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ORIGINAL ARTICLE

In vitro study of bacterial degradation of ethyl glucuronide and ethyl sulphate

Stefanie Baranowski · Annerose Serr · Annette Thierauf · Wolfgang Weinmann · Markus Groβe Perdekamp · Friedrich M. Wurst · Claudia C. Halter

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Abstract Recent studies show that ethyl glucuronide (EtG) can be decomposed by bacteria; whilst so far no degradation of ethyl sulphate (EtS) has been observed. In the present study, in vitro experiments with bacterial colonies were performed. Bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Clostridium sordellii*) were isolated from autopsy material (liver, heart blood, urine, ascites, pericardial fluid, pleural fluid) tested for β-glucuronidase activity, and three bacterial strains were added to nutrient-deficient medium containing EtG and/or EtS and incubated at 36±1°C. Samples were taken after various intervals up to 11 days, and EtG and EtS were determined by electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS). EtG was degraded by *E. coli* and *C. sordellii*—complete degradation occurred in the range of 3–4 days—and these bacteria exhibited β-glucuronidase activity. EtS was not affected within 11 days of incubation.

S. Baranowski · A. Thierauf · W. Weinmann (✉) · M. Groβe Perdekamp · C. C. Halter
Institute of Forensic Medicine,
Freiburg University Medical Center,
Albertstr. 9,
79104 Freiburg, Germany
e-mail: wolfgang.weinmann@uniklinik-freiburg.de

A. Serr
Department of Microbiology and Hygiene, Institute for Medical Microbiology and Hygiene, Freiburg University Medical Center,
Hermann-Herder-Str. 11,
79104 Freiburg, Germany

F. M. Wurst
Christian-Doppler-Clinic, Paracelsus Medical University,
Ignaz-Harrer-Straße 79,
5020 Salzburg, Austria

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Introduction

Ethyl glucuronide (EtG) and ethyl sulphate (EtS) are alcohol consumption markers. In contrast to enzymatic markers, such as GGT or CDT, they are formed even by minor and singular ethanol intake [20, 21]. They are detectable in body liquids and post-mortem tissues and are useful markers for alcohol consumed up to several hours before sampling or death (pre-mortem alcohol consumption markers) [4, 8, 18].

For the correct interpretation of post-mortem EtG and EtS concentrations, knowledge of the stability and degradation or possible formation of these markers is needed. Depending on the time span between death and autopsy, bacterial colonisation of the corpse takes place [2, 11] and microbiological degradation of the analytes can possibly occur. Post-mortem bacterial transmigration, particularly from the visceral organs, is possible, especially if the corpse is not cooled directly after death. Due to their strong protease enzymes, *Staphylococcus* sp. are assumed to be the first to break through the visceral organs, followed by coliform-type organisms which are spore-forming gram-positive bacilli. The last species to leave the gut are a variety of anaerobic species. Depending on the organ submitted for examination, the condition of the corpse, the post-mortem interval before sampling and the enzymatic activity of the respective bacteria, degradation of analytes (i.e. drugs, metabolites, etc.) is possible [16]. As certain microorganisms even have the ability to generate clinically relevant ethanol levels, the stability of EtG and EtS under post-mortem conditions is even more important [1, 19].

Few investigations on the formation and stability of EtG and EtS were performed with differing results: Whilst Helander et al. report on a post-mortem formation of EtG in the presence of ethanol [6], Hoiseth et al. did not find a post-mortem production of EtG, but to a great extent formation of ethanol [9]. Hoiseth et al. and Helander et al. convey the degradation of EtG in blood and urine, respectively [5–7]. These discoveries differ from the result of the research of Schloegl et al. who found EtG to be stable [17, 18], and EtS proved to be stable in corresponding experiments by Dresen et al. [3], but no determination of bacterial contamination had been performed in these experiments.

This present study was designed to test the stability of EtG and EtS under controlled conditions using standardised cultivation medium.

