

Tsivou M<sup>1</sup>, Giannadaki E<sup>1,2</sup>, Lyris M<sup>1</sup>, Georgakopoulos C<sup>3</sup>

# Stabilization efficiency of spray coated chemical mixture on hCG degradation by proteases

Doping Control Laboratory of Athens, OAKA, Maroussi, Greece<sup>1</sup>; Department of Pharmaceutical Chemistry, University of Athens, Athens, Greece<sup>2</sup>; Anti Doping Lab Qatar, Doha, Qatar<sup>3</sup>

# Abstract

In the current study, the efficiency of an in-house chemical stabilization mixture was investigated, against enzymatic breakdown of intact human chorionic gonadotropin (hCG) induced by six proteolytic enzymes (proteases). The mixture, a combination of antibiotics, antimycotic substances, and protease inhibitors, was spray-coated in the interior walls of plastic collection containers. Four-day incubation experiments were conducted at 37°C, in 25 mL-urine aliquots spiked with six proteases and fortified with intact hCG, in the presence and absence of the stabilization mixture. Intact hCG levels were evaluated using the ElAgen Total hCG kit at three different time points. At the end of the incubation period (t = 4), intact hCG levels were decreased due to the presence of proteases. Specifically, the addition of three proteases (subtilisin A, trypsin,  $\alpha$ -chymotrypsin) resulted in undetectable intact hCG levels. In the containers, which were spray-coated with the stabilization mixture, the dissociation of intact hCG was inhibited and intact hCG concentration was higher at the end of the 4-day incubation period at 37°C for five of the six proteases tested, compared to the untreated aliquots. In the presence of subtilisin A (subA), intact hCG levels were undetectable at t = 4, irrespective of the presence or absence of the stabilization mixture.

# Introduction

The in-house chemical stabilization mixture, developed as part of WADA funded projects, has been under continuous evaluation in lab-scale during the last years [1-4]. Before moving forward to an industrial-scale production of stabilized urine collection containers, the selected spray-coated form of the mixture must also be evaluated in terms of efficiency, applicability and absence of analytical interferences. Recently, the spray-coated chemical stabilization mixture was tested in 25 mL urine aliquots against microorganism elimination and steroid glucuronide degradation [5]. The current study investigates, in pre-pilot scale, the efficiency of the chemical stabilization mixture in spray-coated form against enzymatic breakdown of intact hCG, induced by six proteases after 4-day incubation periods at 37°C. Intact hCG levels were evaluated using the ElAgen Total hCG kit.

# Experimental

Two sets of 30-mL polypropylene (PP) medicine cups (Sarstedt, Germany) were prepared; in the first set, the chemical stabilization mixture was spray coated in the interior surface using common plastic spraying devices and excess quantity was dried out in a roller mixer; in the second one, the chemical mixture was not included. The composition of the chemical stabilization mixture has been previously presented [4]. 25-mL pooled urine from a male athlete were added in both sets of cups and 220 mIU/mL of intact hCG, devoid of nicked forms and free subunits (1st International Reference Reagent hCG 99/688, National Institute for Biological Standards and Control, UK) were introduced. Intact hCG stock solution was prepared as described in [6]. The selection of the six proteases applied at a concentration of 200  $\mu$ g/mL has been documented in [2]. Papain from *Carica papaya*, trypsin, pepsin from porcine gastric mucosa and  $\alpha$ -chymotrypsin from bovine pancreas were from Applichem, Germany. SubA isolated from fermentation of *Bacillus licheniformis* was purchased from Sigma-Aldrich, Germany. Bromelain from pineapple stem was from Serva Electrophoresis, Germany. Incubation experiments were



conducted during 4-day periods at 37°C. All samples were processed in triplicate at three different time points: before the spiking of proteolytic enzymes (t = baseline values), after the spiking (t = 0) and at the end of the 4-day incubation period (t = 4). No protease was introduced in two urine aliquots to serve as negative controls in the presence and absence of the chemical stabilization mixture. Intact hCG levels in urine aliquots were evaluated using the ElAgen Total hCG kit (Adaltis, Italy) and the optical densities were measured by an automated ELISA system (DSX, DYNEX Technologies, USA) as described in [3].

#### **Results and Discussion**

Intact hCG levels were comparable in both stabilized and unstabilized urine at t = 0, (i.e. right after the introduction of proteases) for five out of six proteases (**Figures 1 and 2**).

