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Inactivation of microbicidal active halogen compounds by sodium thiosulphate and histidine/methionine for time-kill assays

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ABSTRACT

Rapid inactivation of antimicrobial test agents after exact incubation times with microorganisms is required in time-kill assays. Sodium thiosulphate and a combination of methionine and histidine were compared for neutralisation of active halogen compounds.

Test oxidants were mixed with surplus sodium thiosulphate (3%–6%) or histidine/methionine (1% each) in phosphate-buffered saline and incubated for different times, followed by addition of *Staphylococcus aureus*, *Escherichia coli*, or *Pseudomonas aeruginosa* at 1000 CFU/ml. After further incubation, quantitative cultures were performed.

Thiosulphate did not sufficiently inactivate chlorine and bromine compounds, indicated by a 10-fold (*S. aureus*) up to > 100-fold (*E. coli*, *P. aeruginosa*) reduction of CFU. This was particularly true for high concentrations of the oxidants of about 50 mM, for highly reactive agents (HOCl and bromamine T) more than for chloramine T and *N*-chlorotaurine, and for short pre-incubation times before addition of the bacteria. By contrast, histidine/methionine proved to be suitable for chloramines and bromamine T and for low concentrations of HOCl (0.07%). HOCl at 0.7% could neither be inactivated completely by thiosulphate nor by histidine/methionine. In contrast to chlorine and bromine compounds, iodine was neutralized by thiosulphate, but not by histidine/methionine.

Histidine/methionine is superior to inactivate chlorine and bromine and should replace sodium thiosulphate at least in killing tests with high concentrations of these disinfectants. Inclusion of a short reaction time (maximum one minute) of test oxidant and neutralising substance before addition of bacteria is decisive in inactivation tests to obtain reliable results.

1. Introduction

The activity of microbicidal agents is generally investigated by time-kill assays (European Norm (EN) 1040, 2005; European Norm (EN) 13727, 2012).

The test agent is mixed with test bacteria or fungi at time zero. After defined incubation times, aliquots are removed in which the agent has to be inactivated, followed by quantitative cultures on agar plates. Inactivation is a critical point (Reichel et al., 2008; Rotter et al., 2009). It must occur rapidly and is most time performed by dilution or by a chemical agent. The latter has the advantages of high convenience and lower detection limit and is usually performed in tests with disinfectants (European Norm (EN) 1040, 2005; European Norm (EN) 13727, 2012). Attention must be paid to the completeness of the inactivation of the test agent by its neutraliser (Rotter et al., 2009). Particularly, their reaction should not create new antimicrobial products. Therefore, testing of neutralisers includes incubating the test microorganisms in a mixture of the antimicrobial test agent and its neutraliser, both at the highest concentration applied in the time-kill assay (Reichel et al., 2008).

To inactivate disinfectants/antiseptics from the class of oxidants, the reducing agent sodium thiosulphate is generally recommended in the literature and in guidelines (Arnitz et al., 2009; European Norm (EN) 1040, 2005; European Norm (EN) 13727, 2012). This is due to its rapid action and easy handling. However, in our own plethora of killing assays using chloramines at a high concentration (for review, see (Gottardi and Nagl, 2010; Gottardi et al., 2013)), we observed a reduction of CFU, mainly within 1 $\log_{10}$, in some of the inactivation tests with sodium thiosulphate. Moreover, in recent experiments human granulocytes lost viability in a solution of 1% *N*-chlorotaurine plus 3%

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sodium thiosulphate, although no more oxidizing activity could be found after addition of potassium iodide (Christian Mair and Markus Nagl, unpublished). This could be overcome by using a mixture of 1% histidine and 1% methionine instead of thiosulphate.

These findings prompted us to perform the present detailed study on the inactivation of active chlorine, iodine, and bromine compounds by sodium thiosulphate compared to histidine/methionine, using Gram-positive and Gram-negative bacteria. Special attention was paid to different incubation times of the mixtures before and after addition of the test bacteria, a possibly widely neglected aspect.

2. Materials and methods

2.1. Chemicals

Active oxidizing halogen compounds: chloramine T (CAT) trihydrate, reagent grade, was from Merck (Darmstadt, Germany). *N*-chlorotaurine (NCT) sodium salt was synthesized as reported (Gottardi and Nagl, 2002). Hypochlorous acid solution was from Carl Roth (Karlsruhe, Germany), iodine Suprapur® from Merck, and Betaisodona® from Mundipharma (Limburg, Germany). Bromamine T (BAT) was synthesized from dibromamine T according to Nair et al. (1978). Single substances were dissolved in 0.01 M phosphate-buffered saline (PBS, pH 7.1) or in 0.1 M sodium acetate buffer (pH 4.0) to the concentrations indicated in the results section.

For inactivation, 3% or 6% weight per volume sodium thiosulphate pentahydrate (Merck, Darmstadt, Germany, molecular weight 248.17) in aqueous solution, or a mixture of 1% L-histidine (Serva, Heidelberg, Germany) and 1% DL-methionine (Sigma-Aldrich, Steinheim, Germany) in aqueous solution were applied. These solutions were autoclaved. Lecithine 0.3% (Carl Roth, Karlsruhe, Germany) and 3% Tween 80 (Merck-Schuchardt, Hohenbrunn, Germany) final concentrations (all weight per volume) were added in some experiments to thiosulphate. These solutions were filter-sterilized. Naturally, neutralizers were generally used in molar excess to the halogen compounds to warrant a complete reduction of the oxidation capacity.

