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Analytical and logistic improvements for the doping control analysis at the 2007 Pan-American Games

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1 Introduction

The XV Pan-American Games were held in Rio de Janeiro, Brazil, between July 13th and 31st, 2007. For the first time in the history of the Games the Antidoping activities were planned to start with the opening of the Pan-American villa in July 4th. Out-of-competition (OOC) testing was performed before the completion and throughout the Games, as a full panel in competition (IC) testing, as defined by the organizers. As compared to other Major Games, PAN 2007, with 5,633 athletes and 56 different sports disciplines represented a little over 50% of a Summer Olympiad [1] and more than the 1219 samples analyzed for the 2006 Winter Olympiad [2]).

2 Accreditation, scope, results management

All analytical and managerial procedures were accredited for the ISO/IEC 17,025 norm, by the Brazilian National Metrological Institute (Inmetro) [3], jointly with the World Antidoping Agency (WADA) International Standard for Laboratories (ISL v 4.0 [4]). Target analytes were defined according to the "List of Prohibited Substances 2007" [5] issued annually by WADA. Results were released electronically through the ADAMS system [6], for the first time live in a Major Game. Beforehand it was used at the 2006 Paralympics Winter Games in Turin, Italy and 2006 Asian Summer Games in Doha, Qatar as a parallel system. It was very simple, straightforward and expeditious. This also simplified the task to achieve the goal to deliver results up to 24h after receiving the samples at the laboratory. The Pan-American Sports Organization (PASO) installed a Medical Committee (MC) with 15 members, to evaluate the laboratory results. The PASO-MC agreed, according to WADA´s recommendation, that glucocorticosteroids would be reported only if present in quantities above the Minimum Required Performance Limit (MRPL) set by WADA [7]. This reduced the quantitation burden for the lab, the number of Adverse Analytical Findings (AAF) reported and the administrative burden for the PASO-MC.

3 Logistics and personnel

The main concern was focused on the logistic aspects. It was not feasible to build up a new laboratory to congregate all activities. Therefore, they were dispersed in 7 (seven) different locations on the 5th, 6th and 7th floors of the Chemistry Institute building at the University campus. Part of the 5th and 6th floors (including corridors and bathrooms) were isolated and transfer of sample aliquots and documents outside this restricted area were performed by the laboratory staff, in sealed boxes, with a documented chain of custody, including time of delivery. In all cases, less than 5 minutes were taken for the displacement of secure materials. Five floor security guards were present at all time and exchanged every 8 (eight) hours. Door and floor surveillance cameras were manned 24h / day and registered all displacements to and from each laboratory. The building had its own security detachment as well as the streets within the island of the campus, which were also surveilled by cameras manned by a detachment of the Federal Police Department. To avoid interruption of the activities, all supporting infrastructure had redundancies. Among them, two high speed broad band loops for internet, two connections with separate power stations from the city of Rio de Janeiro; back up cisterns for water supply. Larger instruments had half an hour no-break systems. Compressed air came from 4 compressors and nitrogen gas was delivered either from generators, cylinders and liquid nitrogen 100 L Dewars. Other gases had back up cylinders at the lab and a 24h delivery service from the supplier. All major equipments had service personnel in place or on call to reach the laboratory in less than an hour. All had supplies in stock locally. In total over 200 people collaborated, from which around 80 were directly involved in laboratory activities. Thirteen international experts were released from their WADA laboratories to act as volunteers (Table 1). This was essential since senior staff members from the laboratory would take care of only one of the three shifts. The 10 h length for each shift allowed for the reinforcement of man power in certain key moments and facilitated the transfer of responsibilities among shifts. Also, it was used as the time for eating. Four meals were served every 24 h, in a special canteen service set up for the Games. Coffee, refreshments and snacks were provided all times. Everyone worked for three weeks without rest; therefore there was no need for a spare group of technicians to take over during the resting days. This reduced substantially the number of additional trained personnel engaged in the operation. The distribution of the personnel among the shifts was dictated by the timing of arrival of the samples at the laboratory, taken as 4 to 5 h after the end of the competition, as defined in the program of the PAN. This implied a complex transportation system. For the samples this was taken up by PASO, but for the laboratory personnel it was set up by the lab. Since Great Rio de Janeiro has around 12,000,000 people, the city is widespread and the technicians came from all over. Six

