Introduction of sample tubes with sodium azide as a preservative for ethyl glucuronide in urine

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Abstract

Ethyl glucuronide (EtG) is a direct alcohol marker, which is widely used for clinical and forensic applications, mainly for abstinence control. However, the instability of EtG in urine against bacterial degradation or the post collectional synthesis of EtG in contaminated samples may cause false interpretation of EtG results in urine samples. This study evaluates the potential of sodium azide in tubes used for urine collection to hinder degradation of ethyl glucuronide by bacterial metabolism taking place during growth of bacterial colonies. The tubes are part of a commercial oral-fluid collection device. The sampling system was tested with different gram-positive and gram-negative bacterial species previously observed in urinary tract infections, such as *E. coli, S. aureus, E. faecalis, S. epidermidis, K. pneumoniae, E. cloacae, and P. aeruginosa.* Inhibition of bacterial growth by sodium azide, resulting in lower numbers of colony forming units compared to control samples, was observed for all tested bacterial species.

To test the prevention of EtG degradation by the predominant pathogen in urinary tract infection, sterile-filtered urine and deficient medium were spiked with EtG, and inoculated with *E. coli* prior to incubation for four days at 37°C in tubes with and without sodium azide. Samples were collected every twenty-four hours, during four consecutive days, whereby the colony forming units (CFU) were counted on Columbia blood agar plates, and EtG was analyzed by LC-MS/MS. As expected, EtG degradation was observed when standard polypropylene tubes were used for the storage of contaminated samples. However, urine specimens collected in sodium azide tubes showed no or very limited bacterial growth and no EtG degradation.

As a conclusion, sodium azide is useful to reduce bacterial growth of gram-negative and gram-positive bacteria. It inhibits the degradation of EtG by *E. coli* and can be used for the stabilization of EtG in urine samples.

Keywords. – ethyl glucuronide, microbial degradation, preservative, sodium azide, preanalytical stability

Introduction

In order to prove alcohol consumption or abstinence, various direct alcohol markers are currently analyzed, such as ethyl glucuronide (EtG), ethyl sulfate (EtS) and phosphatidylethanol (PEth). EtG as a sensitive and specific short-term marker is thereby the most versatile, as it can be detected in urine, blood and hair samples. (1) Bacterial degradation as well as neo-formation of EtG from ethanol in urine samples containing bacteria have been reported. (2, 3) World-wide, no preservatives for stabilizing the analyte concentrations are used, although shipment of samples to a laboratory may take several days under non-cooled conditions. The preservation of urine samples was therefore extensively investigated. (4, 5) However, the use of dried urine spots for inhibition of post-sampling EtG degradation is not popular until now, and the use of boric acid tubes turned out to be incompatible with the DRI® immunoassay for EtG detection. (5, 6)

In this article we investigate the preservation of sample integrity by using commercially available sodium azide tubes. Sodium azide is a well-established preservative against microorganisms, which generally inhibits microbial activity. (7, 8) Subsequently, it inhibits the growth of *E. coli*, the predominant pathogen (80%) in urinary tract infections. (9) "Bacterial growth" – which is the increase of numbers of colony forming units (CFU) - within the sample would lead to degradation of EtG, when EtG is used as a carbon source. Inhibition of major processes of the bacterial cell activity - as has been reported for sodium azide - leads to termination of substrate turnover (e.g., inhibition of DNA synthesis and cell division in *Salmonella typhimurium* by azide). (10, 11) As a consequence, the degradation of EtG would be stopped, when the bacterial cell activity stops. Our aim was to investigate, if sodium azide tubes can inhibit the bacterial growth of different gram-positive and gram-negative bacteria. Furthermore, investigation of sodium azide on the stability of EtG in urine inoculated with *E. coli* should be used to test the applicability of this stabilizing agent in analysis for EtG by LC-MS/MS.

