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CASE REPORT

In vitro formation of ethanol in autopsy samples containing fluoride ions

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Abstract We present a case of a death of a diabetic man where the concentration of ethanol in post-mortem blood rose from 0.4 g/l 2 days after autopsy to 3.5 g/l 10 days after autopsy. The presence of fluoride ions in this blood sample was determined with ion chromatography and verified that fluoride ions were added to the vials. The concentrations of free fluoride, corresponding to 0.21 and 0.25% *w/v* potassium fluoride in blood and urine, respectively, were somewhat lower than the recommended 1% *w/v*. However, the amount of fluoride ions bound to calcium, proteins and other compounds in the samples is unknown. The blood sample was also subject to microbiological examination, which revealed growth of bacteria. In addition, a very high concentration of glucose was found in vitreous humour from the deceased. To determine whether the ethanol detected at the first analysis was of ante-mortem origin, ethyl glucuronide was analysed. Its absence, in the blood as well as the urine sample, strongly supported the theory that, in this case, all the ethanol detected was formed post-mortem. This case showed that ethanol may be formed in vitro at a very high concentration, despite the verified presence of fluoride ions. Possible reasons for this unusual formation of ethanol were the abundant presence of bacteria, a high level of glucose and, possibly, an insufficient amount of fluoride added to the vials.

Keywords Ethanol formation · Autopsy · Flouride Ions

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Introduction

Post-mortem formation of ethanol is a frequent problem in forensic toxicology [1–9], and variables like putrefaction [6, 9], sugar availability in the actual medium [10] and trauma [4, 11] may influence the magnitude of ethanol synthesis. After sample collection, the possibility of in vitro ethanol formation is lowered if the samples are handled according to recommended procedures [1]. Numerous studies have shown that formation of ethanol is inhibited or abolished by addition of a preservative like fluoride ions [1, 2, 8, 10, 12–14]. To the authors' knowledge, there is only one published experiment where ethanol was formed in vitro despite the presence of sodium fluoride 1% *w/v*. In that study, the fluoride inhibited ethanol formation in blood by the microorganisms *Proteus vulgaris* and *α-streptococci*, but not with *Candida albicans* [15]. Addition of potassium fluoride to a final concentration of 1% *w/v* as well as storage at 4°C, which also inhibits synthesis of ethanol [2], are the standard routines at the Norwegian Institute of Public Health (NIPH).

If there is uncertainty whether all the ethanol detected is formed post-mortem or if some alcohol was ingested before death, the sample might also be analysed for the non-oxidative ethanol metabolite ethyl glucuronide (EtG). Presence of EtG will indicate that ethanol was ingested before death [16, 17].

We present a case where an unusually high level of ethanol was formed in post-mortem blood in vitro, despite addition of fluoride and where EtG was used to determine if ingestion of ethanol had occurred ante-mortem.

Case report

A 40 year-old man was found dead in an apartment frequented by drug and alcohol addicts. At autopsy, which was performed 1 day after assumed time of death, no injuries or diabetic ulcers were found on external inspection. Investigation of the internal organs, however, revealed extensive bronchopneumonia, as well as diabetic vascular changes in the heart and kidneys. Investigation of vitreous humour revealed a very high level of glucose (996 mg/dl). Peripheral blood and urine, 20 ml of each, were received at the NIPH the same day as the autopsy in 20-ml Sterilin vials (Bibby Sterilin, UK). An 11.5 M potassium fluoride solution was prepared in-house, and 300 μl of this solution was added to all Sterilin vials before they were sent to the pathologists. After arrival at the NIPH laboratory, the samples were stored at 4°C when not transferred to room temperature in relation to analyses.

The concentrations of ethanol and EtG, analysed according to previously published methods [16, 18–20], are shown in Table 1. An enzymatic alcohol dehydrogenase method was used for screening of ethanol in urine. Two headspace gas chromatography flame ionisation detection (HS-GC-FID) methods using two different capillary columns and two different internal standards (*n*-propanol and *tert*-butanol) were used for quantification of ethanol in blood and urine. The two HS-GC-FID blood replicates obtained by the two methods were always analysed on different days. The first determination of ethanol in blood revealed a low level (0.4 g/l). This value had increased markedly 5 days later, and 10 days after autopsy, the level was 3.5 g/l. In urine, the ethanol concentration rose from 0, determined by the enzymatic screening method, to 0.6 g/l, determined by the HS-GC-FID methods 10 days after autopsy. No EtG was detected in blood or urine when liquid chromatography-mass spectrometry analysis was performed 30 days after autopsy. *n*-Propanol, a common putrefactive product, was not present in blood or urine.

Table 1 Concentrations of ethanol and EtG in blood and urine during 30 days of storage

Days after autopsy[1]	Ethanol blood (g/l)	Ethanol urine (g/l)	EtG blood (mg/l)	EtG urine (mg/l)
1	–	0	–	–
2	0.4	–	–	–
7	2.1	–	–	–
10	3.5	0.6[2]	–	–
30	–	–	0	0

–Analysis not performed

[1] Authopsy/sample collection was performed 1 day after assumed time of death.

[2] Mean of two replicates (0.4 and 0.8 g/l)

The blood sample was also subjected to analyses of illegal drugs and 64 medicinal drugs [21], which revealed a high concentration of methadone (0.96 mg/l) as well as low concentrations of methamphetamine, tetrahydrocannabinol and the benzodiazepines diazepam/*N*-desmethyldiazepam and flunitrazepam.

Microbiological analysis of blood [22] performed 8 days after autopsy revealed growth of the facultative bacteria *Escherichia coli* as well as the anaerobic bacteria *Clostridium*, *Bacteroides* and *Prevotella* species. As the first analysis of the urine sample gave no indication of post-mortem formation of ethanol, this sample was not sent to microbiological analysis.