loops covered by mini vans were set to collect and take back the personnel, especially by night and during weekends where there was no frequent public transportation system. The international volunteers had shuttles to and from the laboratory at all times. There was a delay in collecting OOC samples prior to the competitions and this resulted in an overflow of samples while the laboratory was still "warming up". This disrupted the organization and it took several days for the laboratory to catch up with the 24 h release of negative results. The "curve" for results release on the first 10 days (July 13th up to 22nd) followed the "expected" sample arrival. The samples in excess had their results delayed, with a peak of released reports on the 11th day (July 23rd). Then as expected delay occurred for the 15th and 16th days where a larger numbers of samples were collected. As usual there was a reasonable number of samples being reprepared due to the need to confirm the presence of prohibited substances as well as overcome analysis non-conformities (inadequate quality controls, etc…).

4 Experimental

4.1 Preliminary analysis

In agreement with the head of the PASO doping control, pH was not determined at the collection site and density was determined with densitometers. If the density was below 1.004, a second collection was performed. If the urine sample remained diluted the athlete was released and an OOC sample taken. In the collection form it was noted only the specific gravity as "above" or "below" 1.004. This procedure reduced the burden over the athlete and doping control officers, for otherwise hours of waiting for the urine to reach acceptable values. It also simplified the transportation logistics of the samples to the laboratory, with minimum delay. Once received at the laboratory, pH and density were taken. In the event of diluted samples, both samples were analyzed separately.

4.2 Screening and confirmation strategy

The samples were evaluated through 8 (eight) screening procedures (detection by Gas Chromatography (GC) coupled to Mass Spectrometry (MS) and tandem MS (MS^n), High Performance Liquid Chromatography (HPLC) coupled to MS^n, or immunoassays) and applied to all urine samples and a 9th screening applied to the 201 samples collected for EPO analysis (Isoelectric focusing with double blotting). Each suspect sample was re-prepared and then confirmed before release of the AAF. Confirmation procedures were established for each screening / substance; a separate confirmation procedure for endogenous steroid abuse was based on Gas Chromatography coupled to a combustion furnace and an Isotope Ratio Mass Spectrometer (GC/C/IRMS).

4.3 Sample preparation

The main analytical protocols used for urine sample testing in the different analytical screenings are describe elsewhere [8]. Nine screening procedures were done, usually performed in 20 samples batches. The immunoassay analyses were accomplished after procedure of periodical maintenance and calibration according to specifications of the manufacturer. The LC-MS^n techniques being rather sensitive allowed for the analysis of two mixed samples. If there was a suspicious peak, they were re-prepared separately. This permitted a fast turnover of the LC-MS^n analyses to meet the 24 h deadline for results release. EPO screenings were not time enforced and were taken at leisure, most batches being finished in three days. But, as the procedure has three distinct steps, they could be realized in parallel. Therefore all 201 samples including the series of confirmatory analyses for the AAF were finished a week after the end of the Games.

