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Characterisation of human chorionic gonadotropin reference materials by different immunoassays

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Introduction

The misuse of human chorionic gonadotropin (hCG) by athletes is a controversial topic because there are other doping agents with similar effects, which are more difficult to detect, like luteinizing hormone (LH), menotropins, gonadotropin releasing hormone (GnRH). When administered to males, hCG stimulates testicular production of testosterone, which might be suppressed during and after prolonged use of anabolic steroids. For hCG, WADA states that the minimum required performance level (MRPL) should be 5 mIU/ml [1], but characteristics of the method to be used for doping control and recommendations regarding the assay specificity have not been yet defined. The Educational test distributed by WADA in 2009 showed that the results from different laboratories are difficult to compare and that the commercial immunoassays developed for pregnancy evaluation are most frequently used. These are intended to measure the absolute levels of hCG in serum where the distribution of hCG forms differs from urinary hCG. It is known that hCG is excreted in urine in different forms. Some molecules pass the renal barrier and are excreted intact (hCGi), but the majority is excreted as nicked hCG (hCGn), free α- and β-subunits (hCGα, hCGβ), nicked free β-subunit (hCGβn) and the unique core fragment of hCGβ (hCGβcf). Approximately half of the hCG immunoreactivity in urine is due to hCGβcf of the β-subunit of hCG, which most assays do not detect [2]. The necessity of harmonization in the analysis of hCG in doping control is obvious, and a strong recommendation should be made, not only on which method to use but also about pre-analytical conditions. Incorrect transport and storage conditions could lead to the degradation of some forms of hCG in urine; the urinary salt concentration may vary more than 10-fold, which affects the affinity of some antibodies; large variations in urinary flow rate should be compensated for by normalization of urinary creatinine or specific gravity; and reference material (RM) for hCG determination should be recommended. In our study

we investigated the possibility to use commercially available hCG RM from the National Institute for Biological Standards and Control (NIBSC), and we have demonstrated the specificity and sensitivity of immunoassay kits which we use at our laboratory towards those RM.

Materials and Methods

Two automated immunoanalyzers, Adaltis PersonalLab (Adaltis Italia S. p. A, Italy) and Access2 (Beckman Coulter Inc., CA), were used for testing the hCGi, its subunits and fragments RM by three commercially available immunoassay kits – hCG-Eco-Test-Ultra-ELISA (Diatex-EM, Russia), DRG βhCG-ELISA (DRG Instruments GmbH, Germany) and Access Total hCGβ (Beckman Coulter Inc., CA). Reference material of hCGi (99/688), hCGn (99/642), hCGα (99/720), hCGβ (99/650), hCGβn (99/692) and hCGβcf (99/798) were purchased from NIBSC. All standards are WHO Reference Reagents. Phosphate buffer (10 mM) was prepared from phosphate buffered saline (PBS) tablets (Amresco, USA). Bovine serum albumin (BSA) (Sigma, Germany) was used to prepare the solution in PBS (0.1–1%). All above mentioned RMs of hCG were supplied in lyophilized form. Phosphate buffer was used to prepare the stock solutions (1 ml per ampoule). Aliquots were stored at -20^{o}C, and series of dilutions were prepared on the day of the analysis or were stored (in different containers under different temperature) for the stability study of the diluted standards. The concentration of RMs was measured on an open- (Adaltis PersonalLab) and closed-platform (Access 2) type immunoanalyzers in accordance with the kit instruction manuals.

Results and Discussion

There are certain RMs for testing immunoassays designed for hCG measurement [3-5]. As follows from the International Standard for Laboratories [6], it is preferable to use the RMs certified by a body of recognized status (USP, BP, Ph. Eur. or WHO). We have evaluated the possibility to use the above mentioned hCG NIBSC RMs for the detection of hCG misuse. Usually immunoassay kit producers do not indicate the epitope specificity of antibody used for designing of the assay. The kits used by the various doping control laboratories have not been described and no assay has been designed specifically for the purpose of doping control. In our study we used three kits from three different manufacturers. In the respective kit instruction manuals, the following information was provided: hCG-Eco-Test-Ultra is intended for the determination of hCG in human urine and

serum, DRG βhCG-ELISA uses a monoclonal antibody directed toward "unique antigenic site on the βhCG molecule", Access Total βhCG – "two-sites ELISA". We have tested the RMs by all above mentioned kits and found suitable storage conditions, diluent composition and appropriate concentration of a RMs which gave a value close to MRPL (5 mIU/ml). It was found that diluent composition affects the stability of the RM (Table 1), and 1% BSA/PBS was chosen to ensure better stability. Firstly, the necessary quantity of 1% BSA/PBS was added to polypropylene Eppendorf-type micro vials to prevent protein adsorption and then the calculated quantity of hCG RM was added to prepare the required dilution.

