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An overview of the doping control analysis during the Olympic Games of 2004 in Athens, Greece

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Abstract

This study summarizes the results obtained from the doping control analysis during the period of the XXVIII summer Olympic Games (30 July–29 August 2004). The analysis of all doping control samples was performed at the Doping Control Laboratory (DCL)—the World Anti-Doping Agency (WADA) Accredited Laboratory of Athens. Three thousand six hundred and seventeen tests were conducted in total throughout the games. In 23 specimens the presence of a prohibited substance was confirmed. Sixteen of those were related to anabolic agents. The screened results were confirmed with various mass spectrometry analytical techniques, such as gas chromatography/high resolution mass spectrometry (GC/HRMS), gas chromatography/mass spectrometry (GC/MS), gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) and liquid chromatography/mass spectrometry (ion trap) (LC/MS). The results of the first time applied screening and confirmatory procedures for the detection of recombinant human growth hormone in serum were also presented. Besides, 107 therapeutic use exemptions (TUE) were verified for glucocorticosteroid and beta2-agonist use.

Keywords: Doping control analysis; Olympic Games; Urine/blood testing

1. Introduction

The XXVIII summer Olympic Games were held in Athens between the 13 and 29 August 2004 with the participation of 10,864 athletes originating from 202 different countries. Twenty-seven percent of the athletes were selected to go through doping control.

As the full responsibility for laboratory accreditation was taken over by WADA, on behalf of the International Olympic Committee (IOC), on 1 January 2004, the summer Olympic Games in Athens were the first games with a doping laboratory strictly operating under WADA International Standards, and the WADA supervision [1]. As for the Greek part, a collaboration scheme (Memorandum of Understanding, July 2001) between the Athens Organizing Olympic Committee (ATHOC) and the General Secretariat of Sports (GSS), led to the so-called "Olympic Games 2004-Doping Control" work plan, which was launched to ensure the quality of the Doping Control, in accordance to the WADA specifications.

For this purpose the Doping Control Laboratory of Athens (DCL) moved into a new building (total area: 1800 m^2), being constructed at the outskirts of the Athens Olympic Center (OAKA). Having back the successful undertake of the Sixth World Championships of Athletics in Athens, in 1997 [2], the same staff core was used from DCL in these games. The scientific staff and an adequately trained auxiliary staff consisted of 153 people, which worked for the whole Olympic period, with the full attendance of IOC and the WADA Independent Observers. Also, during 2004, the DCL went through a three-times accreditation assessment (ELOT EN ISO/IEC17025) by the National Accreditation Council (ESYD), and was assessed by WADA and the IOC (regarding its renovated infrastructure and processes) for compliance with the World Anti-Doping Code (WADC) [3] and the International Standard for Laboratories (ISL) [4].

According to the IOC, the Period of the Olympic Games started officially on 30 July 2004 and in- and out-competition tests were performed during that time (30 July–29 August 2004),

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all considered as in-competition tests. The requirements for number of sample testing were set to the level of 180 samples/day. The negative samples should be reported in 24 h and for the first time, the positive samples should be reported in 36 h, and the positive EPO samples in 72 h. In total, 3617 samples (2926 urine samples and 691 blood specimens) were tested for all prohibited substances for which accredited methods were operational. Specifically, in blood analysis haemoglobin-based oxygen carriers (HBOCs), recombinant human growth hormone (rGH) and homologous blood transfusion (BT) were tested. Overall, 23 samples (less than 1% of the samples tested) were reported as positive by the DCL, these findings mostly related with anabolic steroid substances and other agents, like the beta2-agonist clenbuterol.

2. Experimental

2.1. Personnel

The DCL personnel for the Olympic Games consisted of 153 people. This was divided in the scientific staff (74 persons) and the technical and support personnel (79 persons). Based on the scheduled sample arrival time at the laboratory, the staff was mainly programmed to work in three rotating shifts, on a 24 h-basis. The shifts were distributed as follows: for the scientific staff, the morning shift lasted 8 h, the afternoon shift lasted 9 h and the night shift lasted 7 h, whereas for the technical staff, this was divided in groups rotating every 8 h. In every analytical sector, there were shifting groups with one supervisor.

2.2. Instrumentation and operation

The main analytical instrumentation used for urine and blood sample testing in the different analytical sectors, is shown in Table 1. A detailed description of the DCL organisation during the Olympic Games period in terms of personnel training, security, power facilities and computer systems is presented elsewhere [5].

Prior to the games, all equipment was serviced. In the GC systems, cleaning of sources, replacement of columns, change of furnace tubes (in GC/C/IRMS) were employed. A maintenance schedule was established during the games, including daily change of septum and liners, as well as precolumn and/or column changes, depending on the instrument performance. Replacement of the inner source in GC/HRMS instruments was also performed. In LC/MS (ion trap) instruments spray chamber cleaning occurred daily, while Electrospray interface cleaning (ESI) was performed only once, during the Olympic Period.

To ensure the quality of screening in the chromatographic procedures, negative and positive quality control samples were included in all batches. For the within batch quality control, sensitivity controls were applied, and these were blank urine samples spiked with reference materials, at the minimum required performance limit (MRPL) or the threshold values (for threshold substances) (Table 2A) [6,7]. To test between batch reproducibility, regarding the retention times, internal standards were spiked in all samples, while a reference sample with a mixture of prohibited substances was introduced in all batches. For the immunological methods, those used in urine and blood testing, there were commercially available quality control samples (Tables 2A and 2B).

