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Stabilization in urine collection containers: reality or science-fiction

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Abstract

The current study - funded by WADA - focuses on finding a suitable application form of an in-house chemical stabilization mixture and on evaluating its efficiency so that it can easily be implemented in the doping control sampling protocol. After taking into consideration existing commercial systems containing preservatives or a mixture of preservatives for the protected collection and transport or urine in clinical laboratories, it was deduced that it would rather be impossible to apply the stabilization mixture as a transparent inner coating in the interior walls of urine collection containers. As a result, the spray coating application form was selected as the more easily acceptable by the end user. In the current work, the efficiency of the chemical stabilization mixture spray coated in the interior walls of urine containers has been tested against steroid glucuronide degradation induced by inoculation with three prokaryotic and two eukaryotic microorganisms during a 7-day incubation period at 37 °C. Microbial growth was assessed immediately after inoculation and at the end of the incubation period. Also, variations in pH and specific gravity (sg) values were recorded. Gas chromatography-mass spectrometry (GC/MS) analysis was performed for the detection of steroids in the free and total fractions. Results showed that cell growth was completely inhibited and degradation of steroid glucuronides was prevented in spray coated urine samples.

Introduction

The efficiency of a generic chemical stabilization mixture consisting mainly of antibiotic, antimycotic substances and protease inhibitors against degradation caused by microorganisms and proteolytic enzymes has already been reported in lab-scale [1-4]. Before implementing the chemical stabilization mixture in an industrial scale, the right application form (liquid, freeze-dried, or spray-coated) should be carefully selected and tested in pilot-scale so as to be easily acceptable by authorities and the athletes' community. In the current study the in-house developed chemical stabilization mixture was spray coated in the interior walls of 30-mL urine collection containers. Its efficiency against microorganism elimination and steroid glucuronide degradation was tested in the presence of five microorganisms carefully selected based on the results of the previous relevant study [1].

Experimental

Experimental tests were conducted in 30-mL polypropylene (PP) cups to simulate the doping urine collection process in pilot-scale. 25 mL of pooled urine were spiked in two sets of plastic cups: those whose interior walls were spray coated with the in-house stabilization mixture [1] and plain plastic cups (Picture 1).

 ISBN 978-3-86884-039-1

Picture 1. a) 30-mL medicine cups spray-coated with stabilization mixture: empty (left), filled with 25 mL urine (right), b) 30-mL medicine cups without stabilization mixture: empty (left), filled with 25 mL urine (right).

Both sets were fortified with steroid conjugates (epitestosterone at 200 ng/mL, androsterone and etiocholanolone glucuronides at 2000 ng/mL) and inoculated with three bacteria (*E. coli, N. simplex, E. faecalis*), one fungus (*A. flavus*) and one yeast (*C. albicans*) at 10^5 - 10^6 cfu/mL. The preparation of inoculum was conducted as described in [1]. Two urine aliquots were not inoculated with microorganisms to serve as negative controls. Inoculated urine samples were incubated at 37 °C for 7 days to stimulate the effect of microbial contamination on the steroid profile. Microbial growth was assessed immediately after inoculation (t = 0) and at the end of the incubation period (t = 7) by spreading 100 µl of the appropriate 10-fold serial dilution on Petri dishes with the specific substrate for each species. Variations in pH and specific gravity values were recorded. All urine samples were processed on days 0 and 7 using the routine analysis screening procedure [5] slightly modified to include the isolation and identification of free and total steroidal fractions. Specifically, sample preparation for the analysis of the free fraction consisted of a liquid–liquid extraction with diethylether and derivatization with N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) / ammonium iodide (NH_4I) / dithioerythritol (DTE) (80 °C, 30 min). Enzymatic hydrolysis of the glucuroconjugates was carried out with β-glucuronidase from *E. coli* (50 °C, 1.5 h). Diethylether was used as extraction solvent using Na_2SO_4 as salting out agent. For the GC/MS analysis the organic layer was dried under nitrogen stream at 50 °C and derivatized with MSTFA / NH_4I / DTE (80 °C, 30 min).