4.4 Quality control strategy

To ensure the quality of screening in the chromatographic procedures, negative and positive quality control samples were included in all batches. The positive controls were blank urine samples spiked with reference materials, at the minimum required performance limit (MRPL) or the threshold values (for threshold substances). A detailed description of the quality control strategy during the Pan-American Games is described elsewhere [8]. Hydrolysis Control (HC) and Derivatization Control (DC) were also included in screenings IV and II, respectively. Screenings I, II, IVC, VIIA, VIIB have internal standards, screenings VI and VIII, external calibration standards and IVB a set of 4 (four) deuterated internal standards and 17α-methyltestosterone. The erythropoietin screening followed WADA´s recommendations and had recombinant EPO as well as NESP as positive controls [9]. For the five low concentration steroids (3´-hydroxystanozolol, 19-norandrosterone, 17alfa-methyl-5beta-androstane-3alfa,17beta-diol, 17β-methyl-5β-androst-1-ene-3α,17α-diol (epimetendiol) and clenbuterol) the batches after screening IVB were analyzed in the fast GC-ITDMS screening IVC. Despite this serial analytical procedure, most batches of "anabolics" screenings (IVB + IVC) ended in less than 20 h. In GC/C/IRMS instrument the stability and reproducibility of measurements was monitored by determining the $\delta^{13}C$ value ($\delta^{13}C$ (‰) = (Rsample $-R$std)/Rstd, where $R = {}^{13}C/{}^{12}C$) of the calibration standard *n*-heneicosane, which remained close to the mean value −30.09 ‰ with a SD value equal to 0.51‰. The alkane standard was injected daily, in triplicate, before any analytical batch. Only when the results were between ± 3 x SD in relation with the historical series values, the analytical batches were injected. Otherwise, the furnace tube was submitted to an oxidation process. In addition, the positive and negative

quality controls injected in the analytical batch were compared with a pre-established historical series. The procedure was considered valid only if the $\delta^{13}C$ values steroids of interest matched with the historical series.

4.5 Instrumental parameters

The instrumental parameters used during the games were described elsewhere [8]. The maintenance of all equipments follows the internal protocol established for each screening in the lab daily routine.

5. Results and Discussion

5.1 Logistics

The logistic aspects were conveniently dealt with through two main administrative tools: a Centralized Administrative Committee and a Crisis Intervention Committee, constituted by representatives of all aspects of the supporting services needed to guarantee the 24h turnover of samples. This allowed to circumvent the initial burden caused by the delay in collecting the OOC samples which would have served as a training set for the laboratory shifts; the delay also resulted in an overwhelming number of samples arriving at the laboratory in the beginning of the Games. All other dozens of unexpected situations were conveniently and timely dealt with by the Committees without further disruption in sample throughput.

5.2 Screening analysis

In total, 1,274 analyses were done in the Pan-American period; 234 Out of Competition and 1040 In Competition. 201 samples analyzed for recombinant erythropoietin (rEPO) and darbepoetin alfa (NESP). Even though several traditional screenings in doping control were adopted thought the Games, it is important to note that the molecular diversity of modern pharmaceutical products shattered the paradigm that similar biological properties were related to similarities in the chemical structures of the drugs. Therefore the main evolution of the screenings II, IVB and VII A & B, was, that they are not related to one pharmaceutical class of drugs anymore. They, in fact, encompass all kind of molecules that are prone to be analyzed by each defined procedure, therefore loosing the direct relationship with the biological effects of the targeted analytes. Considering this, the methods were built respecting mainly the phase 2 excretion profile. Diuretics, usually excreted free in the urine, were put together in the LC-MS-MS (VII B) method with some stimulants that show poor behavior in

the classical GC-MS screening, as modafinil and adrafinil. The tamoxifene metabolites were included in the LC-MS-MS method (VII A) for glucocorticosteroids and anabolic steroids. Analytes as aminoglutethimide, testolactone (aromatase inhibitors) and finasteride metabolite (alpha-reductase inhibitors) were also included in the LC-MS-MS methods. Nowadays, the detection of some anabolic agents as gestrinone and tetrahydrogestrinone by LC-MS-MS is mandatory due the poor behavior of the 4,9,11–trien–3–one moyety in the classical derivatization protocol for steroids [10]. Human chorionic gonadotrofin (β-hCG) showed a similar distribution as the Brazilian athletic population [11]. Only one result was slightly higher than the MRPL of 5 mIU/mL, but, without a clearly established uncertainty and cut off value, it was not released as an AAF. A note was sent to the PASO-MC to perform a medical evaluation of the athlete. Plasma expanders (HES and dextran) were analyzed by a validated Benedict´s test based technique [12] and suspicious samples were confirmed by GC-qMS [13].

