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The Kinetics of Ethanol Markers and the Impact on Steroid Profile upon Bacterial Contamination of Urine Doping Control Samples with *E. coli*

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Abstract

Background: The oral intake of ethanol in large amounts may alter the endogenous anabolic steroid profile parameters in anti-doping testing by slightly increasing Testosterone/Epitestosterone (T/E) and decreasing Androsterone/Testosterone (And/T) ratios. Hence the estimation of ethanol levels in urine specimens is of utmost importance for ensuring proper evaluation of the longitudinal steroid profile of the athletes. World Anti-Doping Agency (WADA) Accredited Laboratories regularly monitor Ethyl glucuronide (EtG), an ethanol metabolite, as a marker to indicate ethanol intake by LC-MS/(MS). Any concentration of EtG above 5 μg/ml should be reported in the Anti-Doping Administration & Management System (ADAMS) as a confounding factor. Previous evidence reports that presence of bacteria and especially glucuronidase activity-possessing microorganisms such as *E. coli* might influence the normal kinetics of EtG, whereas no degradation of ethyl sulphate (EtS) has been reported. The present case-study questions the stability of EtG in the presence of an *E. coli* strain in correlation with the steroid profile parameters. Thus, it was conceivably hypothesized that bacterial contamination of urine samples with microorganisms possessing glucuronidase activity such as *E. coli* induces the enzymatic hydrolysis and hence the degradation of the EtG, which is further linked with alterations of the steroid profile parameters and specifically T/E and And/T ratios. Methods: A metabolic study was conducted using urine specimens collected by one healthy subject (25 years of age) after administration of 200 ml of 40% alcohol. Pooled urine was inoculated with the *E. coli* strain GM00108. Aliquots with and without *E. coli* were incubated at 37°C for 15 days. EtG and EtS levels were measured by direct injection LC-MS. Endogenous anabolic androgenic steroids (EAAS) were processed according to a modified screening procedure and analyzed using GC-MS.

Results: LCQTOF measurement demonstrated significantly higher EtG concentration at t=72h in the inoculated samples compared to the non-inoculated ones which suggests synthesis of EtG. On the other hand, EtS seemed not to lose its stability upon contamination of urine with that specific *E. coli* strain compared to the non-inoculated controls. The current study also reveals that apart from testosterone, total levels of steroid profile parameters were not *E. coli* concentration dependent. The microorganism effectively deconjugated those parameters nonetheless.

Conclusion: Based on the up-to-now results, no conclusion can be drawn that the particular *E. coli* strain degraded EtG, however its post-collection synthesis at t=72 h correlates with previous literature. As to the association of the findings with the steroid profile parameters, reduction of conjugated fraction and subsequent production of free steroids has been observed. In the future, additional representative microorganisms (bacteria, yeasts, fungus) and/or other *E. coli* strains should be investigated.

Keywords: EtG; EtS; Steroid profile; *E. coli*; LCQTOF; GC-MS; WADA; Conjugated fraction; Free fraction

Introduction

The definition and impact of doping

One of the most predominant obstacles in achieving fairness in sporting events worldwide is the global problem which is well-known as doping [1]. The formal definition of doping is described in the first two articles of the World Anti-Doping Code and it includes the presence of a prohibited substance or its metabolite(s) or its markers in an athlete's sample, any attempt of tampering a sample, the denial to provide a doping control sample and the possession of a prohibited substance or method (World Anti-doping Code, 2018). Prohibited substances mainly act by increasing strength or generating a more efficient mechanism of oxygen transport to optimize the aerobic state [2,3]. The compounds which yield performance-enhancing function mainly include anabolic agents, hormones, beta-2-agonists, anti-estrogenic drugs, corticosteroids, stimulants, narcotics and several other compounds with mechanism of action that differs from one another [4,5].

Complications derived from performance-enhancing drug (PED) use

Notwithstanding the performance-enhancing properties of several natural or synthetic compounds which are used in doping, those pharmaceutical agents can trigger detrimental complications to the body [6]. Prior research has thoroughly investigated the repercussions derived from the use of PEDs and the clinical manifestation upon their administration is often accompanied by cardiomyopathy and acute

myocardial infarction [7,8]. Further evidence supports that PEDs users are more prone to atherosclerosis due to increased presence of low-density lipoprotein compared to high- density lipoprotein which is reduced [8,9]. Furthermore, data previously generated, has reported psychiatric clinical manifestation in anabolic steroid users such as aggressiveness, hyperactivity, loss of libido, reckless behavior, as well as depression which is derived from withdrawal [10,11]. However ample support has been provided to the fact that mood disorders triggered by steroid use are dose dependent [8].

Preliminary data provides confirmatory evidence contemplating the complications which anabolic steroids can cause to the neurons. This data is further supported by spatial memory impairment triggered in rats upon androgenic anabolic steroid (AAS) exposure mainly due to increased apoptosis of neurons as well as increased oxidative stress-induced mitochondrial dysfunction [8,12,13].

Research has also consistently shown that the administration of anabolic steroids can induce hypothalamic-pituitary dysfunction causing cessation of natural testosterone production [14,15].

To date there has been some agreement on the fact that PEDs and mainly AAS can cause liver toxicity [16]. However the hepatotoxic complications derive mostly from the use of 17a-alkylated AAS [8,17]. The administration of PEDs can transit to further clinical outcomes such as renal failure. This is grounded on the notion that anabolic steroids can elevate creatinine levels which is associated to kidney toxicity [18].

To make matters worse, the administration of banned substances undermines great values which are taught by sport such as commitment, honest endeavour and fair play [1,19]. To that end, given the fact that the use of performance-enhancing drugs (PEDs) contributes to the development of harmful effects on athletes, as well as corrupting the fair competition in depth, the practice of doping will lead to disqualification from any competition [19].

The use of PEDs has also become a serious public concern. On the basis of the evidence that is currently available the number of participants in youth sports has been tremendously increased, leading to increased stress to perform at high levels mainly due to pressure for parents or coaches and their susceptibility to experimentation and risk- taking behaviours in order to surpass their limitations [20]. Previous work has also focused on contaminated nutritional supplements by doping substances which our found in "black market" making them more accessible by adolescent athletes [21].

World anti-doping agency

Strong evidence suggests that in recent years the market for doping substances has been considerably grown and it has been spreading into schools and health clubs worldwide [4]. The World Anti-Doping Agency (WADA) was established in 1999 and it is an international, independent organization that monitors and regulates the fight against doping in sport at the international level through a uniform set of anti-doping rules, the World Anti-Doping Code (Code). As the Olympic Movement governing body, Internation Olympic Committee (IOC) makes the Code mandatory for the entire Movement. Under its supervision, doping tests were first carried out during the Winter Olympic Games in Grenoble and during the Summer Games in Mexico City in 1968. IOC has set as a top priority the protection of clean athletes and has established a zero- tolerance policy to combat cheating and to make anyone responsible for using or providing doping products accountable. WADA aims to create an environment free of PEDs in order to optimize the welfare of athletes by authorizing an annually updated list of prohibited substances which are banned from any competition [19,22].