Materials and methods

Reagents

Water was produced with a Milli-Q water system from Millipore (Billerica, USA). Acetonitrile (p.a.) was obtained from Acros Organics (Geel, Belgium). Isopropyl alcohol was purchased from Fisher Chemicals (Reinach, Switzerland). EtG was obtained from Medichem Diagnostica (Steinenbronn, Germany) and EtG-d_5 was obtained from Lipomed (Arlesheim, Switzerland). Formic acid (puriss p.a., 98 %) and methanol (spectrophotometric grade, ≥99 %) were purchased from Sigma-Aldrich (Buchs, Switzerland). Sodium azide tubes from the Saliva Collection System (SCS), containing 4 mg of sodium azide per tube, were a gift from Greiner Bio-One International (Kermsmünster, Austria), see Figure 1. Deficient medium glucose solution was obtained from the Institute of Medical Microbiology and Hygiene, Albert-Ludwigs-University, Freiburg. The deficient medium consisted of a 500 mL basic medium (1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, 0.13 g/L $CaCl_2 \cdot H_2O$, 5.0 g/L $(NH_4)_2SO_4$ and 0.5 g/L KNO_3), 2 mL of a trace element solution (5 mg/mL H_3BO_3, 0.4 mg/mL $CuSO_4 \cdot 5\ H_2O$, 1 mg/mL KI, 2 mg/mL $FeCl_3$, 3.6 mg $MnSO_4 \cdot H_2O$ and 7.1 mg $ZnSO_4 \cdot 7\ H_2O$) and 10 mL of a D(+)-glucose solution (0.5 µg/L). Additionally, 500 µL of a growth substance solution was added (2 µg/mL D(+)-biotin, 100 µg/mL Ca-pantothenate, 100 µg/mL p-aminobenzoate, 200 µg/mL thiamine). The rich medium consisted of tryptic soy broth (Merck, Darmstadt, Germany) including casein-peptone, soymeal-peptone and sodium chloride.

Study design bacterial growth study

In addition to *Escherichia coli* (2006/9478), a strain with β-glucuronidase activity which was previously isolated from autopsy material, the growth of the following gram-negative bacterial strains was tested with and without sodium azide: *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* IUK 1230, and *Enterobacter cloacae* IUK 611. Additionally, the growth inhibition by sodium azide of three representative gram-positive bacterial strains was tested: *Staphylococcus aureus* ATCC 29523, *Staphylococcus epidermidis* DSM 1794, and *Enterococcus faecalis* T9. All

tested gram-negative and gram-positive bacterial species are frequently associated with urinary tract infections. The bacterial growth of gram-negative bacteria was examined in the deficient medium for up to two days, whereas the growth of gram-positive bacteria was additionally tested in rich medium for one day due to their inability to grow in the deficient medium. Both culture media are described in the reagents section. The reduction of the bacterial growth caused by sodium azide was calculated in comparison to the bacterial growth in the negative control without sodium azide. The inoculation of the different culture media and the determination of the colony forming units (CFU) over the growth time were conducted in double, as described below in detail for *E. coli* (2006/9478). EtG degradation studies were performed only for *E. coli*.

Study design EtG in sodium azide tubes

Different positive control samples (10 samples, in double), negative control samples (20 samples, in double), and experimental approach samples (10 samples, in double), with a volume of 3.5 mL each, were prepared and measured in a single measurement each, to investigate the degradation of EtG during four days, see table 1. The urine used during the whole experiment was from an alcohol abstinent person, centrifuged at 5000 g for 10 minutes and subsequently sterile filtrated using Millex GP syringe driven filter units (0.22 µm, Millipore, Carrigtwohill, Ireland). The deficient medium glucose solution was prepared by mixing 500 mL basic medium with 2 mL of the trace element solution, 10 ml of the D(+)-glucose solution and 500 µL of the growth substance solution.

For spiking with the bacteria, an overnight culture of *E. coli* (2006/9478) was prepared at MC Farland 0.5 (1.5×10^8 CFU/mL), diluted by 1:10, whereby 10 µL were spiked to each sample. (12) Samples containing EtG were spiked with a concentrated solution, resulting in a concentration of approximately 10 µg/mL each. Samples were incubated at 37 °C (Thermo Scientific HERAcell 150i, Waltham, MA, USA) throughout the whole experiment. The colony forming units (CFU) were cultivated on Columbia blood agar (CBA). (13) The CBA plates were incubated at 37 °C for 3 days under aerobic conditions. The CFU were counted using the Gel Doc EQ Universal Hood (Bio-Rad Life Science Group, Hercules, USA). EtG concentrations were measured by LC-MS/MS. Samples were analyzed at day 0, 1, 2, 3, and 4. Liquid samples were directly frozen at about -18°C after sampling.