5.3 Steroid Profile

The steroid profiles of all samples were evaluated in the light of the WADA's technical document – TD2004EAAS, related to the testosterone, epitestosterone T/E ratio and other endogenous steroids [14]. Aiming to reduce the time of delivery of the results, the steroid profile was preliminary interpreted considering the free plus glucuronide fraction. In that way, the IRMS team received the sample immediately after the screening and the quantification of the glucuronide fraction was done latter. Fifty seven (57) samples were selected for IRMS analysis after interpretation of the steroid profile (free + glucuronide): thirty one (31) with $T/E > 4$ ($4.0 < T/E < 8.2$) and twenty three (23) with DHEA > 100 ng/mL; three (3) samples were included exclusively due to suspicious values for 5α-androstanediol or 5β-androstanediol; four samples presented androsterone and/or etiocholanolone above 10,000 ng/mL, but in these cases other parameters were also suspicious. Only one sample, with $T/E = 5.2$ after quantitation, was confirmed by IRMS and declared after the games as AAF (see 5.6). All suspect samples were declared as AAF so that the respective Federations would evaluate them by the recommended follow up of the athlete´s steroid endogenous profile.

5.4 TUE

A sub-committee of the PASO-MC was in charge of the Therapeutic Use Exemptions (TUEs) [15], granting 126 out of 164 requests. A potential Adverse Analytical Finding due to the detection of terbutaline was delivery to the doping authority. A retroactive TUE was granted because its

administration followed an emergency call to the medical facility at the PAN Villa and the medical doctor wrongly prescribed this forbidden substance without a TUE request.

5.5 Confirmation analysis

Several confirmation methods were available, especially GC-MS alternative methods for GC-qMS screenings (involving different preparation procedures or GC-ITDMS sequential detection). For LC-MS^n confirmation procedures could be GC-qMS, GC-$ITDMS^n$ or LC-MS^n with different LC separation conditions and several additional MRMs. The identification criteria followed WADA´s rules [16].

5.6 Adverse Analytical Findings

Table 2 presents the AAFs that occurred during PAN 2007. Amazingly they represented a good coverage of the main screenings set up for the Pan: two of them represented double blind samples included in the normal flow of samples to the laboratory as agreed between PASO and WADA. These were modafinil and tamoxifen which were detected by our LC-MS^n screenings. Terbutaline was detected in the GC-qMS and LC-MS^n screenings (II and VIIA). The testosterone / testosterone precursor abuse was confirmed by the distinct isotopic difference of 5α-androstan-3α,17β-diol ($\delta\ ^{13}C/^{12}C$ = - 31.42) and the endogenous reference pregnanediol ($\delta\ ^{13}C/^{12}C$ = - 22.25). Fifteen samples showing altered endogenous profiles were reported to the respective International Federations for a longitudinal evaluation [14]. A borderline (by GC-qMS) boldenone/boldenone metabolite was also confirmed by GC/C/IRMS, but in this particular case the sample was sent to the Cologne WADA Accredited Laboratory, since there was no validated methodology in place for the special clean up required for this confirmation. EPO misuse was confirmed as NESP, but it was also clear the presence of rEPO, which may be guessed as a tentative of the athlete to reduce the chances of being caught by administering half the amount of each EPO's pharmaceutical analogue. Only the athlete that had the AAF for NESP requested the "B" sample analysis. It was performed and confirmed the "A" sample result. PASO-MC final recommendations were that 3 (three) AAF (NESP, testosterone / testosterone precursor and boldenone cases) should lead to sanctions of the athlete. Without the knowledge of the laboratory, repetitive evaluations of a swimmer were performed during the PAN 2007. All samples were negative: one with typical normal density and endogenous profile; the other three extremely diluted. The PASO-MC asked the "typical one" as well as one of the diluted samples to be sent to DNA analysis (SONDA - UFRJ laboratory), which disclosed two different DNAs between the two. This possible manipulation of a sample during collection is under scrutiny

by sport and police authorities. The relatively low number of AAF may be accounted for by the pre-testing of the major delegation´s athletes performed before the Games.