Athletes biological passport

A programme on longitudinal profile was developed that analyzes indirect doping biomarkers known as the Athlete's Biological Passport (ABP) [23]. The haematological module of the ABP has been implemented in 2008 by certain international sport federations and since then this indirect methodology has resulted in sanctioning athletes for anti-doping rule violation. This achievement encouraged the expansion of the ABP to establish the intraindividual reference ranges to monitor the steroid profile of an athlete [24]. Thus, WADA accredited laboratories routinely measure EAAS concentrations in each athlete's urine sample and these are subsequently introduced into a global database tool, the steroidal module of ABP creating an individual 'normal' range for the target analytes in every athlete. Following the reporting of the sample's steroid values, the steroid module of the ABP uses the Adaptive Model to identify abnormal values triggering the performance of Confirmation Procedures such as GC-C- IRMS analysis for the unambiguous decision of doping rules violation [25].

Furthermore, an abnormal "steroid profile" (obtained from a single urine Sample) or an atypical "longitudinal steroid profile" (including values obtained from a series of "steroid profiles" collected over a period of time), may be a means to pursue an anti- doping rule violation (ADRV).

Steroid profile as a detection tool

The biotransformation of anabolic steroids in the body is affected by phase I and phase II metabolic reactions before being excreted through the urinary route (26). In phase I, steroids are converted into more polar compounds by enzymatically catalyzed reactions to enhance their polarity and facilitate their excretion. During phase II reactions, steroids or steroid metabolites are coupled with glucuronic acid or sulfate (Figure 1). This conjugation also modulates the elimination of steroids from the body as well as reduces the toxicity of synthetic steroids [26,27].

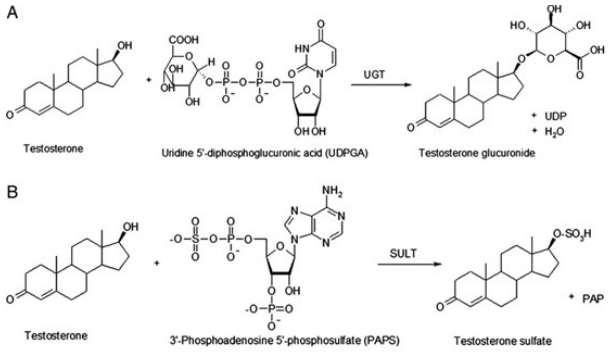

Figure 1: Schematic illustration of phase II modification reactions for testosterone. A) Glucuronidation and B) Sulphoconjugation [28].

Since steroid conjugates analysis is not compatible with GC-MS, the only analytical technique recognized by WADA for endogenous steroids quantification in urine, the deconjugation of the conjugated moiety by enzymatic hydrolysis (β-glucuronidase) is a crucial step during sample preparation and prior to GC-MS measurement.

Steroid profile consists of the quantification of the total fraction, i.e. glucuroconjugated and free, of the urinary compounds linked to T and its metabolism. Steroid profile is a powerful tool to detect drug misuse with endogenous anabolic androgenic steroids. It is composed of the following Markers (as free steroid content obtained from the free steroid fraction plus those released from the conjugated fraction after hydrolysis with β- glucuronidase from *E. coli*): testosterone (T), epitestosterone (E), etiocholanolone (Etio), androsterone (A), 5a-androstane-3a,17β-diol (5αAdiol) and 5β-androstane-3a,17β-diol (5βAdiol) (Figure 2) [30] and the following ratios: testosterone to epitestosterone (T/E); androsterone to testosterone (A/T); androsterone to etiocholanolone (A/Etio); 5α-androstane-3α,17β-diol to 5β-androstane-3α,17β-diol (5αAdiol/5βAdiol); and 5α-androstane-3α,17β-diol to epitestosterone (5αAdiol/E) (Figure 2) [28].

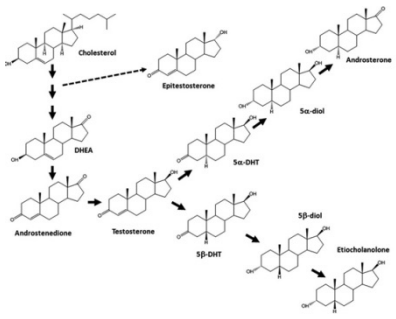

Figure 2: Schematic illustration of the steroid profile parameters and their metabolic pathways [28].

According to the WADA Technical Document – TD2016EAAS, a sample will be indicative of an atypical passport finding (ATPF) which initiates the Confirmation Procedures, if the doping control analysis meets the following criteria:

- T/E>4
- A/T<20
- 5A-diol/5B-diol>2.4
- Concentration of T or E greater than 200 ng/ml
- Concentration of A or Etio greater than 10000 ng/mL
- 5A-diol>250 ng/ml (males), 5A-diol>150ng/ml (females), in combination with 5A-diol/E>10 (in both females and males) (25).

Factors influencing the steroid profile

Much uncertainty still exists concerning the results of the doping control analysis due to the interference of several variables that might influence the quantification or interpretation of the urinary steroid profile(5). The markers of the urinary "steroid profile and/or their ratios can be altered (deceased or increased) by the administration of synthetic forms of EAAS, particularly testosterone or its precursors [for example androstenediol, androstenedione and prasterone (dehydroepiandrosterone or DHEA)], or its active metabolite [dihydrotestosterone (DHT)], as well as epitestosterone (E). Additionally, alteration of the urinary "steroid profile" can occur for a number of reasons including, but not limited to:

- The administration of other anabolic steroids (e.g. stanozolol); can trigger inconsistency in the physiological endogenous hormonal production, which is further substantiated by GC/C/IRMS(29). Evidence regarding anabolic steroid misuse is derived by obtaining higher T and T/E concentration, as well as reduced And/T ratio [5]. Earlier observations support the assertion that testosterone doses higher than 72mg/week increase the urinary excretions of T metabolites and decrease the excretion of E conjugates [30].
- The administration of human chorionic gonadotrophin (hCG) in males misused by some male athletes to stimulate endogenous T production or to prevent testicular atrophy during prolonged administration of AAS;
- The administration of inhibitors of 5α-reductase (e.g. finasteride) which suppress the formation of DHT from T, and thus interfere with the interpretation of the ABP profile;
- A large intake of alcohol (ethanol);
- The administration of antifungals such as ketoconazole or other similar compounds (miconazole, fluconazole) due to their property to inhibit T synthesis;
- The use of masking agents (e.g. probenecid) and diuretics [5,31,32].
- Available evidence has shown that administration of hormonal contraceptives (HC) can significantly reduce the E concentration, leading to an increased T/E value. It is hereby considered as an additional factor affecting the measured biomarkers of steroid profile [33].
- Microbial growth inducing hydrolysis of glucuronide and sulfate conjugates, followed by modifications of the steroid structure by oxidoreductive reactions. Because of the bacterial deconjugation, high amounts of steroids, normally excreted as conjugates (as A and Etio) are observed as aglycons. Another effect of bacterial activity on steroid profiles, which is more rarely observed, is the increase of the T concentration leading to elevated T/E ratios. In relative studies, the alteration of testosterone concentration was not significantly different in the contaminated urine compared to the non-contaminated controls leading to minor changes in the T/E ratio value [34,35]. Elevated amounts of 5a-androstane-3,17-dione and 5b-androstane-3,17-dione in the free fraction are considered typical indicators of microbial degradation. Their formation results mainly from a bacterial deconjugation of androsterone and etiocholanolone glucuronides followed by bacterial 3-hydroxysteroid dehydrogenase activity. The formation of 5a-androstane-3,17-dione and 5b-androstane-3,17-dione in the urine aliquots contaminated with *E. coli*, N. simplex, *A. flavus*, and *C. albicans* was observed in a relative study at the end of the incubation period at 37°C [36]. Hence, acquiring knowledge concerning those factors is a central aspect for achieving optimization of anti-doping techniques.

