M. Tsivou[1)], D. Livadara[1)], D.G. Georgakopoulos[2)], M. Koupparis[3)], J. Atta-Politou[3)], C.G. Georgakopoulos[1)]

Preservation of urine doping control samples – Preliminary results

[1)]Doping Control Laboratory of Athens, OAKA, Maroussi, Greece
[2)]Laboratory of Microbiology, Department of Agricultural Biotechnology, Agricultural University of Athens, Greece
[3)]Laboratory of Analytical Chemistry, Chemistry Department, University of Athens, Greece

Abstract

Sport urine samples transferred to the WADA accredited laboratories may be exposed to high temperatures and improper storage conditions. During this period, microorganisms present in the sample increase in number and may affect sample constituents. Furthermore, the possibility of adulterating samples by masking the abuse of prohibited substances cannot be excluded. Consequently, the need for an effective way of preserving urine specimens prior to the sampling procedure is becoming increasing. A multidisciplinary project funded by WADA, deals with methods of urine stabilization in the collection bottles in order to prevent sample deterioration. The present work summarizes results obtained from the preliminary tests investigating physical and chemical methods of microbial and enzymatic inactivation.

Introduction

Voided urine is often contaminated with urethral, skin, genital or fecal flora (Honour, 1996). Contaminating microorganisms are usually present in low numbers (Hemmersbach et al., 1997) but occasionally the risk of contamination is extremely high, such as in urinary tract infections with levels of microorganisms higher than $1x10^4$ cfu[1]/ml. Once the bacteria are introduced in urine samples, they will continue to multiply in the warm medium of freshly voided urine, as well as under favorable conditions during storage and transportation (e.g. temperatures above 15°C). The minimum time required for contaminants to reach a population of 10^5-10^8 cfu/ml is variable and depends upon the growth rate of the

[1]colony forming units

microorganism, the initial degree of contamination and the ambient temperature (Lauer et al., 1979).

In the presence of microorganisms, steroids conjugates may undergo chemical modifications (cleavage and/or oxido-reductive reactions) (Geyer et al., 1995; Ayotte et al., 1996; Ayotte et al., 1997; Van de Kerkhof et al., 2000; de la Torre et al., 2001). Furthermore, androgen production is possible with a wide variety of bacterial species that can be found in human urine (Bilton, 1995; Kicman et al., 2002). Moreover, testosterone can be converted to boldenone and metabolites by the action of specific microorganisms (Rautenberg et al., 2006; Grosse et al., 2007; Piper et al., 2007). Endogenous androsterone and etiocholanolone may be converted into the corresponding 19-norsteroids (Thieme et al., 2004; Grosse et al., 2005; Rautenberg et al., 2005). Also, enzymes produced by microbial infection of unpreserved samples may cause nicking of hCG molecule and subsequent dissociation of gonadotropins (Cole, 1997; Kardana and Cole, 1997; Muller et al., 1999). The shift in the EPO pattern towards the basic values of the pH gradient has been reported (Lasne et al., 2005; Belalcazar et al., 2006; Desharnais et al., 2006). Finally, in the last couple of years, anti-doping science was concerned about the adulteration of urine by the addition of proteases masking EPO abuse (Maurer et al., 2007; Thevis et al., 2007; Dehnes et al., 2007; Lamon et al., 2007).

According to the WADA Code for the collection of sport urine samples, no stabilization method is applied in the urine collection material. In the present study, the possibility of applying a suitable preservation method to the sample collection vessels was examined and preliminary data are presented. Traditionally applied physical methods of microorganism control include heating, radiation and filtration. All these methods have drawbacks regarding their application in the collection of the large volume urine specimens. As for chemical methods, a wide variety of chemicals are available for controlling microbial growth and enzymatic action. We decided to conduct experiments using a mixture of preservatives instead of testing the efficacy of several antimicrobials one by one. In this way, the action of protein synthesis and enzyme inhibitors was combined in one single mixture with a wide range of activity against eukaryotic, prokaryotic cells and proteolytic enzymes.
The experimental protocol focuses on inoculation of pooled urine under controlled conditions with eleven prokaryotic (bacteria) and eukaryotic microorganisms (fungi, yeasts, protozoa) as well as six proteolytic enzymes. It was considered impossible to carry out tests

with all microorganisms and enzymes that may be present in urine samples. We had to make a choice based on literature and select representative microbial species from various families as well as proteases from different origin. So the eleven species selected for the experimental protocol are related with indoor air, human microbial flora, urinary tract infections and possess the enzymes capable of altering the steroids profile under certain conditions. Moreover, the six proteolytic enzymes involved in the study belong to 3 different classes (serine, cysteine, aspartic) and have various origins (plant, animal, microbial).