For the between batch quality control assurance, quality control charts were constructed for all analytical instruments. As an example, in GC/C/IRMS instruments the stability and reproducibility of measurements was monitored by determining the δ^{13}C value (δ^{13}C (‰) = ($R_{sample} - R_{std}$)/R_{std}, where $R = {}^{13}C/{}^{12}C$) of the calibration standard *n*-octadecane, which remained close to the mean value −30.71‰ with a SD value equal to −0.32‰ (Fig. 1). In two cases represented in Fig. 1, the mean value was exceeded on day 22 and therefore a repairing action needed to be taken, i.e. in a concentrated sample the out of range δ^{13}C value was re-established after sample dilution, while the sensitivity of measurement was improved on day 23 after manipulation of He flow rate. As regards to the GC/HRMS instruments, sensitivity was secured by the sufficiently high signal to noise ratio (S/N) (ratio above 10) of the control substance epimetendiol (methandienone metabolite) measured in the control sample Δ847U (Fig. 2, Table 2A).

2.3. Materials

Solvents used in the urine extraction procedures were of analytical grade (Labscan, Ireland) (Table 3). Standard solutions were made with ion-distilled water. β-Glucuronidase from *Escherichia coli* (Sigma–Aldrich, Germany) was used for enzymatic hydrolysis. HCl (Panreac, Spain) and cysteine (Fluka, Germany) were used for acidic hydrolysis. MSTFA and the MBTFA were purchased from Chem Fabrik Karl Bucher, Germany and DTE from Sigma–Aldrich, Germany. Reference materials used in control samples for procedures I, II and V (Table 2A) were from Sigma–Aldrich, and endogenous anabolic reagents in procedure IV from Steraloids Inc., USA or National Australian Reference Laboratory. Glucocorticosteroid standards were from Sigma–Aldrich, Germany. The calibration standard (*n*-octadecane) in the GC/C/IRMS was from Chiron AS, Norway. The Glucose kit used in plasma expanders procedure were from ABX, France. HCG immunoassay kits were from DPC, LA, USA; Adaltis, Italy. EPO and GH standard suppliers are given in Table 2A. GH antibodies were kindly donated by Dr. M. Bidlingmeier, University Clinic, Munich, Germany. In the blood screening procedures (Table 2B), standard solutions were made with Milli-Q® ultra pure water (Millipore, MA, USA).

2.4. Analytical procedures and protocols

The handling of samples (already transferred to two independently sealed bottles at the doping control stations, usually designated “A” and “B” samples), the preparation of aliquots for testing, the pre-analysis, screening and confirmation procedures, the results management, the documentation and reporting process, were in accordance with the ISL guidelines [4]. In case

Table 1
List of crucial equipment available at the DCL during the Olympic Games

Instrumentation	Manufacturer/model	Quantity	Technical characteristics
Sector I—anabolic steroids, beta-blockers, glucocorticosteroids, beta2-agonists and related substances			
GC/MS (quadrupole)	Agilent Technologies/6890N-5973 inert MSD	8	HP Ultra 1 column (17 m × 0.200 mm i.d. × 0.11 μm)
GC/MS (quadrupole)	Hewlett Packard/6890 Series-5973 MSD	1	HP Ultra 1 column (17 m × 0.200 mm i.d. × 0.11 μm)
GC/HRMS (sector)	Micromass/AutoSpec Ultima	4	HP Ultra 1 methyl silicone gum column (12 m × 0.200 mm i.d. × 0.33 μm)
GC/C/IRMS	GV/Isoprime	2	Supelco SPB-50 column (30 m × 0.250 mm i.d. × 0.25 μm)
LC/MS (ion trap)	Agilent/1100 Series LC/MSD Trap SL	6	Agilent Zorbax Rx-C8 column (2.1 mm × 150 mm i.d., pore size 5)
Sector I—stimulants, narcotics, diuretics and plasma expanders			
Gas chromatograph/nitrogen phosphorus detector (GC/NPD)	Hewlett Packard/5890	2	HP Ultra 1 column (12 m × 0.200 mm i.d. × 0.33 μm)
GC/NPD	Agilent Technologies/6890	2	HP Ultra 1 column (12 m × 0.200 mm i.d. × 0.33 μm)
GC/MS (quadrupole)	Hewlett Packard/5890-5971 Series MSD	1	HP5 column (12 m × 0.250 mm i.d. × 0.25 μm)
GC/MS (quadrupole)	Hewlett Packard/5890-5970 Series MSD	1	HP Ultra 2 column (12 m × 0.200 mm i.d. × 0.33 μm)
GC/MS (quadrupole)	Hewlett Packard/6890 Series-5973 MSD	1	HP Ultra 2 (12 m × 0.200 mm i.d. × 0.33 μm)
GC/MS (quadrupole)	Agilent Technologies/6890N-5973 inert MSD	9	HP Ultra 2 (12 m × 0.200 mm i.d. × 0.33 μm)
Biochemical analyzer	ABX Diagnostics/COBAS MIRA Plus	1	Spectrophotometry, λ = 340 nm (Glucose HK CP)
Sector III—proteins, hormones and blood cell analysis			
Fluorometer	Perkin-Elmer/Victor 3	1	TR-fluorometry (plate reader), λ_{exc} = 340 nm/$\lambda_{emission}$ = 545 nm/572 nm/616 nm/642 nm
Flow cytometer	Beckman Coulter/XL-MCL	1	Argon Laser, λ = 488 nm
Haematology analyzer	SYSMEX/XE2100	1	Fluorescence flow cytometry
Haematology analyzer	SYSMEX/R-500	1	Fluorescence flow cytometry
Haematology analyzer	Beckman Coulter/A^c.T diff	1	Fluorescence flow cytometry
Biochemical analyzer	Diagnostic Product Corporation/Immulite	1	Chemiluminescence, λ = 425–500 nm (Immulite hCG kit)
Enzyme linked immunosorbent assay (ELISA)	BIOKIT, SA/BEST 2000	1	Spectrophotometry, λ = 450 nm/λ_{ref} = 620 nm (EIAgen total hCG kit)
Gamma-counter	Packard/Crystal 5412	1	Gamma radiation, ^{125}I (hCG MAIAClone kit)
Image scanner flatbed	Amersham Pharmacia Biotech/Image Scanner	1	Autoradiography and wet gels/linearity > 3.7OD/full 14-bit pixel depth/optical resolution 2400 dpi × 1200 dpi
Isoelectric focusing (IEF)			
Thermostatic circulator	Amersham Biosciences/MultiTempIII	2	−10 °C up to +90 °C, 200 W cooling capacity, 3l reservoir capacity
Isoelectric flatbed electrophoresis system	Amersham Biosciences/Multiphor II	2	Suitable for gels up to 20 cm × 26 cm
Semidry transfer unit	Amersham Biosciences/TE77 semi-dry transfer unit	2	Perforated platinum-titanium and stainless electrodes; suitable for gels up to 21 cm × 26 cm
Flatbed electrophoresis	SEBIA/Hydragel Hemoglobin K_2O chamber	1	
	SEBIA/Hydragel Hemoglobin K_2O applicator	1	Designed for sample application on HYDRAGEL agarose gels
High performance liquid chromatography (HPLC) (UV/DAD)	Hewlett Packard/Series II 1090 Liquid Chromatograph	2	Superdex prepacked glass column 10/300 GL, exclusion limit ∼1.3 × 10^6 MW