Apart from the exogenous compounds and factors that could induce hormonal alterations associated with the steroid profiling, endogenous factors can also trigger a disturbance in the naturally well-balanced system either by increasing or decreasing the concentrations and ratios of interest.

Concerns have arisen which call into question the stability of steroid profile at different pubertal stages since urinary excretion of

testosterone and epitestosterone was notably increased during puberty as shown in preliminary data [37]. In some cases the concentration of epitestosterone might not increase as rapidly as testosterone and that could lead to unstable T/E during development [28]. However in the vast majority of cases this ratio remains fairly constant [38].

Another key factor that leads to alteration of both estrogenic and androgenic hormones which are involved in the steroid profiling is pregnancy and it mainly affects pregnadiol and T [5,28] In a study conducted in pregnant women by Fabregat et al., the data that was extracted demonstrates increase in estrogen levels and decrease in the androgenic ones, as well as significant rise on epitestosterone glucuronide, which is one of the predominant steroid profile parameters, during the time of the first trimester [39].

Prior studies have also noted the seasonal variation of testosterone concentration among men and normally cycling women. That data had shown that maximum T concentration occurs in the fall and extremely low T levels occur in the summer [40]. However those hormonal alterations do not constrain the validity of doping control testing since they yield concentrations within the normal range of the steroid profile [28].

There is overwhelming evidence for the notion that hypogonadism is linked with several pathological conditions such as cardiovascular disease in men [41]. In spite of the fact that the world of sports mainly involves healthy individuals, current anti-doping techniques conducted by the accredited laboratories should be able to associate the low testosterone levels with the presence of a pathological condition in order to avoid improper steroid profile evaluations [28].

All the above confounding factors are routinely monitored in athletes' urine samples by WADA-accredited laboratories and reported in Anti-Doping Administration & Management System (ADAMS) as part of the steroidal module of ABP. A sample showing signs of microbial degradation or containing any of the substances that may cause an alteration of the "steroid profile" may not be suitable for inclusion in the "longitudinal steroid profile". These findings are to be considered by the Athlete Passport Management Unit (APMU) during the results management process [25].

Impact of ethanol on steroid profiling and bacterial contamination of samples

Setting aside the known physiological effects of ethanol on physical performance, ethanol administration is an additional factor which appears to trigger alterations on steroid profiling. Ethanol intake induces suppression of steroids biotransformation, which results from competitive inhibition of hydroxysteroid dehydrogenases involved in steroid and ethanol metabolism [28]. The main observed effects on steroid profile are elevation of T/E ratios and reduction of urinary concentrations of androsterone and etiocholanolone. Since the quantitation of urinary steroids is influenced by the presence of ethanol in the body, the monitoring of ethanol markers is mandatory for anti-doping laboratories in all urine samples according to WADA Technical Document- TD2016EAAS [25].

Early studies in clinical and forensic toxicology revealed the short detection windows of alcohol presence in the body. This occurs due to the fact that ethanol is rapidly eliminated from the body making its detection time <12h post-drinking [42]. To that end, further approaches that could prove and measure the ethanol consumption for longer periods of time were widely investigated [43]. Ethyl glucuronide (EtG) and Ethyl Sulfate (EtS) were proved to be reliable indicators of ethanol consumption used in forensic applications, since they have longer excretion time windows from their parent compound. The main theoretical premise behind the value of those compounds as markers of ethanol is that they are derived by conjugation reactions catalyzed by Uridine 5'–diphospho-glucuronosyltransferase (UGT) and sulfotransferases (Figure 3) and they are detectable in blood urine and saliva [44,45].

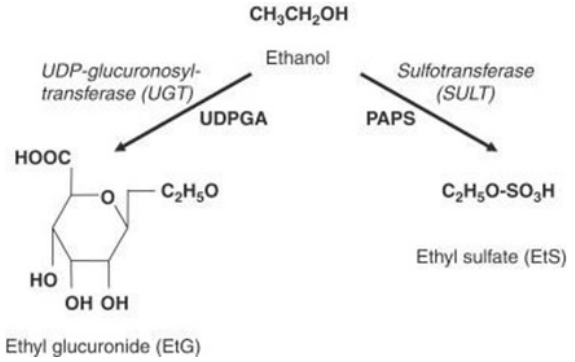

Figure 3: Formation of EtG and EtS from ethanol [46].

The evidence which is currently available supports that the EtG is the most suitable marker for quantitative measurements of ethanol intake in the body and it is detected by liquid chromatography mass spectrometry (LC-MS) [47,48]. This data has led WADA to adopt a shared EtG concentration level in doping control samples above which alcohol could impact significantly the steroid concentration. This is why WADA accredited laboratories report in ADAMS the concentration of EtG if it is estimated to be above 5 μg/ml in urine samples.

Urine specimens arriving at the Doping Control Laboratories are often contaminated by commensal urethral flora, urinary pathogens, and species of the environment. The occurrence of urine samples showing signs of microbial degradation varies during the course of the year with peaks during the warm months. Even if a low initial microbial contamination is present, this can result in a high final microbial level, under conditions of improper storage during transportation. As a result, enzyme activity generated by microorganisms may cause changes in the steroid profile by increasing or decreasing the concentrations of various steroids or by hydrolyzing conjugated metabolites. Additionally, microorganisms possessing glucuronidase activity, such as *E. coli* which is the most predominant bacterium isolated in clinical laboratories and is also the main pathogen in urinary tract infections, can hydrolyse EtG reducing its concentration [49,50]. On the contrary, synthesis of EtG from microorganisms possessing glucuronidase activity, in the presence of free ethanol, has also been reported [51,52]. Hence, EtG cannot be considered as a stable parameter in the presence of microorganisms. While EtS is considered as a stable parameter for the monitoring of ethanol consumption [50,53], there are still concerns about its stability, for samples containing higher bacterial density [54]. Previously reported data showed that EtG degraded fast by *E. coli* and in some cases, disappeared even within 24 h of storage at room temperature. Therefore, knowledge of the stability and degradation [50] or possible formation of conjugated ethanol metabolites, under doping control storage and transportation conditions is needed [49].

Hypothesis and research question

Up to the present time, no documentation is available regarding the stability of ethanol conjugated metabolites, namely EtG and EtS upon storage, in the presence and absence of urinary flora. In the present study, the aim was to study the influence of microbial contamination such as *E. coli* on the fate of EtG in doping control samples and its correlation with steroid profile parameters. It can be conceivably hypothesized that bacterial contamination with microorganisms possessing glucuronidase activity such as *E. coli* induces enzymatic hydrolysis and hence degradation of the EtG, as well as instability in the concentrations of steroid profiling. Therefore, it may be the case that EtS yields a more stable marker for monitoring ethanol. Hence, the current study seeks to acquire knowledge on the stability, degradation or possible formation of conjugated ethanol metabolites, under doping control storage and transportation conditions. To that end, the kinetics of ethanol markers and steroid profile parameters upon bacterial contamination with *E. coli* will be assessed.