Before implementing the experimental protocol, preliminary tests were carried out in order to i) substantiate the ability of two physical methods - membrane filtration and ultraviolet radiation - as sterilizing means for urine samples, ii) evaluate the preservative ability of a mixture of antimicrobial agents on pooled urine inoculated with seven microbial species, and to iii) examine whether the addition of protease inhibitors to urine specimens could prevent the enzymatic digestion of EPO by proteases. Selected results from the preliminary tests are being presented here.

Materials and Methods

Membrane filtration method

Urine samples of 100ml volume and specific gravities within the range 1.005 - 1.010, were forced through sterile plastic disposable syringes with a 50ml capacity (Sartorius AG, Goettingen, Germany) and combined with two different kinds of syringe-driven filter units of a 0.45μm pore-size: the Minisart RC25 syringe-driven filter units (Sartorius AG, Goettingen, Germany) and the sterile Millex-HV filter units with a Low Protein Binding Durapore (PVDF) membrane (Millipore, Cork, Ireland). Each sample was split in 50 ml aliquots and the filtration time was recorded.

Ultraviolet exposure

The potential inactivation of three selected strains of microorganisms in urine samples by ultra violet irradiation emitted by a handheld lamp was examined. More specifically, three urine samples of 100ml volume in sterile disposable Petri dishes with dimensions 12cm x 12cm x 1.2cm, were inoculated with three selected species: the bacterium *Escherichia coli,* the yeast *Candida albicans* and the fungus *Aspergillus niger*. Final concentration of the microorganisms was as follows: $3.3x10^6$ cfu/ml for *E. coli,* $1x10^5$ cfu/ml for *C. albicans* and $4.2x10^4$ cfu/ml for *A. niger.* The types of media used for inoculum preparation were potato

dextrose agar (PDA) for *A. niger*, yeast mannitol glucose broth (YMG) for *C. albicans* and Luria broth (LB) for *E. coli*. One urine sample was inoculated per microorganism. The samples were exposed to shortwave UV radiation (265nm) for ten minutes at a 1cm distance from a shortwave handheld UV lamp (model UVGL-55, UVP Inc., CA, USA). Immediately after exposure, 10-fold serial dilutions were prepared from each set. All plates were incubated at 37ºC for 24h and the numbers of viable cells were determined by the serial dilution method.

Incubation with microorganisms

Another preliminary test was conducted in order to evaluate the sensitivity of various microorganisms to the proposed stabilizing mixture without any addition of reference steroid compounds. Seven species of microorganisms were selected, representative of Gram-negative and positive bacteria, yeasts, fungi. Specifically, *E. coli* was included in the study as it is associated with 70 to 85 per cent of all acquired urinary tract infections (UTI) in young females. *Staphylococcus epidermidis*, commonly found on the surface of the skin, was included because of its ability to induce pH changes, whereas, *Pseudomonas aeruginosa* produced minor changes in the steroid profile in a relevant study (de la Torre et al., 2001). *Nocardioides simplex* is part of the intestinal bacterial flora and possesses high Δ1-steroid dehydrogenase activity (Piper et al., 2007). The yeast *C. albicans* is the most common fungal pathogen isolated from urine samples and was found to convert androst-4-en-dione-3,17 to testosterone under certain circumstances (Kicman et al., 2002). The fungi *Aspergillus* and *Penicillium* are ubiquitous in indoor air.

For inoculum preparation, PDA was used for *A. niger, C. albicans, P. expansum*, LB for *E. coli, N. simplex, P. aeruginosa* and Nutrient agar (NA) for *S. epidermidis*. Aliquots (n=3) were inoculated with seven different microorganisms to the level of $1x10^6$ to $1x10^7$ cfu/ml. Untreated samples and samples containing the preservatives mixture, as depicted in Table 1, were incubated for 7 days at 37ºC. Bacterial growth in urine was assessed immediately after inoculation (t=0) and one week later (t=7) by the tenfold serial dilution method.