of an adverse analytical finding, a Laboratory Documentation Package (LDP) was prepared and signed by the DCL director and two members of the IOC Medical Commission Games Group.

2.4.1. Urine sample testing

All urine samples were imposed into five parallel analytical procedures (I, II, IV–VI, Table 2A) according to the DCL Standard Operating Procedures (SOPs). When specifically stated in the athletes' form, samples were also tested for EPO, plasma expanders or beta-blockers (Table 2A). The sample preparation steps for procedures I, II, IV–VI and plasma expanders are summarized in Table 3. Specifically for procedure IV, sample aliquots were split in three, in order to undertake a different derivatisation or reconstitution process to be used in three different applications (Tables 2A and 3). Remaining sample "A" and intact sample "B" were securely refrigerated. The "B" sample analysis occurred only upon request of the athlete, in order to confirm the "A" sample analysis findings.

Table 2A
Analytical procedures applied on urine samples

a/a	Screening procedures	Instrumentation	Control samples
1	Procedure I: Volatile nitrogen-containing compounds (stimulants, narcotics) (60 analytes)	GC/NPD	CALA: heptaminol, amphetamine, dimethylamphetamine, amphepramone, nikethamide, prolintane, pipradol, strychnine at MRPL, ephedrine at 10 μg/ml, cathine[a] at 5 μg/ml
2	Procedure II: Heavy volatile nitrogen-containing compounds (Stimulants, narcotics) (100 analytes)	GC/MS	CALB: etilefrine, pholedrine, sydnocarb, fenoterol, terbutaline, ethamivan, pentazocine, pethidine, methadone, modafinil, amiphenazole, benzoyl-ecgonine at MRPL, morphine at 1 μg/ml, ephedrine at 10 μg/ml, cathine at 5 μg/ml CALWADA: stimulants and narcotics at MRPL
3	Plasma expanders	Biochemical Analyzer	CALDEXA-CALBEXB (dextran at 1500 and 5000 μg/ml) CALHETA-CALHETB (HES[b] at 1500 and 5000 μg/ml) CALGLU (glucose at 1000 μg/ml)
4	Procedure IV: Combined free and conjugated anabolic agents, beta-blockers and beta2-agonists (107 analytes)	GC/MS	Δ471U: endogenous AAS[c] (androsterone and etiocholanolone at 2500 ng/ml, 5α-androstan-3α,17β-diol at 100 ng/ml, 5β-androstan-3α,17β-diol at 200 ng/ml, DHT[d] at 21 ng/ml, DHEA[e] at 125 ng/ml), salbutamol at 1 μg/ml, T/E = 6, THC-COOH at 15 ng/ml CALMIX10: exogenous AAS and beta2-agonists at 10 ng/ml, beta-blockers at 500 ng/ml, anti-estrogenic substances at 10 ng/ml
5	Combined free and conjugated anabolic agents and beta2-agonists (51 analytes)	GC/HRMS	Δ847U: 19-noretiocholanolone, 5α- and 5β-17α-methyl-androstane-3α,17β-diol, clenbuterol, epimetendiol, 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3α-ol (all at 1 ng/ml), 19-norandrosterone, 3′OH-stanozolol, 4β-OH-stanozolol (all at 2 ng/ml), buprenorphine at 10 ng/ml CALMIX10: exogenous AAS and beta2-agonists at 10 ng/ml, beta-blockers at 500 ng/ml, anti-estrogenic substances at 10 ng/ml
6	Glucocorticosteroids and combined free and conjugated anabolic agents (22 analytes)	LC/MS (ion trap)	Δ471U: betamethasone and flunisolide at 30 ng/ml, epitrenbolone at 10 ng/ml CALMIX10: 13 glucocorticosteroids at 30 ng/ml, trenbolone, formoterol, gestrinone and THG[f] all at 10 ng/ml
7	Procedure V: Diuretics (31 analytes)	GC/MS	CALD (diuretics and other masking agents at 250 ng/ml)
8	Procedure VI: Human chorionic gonadotropin (hCG)	Biochemical Analyzer	Low: CON4 (8.2–12.8 mIU/ml), Medium: CON5 (31–43 mIU/ml), High: CON6 (298–446 mIU/ml)
9	Protein hormone recombinant human erythropoietin (rEPO) (four analytes)	IEF and double blotting appliances	uEPO: endogenous urinary human EPO (NIBSC standard) rEPO: (European Pharmacopoeia standard) NESP: rEPO (darbepoietin α-Amgen)

Note: All positive control samples were prepared using urine as sample matrix.
[a] Cathine: Nor-pseudo-ephedrine.
[b] HES: hydroxyethyl starch.
[c] AAS: anabolic androgenic steroids.
[d] DHT: Dihydrotestosterone.
[e] DHEA: Dehydroepiandrosterone.
[f] THG: Tetrahydrogestrinone.