Materials and Methods

Urine collection

The protocol was approved by the National Research Ethics Service in Greece (Approval number: 1617022983) and one healthy male volunteer of 25 years old enrolled in a controlled drinking experiment upon giving informed consent. In the experiment 8 units of alcohol (corresponding to a mean value of 1,53 g/kg of body weight) was administered in the form of 200ml of whiskey 40% (v/v) diluted in 300ml of sparkling water. Ethanol was administered at 8 p.m. and urine samples were provided 3, 9 and 12 hours post ethanol intake. The urine was pooled reaching an EtG concentration of 84ppm. The pooled urine was equally transferred into two Duran bottles corresponding to the blank urine and the Escherichia coli (*E. coli*) GM00108 inoculum (Figure 4).

Inoculum preparation and bacterial growth

E. coli originated from a urinary infection was supplied by the Department of Microbiology at the Medical School of the University of Athens and a 5 ml liquid master culture was prepared from -80oC stocks: incubation from 24 hours in a rotatory shaker incubator (SI-300R, Fisher Scientific, Korea). Next, the optical density (λ=600 nm) of the suspension was determined in a ultraviolet (UV)-visible spectrophotometer (U-2001, Hitachi, Tokyo, Japan) and the appropriate amount of inoculum was added in 250 ml of urine to reach an initial cell density of 106 cfu/ml. The *E. coli* inoculation occurred at t=0 and 16 ml aliquots of both blank and contaminated urine were collected and stored at -20oC for later measurements of ethanol markers and steroid profile parameters. The extraction of aliquots was carried out at particular time points (t=0, 3, 6, 12, 24, 36, 48, 72, 120, 168, 216, 288, 360 hours). The estimation of *E. coli* growth for the corresponding time points was also part of the experimental design (Figure 4).

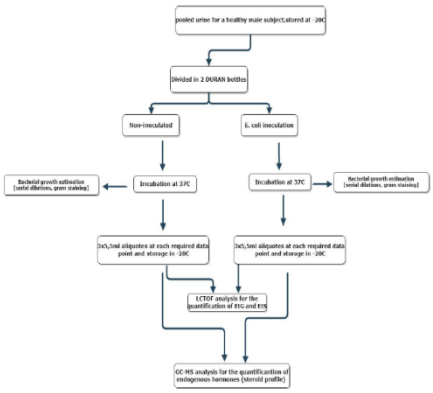

Figure 4: Flow chart of the experimental design.

Viable cell counting-bacterial growth estimation

Serial dilutions: To achieve the most valid statistical practice for estimation of bacterial growth colonies were counted only on plates that have 30-300 colonies and calculate the respective number in colony forming units per milliliter of original sample (CFU/ml). A dilution should occur in order to reach the appropriate colony number for the sample to be counted. We conducted 10-fold (10-1) dilutions by mixing 100μl of our sample with 900μl of distilled water which was the sterile liquid used.

Spread plate method: Upon formation of agar plate by mixing agar (purchased from Biotech Inc., Budapest, Hungary) 20 g/L of distilled H_2O and lysogeny broth (LB) (purchased from Nebotrade Kft., Budapest, Hungary) also known as Luria-Bertani medium 25 g/L of distilled H_2O, volume of 100 μl from the dilutions to be counted was pipette onto the plate surface. A sterile glass spreader was used to accomplish optimal spreading of the sample in the surface of the agar. The plates were then incubated for 24 h in 37°C which is the time required for the *E. coli* colonies to appear (Figure 5). The counting occurred in assumption that each cell can yield one colony. The CFU/ml number of the desired plates was calculated in compliance with the following equation.

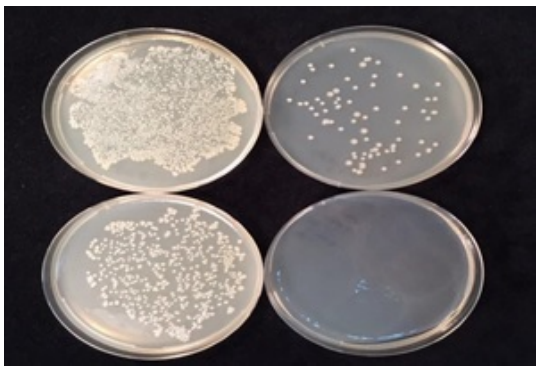

Figure 5: Petri dishes illustrating *E. coli* growth upon serial dilution and incubation process.

Analytical validation

The LC-MS method was validated according to the guidelines of Doping Control Laboratory of Athens, which is accredited by the World Anti-doping Agency, as well as to ISO 17025 for drug testing analysis. The validation process included limit of detection (LOD), limit of quantification (LOQ), and linearity.

The linearity of the calibration curve was established by injecting standard mixtures of known concentrations in urine. The range was considered linear if the regression coefficient r2 measured by a linear regression analysis was higher than 0.995. For our EtG analysis the calibration curve was linear indeed (r2=0.99, P<0.001), LOD was 1.9 μg/mL and LOQ was 6.3 μg/mL.

Measurements

Liquid chromatography quadrupole time-of-flight mass spectrometry (LCQTOF): Measurement of EtG and EtS in urine was carried out by (LCQTOF) (purchased from Agilent Technologies, CA, USA) via the use of selected ion monitoring of m/z 221,0668 for EtG and m/z 226 for the penta-deuterated internal standard (EtG-D5). The routine clinical cut-off value for laboratory is 5 μg/ml. A 200 μL urine sample supplemented with 10 μL of aqueous solution of internal standard (Ethyl-Beta-D—Glucuronide-D5) was centrifuged for 10min at 14000 rpm. The final supernatant was evaporated to dryness under a stream of nitrogen. The final content was dissolved in 100 μL of acetonitrile and transferred to the vial for injection into the LC-MS system.

Gas chromatography mass spectrometry: (a) Solid phase extraction- Samples were extracted according to a modified screening procedure for free steroids, as well as after enzymatic hydrolysis to obtain the conjugated fraction. Urine aliquots of 5 mL were rinsed with 2.0 ml acetate buffer 1 M and the pH was adjusted to 4.8–5.5. Solid phase extraction (SPE) was performed on C-18 cartridges (purchased from Biotage, Uppsala, Sweden) pre-conditioned with 5.0 ml methanol and 5.0 ml acetate buffer (1 M, pH 5.2). After loading the urine, the cartridge was washed with 5.0 ml acetate buffer (1 M, pH 5.2) followed by 5.0 ml n-hexane and the compounds of interest were eluted with 5.0 ml methanol. The eluant was evaporated under nitrogen at (60 ± 5)°C. (See section 6.1.3 for analytical protocol. (b) Urinary steroid profile analysis- Aliquots taken from each time point were analyzed using a modified routine analysis screening procedure following SPE extraction for the isolation and identification of the free and glucuronide fraction of endogenous steroids. All the markers constituing the steroid profile (Epitestosterone, testosterone, etiocholanolone, androsterone, 5α-androstane-3a,17β-diol (5α-DIOL), 5β-androstane-3α, 17β-diol (5β- diol), as well as the ratios T/E, And/Etio, And/T, 5A/5B) were determined in each urine aliquot. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on an Agilent 6890/5973 instrument possessing an Agilent Ultra1 GC column (length 17 mm, i.d. 0.2 mm, film thickness 0.11 μm). The injection volume was 2.0 μl. The injector operated in split mode (1:15 split ratio) and the interface was maintained at 31°C.

Statistical analysis

All values are shown as mean ± SD. For the performance of the statistical analysis GraphPad Prism Software (version 5.02) (La Jolla, CA, USA) was used. Differences between groups were examined for statistical significance with either 1- or 2-way ANOVA as appropriate.