8 Final remarks

Due to a decision from the PASO-MC, taking into account results from previous major games [1, 17], no blood samples were analyzed. Also several peptide hormones were not effectively analyzed during PAN 2007. Proteomics techniques should be included in the panel of screenings to guarantee a better coverage for these substances. Possibly metabolomics will be needed in the future to track down new designer drugs like THG [10] and maybe genomics will have to be applied once the athlete´s will dare going into genetic doping [18]. One may wonder if PAN 2007 has been the last Major Game without “omics”.

9 Conclusions

The WADA Independent Observers concluded that “the Program implemented in Rio is a tremendous step forward in the fight against doping in the region” [19].

Acknowledgements

PAN 2007 anti-doping analysis was made possible through the more then 200 people involved in the laboratory logistics, among them over 70 people from the doping control laboratory (LAB DOP) and volunteers from LADETEC´s associate labs and 13 (thirteen) experts that were kindly released from their duties from other WADA´s accredited laboratories and volunteered to a hard, weird hours, non-stop work, for at least three full weeks. They came from 8 WADA´s accredited labs, more than 20 companies, 10 governmental bodies / departments, several Federal University of Rio de Janeiro (UFRJ) sectors and the University Foundation (FUJB). In-house and abroad training of our staff was performed by Cologne, Barcelona, Lisbon, Seibersdorf and Athens WADA´s accredited labs. Special thanks are due to the Ministry of Sports that financed a large portion of the infrastructure and established a task force to overcome import procedure bureaucracies; as well as to the PAN 2007 Committee (Co-RIO), the Brazilian Olympic Committee (COB) and the PASO authorities for their confidence in the capability of UFRJ to meet all requirements for sample analysis. Support from WADA personnel and its Independent Observer Team, and the PASO-MC is also acknowledged.

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Table 1. Volunteers from WADA accredited laboratories and their function, during PAN 2007 sample analysis, according to their expertise.

Function	Name	Affiliation
Overall Certifying Scientist (CS)	Josep Marcos del Aguila	Barcelona, Spain
	Margarida T. C. Vidal	Havana, Cuba
	Mario Thevis	Cologne, Germany
	Xavier de la Torre	Lisbon, Portugal
CS for EPO	Christian Reichel	Seibersdorf, Austria
CS for GC/C/IRMS	Rodrigo Aguilera	Team Leader Proteomics Analysis from Applied Biosystem
HPLC-MS-MS specialist (sp)	Andreas Thomas	Cologne, Germany
	Koen Deventer	Gent, Belgium
EPO sp	Flaminia Garribba	Rome, Italy
Screening I & II sp	Rodny M. O. do Porto	Havana, Cuba
Screening IVB sp	Dayamin Martinez Brito	Havana, Cuba
	Nadine Haenelt	Cologne, Germany

Table 2. Adverse Analytical Findings that occurred during PAN 2007.

Class of substance*	Analyte	Screening / Confirmation
S1.a. Anabolic Agents	Boldenone/Boldenone metabolite	Screening IV / IRMS
S1.b. Anabolic Agents	Testosterone/ T precursor	Screening IV / IRMS
S2. Hormones and related substances	NESP	Screening X
S3. Beta-2 agonists	Terbutaline	Screening II / IV
Double Blind test from WADA		
S4. Hormone agonists and modulators	Tamoxifen metabolite	Screening VIIA / VIIA
S6. Stimulants	Modafinil	Screening VIIB / VIIB