Table 1. Influence of the diluent composition on the stability of hCG. Results obtained on immunoanalyzer Access 2 (Beckman Coulter, CA) with kit Access Total βhCG.
DP – data from day of preparation; DAP-data from a given day(s) after preparation

Diluent	**PBS**				**0.1% BSA/PBS**				**1% BSA/PBS**	
Day of analys□s	*DP*	*3DAP*	*DP*	*1DAP*	*DP*	*1DAP*	*DP*	*1DAP*	*DP*	*4DAP*
Dilution, times	Measured concentration, mIU/ml									
hCG 1:4000	63.9	14.4	13.5	2.9	153.9	149.2	154.1	153.9	133.5	130.7
hCG 1:8000	31.2	7.9	6.5	1.6	73.0	73.4	73.0	79.9	63.4	60.8
hCG 1:16000	16.1	3.6	3.6	0.9	36.6	36.2	35.9	35.2	32.5	30.4
hCG 1:40000	6.5	1.9	1.4	0.5	14.5	14.9	15.1	14.8	12.7	12.3
hCG 1:80000	3.5	1.2	0.8	0.3	7.3	7.4	7.2	7.4	6.4	6.3
hCG β 1:2000	74.3	16.1	16.4	3.1	271.2	263.5	264.5	258.1	244.2	229.0
hCG β 1:4000	39.7	4.1	13.4	1.6	133.0	131.3	128.4	119.4	119.6	114.1
hCG β 1:8000	20.2	2.7	4.5	0.9	69.9	65.1	61.2	60.2	59.8	57.4
hCG β 1:20000	8.4	0.9	1.6	0.4	25.6	24.8	24.5	23.9	23.5	22.7
hCG β 1:40000	3.9	0.7	0.5	0.4	12.3	12.6	12.9	12.3	12.3	11.8

Our data have shown that the measured concentration is slightly decreased during storage of diluted RMs at 4-8°C The results from RMs diluted in 0.1% BSA/PBS and 1% BSA/PBS are comparable, but 1% BSA/PBS was chosen for the further experiments to prevent unspecific adsorption. At the same concentration of hCG RMs the specificity of kit Access Total βhCG (Beckman Coulter, CA) decreased in the range: hCG, hCGβ, hCGn, hCGβn, hCGβcf and hCGα (data not shown). From the results obtained, the hCG RMs and their dilutions were selected for which selectivity of the kit used was the highest and the concentration measured was closer to the established threshold of 5 mIU/ml: hCGi – dilution 1:40000 and hCGβ – dilution 1:40000. The series of chosen RMs dilutions were analyzed (data not shown) on a fully automated open-platform immunoanalyzer Adaltis PersonalLab by the two different ELISA kits (hCG-Eco-Test-Ultra from Diatex-EM, Russia, and DRG

βhCG-ELISA from DRG Instruments GmbH, Germany). The analysis was repeated on a fully automated closed-platform immunoanalyzer Beckman Coulter Access with special factory recommended kit Total β-hCG Access (Table 2). The data from one assay are not fully compatible with the other, because the antibodies recognize different epitopes, and immunoanalyzers have different detection systems.

Table 2. Comparison of results obtained by different kits and immunoanalyzers

Immunoanalyzer	Adaltis PersonalLab		Access 2
Kit	Diatex-EM	DRG	Total β hCG
	n=7	n=7	n=10
hCG 1:40000			
Mean, mIU/ml	3.0	2.4	4.3
CV, %	8.7	14.6	2.1
hCG β 1:40000			
Mean, mIU/ml	6.5	3.0	7.5
CV, %	6.6	10.0	5.4

The data obtained have shown that the NIBSC hCG RMs can be used for the routine analysis of hCG. The results differ depending on the kit and immunoanalyzer used for analysis. All used kits were specific towards hCGβ and hCGi but also measure hCGβn and hCGn. RM dilutions are stable in 1% BSA/PBS diluent, but nevertheless it is better to prepare them on the day of the analysis from stock solution, considering the slight decrease in activity during storage.

References

[1] World Anti-Doping Agency Technical Document – TD 2009MRPL.
[2] Stenman UH, Hotakainen K, Alfthan H. (2008) Gonadotropins in doping: pharmacological basis and detection of illicit use. British Journal of Pharmacology 154, 569-583.
[3] Stenman UH, Tiitinen A, Alfthan H, Valmu L. (2006) The classification, function and clinical use of different isoforms of hCG. Human Reproduction Update 12 (6), 769-784.
[4] Cole LA, Sutton JM, Higgins TN, Cembrowski GS. (2004) Between-Method Variation in Human Chorionic Gonadotropin Test Results. Clinical Chemistry 50 (5), 874-882.
[5] Laidler P, Cowan D, Hider RC, Kicman AT. (1994) New Decision Limits and Quality-Control Material for Detecting Human Chorionic Gonadotropin Misuse in Sports. Clinical Chemistry 40 (7), 1306-1311.
[6] International Standard for Laboratories. World Anti-Doping Agency. Version 6.0, approved 20 September 2008, part 5.4.6, 59.