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## Stability of ethyl glucuronide in urine, post-mortem tissue and blood samples

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**Abstract** The stability of ethyl glucuronide (EtG) under conditions of degradation was examined in urine samples of nine volunteers and in post-mortem tissue (liver, skeletal muscle) and blood taken from seven corpses at autopsies. Analysis was performed via LC-MS/MS. EtG concentrations in urine samples ranged from 2.5 to 296.5 mg/l. When stored at 4°C in airtight test tubes, EtG concentrations remained relatively constant; when stored at room temperature (RT) for 5 weeks in ventilated vials, variations of EtG concentrations ranged from a 30% decrease to an 80% increase, with an average of 37.5% increase. Liver and skeletal muscle tissue of three corpses with positive blood alcohol concentrations (BAC; ranging from 0.106 to 0.183 g%) were stored for 4 weeks and analysed periodically. EtG concentrations decreased 27.7% on average in 4 weeks storage at RT but EtG was still detectable in all samples with initial EtG concentrations higher than 1 µg/g. Blood and liver samples of four corpses with negative BACs were stored at RT after addition of 0.1 g% ethanol, and no new formation of EtG was observed.

**Keywords** Ethyl glucuronide · Stability · Post-mortem analysis · Alcohol consumption marker · Urine

### Introduction

Ethyl glucuronide (EtG) has been shown to be a useful marker in post-mortem tissue and body liquids for alcohol consumed up to several hours before death [1–3]. As anal-

ysis material in forensic toxicology can be of poor quality, i.e. due to very long storage time or putrefaction [4, 5], the interpretation of post-mortem EtG concentrations requires the knowledge of its stability and its possible new formation. Post-mortem changes to human tissue are known in forensic toxicology [6]. Drugs such as nitrobenzodiazepines [7], cocaine and cocaethylene [8], morphine and its glucuronide metabolites [9] have been reported to decompose or to be biologically degraded in post-mortem analysis material. Ethanol is known to be formed post-mortem [10, 11], and also for other substances, e.g. for gamma-hydroxybutyric acid (GHB) [12], the possibility of a post-mortem formation by bacterial enzyme activity has been observed. Other substances such as methamphetamine, amphetamine [13, 14] and diazepam [15] could still be detected in putrefied tissues after a long storage time.

The stability of EtG has only been proven for a storage time of 4 days in urine samples (change of EtG concentrations was less than 10%). In the same study, no new formation of EtG was observed in EtG-negative urine sample stored for 14 days at 4°C [16].

Our aim was to investigate the stability of EtG for a longer period under conditions of degradation. Therefore urinary samples of volunteers who had consumed different amounts of alcohol during the previous evening were stored up to 5 weeks at room temperature (RT; 22–27°C) and at 4°C; EtG and ethanol concentrations were measured periodically. To investigate the stability of EtG in post-mortem samples, tissue samples of liver, skeletal muscle and blood samples from autopsies with positive and negative EtG concentrations have been stored for up to 4 weeks under conditions of degradation and EtG concentrations were determined periodically.

### Materials and methods

#### Urine samples

About 100 ml of midstream urine was collected from two female and seven male volunteers who described them-

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selves as social drinkers [Alcohol Use Disorders Identification Test (AUDIT) score: 1–7 for men and 1–5 for women]. Volunteers I, II, III, V, VI and IX had consumed between 20 and 50 g of ethanol in the form of beer and wine, while volunteers VII and VIII had consumed a significantly higher amount of alcohol. Volunteer IV had not drunk any alcohol for at least 3 days. Urine samples were collected in the morning. The study has been approved by the ethics committee of the University Hospital Freiburg.

Urine samples were divided into aliquots of 2–3 ml and decanted into PE test tubes. One part of the samples was stored at 4°C, covered with tight plastic screw caps to create realistic analytical storage conditions as in a laboratory (no evaporation could occur). The other part of the samples was covered with a plastic film (Parafilm) with a small hole for ventilation to allow oxidative processes of degradation as well as evaporation of the liquid components; these samples were stored at room temperature (RT, 22–27°C). EtG concentrations and urine alcohol concentrations (UAC) were measured at different time intervals; the creatinine concentrations were measured once at  $t_0$  (see Table 1).

