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Free fatty acids as markers of death from hypothermia

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ABSTRACT

The possibilities of using morphological markers of fatal hypothermia are limited; therefore, other diagnostic criteria of deaths from hypothermia are being researched. The initiation of protective mechanisms against adverse effects of low temperatures results in activation of hormonal systems and development of characteristic biochemical changes that can be impaired by alcohol intoxication. The aim of the study was to assess the usefulness of determinations of the profile of free fatty acid concentrations as potential markers of hypothermia-related deaths, particularly in intoxicated victims.

The study group consisted of blood samples collected during autopsies of 23 victims of hypothermia. The control group included blood samples collected from 34 victims of sudden, violent deaths at the scene of an incident (hangings and traffic accidents) and 10 victims who died because of post-traumatic subdural hematomas with prolonged agony. The study and control groups were divided into three subgroups according to blood alcohol concentrations: 0.0–0.99; 1.0–2.99 and ≥3.0‰.

Statistical analysis in the individual subgroups demonstrated significant increases in concentrations of palmitic, stearic and oleic acids ($P < 0.05$), independent of blood ethanol concentration. Palmitic, stearic and oleic acids can be considered the potential markers of fatal hypothermia, including the cases of intoxicated individuals.

1. Introduction

Death from hypothermia may not cause any specific pathomorphological changes [1–7], however diagnosing fatal hypothermia is particularly difficult in cases where individuals are intoxicated as alcohol accelerates cooling and shortens the time of exposure to cold [8–13]. Biochemical markers of hypothermia are likely to significantly enhance the giving of expert opinions in fatal hypothermia cases [14–22].

A decrease in core body temperature stimulates the hypothalamic–pituitary–adrenal axis [20,23,24], which results in increased levels of catecholamines [25–30] and even more elevated levels of glucocorticosteroids [19]. The hormonal stimulation increases the concentration of ketone bodies [12,31–33], mainly of β-hydroxybutyric acid (β-HBA) as the formation of acetone (Act) is inhibited by ethanol [12,14,16,19,33,34].

Moreover, the low body temperature-induced increase in the level of adrenal hormones [18,19] causes increased concentrations of free fatty acids (FFAs) [28,31,32,35–38]. In cases of induced hypothermia in humans Nesbakken et al. have observed an increase in FFA levels by 200% [37] whereas Okada et al. by 113–267% [35].

Alcohol ingestion can increase the NADH/NAD potential, which is likely to inhibit lipolysis [10,33] and reduce serum FFA levels [39]. Hirvonen et al. [40] as well as Huttunen and Hirvonen [10] have demonstrated that the administration of reserpine and ethanol resulted in decreased levels of FFAs and shortened the survival of guinea pigs exposed to low temperatures (−20 °C).

The studies available only evaluated the effects of hypothermia on the total concentration of particular FFAs in humans and animals [28,31,36,37,40–43] without analysing the percentage content of individual acids of varying degrees of saturation and lengths of the hydrocarbon chain. Moreover, no data regarding threshold FFA values as markers of hypothermia that could be used for routine medico-legal practices have been published.

The aim of the present study was to assess the usefulness of determinations of the profile of concentrations of individual FFAs as potential markers of death from hypothermia, especially in cases of concomitant alcohol intoxication of victims.

2. Material and methods

2.1. Study material

The study group ($N = 23$) and control group ($N = 44$) contained the same cases as the ones in the already published study which focused on the use of glucocorticosteroids for the diagnosis of fatal hypothermia [19]. The health of a given victim in the period

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preceding death was verified using the files from the Prosecutor's Office and autopsy findings.

The study group was selected based on the circumstances of death suggesting fatal hypothermia without other macro- and microscopic changes that could account for the cause of death, and when routine toxicology examinations excluded possible intoxications with substances other than ethanol.

The inclusion criteria in the control group were the cases of violent sudden death at the incident scene (due to hanging or injuries sustained in traffic accidents, $N = 34$) and violent slow death ($N = 10$). Subdural hematomas were chosen as a model of violent slow death (long-term agony makes this group similar to cases of fatal hypothermia, which are classified as violent deaths due to the external traumatic factor, i.e. cold).

Blood samples from the femoral vein were stored at −70 °C until analysed collectively at the end of the winter season. The corpses were kept refrigerated for an average of 3 days, (2–5 days) at about +4 °C until the autopsies were performed.

The effect of time elapsing from death to autopsy was neglected, since the available studies did not indicate fatty acids concentration values to undergo any significant changes in the course of postmortem transformations [44]. However the cases showing symptoms of developing putrefying changes were excluded.

In all the cases, the concentration of glycated haemoglobin (HbA1c, index of long-term control of diabetes) did not exceed that of a healthy individual, i.e. about 6–7%, which indicated normal average glycemia in the period of 120 days preceding death [45,46]. The study and control groups were subdivided into three subgroups according to the blood alcohol concentration (BAC): 0.0–0.99; 1.0–2.99 and ≥ 3.0‰.