Preparation and processing of samples for EtG analysis

By adding 150 µL of acetonitrile (containing the internal standard) to 50 µL of diluted sample (40 µL of urine and 10 µL of water) in a 2 mL Eppendorf tube, proteins were precipitated. The samples were then vigorously shaken for five minutes on a VIBRAX VXR basic (IKA, Staufen, Germany). Afterward, the samples were centrifuged for 10 min at 16,000 g on a Mikro 220 R (Hettich, Switzerland), before 100 µL of the supernatant solution was transferred into a glass vial with insert (0.35 mL, Infochroma, Zug, Switzerland). The organic phase was evaporated to dryness at 50 °C under a gentle stream of nitrogen. The residue was dissolved in 100 µL of reconstitution solution (water/acetonitrile, 95/5 + 0.1% formic acid, (v/v)). An aliquot of 10 µL was injected into the LC-MS/MS system. A blank urine and a zero sample (from an abstinent person) with, and without internal standard were included. After the measurement of the diluted sample, in order to remain within the calibration range, the sample concentration was back calculated to a non-diluted concentration. The chromatographic and mass spectrometric system and conditions employed for the analysis were described earlier with some modifications. (4, 14-16) The analytical method is based on a LC-MS/MS method on a 3200 QTrap instrument (Sciex, Toronto, Canada) with post column addition of isopropyl alcohol, whereby chromatographic separation was performed on a Synergi Polar RP 4 µm, 150 x 2.0 mm, with a Polar RP, 4 x 2.0 mm, precolumn (Phenomenex, Torrance, USA). Samples are quantified based on a six point calibration from 0.1-10 µg/mL. The LOD was established at 0.04 µg/mL (signal to noise ratio of 3:1), the LLOQ at 0.1 µg/mL. The method was fully validated at our institute and is currently used for routine analysis. By evaluating urine samples from six different subjects for selectivity and specificity, no significant interferences were observed (intra assay imprecision of 8.8%CV and intra-assay accuracy of 99.5%). By analyzing three series of six quality control samples at each concentration (0.1, 0.3, 1, 10 µg/mL) on three different days, EtG showed a mean inter-assay precision of 4.9- 10.0%CV and an inter-assay accuracy of 101.9-114.6%.

Results

Bacterial growth study

The growth of all tested bacterial strains in the deficient medium with or without sodium azide is depicted in figure 2. The growth of all gram-negative bacteria was inhibited by sodium azide compared to positive control samples in standard polypropylene tubes without sodium azide (13 mL, 100×16 mm Ø, Sarstedt, Nümbrecht, Germany). The CFU numbers of *K. pneumonia* and *E. cloacae* were 1-2 log10 higher than the initial value after growth for one or two days without sodium azide. This difference in the log10 CFU was even 2-3 for *P. aeruginosa* compared to the incubation with sodium azide. All gram-positive bacteria did not grow in the deficient medium. Nevertheless, sodium azide highly reduced the number of the CFU values of *E. faecalis* and *S. aureus* after one day of incubation in the deficient medium. The CFU values of *S. epidermidis* were reduced in the deficient medium even without sodium azide. According to these results an inhibitory effect could only be observed for *E. faecalis* and *S. aureus*. To evaluate this, the antimicrobial effects of sodium azide against all three gram-positive bacterial strains were tested in rich medium. The growth of all gram-positive bacteria was strongly reduced by sodium azide at a level of 5-6 log10 after one day of incubation when compared to positive control samples in standard tubes (see figure 3).

EtG in sodium azide tubes

EtG concentration changes for the two matrices, deficient medium and urine, are shown in figure 4 and figure 5, respectively. The mean number (n=2) of the colony forming units (CFU) in the various samples are shown in figure 6. In agreement with literature reports, a degradation of EtG in both matrices was observed, when bacteria were mixed and stored with EtG in untreated plastic tubes. (4) After the first day, about 90% of the initial EtG concentration had been metabolized by bacteria in deficient medium, compared to 100% degradation in urine after the first day. In deficient medium, EtG concentrations decreased to concentrations below LOD after two days of incubation. A clear increase in bacterial growth could be observed for both sample types. A closer look at the number of colony forming units in the different matrices revealed that the bacterial growth had been slower in deficient medium compared to urine. No degradation of EtG and no bacterial growth were observed when samples were frozen. Samples stored in sodium azide tubes showed no signs of EtG hydrolysis during the four days of measurement, as the quantification results remained within the confidence interval of ± 15% of the initial concentration (17). Negative control samples, without any EtG, showed no signs of EtG formation. Sterility control of urine and deficient medium showed no increase in CFU and thus, no signs of bacterial growth.