#### Post-mortem samples

All post-mortem samples of liver, skeletal muscle tissue (iliopsoas muscle) and blood (femoral blood) were collected from autopsies. Each sample was divided into aliquots of about 1 g and put into PE test tubes which were covered with loose screw caps that allowed the passage of air. All samples were stored at RT (22–27°C) in a laboratory hood. Two different experimental designs were performed.

To determine the stability of EtG in degraded post-mortem tissues, liver and muscle tissue samples from corpses with positive blood alcohol concentrations (BAC) were taken (for a list of samples see Table 2). EtG concentrations were quantified at the beginning and periodically during 4 weeks of storage.

To investigate a possible post-mortem formation of EtG, liver and blood samples of corpses without indications for

**Table 1** Time schedule for analysis of EtG, creatinine and alcohol concentration in nine urine samples after storage at given temperatures

Storage (weeks)	RT; creatinine	4°C; EtG	RT; EtG	4°C; UAC	RT; UAC
0	×	×	×	×	×
1		×	×	×	×
2			×		×
3		×	×	×	×
4			×		×
5		×	×	×	×

UAC Urine alcohol concentration, RT room temperature, × analysis was conducted at this point, EtG ethyl glucuronide

**Table 2** Post-mortem tissue samples for stability assay

Case no.	BAC (g%)	Tissue
1	0.183	Liver Muscle
2	0.106 (Alcohol formed by putrefaction?)	Liver
3	0.134	Liver Muscle

BAC Blood alcohol concentration (g%)

recent alcohol consumption and with negative BACs were stored and analysed (for case histories, see Table 3). On the day of the autopsy, each liver sample was divided into six aliquots and each blood sample into five aliquots. Two of the liver aliquots and one of the blood aliquots were topped up with an equivalent weight of deionised water, to the other four aliquots an equivalent weight of 0.2 g% ethyl alcohol was added, so that the whole sample had an alcohol concentration of 0.1 g%. Samples were periodically analysed for EtG during 4 weeks (for time schedule, see Table 4).

#### EtG extraction and quantification

Liquid samples (urine and post-mortem blood) were prepared according to a previously described method [17], and 10 µl of pentadeuterated internal standard (5 µg/ml solution in water) was added to 100 µl of the respective body fluid and mixed with 250 µl of methanol. The sample was centrifuged; 250 µl of the supernatant was freeze-dried in a vacuum centrifuge (Alpha RVC, Christ, Osterode, Germany) for 45 min, operated at 50°C and 1 mbar vacuum. The freeze-dried residue was redissolved in 140 µl of 0.1% formic acid.

For post-mortem tissue samples (liver and muscle) an extensive clean-up method was used. After the storage episode, to each aliquot (1 g of the fresh tissue at  $t_0$ ) 5 µg pentadeuterated EtG (0.1 mg/ml) and one weight equivalent of deionised water were added and the tissue was homogenised (Ultra-turrax homogeniser, Ika, Staufen, Germany). The homogenate was treated for 1.5 h in an ultrasonic bath (Sonorex Super RK 103H, Bandelin, Berlin, Germany).

After centrifugation at 4,000 rpm (relative centrifugal force, RCF of 6,240×g) for 10 min the liquid phase was removed and pressed through a syringe filter (Chromafil P-45/25 PVDF, 0.45-µm pore diameter, Macherey-Nagel, Düren, Germany). After precipitation with 1 ml of methanol and centrifugation for 10 min, the liquid phase was freeze-dried in a vacuum centrifuge.

The freeze-dried residue was redissolved in acetonitrile/hydrochloric acid solution (acetonitrile/0.1 M HCl, 70:30, v/v) and a solid phase extraction was performed with an aminopropyl-extraction column (Isolute-NH<sub>2</sub>, 3 ml/500 mg, Separtis, Grenzach-Wyhlen, Germany). The column was rinsed with 2.5 ml of methanol, 2.5 ml of water and

**Table 3** Case histories of the deceased where liver/blood samples were investigated for the presence of EtG

Case no.	Age, sex	Cause of death	Indications for alcohol abuse	BAC	Analysed tissue
4	Newborn, male	Congenitive cardiovascular heart malformation	Mother during pregnancy “extreme rarely” glass of champagne	neg.	Liver
5	53 years, male	Myocardial inflammation, dilatation of the left ventricle	No indications for heavy consumption	neg.	Liver, blood
6	41 years, male	Death by burning, thermal shock	No indications for heavy consumption	neg.	Liver, blood
7	32 years, female	Suicide. Bleeding to death after cut in right femoral artery	No indications for heavy consumption	neg.	Liver