Materials and methods

Isolation of bacteria

For identification of the general bacterial colonisation of corpses, samples (liver, heart blood, urine, ascites, pericardial fluid, pleural fluid) were taken from 13 non-preselected corpses during forensic autopsies. Identification of all bacterial isolates was performed according to standard procedures [12] at the Institute for Medical Microbiology and Hygiene in Freiburg. Presumptive identification of aerobic bacterial isolates was achieved using gram staining and cytochrome oxidase reaction. Gram-negative bacilli belonging to the Enterobacteriaceae were differentiated by the carbohydrates they metabolise in a commercially available kit, Enterotube II, Becton Dickinson, Heidelberg, Germany. Non-fermentative gram-negative bacilli, enterococci, streptococci and staphylococci were also identified by commercially available kits, API 20 NE, API 20 Strep and API 20 Staph (BioMérieux, La Balme les Grottes, France), respectively. These are identification systems which use miniaturised biochemical tests and specific databases. They allow the identification of most non-fermentative gram-negative bacilli, streptococci, staphylococci and members of related genera encountered in medical and veterinary bacteriology. *Staphylococcus aureus* was identified by the clumping factor test and the tube coagulase test. One *Bacillus* species and two *Lactobacillus* species were identified to the genus level only. Anaerobic isolates were incubated in an anaerobic jar containing a gas-generating kit and were further identified by 16S rRNA PCR and sequencing.

For further experiments, *Escherichia coli*, *Clostridium sordellii* and *Klebsiella pneumoniae* were chosen.

β-Glucuronidase test

The β-glucuronidase test (Rosco DiagnosticaTM, Taastrup, Denmark) was performed according to the manufacturer's instructions. This test is based on the enzymatic cleavage of *p*-nitrophenyl-β-D-glucuronide into glucuronic acid and the intensely yellow coloured chromophore *p*-nitrophenol. Briefly, a suspension of the bacterial isolate with a turbidity adjusted to at least McFarland no. 4 was prepared. After the addition of one test tablet (Rosco Diatabs™ β-glucuronidase), the reaction was incubated at 35–37°C overnight. Glucuronidase activity was indicated by a visibly distinguishable colour change from uncoloured to yellow. *E. coli* ATCC 25922 served as positive and *K. pneumoniae* ATCC 13883 as negative control organisms.

Degradation stability tests

For each strain of bacteria, five cultures were incubated on a shaker for 11 days at 36±1°C. Aliquots were collected daily for determination of EtG and EtS by LC-MS/MS.

Cultures consisted of 10 ml nutrient-deficient medium (NDM, liquid medium without source of carbon) and variable constellations of bacteria (10 µl of a solution with approximately 1×10^4 colony forming units/ml), EtG and EtS solutions (final concentration approximately 10 mg/l). The cultures for the actual degradation stability test consisted of NDM, bacteria and (a) EtG only, (b) EtS only and (c) both EtG and EtS. One control sample contained NDM, EtG and EtS, another control sample consisted of NDM and bacteria. Preliminary tests were conducted with both rich medium (brain heart infusion) and NDM. Comparable results were obtained for both media, NDM was chosen for further experiments.

During incubation, condensation of solvent occurred on the walls of the incubation flasks; therefore, a normalisation of the volume loss due to condensation of water was performed by the use of the negative control sample.

Reagents and instrumentation

Media used for the cultivation of bacteria were liquid NDM, liquid brain heart infusion medium, Columbia blood agar plates and yeast cysteine blood agar plates prepared in the Institute for Medical Microbiology and Hygiene, Freiburg University Medical Center.

Testing for β-glucuronidase activity was performed with Rosco Diatabs™ β-glucuronidase (PGUA) (Rosco Diagnostica, Taastrup, Denmark).

For generation of the correct atmosphere in anaerobic culture, GENbox anaer sachets (bioMérieux, Nuertingen, Germany) were used.

High-performance liquid chromatography (HPLC) grade acetonitrile and formic acid (analytical grade) were obtained from Merck (Darmstadt, Germany). EtG and pentadeuterated EtG (D5-EtG) were purchased from Medichem (Stuttgart, Germany). Sodium ethyl sulphate was obtained from ABCR (Karlsruhe, Germany). Deuterated ethyl sulphate (D5-EtS) was prepared by an in-house procedure [3].

Mass spectrometric analyses were performed with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system consisting of an API 365 triple quadrupole tandem mass spectrometer with a Turbo IonSpray interface (Applied Biosystems/Sciex, Darmstadt, Germany) and HPLC system (three pumps LC-10AD; system controller SCL-10A Shimadzu, Duisburg, Germany). HPLC separation was achieved at 40°C with a polar-endcapped phenylpropyl reversed phase column (Synergy Polar-RP 250×2 mm, 4 μm) with a guard column (4×2 mm, same packing material; Phenomenex, Aschaffenburg, Germany). For isocratic elution, 0.1% formic acid was used with a flow rate of 0.2 ml/min. Acetonitrile was added post-column at a flow rate of 0.2 ml via a T-union to enhance the volatility of the eluent. Both EtG and EtS were analysed in one run by a validated MS/MS method with electrospray ionisation (ESI) [19].