Discussion

Compared to standard polypropylene tubes for urine storage, suppressed bacterial growth was observed in all experiments, when sodium azide tubes were used. We could efficiently prevent the growth of bacteria of different species, gram- negative as well as gram-positive species. The presented bacterial growth study showed that the composition of the medium plays a crucial role, when it comes to bacterial growth. Thereby the investigated gram-positive bacteria proved to be demanding and did not grow in deficient medium. In the study of Helander et al., no EtG degrading gram-positive bacteria were found in urine samples. (9) One reason might be, that urine is not the appropriate medium for gram-positive bacteria due to the lack of essential nutrients, necessary for the higher demand of gram-positive bacteria to multiply. Baranowski et al. found gram-positive species which showed β-glucuronidase activity in autopsy material, such as *Clostridium sordellii* or *Clostridium perfringens*. (3) The use of sodium azide does not completely eliminate bacterial load within the sample. Nevertheless, bacterial activity and growth can be inhibited (bacteriostatic effects) by sodium azide, leading subsequently to inhibition of biological degradation of organic substrates in microbial niches. Such bacteriostatic effects have also been reported for some antibiotics which keep pathogens number under the infectious level. (18) Bacterial growth would lead to degradation of EtG, as it is used as a carbon source. Inhibition of major processes of the bacterial cell activity as has been reported for sodium azide, leads to termination of substrate turnover. (10, 11) In our EtG degradation experiment with *E. coli*, sodium azide successfully prevented bacterial growth and thus inhibited the degradation of EtG.

It should be mentioned, that in contrast to boric acid tubes, which have been tested in previous work, the sodium azide tubes from Greiner Bio-One (saliva collection system), used here for urine sampling, are compatible with the DRI® immunoassay for EtG (Thermo Fisher Scientific) – data not shown here. (5, 6) In the unlikely case of EtG degradation within a sodium azide preserved tube, a solely quantification of EtS would remain the only option. Considering the toxicity of sodium azide as a chemical, it is important to mention, that the vacutainer-type collection tubes do not have to be opened for filling with urine, since they can be combined with the urine collection device from Greiner-Bio-One. This minimizes the potential contact with sodium azide by medical staff. For disposal, the sample tubes should be directed to waste combustion, since sodium azide decomposes at temperatures above 300 °C.

Conclusion

Our study shows, that urine collection in sodium azide containing vacutainer-type tube tubes can prevent bacterial growth and thus prevent the degradation of EtG by bacteria during sample transportation and storage. Sodium azide proved to be reliable for the protection against preanalytical EtG degradation by *E. coli*, a gram-negative bacterium, which has been observed previously as a major source of bacterial contamination of urine specimens due to urinary tract infection. The growth of gram-negative and gram-positive bacteria, which is necessary for bacterial degradation of EtG, was effectively inhibited in different cultivation media upon use of sodium azide tubes, whereas in control tubes without sodium azide bacterial growth took place. By the use of sodium azide, degradation of EtG can be effectively hindered, even when samples are not cooled during transportation, thus eliminating the risk of having lower or even false-negative results for EtG. Based on our results, the utilization of sodium azide tubes enhances the reliability of EtG analysis for the detection of recent alcohol consumption.

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Conflict of interest

The authors declare that they have no conflict of interest.

Table 1: Positive and negative control samples and their composition.

Sample Name	**Sample type**	**Medium**	**EtG [µg/mL]**	**Bacteria**	**Type of tube**
A1	Positive control	Deficient medium	10	*E. coli*	Untreated
B1	Positive control	Urine	10	*E. coli*	Untreated
A2	Negative control	Deficient medium	0	*E. coli*	Untreated
B2	Negative control	Urine	0	*E. coli*	Untreated
A3	Negative control	Deficient medium	0	*E. coli*	Sodium azide
B3	Negative control	Urine	0	*E. coli*	Sodium azide
A4	Experimental approach	Deficient medium	10	*E. coli*	Sodium azide
B4	Experimental approach	Urine	10	*E. coli*	Sodium azide
A5	Sterility control	Deficient medium	0	-	Untreated
B5	Sterility control	Urine	0	-	Untreated

Figure 1. Urine collection cup with sodium azide sample tube. The sodium azide tube can be filled by pushing the orange cap through the valve closure in the collection cup: The vacuum within the tube will automatically suck 2.8-3.5 mL of sample into the tube.