The process of confirmation of the adverse analytical findings[1] was a modified version of screening, generally involving, A-sample aliquot, blank urine and positive control extracts. In the case of a distorted endogenous steroid profile found in the screening procedure IV (androsterone, etiocholanolone concentration > 10,000 ng/ml, DHEA > 100 ng/ml, testosterone or epitestosterone concentration > 200 ng/ml, testosterone/epitestosterone > 6), a preconfirmatory GC/C/IRMS stage was applied. Depending on the above analysis data, the samples proceeded to confirmatory analysis, which involved GC/MS analysis with GC/C/IRMS evaluation. Besides, the analytical technique of LC/MS (ion trap) was applied for the confirmation and/or quantification of threshold substances like salbutamol, ephedrines, morphine and HBOCs (unpublished data).

The screening for the modified glucose polymer (HES) and dextran was based on an enzymatic hexokinase glucose assay (D. de Böer, pers. commun., Laboratory for Doping Analysis and Biochemistry, Lisbon, Portugal). The ‘total’ glucose concentration (after an acid hydrolysis step) versus the ‘free’ glucose concentration (without hydrolysis) from a reference population ($n = 286$ urine samples) were plotted and a standard curve was created. The cut-off curve was the result of a positive 3.3 shift of standard deviation (i.e. +3.3SD) of the standard

[1] “Adverse Analytical Finding” is defined in the World Anti-Doping Code as “a report from a laboratory or approved testing entity that identifies in a specimen the presence of a prohibited substance or its metabolites or markers (including elevated quantities of endogenous substances) or evidence of the use of a prohibited method” [3].

Table 2B
Screening tests performed on whole blood or blood serum samples

Blood testing parameters	Instrumentation	Control samples
Blood serum		
Recombinant human growth hormone (rGH)	Time resolved fluorometer	Pituitary extract (NIBSC standard) (0–50 ng/ml) Human serum spiked with genotropin (Pharmacia) (10 ng/ml) Human serum (Pharmacia)
Haemoglobin-based oxygen carriers (HBOCs) (five analytes)	Optical analysis	Colorimetric scale made from blank human serum spiked from 0–2 mg/ml with Oxyglobin (Biopure corp.)–cut-off ref. value: 1.5 g/l
Whole blood		
Haematocrit (Hct), haemoglobin (Hb), reticulocytes (RET) (Health tests)	Haematology analyzer	*e*CHECK # 42190802 (all control kits were supplied by SYSMEX) Hct (%): 34.7–37.9 Hgb (g/dl): 12.3–13.1 RET (%): 2.53–3.79
Homologous blood transfusion (BT)	Flow cytometer	Isotype control mouse IgG_1, FITC (DAKO) α-Glycophorin A, FITC (DAKO) Mouse anti human IgG, FITC (Immucor Inc.; Gamma Biol.) Mouse anti human IgM, FITC (Immucor Inc.; Gamma Biol.) Control cells mixed positive (Panocell; Immucor Inc.; Gamma Biol.) Control cells mixed negative (Panocell; Immucor Inc.; Gamma Biol.)

curve (Fig. 3). The outliers were considered as suspect samples and were analyzed with a GC/MS procedure combined with a pertrimethylsilyl derivatisation [8]. If the findings from the above method suggested a possible HES or dextran misuse, a GC/MS confirmatory procedure was subsequently applied. This procedure included four steps: permethylation, acid hydrolysis, reduction and acetylation [9].

The urinary EPO test was based upon IEF of a protein concentrate in a gel featuring a pH 2–6 gradient (i.e. an electrophoretic separation based upon differences in isoelectric points of the isoforms), followed by double blotting technique with the primary monoclonal antibody (mAb) AE7A5 (Minneapolis, USA) against human urinary EPO, and final immunochemical detection, which maps the original position and quantity of EPO [10–12]. When a urine sample was suspected for rEPO abuse, the confirmation procedure followed was the same as for the screening, together with a stability test, in order to ensure the stability of the profile found in the sample. The criteria applied by the DCL for the evaluation of screening and confirmation data of the urine EPO tests are those referred in Ref. [13].