Results and Discussion

Microbial growth results in the presence and absence of the in-house chemical stabilization mixture in spray-coated containers are presented in Figure 1 at t = 0 and t = 7.
Cell growth was completely inhibited in spray coated urine containers after a 7-day incubation period at 37 °C (Picture 2).
In the unstabilized aliquots the number of colonies increased over time under the favorable temperature of 37 °C except for *C. albicans*. The mean sg values at t = 0 measured in spray coated urine containers ranged from 1.010 to 1.013 whereas the respective values in unstabilized urine were 1.006 - 1.009 (n = 4). Statistically significant variations with respect to the standard deviation (SD) of the sg of the blank value were found in stabilized urine aliquots ($\Delta sg > 2SD_{blank}$). The mean pH values (n = 4) at t = 0 in spray coated urine containers ranged from 7.7 to 7.9 at t = 0 whereas the respective values in unstabilized urine ranged from 6.7 to 6.8. Statistically significant differences were found in stabilized urine aliquots at t = 0 with respect to the SD of the pH values recorded in blank samples ($\Delta pH > 2SD_{blank}$).

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Deconjugation of epitestosterone glucuronide induced by *E. coli* and *A. flavus* at t = 7 d was prevented in spray coated urine containers. No formation of 5α-/5β-androstane-3,17-diones in the free and total fractions at t = 7 d was observed in spray coated urine containers inoculated with *E. coli*, *N. simplex* and *A. flavus.* Table 1 outlines the alterations in the steroid profile observed after 7 days at 37 °C in the absence of the chemical stabilization mixture.

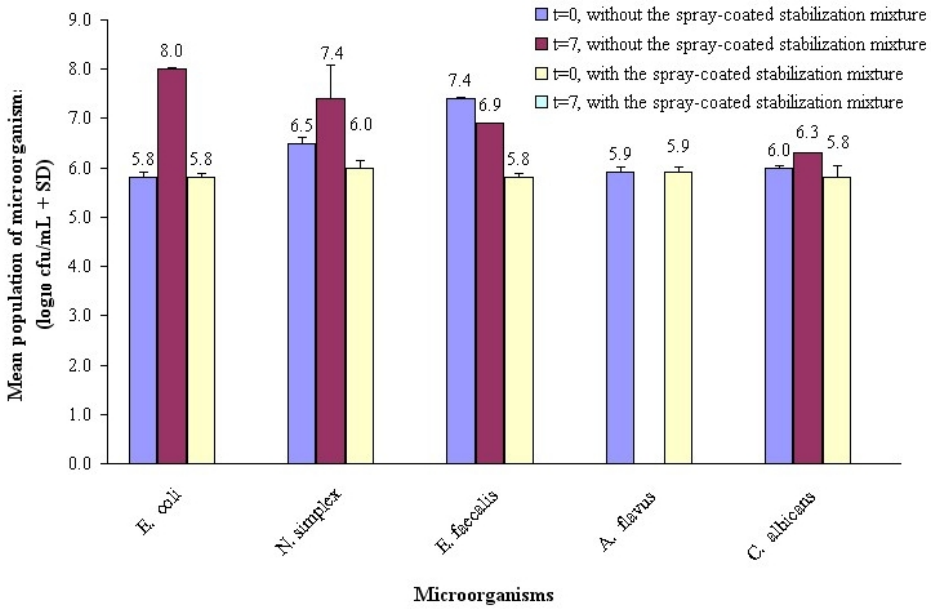
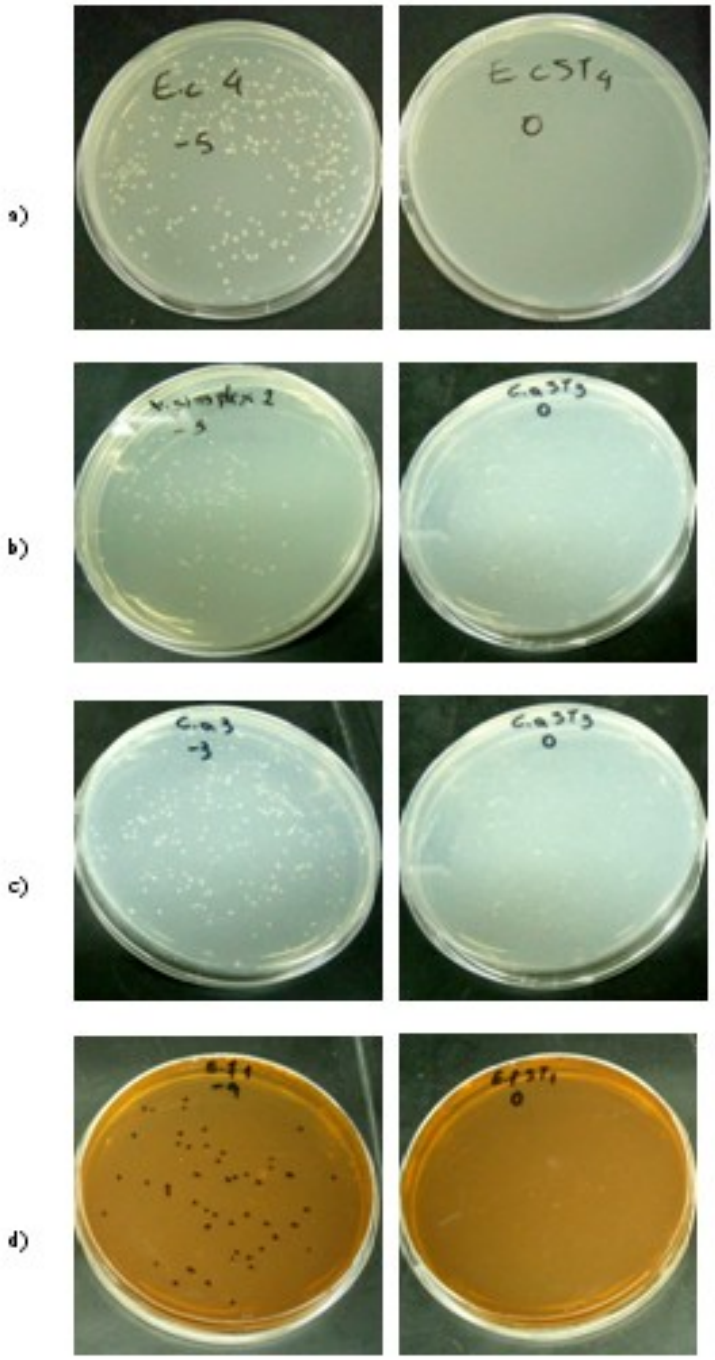