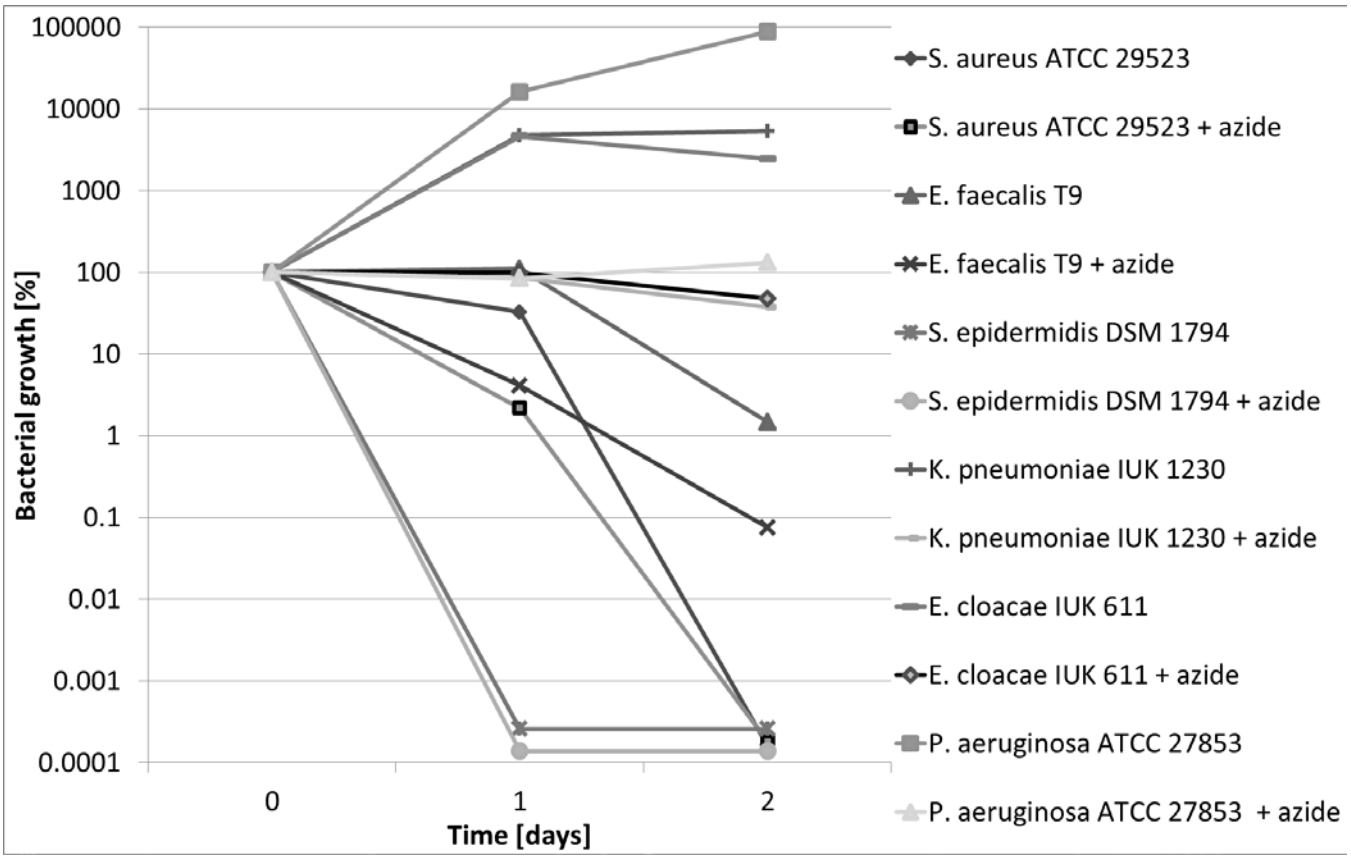

Figure 2. Mean colony forming units (CFU) in percent of the initial value (day 0) measured after growth of different bacteria in the deficient medium with or without sodium azide. Each data point shown represents the mean CFU out of two measurements.