Results and Discussion

E. coli and ethanol consumption markers

To assess the stability of EtG and EtS upon bacterial contamination with *E. coli* the bacterial growth during the incubation period of two weeks was initially estimated. The time-response curve of log10 [*E. coli*] (Figure 6) indicates that the loading phase of *E. coli* was terminated at t=12 hours when *E. coli* reached its peak urine concentration before the stationary phase was initiated and lasted until t=48 hours of incubation with the microorganism. The stability of the population number in the latter stages is justified by the fact that the formation of bacteria was accompanied by simultaneous death. In accordance with the present result, previous papers have demonstrated that bacteria generally reach their maximal concentration in a very short period of time indicating the significance of urine collection and transportation conditions [34].

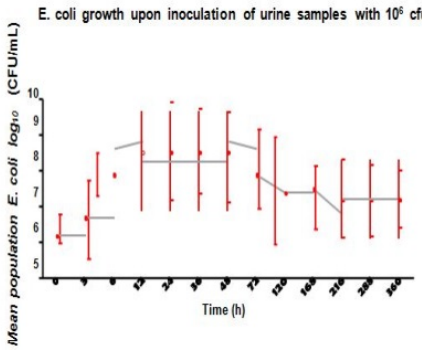

Figure 6: Bacterial growth of *E. coli* population during storage in 37°C from t=0 h until t=360 h.

To measure the expression of ethanol markers upon bacterial contamination with *E. coli*, both EtG and relative EtS area were quantified with LCQTOF (Figure 7). Measurement of EtG highlighted its synthesis at t=72 h in the contaminated samples which exhibit significantly higher EtG concentration compared to the non-inoculated controls (Figure 7A). Similarly, EtS seemed not to lose its stability upon contamination of urine with that specific *E. coli* strain compared to the non-inoculated controls. EtS was not detected after t=120 h revealing its shorter half-life compared to EtG (Figure 7B). The kinetics of EtG were also measured with GC-MS (see supplemental information). The synthesis of EtG substantiates with previous findings of Helander et al. which proffer that EtG is synthesized in the presence of *E. coli* [34,51]. A drawback of this framework is that only a single bacterial *E. coli* strain was investigated, underlining that this strain was not aggressive.

Steroid profile parameters in the presence of *E. coli*

The T/E ratio, testosterone and epitestosterone: To observe the deconjugation that was induced by the β-glucuronidase enzyme of the *E. coli* strain in the steroid profile, all endogenous steroid profile markers as well as indicative ratios were measured (both conjugate and free fraction as well as total).

Initially we determined the impact of *E. coli* to the total T/E ratio (free+conjugate fraction). It is evident that T/E was notably elevated

(>4) along with the loading phase of *E. coli* proliferation and reached its peak urine concentration in the phase when *E. coli* was most abundant compared to the non-inoculated urine (Figure 8A). From this finding we deduce that *E. coli* when in exponential phase effectively deconjugated the total fraction of T/E, resulting in production of free T/E as opposed to the stable kinetics of its respective conjugated fraction (Figure 8B). The increase of T/E ratio that we observed is in agreement with the paper of Van de Kerkhof et al. as well as Mareck et al. who also corroborates this finding [5,55].

The rise of T/E triggered by the high concentration of the microorganisms raises questions concerning the kinetics of free testosterone alone. Hence, the measurement of testosterone independently, provided an insight of free testosterone produced upon deconjugation of testosterone glucuronide in doping control samples infected with the same *E. coli* strain. The concentration of total testosterone was measured in *E. coli* - inoculated and non-inoculated samples that were both stored in 37°C for two weeks.

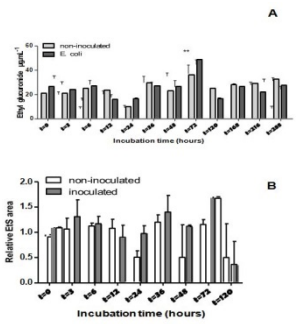

Figure 7: Effect of bacterial contamination with *E. coli* on ethanol markers EtG and EtS. (A) EtG concentration of inoculated and blank urine samples. Synthesis of EtG was observed at t=72 h of the corresponding *E. coli* –inoculated sample. (B) Relative abundance of EtS in both *E. coli*-inoculated and blank samples. No significant alteration induced by *E. coli*. *p<0,05, **p<0,01 and ***p<0,001. (2-way ANOVA). Data are representative of three independent experiments (mean and SD).

Quantification of total testosterone revealed a significant increase of hormonal levels compared to non-inoculated urine samples when *E. coli* reached its peak concentration (t=36-t=48) (Figure 9A). As may be seen in Figure 9B, *E. coli* intervention exhibited high deconjugation impact to testosterone glucuronide producing free fraction during incubation with *E. coli*. Total testosterone seemed to be linked with the incubation time with *E. coli* demonstrating an increase after t=120 regardless of the reduction of *E. coli* population (Figure 6 and 7C). The evidence from the testosterone measurements suggests that even though *E. coli* is degraded as soon as its stationary phase is terminated bacterial debris from the dead bacterium secretes β-glucuronidase continuing the deconjugation process and producing free testosterone. The fact that peak free testosterone concentration is higher than 10% of total testosterone (Figure 7B and C) confirms the hydrolyzing activity of the specific *E. coli* strain and is indicative of successful bacterial contamination in agreement with previous evidence found by de la Torre et al. [34].

For estimating the implication of *E. coli* presence in epitestosterone concentration, the same approach was used and epitestosterone levels were quantified using GC-MS.

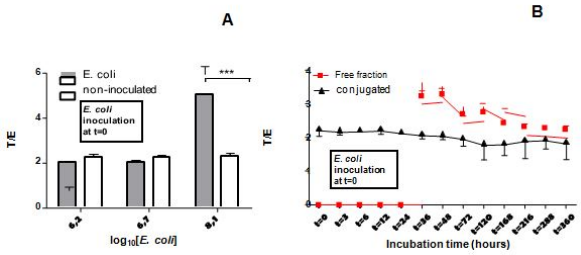

Figure 8: Rise in T/E value when *E. coli* reached its peak concentration. (A) Impact of *E. coli* inoculation in total T/E levels compared to the non-inoculated controls. Measurements of T/E correspond to t=0 hours (log10[*E. coli*]=6.2), t=3 hours ((log10[*E. coli*]=6.7) and t=12-48 hours (log10[*E. coli*]=8.1) post to *E. coli* inoculation. (B) Deconjugation activity of 106 CFU/ml *E. coli* that led to the formation of free T/E and stable kinetics of conjugated T/E. *p<0,05, **p<0,01 and ***p<0,001. (2-way ANOVA). Data are representative of three independent experiments (mean and SD).