Table 1. Proposed mixture of preservatives

Name	Substance	Company	Concentration
Sodium azide	Sodium azide	Sigma	10mg/ml
Antibiotic/antimycotic mixture	Penicillin G sodium Streptomycin sulfate Amphotericin B	Gibco/Invitrogen	2ml/100ml urine
Anti-protease mixture	AEBSF[2] E-64[3] Bestatin.HCl Leupeptin.HCl Aprotinin EDTA.Na	Sigma	1g cocktail/g protease
Specific serine protease inhibitor	PMSF[4]	Sigma	1mM
Pepstatin	Pepstatin	Sigma	2.4mM
Specific trypsin inhibitor	Soybean trypsin inhibitor	Sigma	2g trypsin inhibitor/g trypsin

[2] (4-(2-aminoethyl) benzenesulfonyl fluoride
[3][N-[N-[L-3-trans-carboxirane-2-carboxyl]-L-leucyl]-agmatine]
[4]phenylmethylsulfonyl fluoride

Addition of proteases

The possibility of preventing EPO digestion in urine samples by different animal, plant and microbial proteases was investigated using various commercial protease inhibitors. A series of 10ml-urine specimens enriched with rEPO BRP (Biological Reference Preparation of the European Pharmacopoeia Commission) were spiked with 100µg/ml of common proteases with high enzymatic activity, all purchased from Sigma-Aldrich, Germany, in powder form (Table 2). Prior to the addition of proteases, urine samples were fortified with different proteases inhibitors at concentrations two to three-fold higher than the ones recommended by the manufacturing company. After incubation at 37°C for 1 hour, all aliquots were analyzed for total EPO content using the automated chemiluminescent Immulite EPO kit (Diagnostics Products Corporation, LA, USA).

Another experiment was conducted the same way as the first, though a longer incubation period was applied (1 week at 37°C) for three out of the six selected proteases.

Table 2. Proteolytic enzymes selected

Classification	Proteases	Origin	Enzymatic activity (units/mg protein)
		ANIMAL	
Serine	α-chymotrypsin	bovine pancreas	98.7
Serine	trypsin	bovine pancreas	8801
Aspartic	pepsin	porcine gastric mucosa	3200-4500
		PLANT	
Cysteine	bromelain	pineapple stem	11.5
Cysteine	papain	*Carica papaya*	10.5
		MICROBIAL	
Serine	subtilisin A	*Bacillus licheniformis*	7.8

Results and Discussion

Membrane filtration method

The results from the membrane filtration preliminary test showed that the filtration process cannot be applied for the removal of bacteria from the large-volume doping-control specimens because it was found impossible to force urine samples through the syringe in a reasonable time due to the clogging of the filter.

Ultraviolet exposure

No decrease whatsoever was observed in the cell concentration of *E. coli, C. albicans, A. niger* in UV-exposed urine (Table 3), under the experimental conditions of the test. The absence of any germicidal effects of UV radiation might be attributed to the fact that the UV dose applied was inadequate or the exposure time too short to kill microorganisms.

Table 3. Effect of UV exposure on the population of selected microorganisms

Microorganism	Initial population (cfu/ml)	Final population (cfu/ml)
Escherichia coli	$3.3x10^6$	$3.3x10^6$
Candida albicans	$1.0x10^5$	$1.3x10^5$
Aspergillus niger	$4.2x10^4$	$8.6x10^4$

Incubation with microorganisms

Table 4 shows the results of bacterial counts for the seven microorganisms inoculated into urine samples containing or not the stabilizers mixture. Cell growth was completely inhibited in urine samples treated with the antimicrobial substances, whereas microorganisms survived in all untreated samples.

Table 4. Population of microorganisms incubated for 7 days at 37ºC with and without stabilizers

Microorganisms	Treatment	Initial population cfu/ml (t=0)	Final population cfu/ml (t=7days)
Gram-negative bacteria			
E. coli	without stabilizers	$2.0x10^7$	$3.6x10^7$
E. coli	**stabilizers mixture**	**$2.2x10^7$**	**0**
P. aeruginosa	without stabilizers	$5.5x10^7$	$2.8x10^8$
P. aeruginosa	**stabilizers mixture**	**$1.3x10^7$**	**0**
Gram-positive bacteria			
S. epidermidis	without stabilizers	$5.8x10^6$	$4.9x10^7$
S. epidermidis	**stabilizers mixture**	**$1.0x10^6$**	**0**
N. simplex[5]	without stabilizers	$5.1x10^7$	$2.6x10^2$
N. simplex	**stabilizers mixture**	**$1.0x10^7$**	**0**
Yeast			
C. albicans	without stabilizers	$1.0x10^6$	$9.0x10^8$
C. albicans	**stabilizers mixture**	**$2.0x10^6$**	**0**
Fungi			
A. niger	without stabilizers	$1.3x10^7$	$2.5x10^2$
A. niger	**stabilizers mixture**	**$1.3x10^6$**	**0**
P. expansum	without stabilizers	$1.7x10^7$	$1.4x10^3$
P. expansum	**stabilizers mixture**	**$1.3x10^7$**	**0**