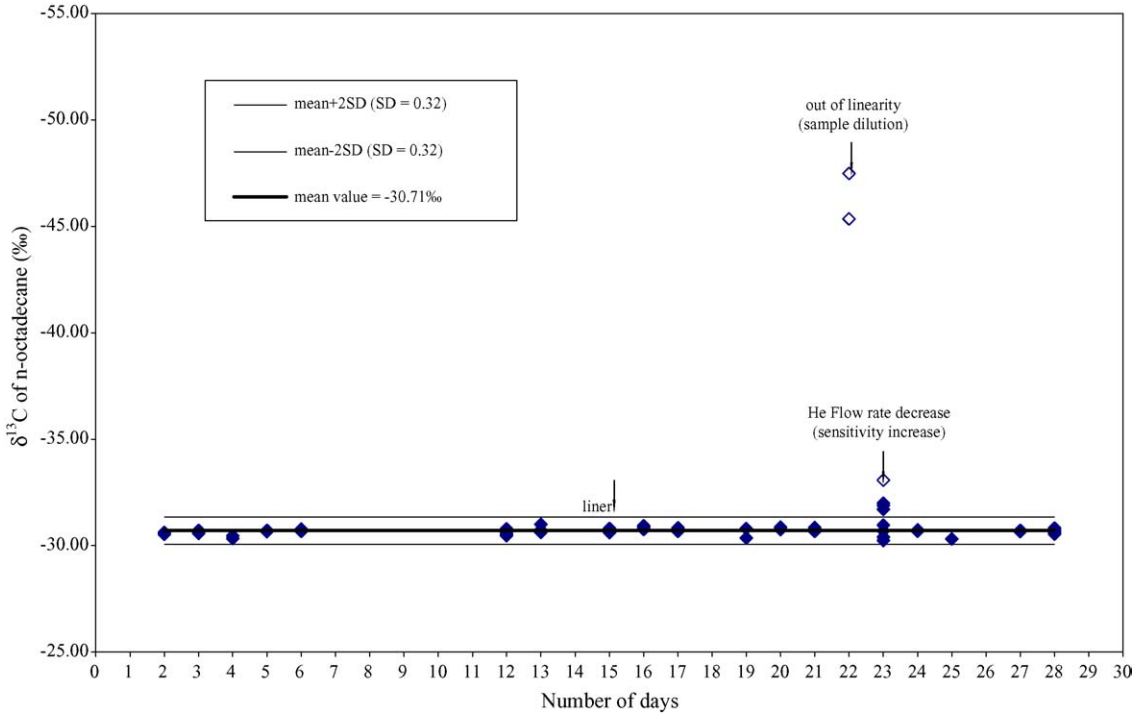

Fig. 1. $\delta^{13}C$ values/sample batch measured in control sample (*n*-octadecane) in one GC/C/IRMS instrument. Data taken from days 2 to 28 of the Olympic Games period. The open symbols refer to data, which have been modified after a mending action in the GC/C/IRMS.

Table 3
Sample preparation for screening procedures I, II, IV–VI and plasma expanders

	Screening procedures in urine samples							
	I	II	IV			V	VI	Plasma expanders
Sample volume	2.5 ml	2.5 ml	5.0 ml			2.5 ml	450 μl	250 μl
ISTD[a]	Diphenylamine	Codeine	Methyltestosterone	Methyltestosterone	Methyltestosterone	Mefruside	Automated chemiluminescence procedure	–
Hydrolysis	–	Acidic, cysteine	Enzymatic, *E. coli* β-glucuronidase	Enzymatic, *E. coli* β-glucuronidase	Enzymatic, *E. coli* β-glucuronidase	–		(i) No hydrolysis, (ii) acidic (HCl, 100 °C, 1 h)
Extraction solvent	*Tert*-butylmethylether	Diethylether-isopropanol	Diethylether	Diethylether	Diethylether	Ethyl acetate double extraction		Two automated consecutive enzymatic reactions
pH	14	9.5–10	9–10	9–10	9–10	9–9.5		
Reconstitution reagent	–	–	–	–	CH_3CN–H_2OCH_3COOH	–		
Derivatisation reagent	–	MSTFA[b]/MBTFA[c]	MSTFA/NH_4I/DTE[d]	MSTFA/NH_4I/DTE	–	$CH_3COCH_3CH_3IK_2CO_3$		
Incubation conditions	–	10 min, 80 °C/5min, 80 °C	30 min, 80 °C	30 min, 80 °C	–	20 min, 900 W	–	–
Injection mode	Splitless	Split 1:10	Split 1:15	Split 1:10	–	Split 1:15	–	–
Injection volume (μl)	2	2	2	1	10	1	–	–
Acquisition mode	–	Full scan	SIM[e]	SIR[f]	MS/MS or $MS^{(3)}$	SIM	–	–
Analysis	GC/NPD	GC/MSD	GC/MSD	GC/HRMS	LC/MSD	GC/MSD	Immulite analyzer	Biochemical analyzer

[a] Internal standard.
[b] *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide.
[c] *N*-Methyl-bis(trifluoroacetamide).
[d] Dithioerythritol.
[e] Selective ion monitoring.
[f] Selective ion recording.