Tryptic soy broth, PBS, sodium acetate, and potassium iodide were from Merck.

2.2. Bacteria

Staphylococcus aureus ATCC 6538, *Escherichia coli* ATCC 11229 and *Pseudomonas aeruginosa* ATCC 27853 deep frozen for storage were grown on Mueller-Hinton agar plates, from which they were cultivated for 16 h at 37°C in tryptic soy broth (Merck), centrifuged for 10 min at 1800 × *g* and washed twice with sterile 0.9% NaCl. These suspensions used for the experiments described in Section 2.4. contained $1-3 \times 10^9$ colony forming units (CFU)/ml, determined by quantitative cultures after appropriate dilution in saline.

2.3. Photometric evaluation of inactivation of the active halogen compounds

An aliquot of each disinfecting solution (500 µl) was mixed with 500 µl of the neutralising solution. Subsequently, 100 µl of 50% acetic acid was added, followed by 5 mg potassium iodide (KI). The absence of a brown colour and the absence of a triiodide peak at 350 nm in a DU 800 Beckman spectrophotometer was documented (Alexander, 1962). For comparison, an aliquot of each disinfecting solution without neutraliser was treated with KI, which caused an intensive brown colourisation in all cases.

2.4. Antimicrobial activity of the inactivation solution

First, 2 ml of the oxidant solution was mixed with 2 ml of the neutralising solution and incubated at 20–22 °C for 1, 30, 60, and 120 min. Subsequently, 40 µl of the bacterial suspension were added to a final concentration of about 1000 CFU/ml and incubated at 20–22 °C for additional 1, 3, 5, 15, and 30 min. Each strain was tested separately. Controls were incubated in plain PBS and in mixtures of 2 ml PBS with 2 ml neutralising solution without oxidants to rule out a microbicidal activity of the neutralisers.

After incubation, aliquots (50 µl) of the samples were spread in duplicate on tryptic soy agar plates with an automatic spiral plater (model WASP 2, Don Whitley Scientific, Shipley, UK), allowing a detection limit of 10 CFU/ml for all tested microorganisms taking into account both plates. The plates were incubated at 37 °C, and CFU were counted after 24 h. In case of no or weak (small colonies < 1 mm) bacterial growth, the plates were stored at 37 °C for a minimum of 72 h before evaluation. Sufficient inactivation was considered as no reduction in CFU compared to the controls.

2.5. Mass spectrometry of NCT plus sodium thiosulphate

To identify the products of the reaction between NCT and sodium thiosulphate, which showed antimicrobial activity, we applied ESI mass spectrometry (MS). A Thermo Scientific LTQ Orbitrap XL equipped with an electrospray source was used. The electrospray voltage was set at 4.0 kV and negative polarity. The heated capillary was held at 275 °C, the resolution was 30.000. MS/MS were performed with collision induced dissociation, normalized collision energy was set to 35. Samples were introduced to the MS via direct infusion applying a flow rate of 5 µl/min. Reaction partners 1% NCT and 6% sodium thiosulfate were infused individually, as well as the expected product of taurine at a concentration of 1%, dissolved in distilled water. Analytes were diluted 1:1 with 0.5% ammonium hydroxide to enhance ionization in negative polarity. 200 µl of 1% NCT plus 200 µl of 6% sodium thiosulphate were incubated for 1 min and 30 min at 20–22 °C, similar to the tests on antimicrobial activity. Again, ammonium hydroxide was added to aid MS-analysis.

2.6. Statistics

Data are presented as mean values and standard deviations (SD) of a minimum of three independent experiments. Student's unpaired *t*-test in case of two groups, or one-way ANOVA and Bonferroni's and Dunnett's multiple comparison test in case of more than two groups were used to test for a difference between the test and control group. $P < 0.05$ was considered significant. Calculations were done with GraphPad Prism 6.01 software (GraphPad Inc., La Jolla, CA, USA).

3. Results

3.1. Antimicrobial activity of oxidants plus sodium thiosulphate

First tests were done using the mild oxidant NCT (1% = 55 mM) and the standard neutralizer sodium thiosulfate (6% = 242 mM). Both (2 ml each) were mixed, and after undefined time between 3 and 30 min, bacteria were added and aliquots for quantitative cultures were removed after further 5, 30, and 60 min. While controls consisting of thiosulphate without NCT did not reduce the bacterial count compared to PBS, the results of NCT plus thiosulphate in independent repetition experiments surprisingly were not consistent. In some tests, there was no reduction of the CFU count compared to controls in PBS, while in others a significant reduction from the starting value (3 $\log_{10}$) to the detection limit (1 $\log_{10}$) occurred. This was particularly true for longer incubation times of 30 and 60 min with no difference between these two time points. Notably, the solution of NCT plus thiosulphate remained colourless after addition of potassium iodide a few seconds later, and there was no photometric peak visible at 350 nm, indicating that the reduction of oxidation capacity was complete, at least to a non-iodide oxidizing level.

These inconsistencies prompted us to define the storage time of the

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