[5]former *Corynebacterium simplex*

Addition of proteases

As reported previously (Lamon et al., 2007), the presence even of 5µg/ml of protease in sport urine samples is sufficient to eliminate EPO signal. We decided to conduct tests using higher concentration of proteases (100µg/ml) so as to investigate whether the selected protease inhibitors could inhibit relatively high enzymatic activities. From the examination of Figure 1, illustrating the results of the various tests conducted, the addition of six proteases in urine aliquots did result in low EPO concentrations. However, we demonstrated that the inclusion of the right combination of protease inhibitors into the urine containers prevented EPO degradation in a great extent. This conclusion was further confirmed for 3 of the proteases tested after a longer incubation period (Table 5).

Figure 1. Effect of protease addition (100µg/ml) in rEPO concentration (t = 1hr at 37°C)

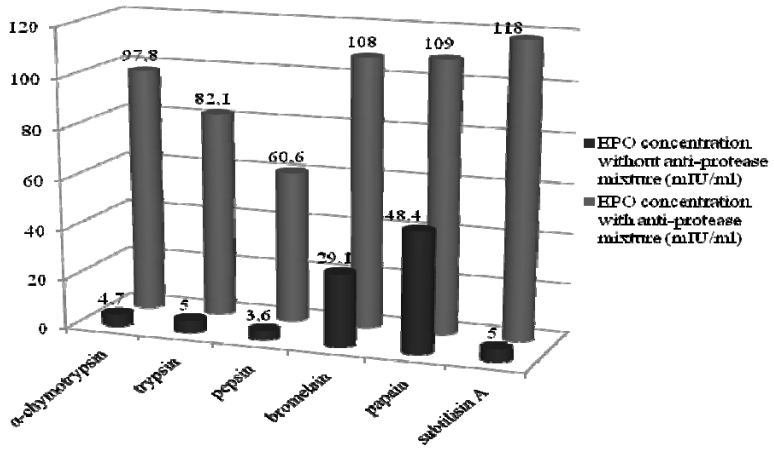

Table 5. Effect of protease addition (100µg/ml) in rEPO concentration (t = 7 days at 37°C) (n=3)

Protease	EPO concentration (mIU/ml) t=0	EPO concentration (mIU/ml) t=7 days
papain	14.6	4.3
papain + stabilizers mixture	**>200**	**>200**
bromelain	24.2	4.4
bromelain + stabilizers mixture	**>200**	**>200**
trypsin	4.6	3.5
trypsin + stabilizers mixture	**>200**	**192.3**

Conclusions

Two physical methods of microorganism control (UV radiation and membrane filtration) were carefully considered but were found not to work properly for the preservation of sport urine samples. Thus, a chemical mixture is proposed as a stabilization treatment for urine samples consisting basically of three main components: sodium azide, an antibiotic/antimycotic solution and a cocktail of various protease inhibitors. Preliminary experiments showed that microbial growth was completely inhibited in the presence of the preservatives mixture. Moreover, the addition of protease inhibitors prevented EPO degradation to a great extent. The results obtained from the different preliminary tests show that the addition of the suitable preservation mixture to urine collection vessels may be applicable for use in the sampling protocol.

Acknowledgements

This study is supported by a WADA research grant (05D6CG). Part of this work is the subject of the Master's Degree of Miss D. Livadara. The authors are also grateful to

Professor N. Legakis (Department of Microbiology, Faculty of Medicine) for the fruitful discussions. The cooperation of Professor Ath. Tsakris, Assistant Professor Ar. Velegraki and Lecturer J. Papaparaskevas (Department of Microbiology, Faculty of Medicine) in the provision of selected microorganisms is highly acknowledged.

References

Ayotte C, Goudreault D, Charlebois. A. (1996) Testing for natural and synthetic anabolic agents in human urine. J Chromatogr B **687**, 3-25.

Ayotte C, Charlebois A, Lapointe S, Barriault D, Sylvestre M. (1997) Validity of urine samples: microbial degradation. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U. (eds.). *Recent Advances in Doping Analysis (4)*, Köln, pp 127-137.

Belalcazar V, Gallego RG, Llop E, Segura J, Pascual JA (2006) Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine. Electrophoresis **27**, 4387-4395.