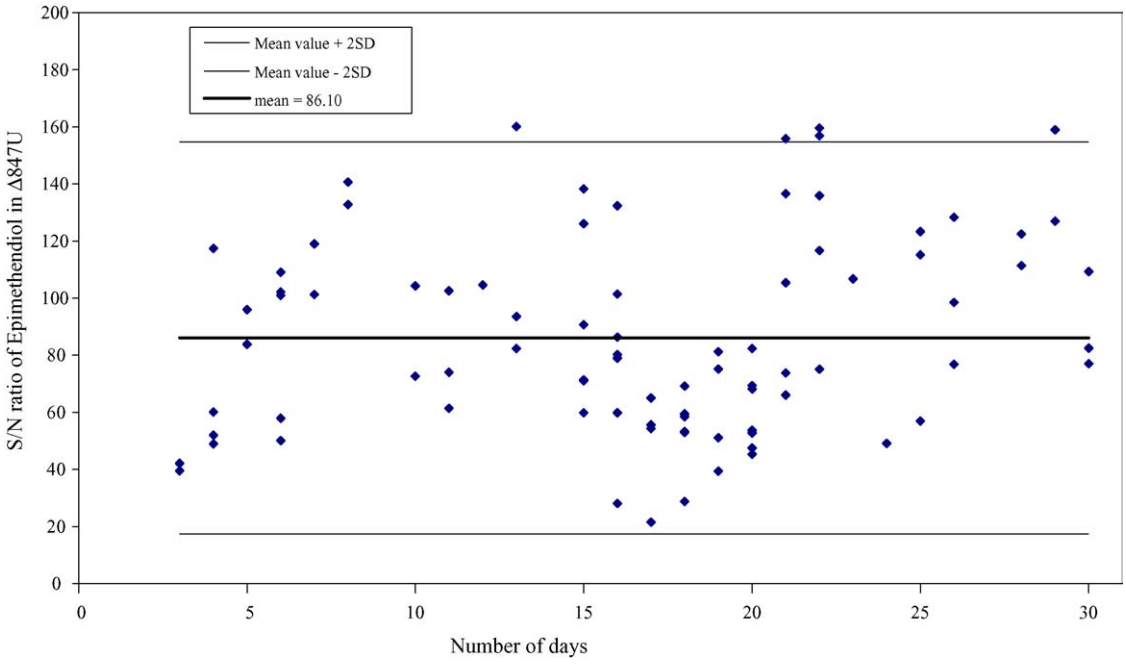

Fig. 2. S/N ratio/sample batch of epimethendiol (methandienone metabolite) measured in the control sample (Δ847U) in one GC/HRMS instrument. Data start on day 3 of the Olympic Games period.

2.5. Blood sample testing

In the blood sample analysis three new parameters were introduced: the HBOC, the hGH and the homologous BT (Table 2B). A number of health tests involving the determination of the percentage of reticulocytes (RET), the haematocrit (Hct) and haemoglobin (Hb), were also conducted initially, in order to exclude the athletes participation.

The method used at the DCL for the detection of GH concentration was based on two specific sandwich-type fluorescent immunoassays [14]. Assay 1, so-called Rec Assay, used mAb 5D7 or mAb 8B11, which preferentially recognized the 22 kDa fractions, identical to those of the recombinant human growth hormone (rGH). Assay 2, so-called Pit assay, used mAb 1B3 or mAb 8A9, which recognized all GH isoforms, namely the total human growth hormone (hGH). MAb 5D7 was always used in

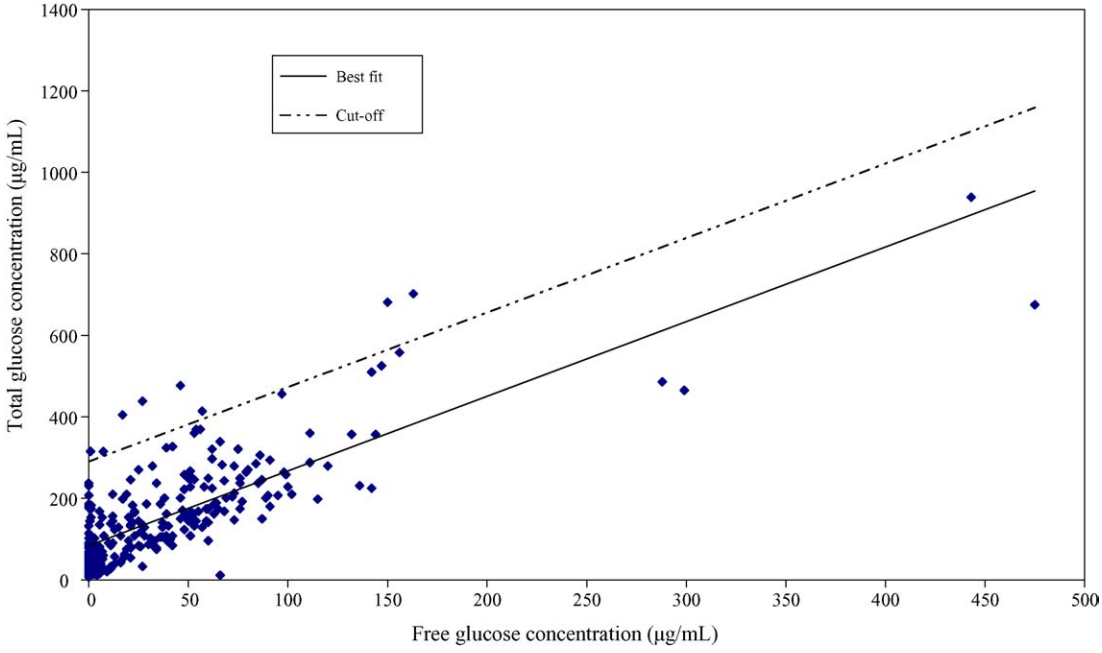

Fig. 3. Relation between “free” and “total” glucose concentration in a random-wise selected population. Cut-off curve: [glu after hydrolysis] = [glu before hydrolysis] × 1,1092 + 109 + 205 (Eq. (1)), where the value “205” is obtained by the following way: (3.3 × [glu concentration measured after hydrolysis] − [glucose concentration after hydrolysis calculated by Eq. (1)]).

conjunction with mAb 1B3 and mAb 8B11 with mAb 8A9. As a detection mAb, the biotinylated mAb 10A7 was used. Both assays were performed for each serum sample and the GH concentration ratio given by the Rec/Pit assays was determined. The ratio given by a number of sera from untreated with rGH normal human subjects had been previously estimated [14], and thus it was possible to discriminate between normal subjects and subjects that had been administered rGH.

The pre-screening of the HBOCs was conducted by comparison of the color of the serum sample, in a colorimetric scale where the decision cut-off level was set at 1.5 g/l. LC/MS/MS [15], electrophoretic [16] and size exclusion HPLC (SEC-HPLC) [17] methods were accredited and used as the screening and confirmatory methods. An improved protocol for the electrophoretic and SEC-HPLC detection of HBOC was developed in the DCL, allowing safe discrimination between HBOCs and hemolysed Hb, even in the absence of reference HBOC material, or severe sample haemolysis (unpublished data).