BAC Blood alcohol concentration [g%], *neg.* negative

2.5 ml of acetonitrile/hydrochloric acid solution. The re-dissolved sample was applied onto the column and washed with 2 ml *n*-hexane. Finally, the column was eluted with 1.8 ml of aqueous ammonia solution (1%). The eluate was dried in the vacuum centrifuge and redissolved in 1 ml of 0.1% formic acid and vortex-mixed. Aliquots of 10 µl were injected into the LC-MS/MS system (Sciex/Applied Biosystems, Darmstadt, Germany) with negative electrospray ionisation (turbo ion spray) using a Synergi Polar RP column (250×2 mm, 4 µm; Phenomenex, Aschaffenburg, Germany) as previously reported [17]. Elution was performed with 0.1% formic acid with post-column addition of acetonitrile, and a short gradient to 90% acetonitrile was run to clean the column after each analysis of post-mortem tissue samples. The Limit of detection (LOD) and the Limit of quantitation (LOQ) of the used method were 0.052 and 0.152 µg/ml, respectively [17].

Although pentadeuterated EtG was added to the samples before extraction, and would make up for any losses or degradation of EtG during the clean-up procedure, the stability of EtG to ultrasonic treatment—which had to be used for the extraction steps—was investigated. Three EtG positive blood samples and three spiked aqueous solutions were analysed before and after ultrasonication with the same conditions as used for the extraction procedure, described above. Concentrations of samples with ultrasonic treatment were 8% smaller than the respective concentra-

tions of samples without treatment. We therefore conclude that EtG is stable in 1.5 h ultrasonic treatment.

## Results and discussion

### Urine samples

Stability in a biological fluid is a function of the storage conditions, the chemical properties of the substance, the matrix and the storage system. Therefore the stability of this analyte is only valid in the tested matrix with the tested storage system and should not be extrapolated to other conditions [18].

EtG concentrations of nine urine samples stored at room temperature were compared with urine samples stored at 4°C. Positive absolute EtG concentrations ranged from 2.5 to 296.5 mg/ml, creatinine concentrations ranged from 43.6 to 216.1 mg/dl and the highest measured UAC was 0.15 g%.

Normalisation of urinary EtG concentrations to a 100-mg/dl creatinine concentration was useful in an earlier work to compensate for urine dilution [19–21] and is commonly used for EtG concentrations in urine. In this study, the creatinine concentrations of every fresh urine sample at the beginning of the storage experiment ( $t_0$ ) were measured. In the following, every EtG value of the stored specimens were divided by the creatinine concentration of

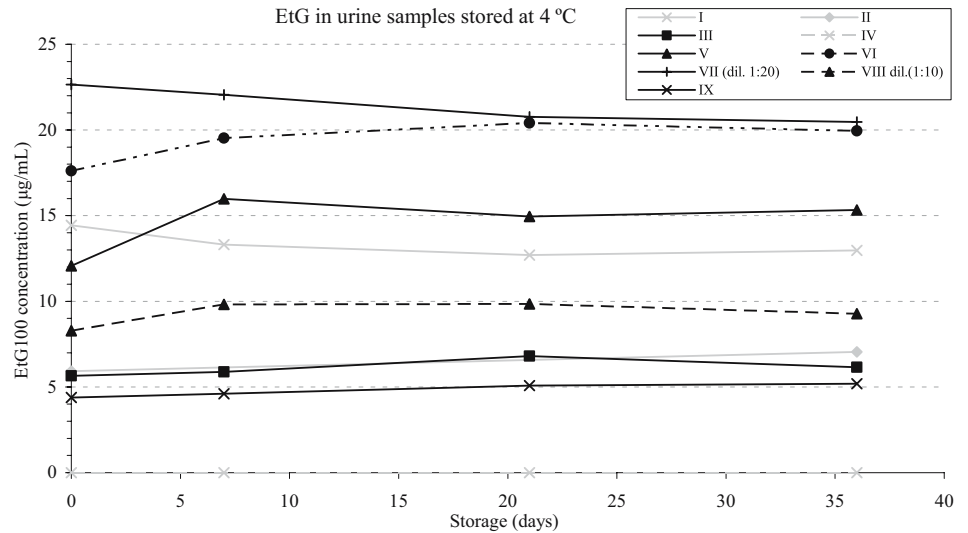
**Table 4** EtG concentrations of postmortem liver and blood samples stored at RT