3. Analytical methods

BAC was determined using headspace gas chromatography with a flame ionisation detector (GC–FID) [47]. To identify and determine 19 fatty acids during a one-step analytical procedure, gas chromatography coupled to mass spectrometry (GC–MS) with a negative chemical ionisation was used, which was designed and validated for the purposes of the present study. The Trace GC Ultra (Thermo Finningan, USA) device coupled to a quadrupole mass spectrometer and TriPlus Autosampler (Thermo Finningan, USA) were used. GC–FID and GC–MS were operated under X'Calibur software. All 19 FFAs from the blood samples were isolated by solid phase extraction (SPE) using the GX-274 ASPEC (Gilson, USA) automated system. The Merck RP–C_{18} (Merck, Germany) extraction columns were applied. Pelargonic and margaric acids (10 μg/ml) and 5 ml of deionised water were added to 0.5 ml of blood, ultrasonicated for 15 min and centrifuged at 5000 RPM. SPE columns were conditioned with 1 ml of methanol plus 2 ml of deionised water and 4 ml of blood supernatant was injected onto the column. The columns were washed with 1 ml of acetic acid followed by 2 ml of deionised water and dried with nitrogen for 5 min. FFAs were eluted using 2 ml of acetone. Eluates were evaporated with the nitrogen stream at 50 °C in the RapidVap concentrator (Labconco, USA). The dry residue was derivatised using 50 μl of 10% of pentafluorobenzyl bromide in acetonitrile (15 min at room temperature) with the addition of 10 μl of triethylamine as a catalyst. The solvent was evaporated, the dry residue dissolved in 50 μl of ethyl acetate and placed in the Autosampler vials (2 ml). The use of negative chemical ionization (NCI) enabled to decrease fragmentation and form stable ions derived from carboxylate anions $[R^n–COO]^-$. A quantifier ion and a qualifier ion were selected for each of the 19 fatty acids and two internal standards.

4. Statistical procedures

The study results were statistically analysed using Statistica® 6.0 and non-parametric tests. The significance of inter-group differences in FFA concentrations was assessed using the tests of independent variables: Mann–Whitney U, Wald–Wolfowitz and Kolmogorov–Smirnov whereas the intra-group differences in FFA concentrations depending on BAC were evaluated with the ANOVA Kruskal–Wallis and median tests. $P < 0.05$ was considered as statistically significant.

5. Results

Tables 1 and 2 present the concentrations of individual saturated and unsaturated FFAs in the control and study groups with the BAC taken into account. The mean concentration of saturated and unsaturated FFAs in hypothermic individuals compared to controls increased by 425%; by 487% for saturated and 413% for unsaturated acids. Considering the above, the effect of hypothermia on the increase in total FFA concentration is obvious, however the involvement of individual acids in this increase markedly varies (Tables 3 and 4).

Amongst the hypothermia victims, elevated median concentrations of six saturated acids, i.e. caprylic, capric, lauric, myristic, palmitic and stearic ones, were observed at each BAC range (0.0–0.99; 1.0–2.99 and ≥ 3.0 g/l). Otherwise, the mean concentrations of arachidic, behenic and lignoceric acids were found to be slightly reduced. However, these three acids together constitute only about 0.4% (study group) and about 3.4% (control) of the total pool of saturated FFAs, thus their effect on total lipidaemia was irrelevant. The highest percentages in the pool mentioned regarded two acids, i.e. palmitic (over 53% in the control and about 42% in the study group) and stearic (about 29% and over 34%, respectively). Moreover, increased concentrations of both these acids in hypothermia victims were statistically significant, irrespective of the blood alcohol concentration. Similar results were obtained for caprylic acid, which constituted about 5–7% of FFA pool (the third in terms of concentration amongst saturated acids). The increases in concentrations of the remaining saturated acids were statistically significant only at BAC below 1.0 g/l; for capric acid also at BAC 1.0–3.0 g/l.

Amongst unsaturated FFAs, the concentration of nervonic acid (with the longest carbon chain, C24) and of erucic acid (C22) did not change significantly due to hypothermia in all ranges of ethanol concentration (from 0.0 to >3.0 g/l). The concentrations of arachidonic and cervonic acids (C20 and C22) significantly increased only at BAC ≤ 1.0 g/l. The concentrations at BAC > 1.0 g/l did not alter or were non-significantly reduced (or at the border of significance). In the pool of unsaturated FFAs, erucic and nervonic acids together constitute only about 0.007–0.07% (Table 4) and similarly to saturated FFAs of the longest carbon chains, i.e. arachidic, behenic and lignoceric acids (C20, C22 and C24) did not significantly affect the total lipidaemia. The mean concentrations of the remaining unsaturated FFAs (palmitoleic, petroselinic, oleic, elaidic, linoleic and linolenic) showed a statistically significant increase in the study group (i.e. amongst hypothermic individuals) at BAC 0.0–3.0 g/l, whereas at BAC > 3.0 g/l a statistically significant increase was observed only for petroselinic and oleic acids. However, only 4 acids were most highly involved in the generation of the total increase, i.e. oleic (constituting about 26–38% of the pool of unsaturated FFAs in the study group and about 25% in the control group), linolenic (about 21–24% and about 22–25%), palmitoleic (about 20–26% and about 15–21%) and arachidonic acid (about 15% and about 16%, respectively), yet only at BAC 0.0–1.0 g/l. In the study group, the content of arachidonic acid in the pool of unsaturated FFAs