The testing for blood transfusion was based on the differentiation of minor red blood cell antigen populations present in the sample. Red blood cells (RBC) were specifically labeled with immunochemical fluorescence and sorted with laser flow cytometry [18]. For a particular antigen, the presence of two (or more) RBC populations on the same histogram was considered evidence of homologous blood transfusion.

3. Results and discussion

An analytical flow chart from sample receipt to reporting, along with the number of samples tested are given in Fig. 4. From the total of 3617 samples analyzed during the Olympic Period, 2926 samples corresponded to urines for screening procedures I, II, IV–VI and 691 to blood samples. Additionally to the above screening, 375 of the urine samples were screened for rEPO and plasma expander abuse, while 298 blood samples were tested for BT, and 397 were tested for rGH and HBOCs. Besides, 64 health tests were conducted in modern pentathlon athletes. The majority of the analyzed urine samples came from sports such as athletics (15%), aquatics (12%), football (7%) and weightlifting (7%) (Table 4).

In general, no major problems were encountered during the games, in terms of the quality/quantity of sample processing and the time commitment from sample receipt to result reporting. The demanding task for a daily analysis of 180 samples/day was occasionally over met, due to the incremental increase of sample number, up to 250 samples/day, especially on the days 11, 15 and 21 of the Olympic Games period (Fig. 5). From 2926 urine samples, referred to the I, II, IV–VI screening procedures, 2070 were completed in between 24 and 48 h, while about 350 samples were analyzed and reported between 48 and 72 h, with the rest being marginal cases (Fig. 6). As for the processing time of sample results from EPO urine, GH, HBOC, and BT blood testing, this occurred between 36 and 48 h in the majority of samples.

3.1. Anabolic steroids

Anabolic steroid screening (procedure IV) led to the majority of the reported positive cases, revealing that the “old fashioned synthetic steroids” are still in use. Special notice should be given to the Micromass Autospec HRMS instruments, that run without a single case of loss in the lock mass, using perfluorokerosine as

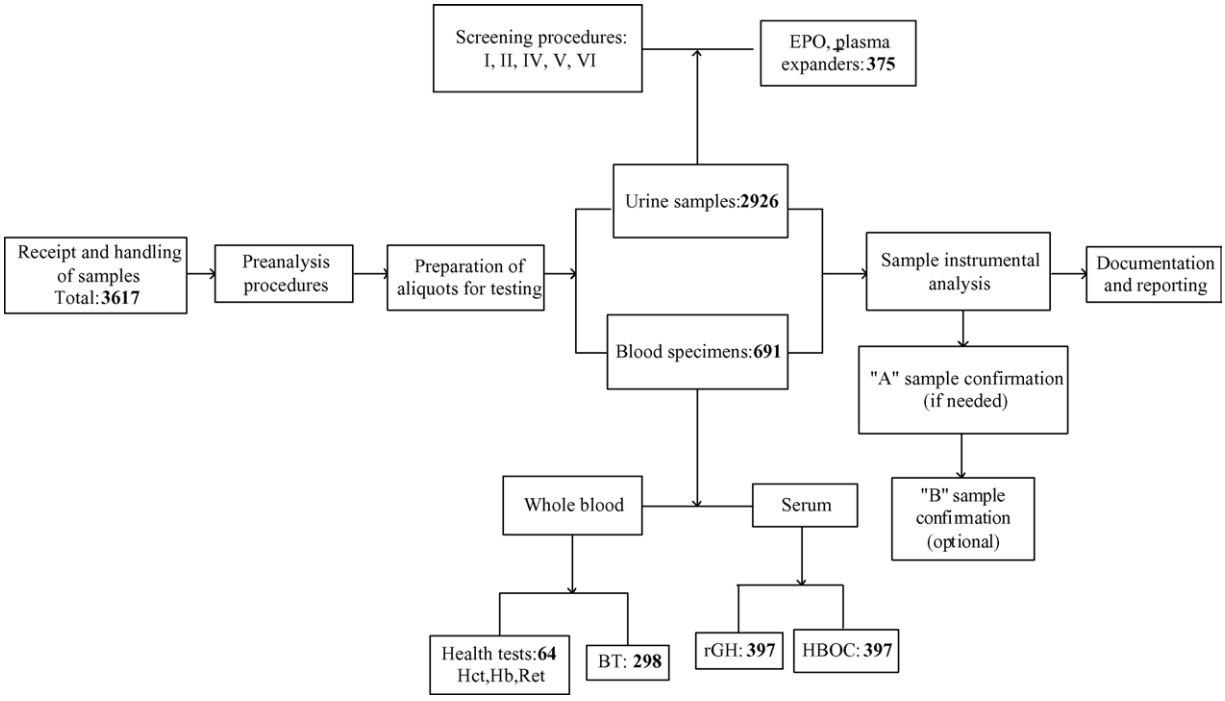

Fig. 4. Flow chart for sample processing and number of samples during the Olympic period at the DCL.