Tissue	Aliquot	Ethanol concentration (g%)	Storage (weeks)	Case 4	Case 5	Case 6 (µg/g)	Case 7
Blood	1	0.0	–	<sup>a</sup>	neg.	neg.	<sup>a</sup>
	2	0.1	–	<sup>a</sup>	neg.	neg.	<sup>a</sup>
	3	0.1	1	<sup>a</sup>	neg.	neg.	<sup>a</sup>
	4	0.1	2	<sup>a</sup>	neg.	neg.	<sup>a</sup>
	5	0.1	3	<sup>a</sup>	neg.	neg.	<sup>a</sup>
Liver	1	0.0	–	neg.	neg.	0.6	neg.
	2	0.1	–	neg.	neg.	0.5	neg.
	3	0.1	1	neg.	neg.	0.4	neg.
	4	0.1	2	neg.	neg.	0.5	neg.
	5	0.1	3	neg.	neg.	0.9	neg.
	6	0.0	4	neg.	neg.	<sup>a</sup>	neg.

*neg.* Negative, *RT* room temperature, – samples were immediately analysed without storage

<sup>a</sup>No material available

**Fig. 1** Changes in urine ethyl glucuronide (EtG) concentration stored in airtight plastic tubes at 4°C over 5 weeks. *Dil.* Diluted before processing. EtG concentrations of the undiluted samples at  $t_0$  before normalising to 100 mg/dl creatinine concentration were as follows: I, 20.8 µg/ml; II, 3.6 µg/ml; III, 9 µg/ml; IV, 0 µg/ml; V, 8.8 µg/ml; VI, 38.1 µg/ml; VII, 208.4 µg/ml; VIII, 36.14 µg/ml; IX, 4.39 µg/ml



the respective sample at  $t_0$  and then multiplied by 100. Results are given as EtG100 in all figures.

Airtight test tubes with urine samples stored at 4°C showed small variations of the EtG concentrations, alterations compared to the EtG concentration at  $t_0$  ranged from -12 to +32%, with an average value of +9.2%. The total increase after 5 weeks was 9.8% on average. Under these conditions (which had been chosen to imitate pre-analytical storage conditions in a laboratory), EtG can be regarded as stable (see Fig. 1).

In ventilated samples stored at RT (22–27°C), larger changes in EtG concentrations were observed, ranging from -30 to +83% (average of +22.5%; see Fig. 2). In the aliquots that had been stored for 5 weeks, EtG concentrations increased by +37.5% in average compared to EtG determined at  $t_0$ . One reason for this increase is the evaporation of water, because test tubes were not airtight to allow oxidative degradation processes; however, this rate was not constant for each sample and has not been determined in detail. The decrease of -30% found in only

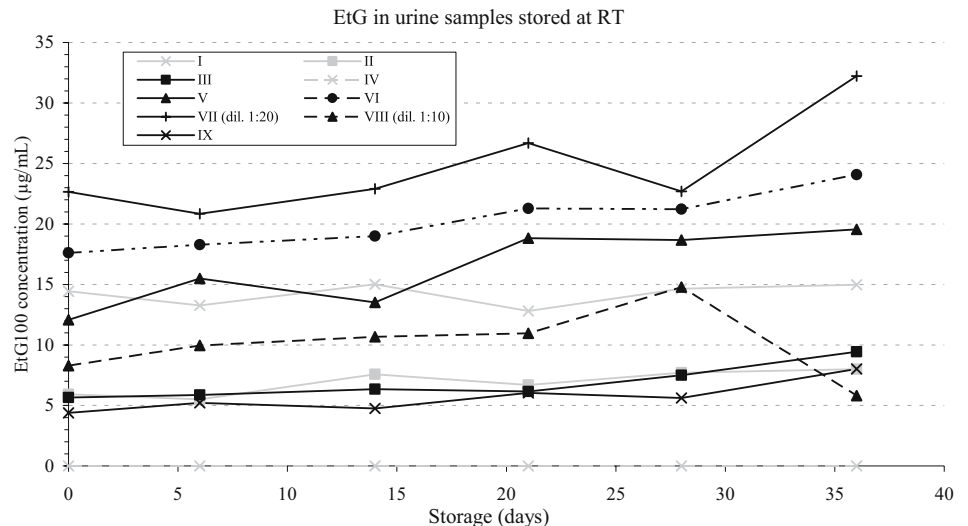
one sample (sample VIII after 36 days; see Fig. 2) is not representative and there is no obvious explanation for this decrease.