Figure 1. Mean population of microorganisms (log_{10} cfu/mL + SD) in the beginning and at the end of the 7-day incubation period at 37 °C, in the presence and absence of the in-house chemical stabilization mixture in spray-coated form. Bars for t = 7 with the spray-coated stabilization mixture do not appear due to zero values. It was not possible to quantify the population of *A. flavus* at t = 7 with the serial dilution procedure because of mycelial growth in the samples.

Microorganism	Observations
Gram-negative bacteria	t = 7 days at 37 °C
E. coli	1. Formation of 5β-androstane-3,17-dione in the free fraction and 5α-androstane-3,17-dione in the total fraction 2. Deconjugation of epitestosterone glucuronide
Gram-positive bacteria	
N. simplex	Formation of 5α / 5β-androstane-3,17-dione in the free and total fraction
E. faecalis	No changes observed
Fungus	
A. flavus	1. Formation of 5α / 5β-androstane-3,17-dione in the free and total fraction 2. Deconjugation of epitestosterone glucuronide
Yeast	
C. albicans	No changes observed

Table 1. Alterations in the steroid profile of the unstabilized urine samples induced by the microorganisms of the current protocol.

ISBN 978-3-86884-039-1



Picture 2. *E. coli* (a), *N. simplex* (b), *C. albicans* (c) and *E. faecalis* (d) colonies in Petri dishes from counts of the respective bacterial populations in urine stored in spray-coated containers (right) and in simple urine collection containers (left) (t = 7 days). *E. coli* and *N. simplex* were grown in Luria-Bertani broth (LB). *C. albicans* was cultured on potato dextrose agar (PDA) and *E. faecalis* on tryptic soy agar (TSA).

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Conclusions

The addition of the chemical stabilization mixture in spray-coated form in the interior surface of plastic urine containers inhibited microbial growth and prevented steroid degradation at the end of a 7-day incubation period at 37 °C. The spray-coated stabilization mixture has also been tested against intact human chorionic gonadotropin (hCG) degradation by proteases (unpublished data). The implementation of a stabilization mixture in the plastic urine collection containers seems more realistic than it was a few years ago. The evaluation of the efficiency of the spray coated stabilization mixture against recombinant erythropoietin (rEPO) degradation by proteases as well as an estimation of analytical interferences should have to be completed before moving forward to an industrial scale production of sampling containers spray-coated with the stabilization mixture.

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Acknowledgements

This study is supported by a WADA research grant (10A13CG).