Analysis of free fluoride ions in blood and urine was performed at SINTEF (Trondheim, Norway) with a Dow Ion Exchange (DIONEX) ion chromatograph (Sunnyvale, CA, USA) consisting of a IP25 pump and a CD20 conductivity detector coupled with a ASRS conductivity suppressor. The sample was diluted 1:50 with distilled water, filtrated using a 0.22-μm filter before 20 μl was injected on a IonPac AS9-HC column (4×250 mm, 9 μm) that was protected by a AG9-HC guard column (4×50 mm, 9 μm) from DIONEX. The mobile phase consisted of 19 mM sodium carbonate and a 1 ml/min flow rate was used. For quantification, the method of standard additions of sodium fluoride to the diluted sample was performed for the calibration range of 0.001–0.3 mg/ml (0.05–15 mg/ml corrected for dilution factor). The free fluoride concentrations in blood and urine were determined by extrapolation. These analyses verified the presence of fluoride ions. The concentration of free fluoride ions was 0.675 mg/ml in blood, corresponding to 2.1 mg/ml potassium fluoride (0.21% *w*/*v*). In urine, the concentration of free fluoride ions was 0.812 mg/ml, corresponding to 2.5 mg/ml potassium fluoride (0.25% *w*/*v*).

Discussion

In this case report, we have documented in vitro formation of ethanol reaching a concentration of 3.5 g/l in a post-mortem blood sample, despite the verified presence of fluoride ions. Such a finding was unexpected and not consistent with the numerous previous publications indicating that presence of fluoride ions inhibits the in vitro formation of ethanol [1, 2]. We have also used the non-oxidative ethanol metabolite EtG to substantiate that no alcohol was ingested ante-mortem [16].

The cause of death, in this case, as stated by the pathologist, was bronchopneumonia in combination with methadone intoxication. The deceased suffered from diabetes, and the finding of an extremely high level of glucose in vitreous humour post-mortem probably reflects a high level of glucose in blood at the time of death, although a direct relationship between vitreous humour and blood glucose is not documented [23].

The microbiological analysis showed the presence of four different bacteria. Growth of *E. coli* from the blood may reflect a state of bacteraemia before death, for instance, originating from a urinary tract infection, but is more likely to represent contamination from the normal flora in the gastrointestinal tract. The anaerobic bacteria present in blood most likely represented post-mortem contamination, as they rarely cause septicaemia in living subjects, and the case history also gave no suspicion of anaerobe septicaemia. *C. albicans*, which formed ethanol in the presence of fluoride in a previous study [15], was not detected in this blood sample.

The presence of bacteria and glucose probably made the conditions for in vitro formation of ethanol optimal [3, 10, 24], but the presence of fluoride as well as storage at 4°C should still be expected to inhibit formation of ethanol. The detected concentrations of free fluoride ions corresponding to 0.21% and 0.25% *w/v* potassium fluoride in blood and urine, respectively, were somewhat lower than the recommended 1% *w/v*. We cannot exclude that the vials, in this case, contained a smaller amount of fluoride. On the other hand, the ion chromatograph only detected free and not bound fluoride ions. A falsely low concentration of fluoride ions was therefore expected. The concentration of calcium usually present in these fluids is not able to fully explain the lowered level of fluoride, but it could not be excluded that drugs or other molecules present in post-mortem blood and urine could bind the actual amount of fluoride.

The very first analysis of ethanol in blood, which was performed only 2 days after autopsy, showed an ethanol concentration of 0.4 g/l. It was uncertain whether this was also formed post-mortem or originated from an ante-mortem ingestion of ethanol, although the negative ethanol in urine supported the theory of post-mortem formation. Previous studies have indicated that the ethanol metabolite EtG could distinguish ingested ethanol from that formed post-mortem [16, 17]. Instability of EtG, especially in the presence of bacteria, might lead to false negative results [25, 26], but this instability is also prevented by fluoride ions (own data, unpublished results). As the levels of EtG are much higher in urine than in blood [27, 28], instability is less likely to lead to a negative EtG result in urine, and therefore, a negative EtG in both blood and urine is more reliable. According to this, the ethanol detected, in this case, was probably only formed post-mortem, starting maybe before sample collection, but with the majority of the formation occurring in the laboratory, after addition of fluoride.

In this case, the first analysis of ethanol was made early enough to reveal the low level of ethanol. If there had been a few days delay before the first analysis was made, an incorrect conclusion of a high level of ethanol at the time of death could have been made, and the deceased even might have been thought to have died from alcohol intoxication. This would also have been in accordance with the case history. On the other hand, this mistake would also have been avoided by the analysis of EtG.

There was a smaller rise in ethanol values in urine than in blood. The amount of fluoride was nearly the same, but other conditions, like glucose and bacteria, were probably different in blood and urine. One additional possible explanation for the less formation in urine could be that the urine sample was stored almost the whole time period at 4°C. On the contrary to this, due to the more numerous analyses performed in blood, this sample was stored at room temperature for a few hours during pipetting, and hence, more favourable conditions for ethanol synthesis were present.

This case was detected due to a combination of several factors. Firstly, the ethanol determinations at the NIPH are carried out at different days with strict requirements for the deviation between ethanol results. Secondly, the difference between results in two different matrices and, thirdly, the possibility to measure EtG in both blood and urine did lead to the disclosure of this in vitro formation of ethanol.

We have no perfect explanation for this unusually pronounced ethanol formation in presence of fluoride. Our suggestions are the combination of a high level of glucose, abundant presence of bacteria and possibly an insufficient amount of fluoride.

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