For EtG, the MS/MS transition with *m/z* 221/75 (precursor ion/product ion) was used as quantifier, *m/z* 221/203, 221/113 and 221/85 were used as qualifiers and *m/z* 226/75 represented the deuterated standard. The transitions for EtS were *m/z* 125/97 (quantifier), 125/80 (qualifier) and 130/98 for the deuterated standard.

Table 1 Bacteria isolated from autopsy material and β-glucuronidase activity test results (49 samples in total, 22 infected with bacteria yielding 37 microbiological isolates)

Bacteria identified	β-glucuronidase activity	Number of samples where present
Bacteroides fragilis	+	1
Bacteroides vulgatus	+	2
Clostridium perfringens	+	2
Clostridium sordellii	+	3
Escherichia coli	+	7
Clostridium sordellii	−	2
Clostridium tertium	−	2
Enterococcus cloacae	−	1
Enterococcus faecalis	−	2
Enterococcus sp.	−	1
Hafnia alvei	−	2
Klebsiella pneumoniae	−	1
Lactobacillus gasseri	−	2
Proteus mirabilis	−	3
Staphylococcus aureus	−	2
coagulase negative Staphyloccus	−	1
Stenotrophomonas maltophilia	−	1
Streptococcus anginosus	−	1
Streptococcus viridans	−	1

Results

During the autopsies, 49 specimens were taken from 13 corpses, 27 of the samples were free of bacteria and 22 (45%) contained 1 or more strains of bacteria. An overall number of 37 isolates was found. Identification of bacteria from autopsy material and subsequent testing for β-glucuronidase activity yielded 5 glucuronidase-positive strains (see Table 1) in 15 isolates and 14 strains in 22 isolates showed no glucuronidase activity, an overall number of 19 different strains of bacteria.

Bacteria selected for further experiments were *E. coli* because of the presence in post-mortem tissue and also in clinical urine samples, as previously published [5]. *C. sordellii* appeared frequently in post-mortem tissue samples; the strain utilised was tested positive for glucuronidase activity although there were also glucuronidase-negative *C. sordellii* strains isolated in this experiment. *K. pneumoniae* was chosen due to the easy cultivability and lack of β-glucuronidase activity.

The degradation of EtG and EtS was followed-up for up to 11 days, and the results are shown in Figs. 1 and 2. Figure 1 shows the time dependence of the concentration of EtG incubated with bacteria and NDM. After 96 h, EtG was degraded by *E. coli* and *C. sordellii*, but not by *K. pneumoniae*.

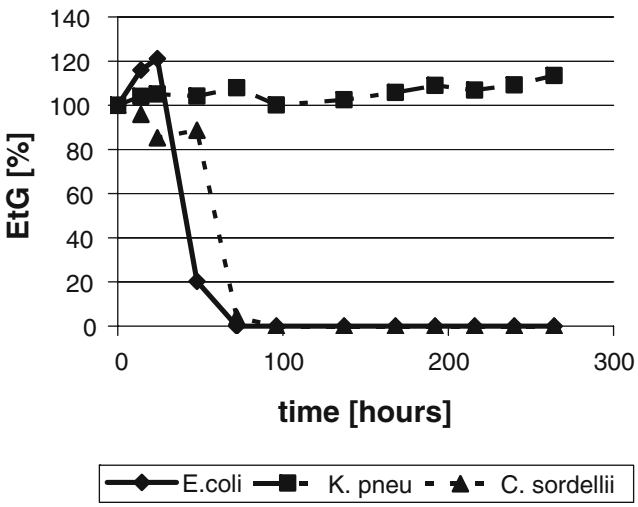

Fig. 1 EtG concentration with *E. coli*, *K. pneumoniae* and *Clostridium* sp. in NDM incubated at 36±1°C for 11 days, initial EtG concentration 10 mg/l

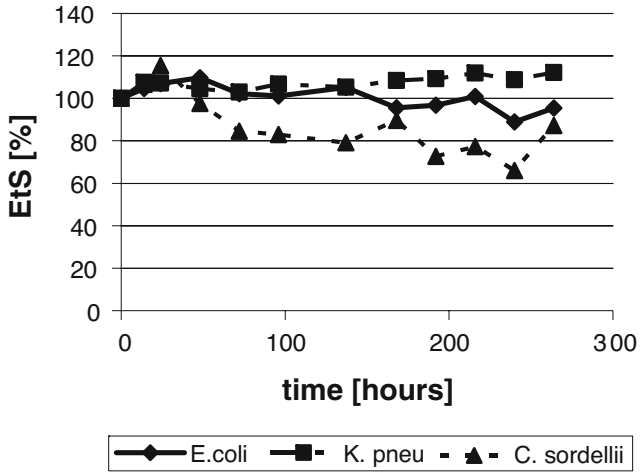

Fig. 2 EtS concentration with *E. coli*, *K. pneumoniae* and *Clostridium* sp. in NDM incubated at 36±1°C for 11 days, initial EtS concentration 10 mg/l