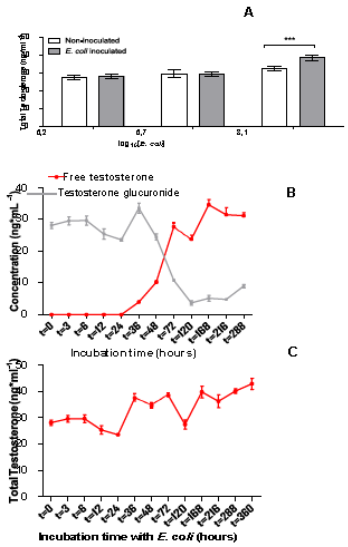

Figure 9: *E. coli* induced testosterone production. (A) *E. coli* influence on total testosterone. (B) Deconjugation effect of *E. coli*. Measurements of T/E correspond to t=0 hours (log10[*E. coli*]=6.2), t=3 hours ((log10[*E. coli*]=6.7) and t=12-48 hours (log10[*E. coli*]=8.1) post to *E. coli* inoculation. Production of free testosterone upon subsequent reduction of testosterone glucuronide. (C) Schematic representation of time-response curve, showing the kinetics of total testosterone upon *E. coli* inoculation (t=0h) until t=360h. *p<0,05, **p<0,01 and ***p<0,001. (2-way ANOVA). Data are representative of three independent experiments (mean and SD).

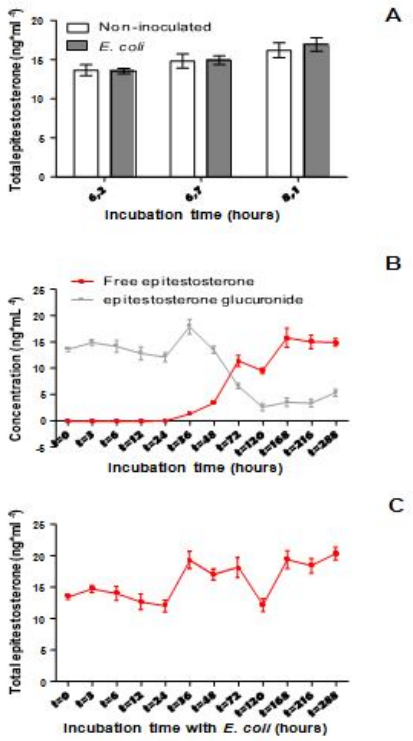

Figure 10: *E. coli* effect on epitestosterone. (A) Comparison of total epitestosterone exhibited by *E. coli* compared to the non-infected samples. Measurements of epitestosterone correspond to t=0 hours (log10[*E. coli*]=6.2), t=3 hours ((log10[*E. coli*]=6.7) and t=12-48 hours (log10[*E. coli*]=8.1) post to *E. coli* inoculation. No significant difference between samples was established. (B) Impact of *E. coli* on free epitestosterone and conjugated epitestosterone fraction. The hormones were quantified separately. (C) Kinetics of total epitestosterone upon inoculation with 106 cfu/ml of *E. coli* t=0 until t=360 hours. *p<0,05, **p<0,01 and ***p<0,001. (2-way and 1-way ANOVA). Data are representative of three independent experiments (mean and SD).

The data which is shown in Figure 10 appears to suggest that *E. coli* did not induce any production of total epitestosterone compared to the non-inoculated controls (Figure 10A). The significant elevation of free compared to the conjugated fraction lends support to the deglucuronidation efficacy of β-glucuronidase that *E. coli* yields against epitestosterone (Figure 10B). Thereby it is safe to conclude the significant increase of total T/E value (when *E. coli* reached its peak concentration, rather depends on total testosterone elevation when identical conditions were applied and it is not influence by epitestosterone. We also demonstrate that no considerable alteration occurs in the concentration of total epitestosterone upon incubation with *E. coli* (Figure 9C). Mareck [2008] has already noted that epitestosterone glucuronide is cleaved rapidly by the glucuronidase activity of *E. coli* which is supported by the spike of free testosterone in the final incubation time points found in our samples [5]. Thereby deterioration of epitestosterone glucuronide and subsequent production of free epitestosterone revealed that the fractions of that hormone are incubation time with *E. coli*-dependent rather than *E. coli* dependent.

The impact of *E. coli* on A/Etio. androsterone and etiocholanolone: To establish the relationship between *E. coli* and Androsterone/Etiocholanolone ratio in urine, we investigated the impact of *E. coli* and thus β-glucuronidase to And/Etio ratio.

As Figure 11 illustrates, the total And/Etio ratio did not exhibit any significant effect upon inoculation with *E. coli*. Even when *E. coli* reached its peak concentration its impact in total And/Etio ratio was not significant (Figure 11A). Therefore the considerable reduction of A/Etio ratio that is supported by Mareck et al. was not supported by the current findings [5].

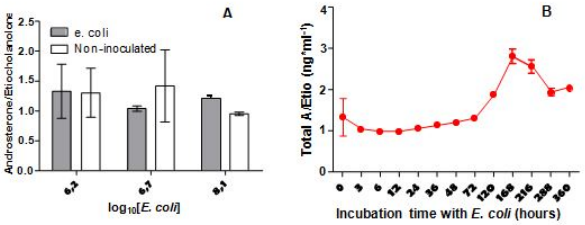

Figure 11: Effect of *E. coli* inoculation (106 cfu/ml) on A/Etio value. (A) Impact of *E. coli* growth on And/Etio ratio value. Measurements of A/Etio correspond to t=0 hours (log10[*E. coli*]=6.2), t=3 hours ((log10[*E. coli*]=6.7) and t=12-48 hours (log10[*E. coli*]=8.1) post to *E. coli* inoculation. (B) Kinetics of A/Etio after inoculation with *E. coli* from t=0h until t=360h. No significant alteration of total A/Etio value based on 1-way ANOVA. *p<0,05, **p<0,01 and ***p<0,001. (2-way and 1-way ANOVA). Data are representative of three independent experiments (mean and SD).

Next, we proceeded to the quantification of androsterone which is an additional steroid profile parameter in order to determine the effect of our bacterium of interest on kinetics of that particular endogenous hormone. Inoculation of samples with *E. coli* did not induce any significant alteration in the total androsterone levels (Figure 12). Notwithstanding the fact that *E. coli* exhibited its death face after 72 h of incubation, this is the time point when the deconjugation process of androsterone glucuronide was initiated.

The current study did not provide any evidence for significant alteration of total androsterone dependent to bacterial growth. However the kinetics of total androsterone support that prolonged incubation time with *E. coli* causes cleavage of androsterone glucuronide, which corroborates the findings of Tsivou et al. concerning the effective deconjugation of epitestosterone by *E. coli* [56]. The fall of androsterone glucuronide was initiated at t=36 hours (*E. coli* exhibited each stationary phase upon reaching each peak concentration) with concurrent increase in free androsterone. The identical kinetics of conjugated and total androsterone reveal that the total steroid concentration is predominantly consisted by conjugate androsterone fraction. The gradual reduction of total androsterone after t=36h accords with earlier observations of Mareck et al. which showed that concentration of And is reduced by β-glucuronidase (5).

In Ref. [56] the authors support the efficacy of *E. coli* in deconjugating testosterone, epitestosterone and androsterone glucuronides which is consistent with our findings.

The following step was to quantify the concentration of etiocholanolone to estimate the influence of *E. coli* to that endogenous steroid as well.