Bilton RF. (1995) Microbial production of testosterone. Lancet **345**, 1186-1187.

Cole LA (1997) Stability of hCG free β-subunit and β-core fragment in urine. Prenat Diagn **17**(2), 185-189.

Desharnais P, Groleau PE, Hamelin C, Ayotte C. (2006) Intermediate neuraminidase activity: a possible link toward suspicious or "effort-like" EPO isoelectric profiles. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (14),* Köln, pp 435-438.

Dehnes Y, Borgen M, Hemmersbach P. (2007) Enzymatic digestion of EPO. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (15)*, Köln, pp 405-408.

de la Torre R, de la Torre X, Alia C, Segura J, Baro T, Torres-Rodriguez, JM. (2001) Changes in androgenic steroid profile due to urine contamination by microorganisms: A prospective study. Anal Biochem **289**, 116-122.

Geyer H, Schanzer W, Mareck-Engelke U, Donike M. (1995) Factors influencing the steroid profile. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U. (eds.) *Recent Advances in Doping Analysis (3),* Köln, pp 95-113.

Grosse J, Anielski P, Hemmersbach P, Lund H, Mueller RK, Rautenberg C, Thieme D (2005) Formation of 19-norsteroids by in situ demethylation of endogenous steroids in stored urine samples. Steroids **70**, 499-506.

Grosse J, Rautenberg C, Wassill L, Ganghofner D, Thieme D. (2007) Degradation of doping-relevant steroids by *Rh. erythropolis*. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (15)*, Köln, pp 385-388.

Hemmersbach P, Birkeland KI, Norli HR, Ringertz SH. (1997) Urine storage conditions and steroid profile analysis. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U. (eds.) *Recent Advances in Doping Analysis (4)*, Köln, pp 99-106.

Honour JW. (1996) Testing for drug abuse. Lancet **348**, 41-43.

Kardana A and Cole LA. (1997) The stability of hCG and free β-subunit in serum samples. Prenat Diagn **17**(2), 141-147.

Kicman AT, Fallon JK, Cowan DA, Walker C, Easmon S, Mackintosh D. (2002) *Candida albicans* in urine can produce testosterone: Impact on the Testosterone/Epitestosterone Sports Drug Test. Clin Chem **48**(10), 1799-1801.

Lamon S, Robinson N, Sottas P-E, Henry H, Kamber M, Mangin, P, Saugy M (2007) Possible origin of undetectable EPO in urine samples. Clin Chim Acta **385**, 61-66.

Lasne F, Martin L, de Ceaurriz J. (2005) Active urine and detection of recombinant erythropoietin. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (13)*, Köln, pp 297-304.

Lauer BA, Reller LB, Mirrett S. (1979) Evaluation of preservative fluid for urine collected for culture. J Clin Microbiol **10**(1), 42-45.

Maurer J, Kohler M, Schänzer W, Thevis M. (2007) Identification of proteolytic enzymes in human urine. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (15)*, Köln, pp 283-291.

Muller F, Doche C, Ngo S, Faïna S, Charvin M-A, Rebiffe M, Taguel V, Dingeon B. (1999) Stability of the free β-subunit in routine practice for trisomy 21 maternal serum screening. Prenat Diagn **19**, 85-88.

Piper T, Hebestreit M, Flenker U, Geyer H, Schänzer W. (2007) A new method to determine $\delta^{13}C_{VPDB}$ values of trace amounts of boldenone and its main metabolite. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (15)*, Köln, pp 169-178.

Rautenberg C, Grosse J, Anielski P, Thieme D, Hemmersbach P, Lund HS, Mueller RK. (2005) Aspects of in situ formation of 19-norsteroids. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (13)*, Köln, pp 449-452.

Rautenberg C, Grosse J, Thieme D, Wassill L, Ganghofner D, Mueller RK. (2006) Testosterone degradation induced by *Rh. erythropolis*. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (14)*, Köln, pp 379-382.

Thevis M, Maurer J, Kohler M, Schänzer W. (2007) Proteases in doping control analysis. Int J Sports Med **28**, 545-549.

Thieme D, Anielski P, Grosse J, Hemmersbach P, Lund H and Rautenberg C. (2004) Kinetic of in-situ demethylation of deuterated endogenous steroids in urine samples. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (12)*, Köln, pp 177-188.

Van de Kerkhof, DH, de Boer D, Thijssen JHH, Maes RAA. (2000) Evaluation of Testosterone/Epitestosterone ratio influential factors as determined in doping analysis. J Anal Toxicol **24**, 102-115.