Figure 2 displays the time dependence of the EtS concentration with bacteria and NDM. No degradation has taken place over the observation period of 11 days in the experimental setup. The additional presence of the respective corresponding ethanol consumption marker (EtS in the EtG setup, EtG in the EtS setup) did not change the results considerably.

In the controls (without bacteria or EtG/EtS), neither an increase nor decrease of EtG and EtS concentrations was observed.

Discussion

The results show that EtG was degraded after 96 h by *E. coli* and *Clostridium* sp., no matter whether only EtG was present or both EtG and EtS. The presence of *K. pneumoniae* did not affect the concentration of EtG (see Fig. 1). In none of the experiments EtS was degraded by any of the bacteria studied. Only 45% of the isolated samples showed bacterial growth. The percentage of infected samples is within the normal range of bacterial infection reported in other studies [2]. As more than half of the samples were free of bacteria, this can be seen as proof of a correct contamination-free working technique with bacteria coming from the autopsy material and not from contamination during the autopsy process. To enable bacterial degradation, bacterial growth is necessary. Bacterial growth could be noticed in rich medium and in nutrient-deficient medium with or without glucose and independent of the presence or absence of EtG and EtS. This means that these two metabolites do not have antimicrobial effects on the chosen organisms in the concentrations studied.

In none of the experiments formation of EtG or EtS could be observed, but there was no ethanol present as a substrate for the bacteria to form ethanol metabolites, unlike in the experiments of Helander et al. which showed bacterial EtG formation [6]. As this was a study on EtG and EtS degradation, this aspect was not investigated.

Although a real corpse cannot be compared to nutrient-deficient media, this study shows that *E. coli* and *C. sordellii* are in principle able to degrade ethyl glucuronide. This has to be taken into account when judging negative EtG results from post-mortem samples. In forensic cases, the corpses are most often not cooled immediately after death. This circumstance favours the growth of bacteria and their transmigration through the gut into other parts of the body. Screening the post-mortem samples for bacterial colonisation would not be a solution, as glucuronidase-positive bacteria might already have disappeared from the sample by suppression from other glucuronidase-negative bacteria. The presence of only glucuronidase-negative bacteria might mimic the impossibility of bacterial EtG decay although bacterial EtG degradation might have occurred earlier in time.

In comparison to the study of Helander and Dahl [5], an overlap can be seen as both studies yielded four similar types of bacteria. But there were also strains of bacteria found in the study of Helander and Dahl ($n=5$ plus "Enterococcus sp." and "Group B streptococci" that were not closely specified), but not in the present study and vice versa ($n=15$). Furthermore, Helander and Dahl did not study the β-glucuronidase activity of the bacteria. Helander and Dahl found EtG degradation in 68% of the samples infected with *E. coli*, which can be confirmed by the present results as *E. coli* is positive for β-glucuronidase. They also found loss of EtG in one sample infected with *K. pneumoniae* (one out of three) and with *E. cloacae* (one out of one), although β-glucuronidase activity was not found in these bacteria in the present study. The reason for this discrepancy might be based either on misidentification of the bacteria, on the intermittent disappearance of the relevant glucuronidase-positive bacteria or on the presence of different sub-species that possess or lack β-glucuronidase activity. This phenomenon was found in *C. sordellii* strains in this study where three isolates had glucuronidase activity and two isolates did not.

In the light of the ability of some bacterial strains to degrade EtG, studies on the use of preservatives, such as sodium fluoride [5], potassium oxalate [1, 13], boric acid [14] or chlorhexidine [10, 15] should be performed. Furthermore, the bacterial degradation of EtS, which so far seems to be rather stable, should be tested. To the author's knowledge, there are no commercially available kits for testing sulphatase activity.

As to the actual knowledge, EtS is not degraded or newly formed by bacteria; determination of both EtG and EtS is advantageous for the assessment of pre-mortem ingestion of ethanol in samples which are colonised by bacteria, especially as this will cause almost no additional effort if an LC-MS/MS method for detection of both analytes is used.

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