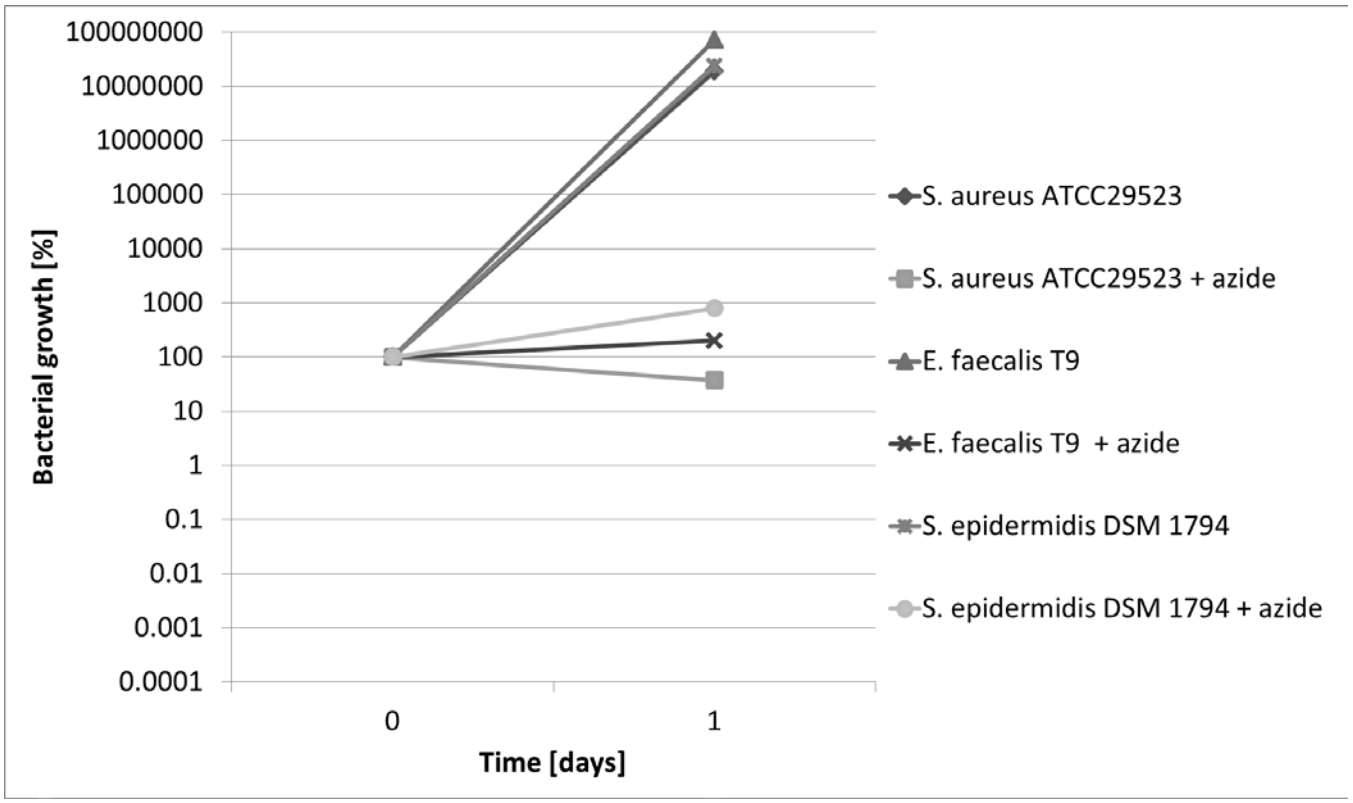

Figure 3. Mean number of colony forming units (CFU) in percent of the initial value (day 0) measured after growth of the gram-positive bacteria in the rich medium with or without sodium azide. Each data point shown represents the mean number of CFU out of two measurements.