Following incubation at 37°C, the presence of the six proteolytic enzymes in the unstabilized aliquots resulted in decreased intact hCG levels over time. Intact hCG was undetectable at the end of the incubation period in aliquots containing trypsin and  $\alpha$ -chymotrypsin, but was detectable in the corresponding aliquots in the spay-coated cups. For five out of six proteases tested, (papain, bromelain, pepsin,  $\alpha$ -chymotrypsin, trypsin), intact hCG levels were higher at t = 4 in stabilized aliquots compared to the unstabilized ones (**Figures 1, 2**). In case of sub A, an immediate degradation of intact hCG (66 mIU/mL) was observed at t = 0 in unstabilized urine, whereas the respective intact hCG levels were practically unaffected (210 mIU/mL) due to the presence of the stabilization mixture (**Figure 1**). However, at t = 4, intact hCG levels were undetectable in aliquots spiked with sub A, irrespective of the presence or absence of the stabilization mixture (**Figure 1**).



**Figure 1**. Changes in intact hCG levels (mean mIU/mL + SD, n = 3) due to the addition of 200  $\mu$ g/mL of bromelain, papain, subtilisin A (Sub A) and pepsin, with and without the chemical stabilization mixture in spray-coated form, following 4-day incubation periods at 37°C (some bars do not appear at t = 4 due to zero values). ST denotes the presence of the spray-coated chemical stabilization mixture in urine collection containers.

Poster





**Figure 2**. Changes in intact hCG levels (mean mlU/mL + SD, n = 3) due to the addition of 200  $\mu$ g/mL of  $\alpha$ -chymotrypsin and trypsin, with and without the chemical stabilization mixture in spray-coated form, following 4-day incubation periods at 37°C (some bars do not appear at t = 4 due to zero values). ST denotes the presence of the spray-coated chemical stabilization mixture in urine collection containers.

# Conclusions

The results presented here, combined with those reported in a recent study [5] and unpublished data, demonstrate that the chemical stabilization mixture can be applied in spray-coated form in the interior walls of plastic urine collection containers for the inactivation of microbial and enzymatic action, thus improving the quality of athletes' urine samples. The evaluation process of the spray-coated stabilization mixture in pre-pilot scale is still on-going, compiling data from two WADA accredited labs (Rome and Ghent) regarding the degradation of recombinant erythropoietin (rEPO) by proteases, as well as the estimation of analytical interferences during the routine screening procedures.

# References

[1] Tsivou M, Livadara D, Georgakopoulos DG, Koupparis M, Atta-Politou J, Georgakopoulos CG. (2009) Stabilization of human urine doping control samples: II. Microbial degradation of steroids. *Anal Biochem* **388**, 146-154.

[2] Tsivou M, Dimopoulou HA, Georgakopoulos DG, Koupparis M, Atta-Politou J, Georgakopoulos CG. (2010) Stabilization of human urine doping control samples: III. Recombinant human erythropoietin. *Clin Chim Acta* **411**, 448-452.

[3] Tsivou M, Dimopoulou HA, Georgakopoulos DG, Koupparis MA, Atta-Politou J, Georgakopoulos CG. (2010) Stabilization of human urine doping control samples: IV. Human Chorionic Gonadotropin. *Anal Bioanal Chem* **398**, 1313-1318.

[4] Tsivou M, Georgakopoulos DG, Dimopoulou HA, Koupparis MA, Atta-Politou J, Georgakopoulos CG. (2011) Stabilization of human urine doping control samples: a current opinion. *Anal Bioanal Chem* **401**, 553-561.

[5] Tsivou M, Giannadaki E, Georgakopoulos DG, Lyris E, Georgakopoulos CG. (2013) Stabilization in urine collection containers: reality or science-fiction? In: Schänzer W, Thevis M, Geyer H, Mareck U (eds), *Recent Advances in Doping Analysis (21)*, Köln, pp 242-246.

[6] Grebescenko AS, Dykhal YI, Rodchenkov GM. (2011) Characterisation of human chorionic gonadotropin reference materials by different immunoassays. In: Schänzer W, Geyer H, Gotzmann A, Mareck U (eds) *Recent Advances in Doping Analysis (19)*, Köln, pp 227-230.



# Acknowledgements

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