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Fluorescence flow cytometer to determine urine particle reference intervals in doping control samples☆

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ABSTRACT

Background: Urine is still the matrix of choice to fight against doping, because it can be collected non-invasively during anti-doping tests. Most of the World Anti-Doping Agency's accredited laboratories have more than 20 years experience in analyzing this biological fluid and the majority of the compounds listed in the 2010 Prohibited List – International Standard are eliminated through the urinary apparatus. Storing and transporting urine samples for doping analyses does not include a specific protocol to prevent microbial and thermal degradation. The use of a rapid and reliable screening method could enable determine reference intervals for urine specimens in doping control samples and evaluate notably the prevalence of microbial contamination known to be responsible for the degradation of chemical substances in urine.
Methods: The Sysmex® UF-500i is a recent urine flow cytometer analyzer capable of quantifying BACT and other urinary particles such as RBC, WBC, EC, DEBRIS, CAST, PATH.CAST, YLC, SRC as well as measuring urine conductivity. To determine urine anti-doping reference intervals, 501 samples received in our laboratory over a period of two months were submitted to an immediate examination. All samples were collected and then transported at room temperature. Analysis of variance was performed to test the effects of factors such as gender, test type [in-competition, out-of-competition] and delivery time.
Results: The data obtained showed that most of the urine samples were highly contaminated with bacteria. The other urine particles were also very different according to the factors.
Conclusions: The Sysmex® UF-500i was capable of providing a snapshot of urine particles present in the samples at the time of the delivery to the laboratory. These particles, BACT in particular, gave a good idea of the possible microbial degradation which had and/or could have occurred in the sample. This information could be used as the first quality control set up in WADA (World Anti-Doping Agency) accredited laboratories to determine if steroid profiles, endogenous and prohibited substances have possibly been altered.

1. Introduction

Urine samples have been collected for decades for anti-doping purposes [1,2]. Urine is still the most common matrix to detect the majority of the prohibited doping agents commonly used to improve performances. Unfortunately, because there are no specific pre-analytical protocols, nearly all of the samples are contaminated by microorganisms [3]. These organisms usually come from the urethral and the bladder flora or from environmental contamination [4]. Very rapidly, these organisms multiply especially if urine samples are not kept properly. Consequently, urine samples get degraded over time and temperature. Several publications have notably reported some of the degradation effects. There is notably the hydrolysis of steroid conjugates, increase of testosterone in the free fraction, formation of metabolic by-products, production of boldenone, degradation of hCG (human chorionic gonadotrophin), LH (luteinizing hormone) or EPO [5–9]. To prevent these possible degradations, various strategies have suggested. There are notably physical and chemical methods. The most common physical method to prevent microbial growth is to keep urine samples refrigerated or frozen. Other physical methods have been suggested such as heat sterilization, membrane

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Abbreviations: WADA, World Anti-Doping Agency; EPO, erythropoietin; BACT, bacteria; RBC, red blood cell; WBC, white blood cell; EC, epithelial cell; DEBRIS, debris; CAST, casts; YLC, yeast like cells; SRC, small round cell; PATH.CAST, pathological cast; COND, conductivity; X'TAL, crystal; CI, confidence interval; hCG, human chorionic gonadotrophin; LH, luteinizing hormone; K2EDTA, di-potassium ethylenediaminetetraacetic acid; IC, in-competition test; OOC, out-of-competition test; RBP, retinol binding protein; pH, potential of hydrogen; SD, standard deviation.

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filtration, ultraviolet irradiation and ultrasonication. Unfortunately most of these methods are impractical with respect to the anti-doping analyses sampling protocol; furthermore, they are incompatible with anti-doping analyses as they are usually too expensive and can be applicable only to thermostable molecules. Another option to prevent urine degradation is the use of chemical methods such as sodium azide, antibiotic and antimycotic as well as antiprotease mixtures [6]. Unfortunately and until now, this approach is regarded by athletes with some suspicion as introducing the possibility for tampering with the sample. The use of coated bottles could be an option to dispel any doubts – this is already the case for blood samples used for anti-doping purposed as the collection tubes are coated with K2EDTA – but unfortunately most of the stabilizing agents interfere with some of the analyses. Furthermore, none of the mentioned chemical stabilizing agents possesses the wide antimicrobial and antifungal spectrum as well as broad specificity against proteolytic enzymes. As a result, a cocktail of chemical agents would be needed to cover the required needs. Some very encouraging tests have been conducted recently and a cocktail of chemical agents may be used in a close future to stabilize athletes urine samples [10].