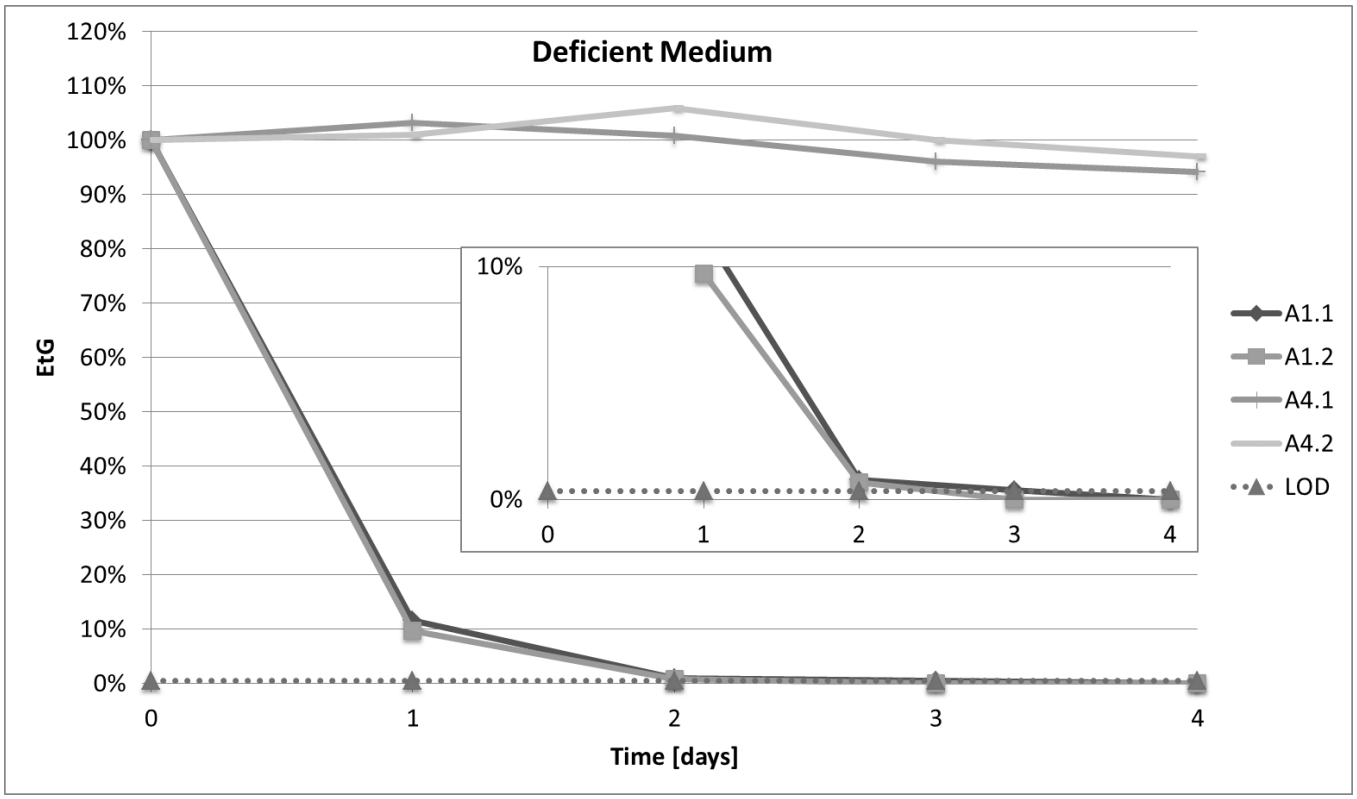

Figure 4. EtG concentration changes over time for deficient medium samples: It is visible, that samples stored in tubes without sodium azide (performed in double, A1.1 and A1.2, see table 1) show EtG degradation. Samples stored in sodium azide tubes (performed in double, A4.1 and A4.2, see table 1) show no EtG degradation. No EtG was measurable in negative control samples, not displayed here.