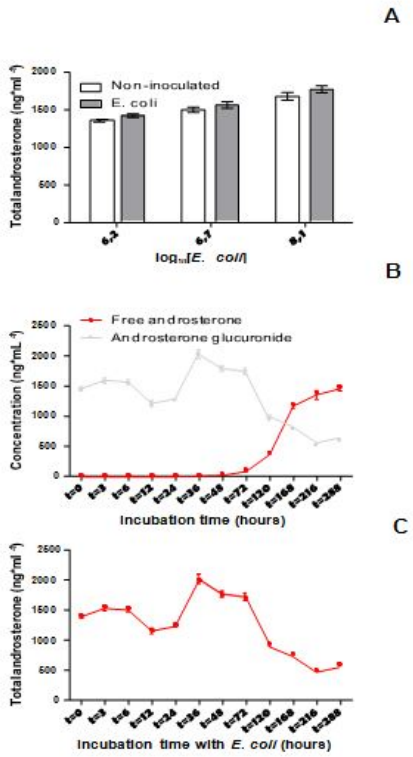

Figure 12: Deconjugation of androsterone. (A) Effect of *E. coli* growth to the concentration of total androsterone. No significant alteration compared to the controls. (B) Quantificantion of free and conjugated androsterone upon *E. coli* inoculation. Free androsterone production along with incubation occurred with *E. coli*. The fractions were measured separately. (C) Schematic representation of total androsterone concentration in response with time during storage with *E. coli*. *p<0,05, **p<0,01 and ***p<0,001. (2-way and 1-way ANOVA). Data are representative of three independent experiments (mean and SD).

Based on the results derived from the use of GC-MS, no considerable effect was identified upon testing different concentrations of the microorganism to the concentration of that hormone compared to the non-infected control samples (Figure 13A). Along similar lines with the glucoronide fractions of T, E and A which were previously quantified conjugated etiocholanolone was diminished and free fraction was produced during the incubation upon inoculating 106 cfu/ml of the microorganism (Figure 13B). By observing the kinetics of free and conjugated etiocholanolone (Figure 13B) as well as total etiocholanone (Figure 13C), it is safe to suggest that etiocholanolone was completely deconjugated. With regard to the effect of *E. coli* to total etiocholanolone, the current finding does not support previous research in this area. In fact in contrast to earlier findings which reported an etiocholanone reduction induced by β-glucuronidase of *E. coli* [5], we do not demonstrate any notable decrease of total etiocholanolone levels caused by the contamination.

According to WADA Technical Document (25), the And/T ratio yields a significant marker during doping control testing. Via GC-MS the total And/T value was measured for each incubation time point required in order to assess the kinetics of the ratio.

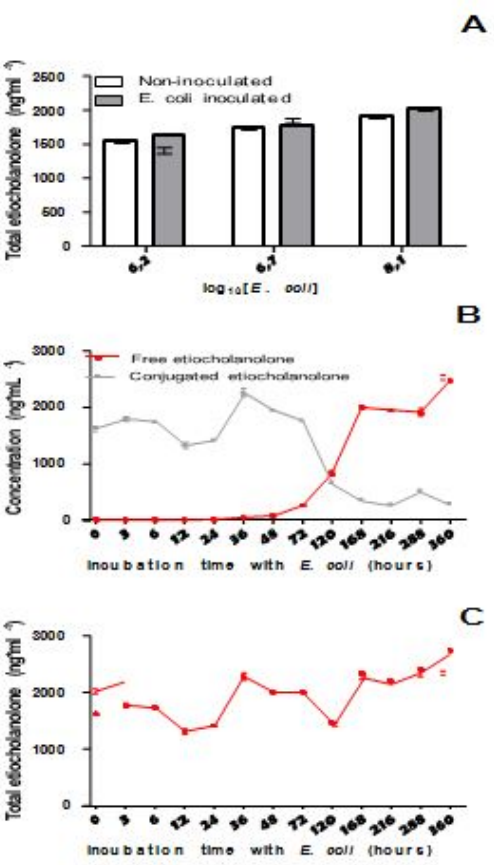

Figure 13: Response of etiocholanolone triggered by inoculation of urine samples with 10^6 cfu/ml *E. coli*. (A) Comparison of total etiocholanolone response triggered by *E. coli* inoculation compared to the non-infected samples. Measurements of etiocholanolone correspond to t=0 hours (log10[*E. coli*]=6.2), t=3 hours ((log10[*E. coli*]=6.7) and t=12-48 hours (log10[*E. coli*]=8.1) post to *E. coli* inoculation. No significant difference between samples was established. (B) Quantification of free and conjugated etiocholanolone during incubation with *E. coli* from t=0 to t=360 hours. The fractions were measured independently. (C) Total etiocholanolone response in urine during incubation from t=0 to t=360 hours upon inoculation of urine samples with the *E. coli* strain. *p<0,05, **p<0,01 and ***p<0,001. (2-way and 1-way ANOVA). Data are representative of three independent experiments (mean and SD).

This data (Figure 14) confirms the rise of And/T ratio between t=36 until t=120 of the incubation process with the microorganism. However, after t=120 the ratio was reduced which concurs with previous observations [5]. To establish the effect of *E. coli* on 5αAdiol which is additional steroid profile parameter, measurements of the concentrations of this particular steroid obtained by bacterial contamination were carried out.

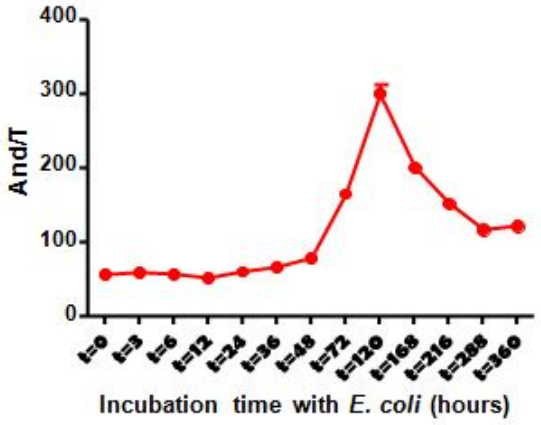

Figure 14: Schematic representation of And/T ratio kinetics upon bacterial contamination with *E. coli*.

As Figure 15 shows, when urine was inoculated with *E. coli*, no significant difference in the concentration of total 5A-diol was detected compared to the non-inoculated samples in correlation to the bacterial growth (Figure 15A). It was also shown that there was not a major spike in the total concentration of that specific steroid in connection with the incubation time with the bacterium (Figure 15C). However *E. coli* seems to effectively deconjugate 5A-diol glucuronide producing free fraction (Figure 15B). Taken together, these results indicate that *E. coli* exhibits deglucuronidation activity against 5A-diol regardless of its not significant effect in total hormone's levels.

Next, the quantification of 5β-diol levels took place in order to estimate the impact that *E. coli* yielded to the hormone. As seen in Figure 15A there was no correlation between changes in *E. coli* population and concentration of total 5β-Adiol. However prolonged incubation time with the bacterium had been shown to effectively deconjugate the glucuronide fraction of the hormone producing free fraction. Eventually total 5β-Adiol was mainly consisted by free fraction (Figure 16B and C).

Observing the concentration values of total 5αAdiol and total 5βAdiol (Figure 15 and 16), it safe to conclude that neither parameter exhibited values higher than 250ng/ml which indicates suspicious steroid profile. Thereby, no influence by *E. coli* is reported in the quantification of those hormones.

To determine whether bacterial contamination with the present strain of *E. coli* leads to false interpretation of doping control test results as far as the ratio 5αAdiol/5βAdiol knowing that yielding a value higher than 2,4 the Laboratory receives a Suspicious Steroid Profile Confirmation Procedure, the value of the particular ratio was quantified via the use of GC-MS.

