcohol was consumed before death. However, according to recent case reports and the results of *in vivo* experiments using rats, there have been cases where the ethanol or *n*-propanol concentration ratio greatly exceeded 20:1, even when ethanol had not been consumed [5]. In addition, in *in vitro* experiments where *Candida albicans* was added to a normal blood sample containing no alcohol before decomposition at room temperature, it was demonstrated that the concentration of ethanol produced during the decomposition process exceeded 20 times that of *n*-propanol and in some samples, *n*-propanol could not be detected [11].

In this study, although police investigations found that none of the deceased were drinking before their death, the ethanol/n-propanol ratio exceeded 20:1 in six cases in the decomposed group (Nos. 4, 5, 6, 8, 13, and 14) (Table 2). Therefore, *n*-propanol was often detected at high concentrations in decomposed cases, but in some cases, the concentration of *n*-propanol was not a good indicator of blood decomposition or postmortem ethanol production.

There was a clear, significant difference in the *n*-butyric acid concentration in the blood between the decomposed and non-decomposed groups. *n*-Butyric acid was detected at ≥ 0.030 mg/mL in blood collected from the decomposed group (Table 2). *n*-Butyric acid was also detected in samples from the non-decomposed group but in significantly lower concentrations. Our results demonstrated that the concentration of *n*-butyric acid was much greater in samples from the decomposed group than in those from the non-decomposed group (Table 1).

n-Butyric acid is hypothesized to be produced by anaerobic bacteria in the human intestine [12]. As blood decomposition progresses after death, these intestinal bacteria transfer from the intestinal tract to the circulatory system, and it is hypothesized that the production of *n*-butyric acid in the blood promotes this process. For all cases, the concentration of *n*-propanol produced after death was lower than that of ethanol produced after death. However, the *n*-butyric acid concentration in six cases (Nos. 1, 3, 6, 9, 10, and 19) in the decomposed group exceeded the ethanol concentration (Table 2). In addition, the *n*-butyric acid concentration was higher than the *n*-propanol concentration in 19 cases.

When the concentration of *n*-propanol is ≤ 0.010 mg/mL, it is possible to conclude that a sample has not been affected by decomposition [5,11]. Cases 6 and 14 (Table 2) of the decomposition group had high ethanol concentrations (0.169 and 0.319 mg/mL, respectively), and low *n*-propanol concentrations (0.004 and 0.005 mg/mL, respectively). Moreover, the ethanol to *n*-propanol ratios of these cases (Nos. 6 and 14) were approximately 42:1 and 64:1, respectively. In contrast, the *n*-butyric acid concentrations in these cases were 0.400 and 0.260 mg/mL, respectively. Furthermore, in Cases 1 and 12, the amount of *n*-propanol slightly exceeded 0.010 mg/mL, but the amount of *n*-butyric acid was significantly higher.

Therefore, it may be possible to prove that the sample was decomposed by measuring the *n*-butyric acid concentration in the blood.

In this study, a high concentration of *n*-butyric acid was detected when blood decomposed. Future studies should increase the number of cases and further investigate parameters, such as when *n*-butyric acid begins to be produced in the blood after death and when the amount of *n*-butyric acid increases significantly. In conclusion, because *n*-butyric acid is consistently detected in decomposed blood, it could more sensitively reflect blood decomposition than *n*-propanol.

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Tables

Table 1. Summary of the examined cases

	Decomposed group	Non-decomposed group
Number of samples	20	20
Male	14	7
Female	6	13
Age	70.9 ± 17.8 (31–92)	57.4 ± 22.9 (4–90)
PMI (days)	15.5 ± 13.3 (3–60)	1.7 ± 0.8 (0.4–3)
Ethanol (mg/mL)	0.757 ± 0.853	0.013 ± 0.007 (0.005–0.028)
n-Propanol (mg/mL)	0.047 ± 0.033	0.0002 ± 0.0004 (0–0.002)
n-Butyric acid (mg/mL)	0.343 ± 0.259	0.003 ± 0.002 (0.001–0.007)

PMI, postmortem interval

Table 2. Ethanol, propanol, and *n*-butyric acid concentrations in blood samples of decomposed group

Case No.	Ethanol (mg/mL)	*n*-Propanol (mg/mL)	Ratio of EtOH/***n***-PrOH	*n*-Butyric acid (mg/mL)	Ratio of EtOH/*n*-Butyric acid	PMI (days)	**Season***	**Environment****
1	0.134	0.011	12.2	0.162	0.8	7	WTR	IN
2	0.895	0.086	10.4	0.133	6.7	30	WTR	IN
3	0.335	0.024	14.0	0.973	0.3	30	SPR	OUT
4	1.082	0.033	32.8	0.184	5.9	6	FAL	IN
5	1.798	0.062	29.0	0.844	2.1	25	WTR	IN
6	0.169	0.004	42.3	0.400	0.4	20	SPR	OUT
7	0.546	0.074	7.4	0.063	8.7	60	SPR	IN
8	1.209	0.051	23.7	0.526	2.3	3	SPR	IN
9	0.395	0.100	4.0	0.574	0.7	10	SMR	OUT
10	0.353	0.052	6.8	0.176	2.0	21	SMR	IN
11	0.925	0.103	9.0	0.367	2.5	10	SMR	IN
12	0.191	0.012	15.9	0.285	0.7	5	FAL	IN
13	3.903	0.016	243.9	0.250	15.6	14	FAL	OUT
14	0.319	0.005	63.8	0.260	1.2	7	WTR	IN
15	0.722	0.060	12.0	0.579	1.2	7	SMR	IN
16	0.792	0.094	8.4	0.102	7.8	14	FAL	IN
17	0.218	0.025	8.7	0.154	1.4	12	FAL	IN
18	0.462	0.060	7.7	0.232	2.0	16	FAL	IN
19	0.276	0.016	17.3	0.568	0.5	3	FAL	IN

20	0.415	0.045	9.2	0.030	13.8	10	FAL	IN

*Abbreviations of seasons: SPR, spring; SMR, summer; FAL, fall; WTR, winter

**Abbreviations of environments: IN, inside; OUT, outside

PMI, postmortem interval