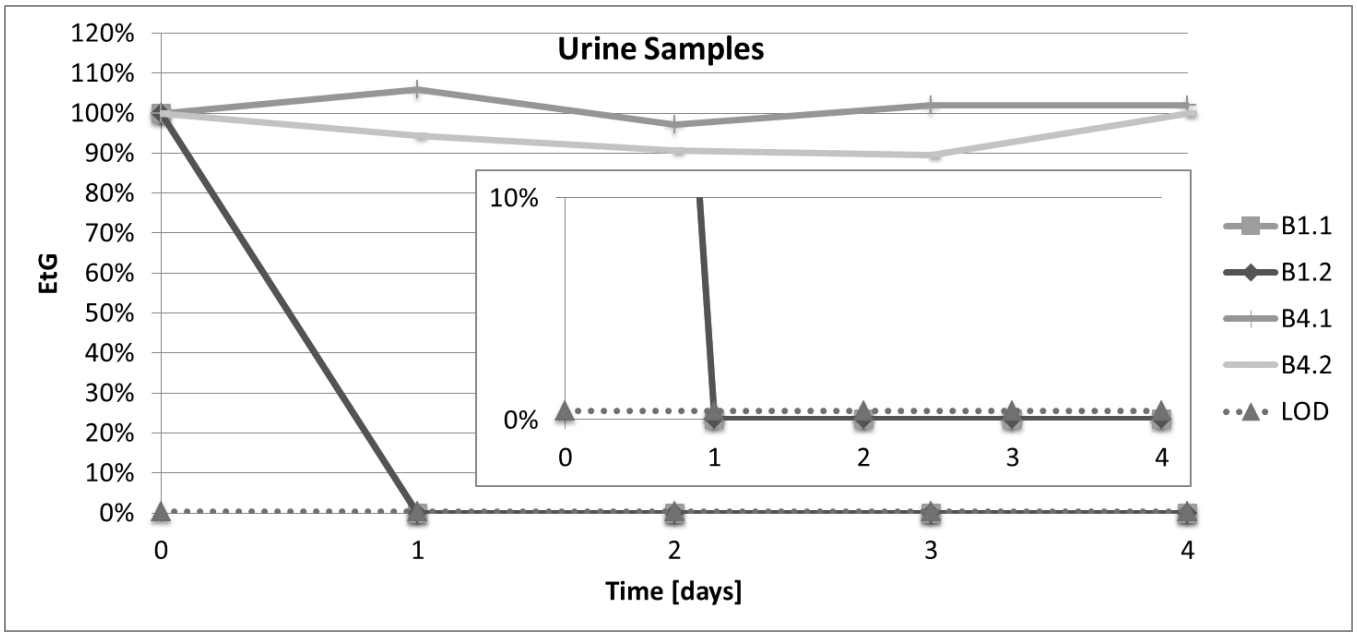

Figure 5. EtG concentration changes over time for urine samples: It is visible, that urine samples stored in tubes without sodium azide (perfomed in double, B1.1 and B1.2, see table 1) show EtG degradation. Urine samples stored in sodium

azide tubes (performed in double, B4.1 and B4.2, see table 1) show no EtG degradation. No EtG was measurable in negative control samples, not displayed here.

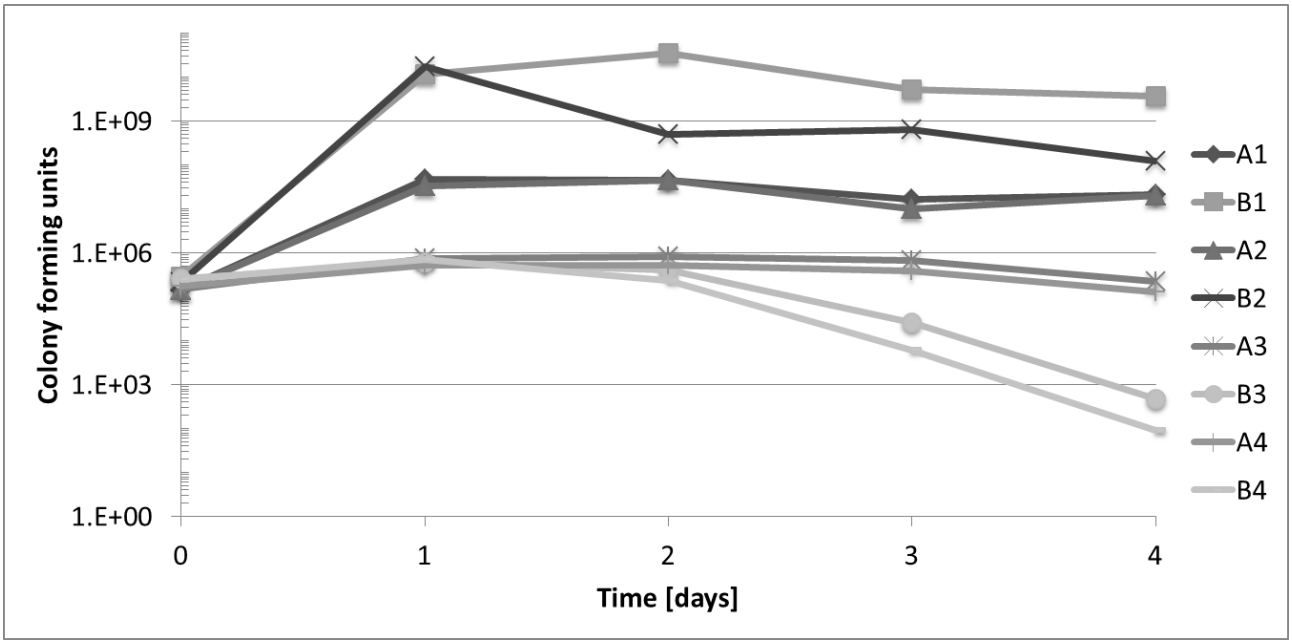

Figure 6. Mean number of colony forming units measured during the time course of the experiment. Each data point shown represents the mean No. of CFU out of two measurements. In contrast to samples treated with sodium azide (A3, B3, A4, B4, see table 1), large increases in No. of CFU are visible for samples stored in tubes without sodium azide (A1, B1, A2, B2, see table 1).

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