Urine particle analyzers such as the Sysmex® UF-500i are fluorescent flow cytometers mainly intended for microbiological diagnostics (BACT) [11–14]. Additional variables are measured and provide valuable quantitative information on the size and staining properties of the particles. They are classified as RBC, WBC, EC and CAST. Apart these variables, research variables are also evaluated such as COND, X'TAL, YLC, SRC, PATH.CAST, MUCUS and DEBRIS. Sperm concentration can be determined, but it was not examined as it could be considered as an invasion of athlete privacy and especially it is not useful in an anti-doping context. The aim of this study was to establish urine particle reference intervals in doping control samples. In that way, it is possible to evaluate the urine contamination level after uncontrolled pre-analytical collection and transport conditions. The data obtained suggest that doping control urine samples should be stabilized in the future using a mixture of chemical agents. Meanwhile, the level of BACT and/or YLC contamination could provide an insight of the possible alteration of urine samples. Thus, acceptance criteria should be set up accordingly. All samples with elevated BACT and/or YLC contamination should be discarded to avoid the misinterpretation of analytical data. The other variables could be of some interest to evaluate the hydration status of athletes, the preservation conditions of urine samples or eventually provide additional variables to identify what is so called "effort urines" [10,15,16]. These latter are urine samples collected immediately after an intense physical effort and contain a lot of proteins due to an exercise-induced transient renal dysfunction.

2. Materials and methods

2.1. Urine samples

The study was conducted according to the Declaration of Helsinki as amended in the 41st World Medical Assembly. Prior to routine anti-doping analyses, all urine samples were vigorously agitated and then 3 ml were deposited into an anonymized tube (Sarstedt, Nümbrecht, ref. 60.549). This aliquot was then used to perform the research described in this manuscript. Most of the anti-doping urine samples ($n = 501$) received in our laboratory over a period of two months (March until Mail 2010) were submitted to an immediate cytobacteriological exam with a Sysmex® UF-500i automated urinalysis analyzer (Sysmex Europe GmbH, Norderstedt, Germany). All samples were transported from the place of urine sampling to our laboratory at room temperature and there was no indication of temperature follow up during the entire transport (no datalogger). It was decided to classify the time necessary to deliver the urine samples to the laboratory into two arbitrary periods, the short and long period. The short period corresponded to a delivery time period of less than 3840 min (3840 min corresponded to the median delivery time period of all urine samples). All other urines samples were classify into the long period. Gender and test type [in-competition (IC), out-of-competition (OOC)] were collected from official doping control forms.

2.2. Sysmex® UF-500i

The Sysmex® UF-500i is a fully automated fluorescent flow cytometer able to classify and count cells and formed particles in native (unthawn) uncentrifuged urine samples [17]. This instrument can theoretically analyze up to 50 samples/h. All urine anti-doping samples were analyzed in manual mode in accordance to the manufacturer's recommendations. To avoid any possible carryover, the number of rinses was set in the software as the following according to the BACT concentration: 1.0×10^4 BACT/ml: 0 rinses; 1.0×10^5 BACT/ml: 1 rinse; 1.0×10^6 BACT/ml: 2 rinses; 1.0×10^7 BACT/ml: 2 rinses; 1.0×10^8 BACT/ml: 2 rinses. The 3 ml urine aliquots were homogenized for 15 min. on a roller mixer and then inverted manually at least 10 times prior to analyses.

2.3. Data analysis

All statistical analyses were performed on Matlab® Version 6.1.0 with Statistics Toolbox Version 3.0. A multiple way analysis of variance was performed for testing the effects of factors gender [male, female], test type [IC, OOC] and delivery time [short, long] on the variables RBC, WBC, EC, CAST, BACT, COND, X'TAL, YLC, SRC, PATH.CAST, MUCUS and DEBRIS (501 readings per variable).

3. Results

The sharing out of the samples is given in detail in Table 1. In summary, there were 405 male urine samples, 96 female urine samples, 292 OOC (out-of-competition tests) urine tests and finally 209 IC (in-competition tests). The median period to deliver the 501 urine samples was equal to 3840 min with a mean value of 4770 min. The overall distribution time necessary to deliver all urine samples to our laboratory is given in Fig. 1. Table 2 provides in detail the sharing out of the delivery periods according to the various test types (OOC versus IC).

Table 3 summarizes the statistical significance of heterogeneous factors (gender, test type and delivery time) on the variables measured on the 501 anti-doping urine samples. All p-values <0.05 are highlighted in bold. RBC, WBC, CAST, BACT, SRC and PATH.CAST were significantly different between male and female urine samples. EC, CAST, COND, X'TAL, SRC, PATH.CAST and DEBRIS were significantly different between IC and OOC tests. Finally, BACT and COND were significantly different between short and long delivery time periods.

The reference values of the variables showing significant differences (p-value <0.01, RBC excepted) due to heterogeneous factors are depicted in Fig. 2A and B. All box plots show the five statistics (minimum, first quartile, median, third quartile and maximum) and each individual legend give the number of observations, the mean and the 95% CI of the various variables.

Table 1
Sharing out of the 501 anti-doping urine samples according to gender, test type and delivery time.

	Short			Long			Total male	Total female	Total
	Male	Female	Total	Male	Female	Total			
OOC	131	23	154	82	56	138	213	79	292
IC	97	10	107	95	7	102	192	17	209
Total	228	33	261	177	63	240	405	96	501