UACs of ventilated urine samples stored at RT showed a total loss of ethanol after 5 weeks of storage (see Fig. 3). UAC in closed samples stored at 4°C remained relatively constant during the whole test period.

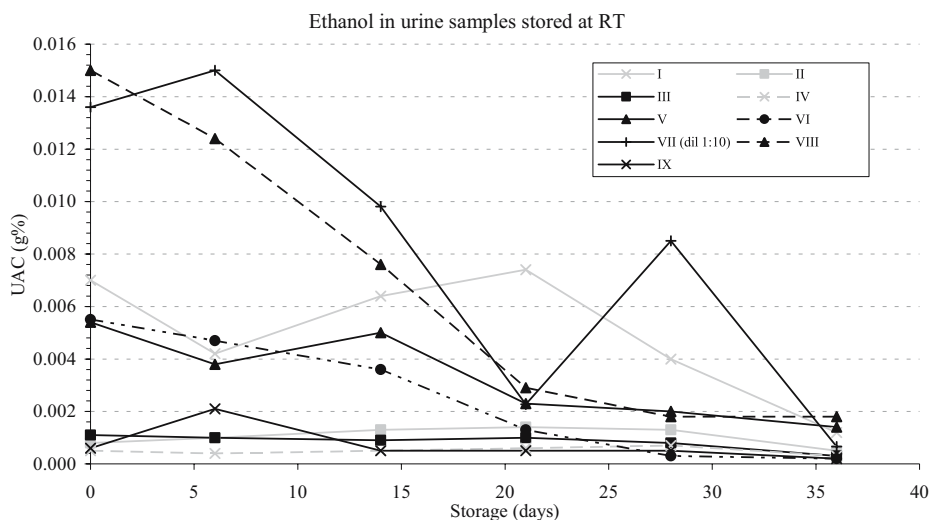
The EtG and BAC negative control sample (sample IV) remained negative for both parameters for the whole observation period under both sets of storing conditions.

In urine samples, EtG was a stable marker when stored at 4°C for 5 weeks. When stored at RT in ventilated vials, a significant increase in EtG concentration was observed due to evaporation of water, whereas ethanol had totally disappeared. Despite the alterations of the measured concentrations, there was no evidence of decomposition of the analyte EtG. Qualitative information about EtG concentrations (positive/negative) was still obtainable for all samples after 5 weeks of storage.

**Fig. 2** Changes in urine EtG concentration stored in ventilated plastic tubes at room temperature (RT; 22–27°C) over 5 weeks. *Dil.* Diluted before processing. For EtG concentrations at  $t_0$  before normalising to 100 mg/dl creatinine concentration, see legend of Fig. 1



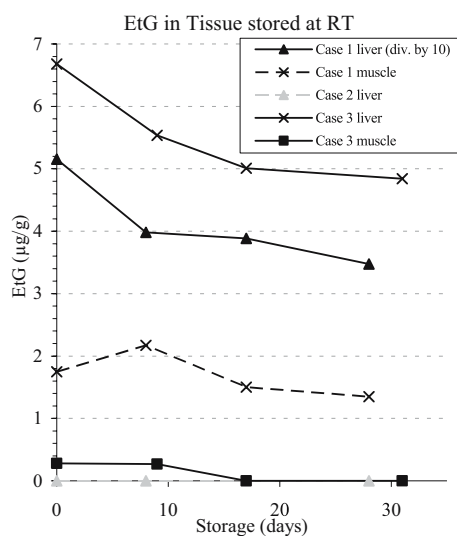
**Fig. 3** Changes in urine ethanol concentration stored in ventilated plastic tubes at room temperature (*RT*; 22–27°C) over 5 weeks. *Dil.* Diluted before processing



### Post-mortem samples

One aim of the post-mortem assay was to examine the stability of EtG in tissue samples stored at room temperature. Therefore liver and skeletal muscle tissue were taken from three corpses for analysis. BACs of the victims ranged from 0.106 to 0.183 g% (see Table 2).