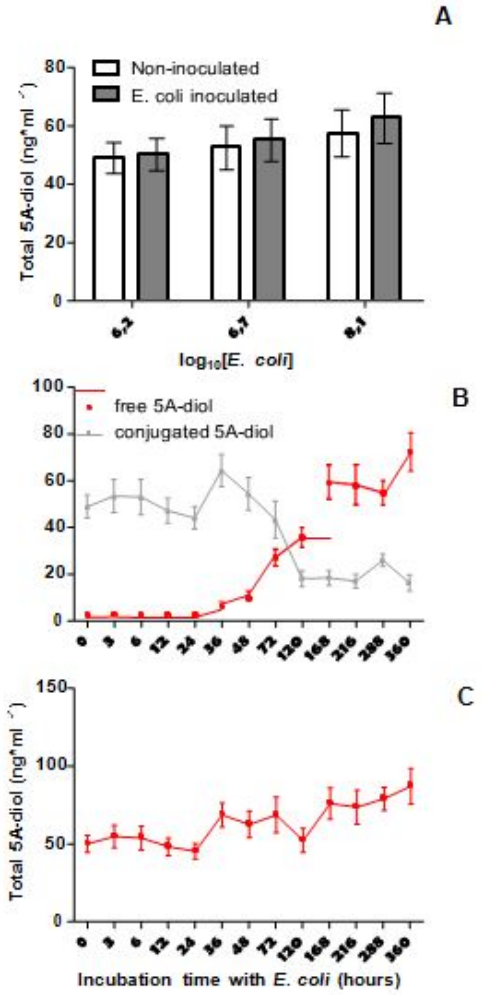

Figure 15: The effect of *E. coli* in 5α-diol steroid levels. (A) Total 5α-Adiol levels in the presence of *E. coli* compared to non-inoculated controls. No significant change observed. (B) Kinetics of free and conjugated 5α-diol upon inoculation with *E. coli* from t=0 until t=360. (C) Kinetics of total 5α-diol upon inoculation with *E. coli* from t=0 until t=360h. *p<0,05, **p<0,01 and ***p<0,001. (2-way and 1-way ANOVA). Data are representative of three independent experiments (mean and SD).

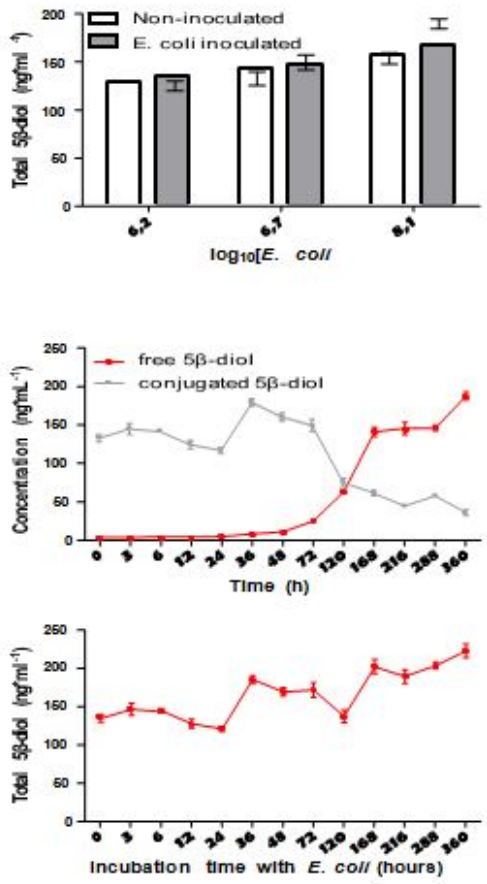

Figure 16: Fate of 5β-diol upon bacterial contamination with *E. coli.* (A) Concentration of total 5β-Adiol upon treatment with *E. coli* compared to non-inoculated controls. No significant effect observed by the presence of different *E. coli* concentrations. (B) Kinetics of free and 5β-diol glucuronide after inoculation with *E. coli* at t=0. Abrogation of conjugated fraction and production of free fraction by β-glucuronidase. (C) Kinetics of total 5β-diol after inoculation with *E. coli.* Neither increase nor reduction of total levels were observed, demonstrating that reduction of conjugated fraction was proportional to the production of free fraction. *p<0,05, **p<0,01 and ***p<0,001. (2-way and 1-way ANOVA). Data are representative of three independent experiments (mean and SD).

The graph (Figure 17) illustrates a steady curve exhibiting values lower than 0.5<2.4, which indicated that this ratio is not affected by contamination of urine samples with that strain and thus not decreasing the validity of steroid profiling with regard to this parameter.

Taken together, with a few exceptions, the results reveal that total levels of steroid profile parameters were not *E. coli* concentration dependent. In spite of the inefficacy of this *E. coli* strain to induce a significant effect in the levels of the steroids mentioned above, the microorganism effectively deconjugated the hormones that the steroid profile consists.

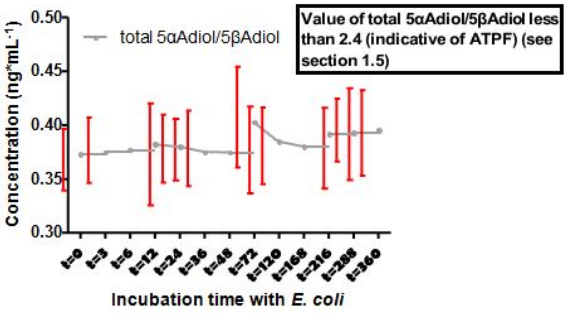

Figure 17: 5αAdiol/5βAdiol kinetics upon inoculation with 10^6 cfu/ml of *E. coli.*

Conclusion

Currently, an elevated percentage of diagnostic samples are not analyzed in local laboratories, but shipped for testing worldwide. Storage and transportation conditions facilitate the bacterial contamination with *E. coli* that possesses β-glucuronidase. Concentrations and ratios of various endogenously produced steroidal hormones that represent the steroid profile and serve as a valuable tool in doping control testing, as well as EtG and EtS which are indicators of ethanol consumption are known to lose their stability due this enzyme. This study has gone some way towards enhancing our understanding of the impact of *E. coli* in steroid and ethanol profiling in doping. Whilst this study did not confirm the initial hypothesis concerning the enzymatic hydrolysis of EtG and thus degradation by *E. coli*, it indicates that EtG is synthesized due to *E. coli* presence at t=72 h and not maintaining its stability during the whole incubation process. The results also substantiate the deconjugation effect of *E. coli* on steroid profile parameters and suggested that T/E marker is influenced by the contamination with the particular strain yielding a total concentration higher than 4 and thus meeting the criteria for ATPF. The obtained results are comprehensive and prove that this *E. coli* strain may act as a negative factor leading to FP doping control analysis.

Finally a number of important limitations need to be acknowledged. First, the major weakness of this study is the fact that it is based on one subject and intrinsic variation of hormonal concentration among individuals might occur. Thereby, it is recommended that further research should be undertaken in this area including a larger sample size to establish more precision. If the debate is to be moved forward, applying our study design in both enhanced and natural athletes would be a reasonable approach to tackle the issue of bacterial contamination impact in doping and could provide more definitive evidence. It is also unfortunate that our laboratory had no access to deuterated EtS. Consequently, more work will need to be done to establish the maintenance of the stability of EtS in doping control samples upon bacterial contamination with *E. coli.* Further experimental investigations including additional *E. coli* strains are strongly recommended and will provide a more clear understanding, since each strain exhibits different degree of aggression.

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