The highest measured EtG concentration at  $t_0$  was 51.53  $\mu\text{g/g}$  (liver sample 1). All positive samples at  $t_0$  showed a decrease in EtG concentration during the assay; after 4 weeks the average decrease was  $-27.7\%$ . In muscle sample 3, which only had a very small EtG concentration of 0.28  $\mu\text{g/g}$  at  $t_0$ , no sign of EtG was further detected after 3 and 4 weeks of storage. The decrease in EtG concentration in the samples could have resulted from the degradation of the analyte. In liver sample 2 with a BAC of 1.06 g%, no EtG was observed during the whole observation period.



**Fig. 4** Changes in EtG concentration in tissue samples stored in ventilated plastic tubes at room temperature (*RT*; 22–27°C) over 30 days. Values of case 1 liver were divided by 10 for better presentation. *Div.* divided

This corpse was heavily putrefied after it had been lying for about 6 months in the victim's apartment. It remains unclear if the ethanol had been generated post-mortem or if EtG had degraded after that prolonged storage time.

Despite the decrease in EtG concentrations, all samples with EtG higher than 1  $\mu\text{g/g}$  at  $t_0$  still had detectable EtG concentrations after 4 weeks. In an earlier work, victims with high BACs who had consumed alcohol long enough before death to be resorbed and metabolised by the body, were shown to have high EtG concentrations in urine, liver and blood at the time of autopsy [1]. After these *in vitro* experiments, it can be assumed that EtG should be detectable even after weeks of post-mortem degradation of the body. If no EtG can be found under these circumstances, a post-mortem genesis of the alcohol by putrefaction should be considered. However, after several months, EtG might no longer be detectable due to prolonged degradation time.

The graphs are shown in Fig. 4.

Another question in post-mortem analysis is whether EtG can be produced in tissues and in blood post-mortem or by putrefaction. Liver is the organ of main EtG production in the living, and no pre-analytical EtG production in blood has been reported as yet. But as samples were not stored sterile but ventilated, a bacterial contamination could not be excluded, which might lead to a post-mortem EtG formation. Therefore, we decided to test liver and blood samples of corpses with negative BACs for new formation of EtG during storage at *RT* for up to 4 weeks after spiking with ethanol.

All tissue samples which were negative for EtG at  $t_0$  ( $n=5$ ) remained negative for up to 4 weeks of storage.

One sample (liver sample 6) had a low EtG concentration at  $t_0$  of 0.6  $\mu\text{g/g}$ . Within 3 weeks of storage the determined concentrations varied from 0.4 to 0.9  $\mu\text{g/g}$ . In contrast to muscle sample 3 (see above) with low EtG concentrations, liver sample 6 still showed detectable EtG concentrations after 3 weeks. The results are presented in Table 4.

## Conclusions

EtG in urine samples stored at 4°C proved to be stable for 5 weeks. In samples stored at higher temperatures (22–27°C), larger variations of EtG concentrations were detected during 5 weeks of storage. An increase in EtG could be explained by evaporation of water, since sample vials were ventilated, but the higher variations (decrease by 30% and increase by 80%, respectively) remain unclear. Cooling at 4°C can be recommended for pre-analytical storing of urine samples prior to EtG analysis.

In EtG-positive tissue material, which was stored under conditions where the sample slowly decomposed at room temperature, a slow decrease in EtG concentrations over 4 weeks of storage was observed. However, in all samples with initial EtG concentrations higher than 1 µg/g, EtG was still positive after 4 weeks.

These in vitro experiments suggest that a negative result for EtG after 4 weeks of decomposition in a tissue or blood sample excludes a high EtG concentration at the time of death.

A post-mortem formation of EtG was not found in these in vitro experiments, which would support the hypothesis that EtG concentrations in body liquids or tissues like liver and skeletal muscle prove an alcohol consumption of the victim prior to death.

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