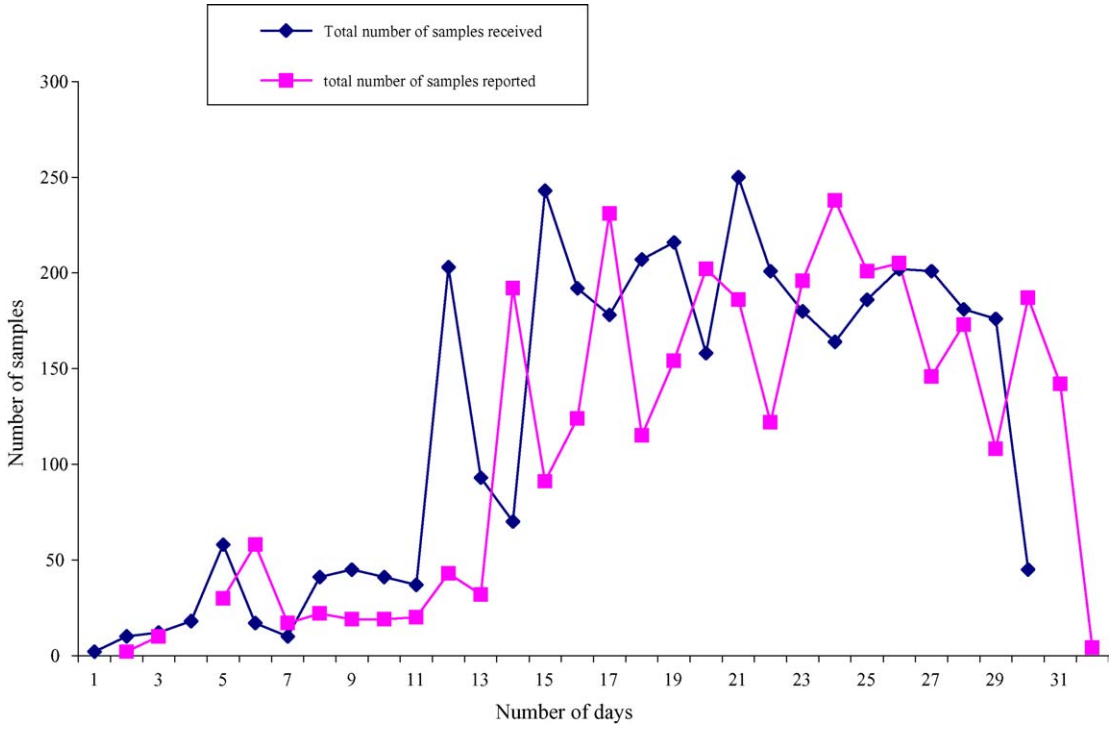

Fig. 5. Daily distribution of number of samples received and reported from the DCL for the Olympic Games period. There were no results reported on 4/8/2004.

calibrating material. The HRMS screening conditions (achieving a resolution of 10,000) allowed the screening of samples at a sensitivity level, 10 times lower than the MRPL [7]. Besides, their use proved to be a valuable tool for the fast, highly reliable and sensitive confirmations, using a resolution of 20,000.

Indicative data for testosterone/epitestosterone (T/E) and androsterone/etiocholanolone (A/E) ratios from two GC/MS instruments, during screening procedure IV, are presented in Fig. 7. It can be seen, that the majority of samples analyzed (male and female athletes) gave T/E ratios ranging from 0.5 to 1. At the same time maximum (A/E) ratios were between 1 and 2. Cases where T/E ratios were between 4 and 6 were marginal. In the case where the T/E ratio was close to 6, taking into consideration the uncertainty of the method (8.5%), there was no further examination. In case that T/E was well above 6, these data pointed to positive samples (Fig. 6, Table 6) [19].

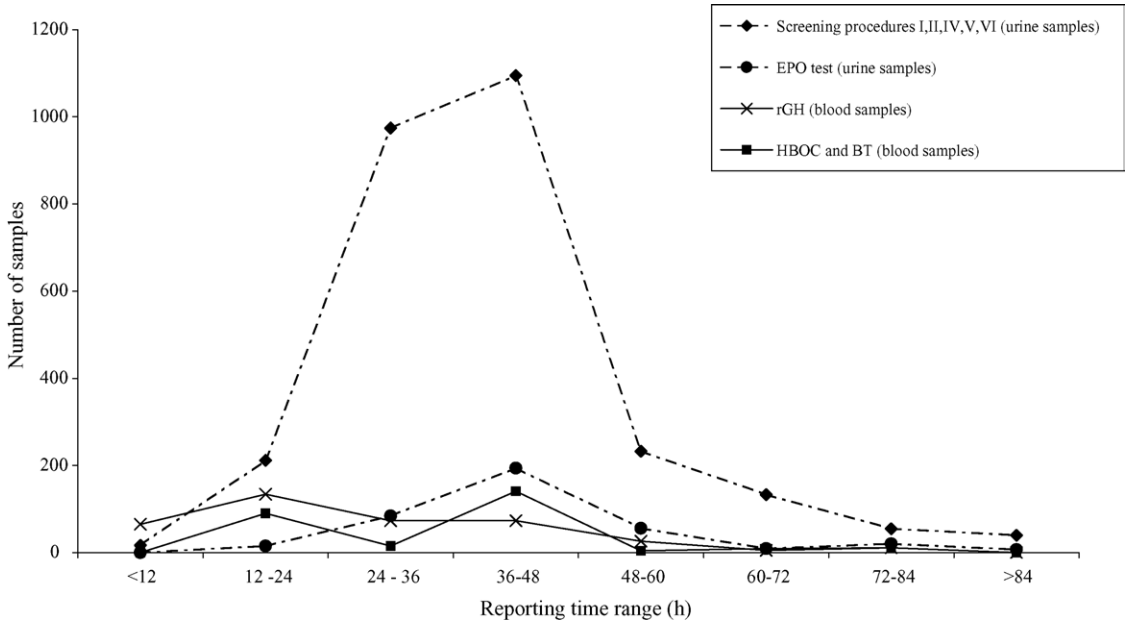

Fig. 6. Number of samples reported at different time ranges in the different screening procedures.

Table 4
Distribution of urine samples per sport

Sport	Total (%)	Number of urine samples
Athletics	15	432
Aquatics[a]	12	344
Weightlifting	7	219
Football	7	207
Cycling[b]	4	124
Rowing	4	122
Volleyball[c]	4	120
Boxing	4	112
Wrestling	4	109
Shooting	3	100
Judo	3	89
Basketball	3	88
Canoeing	3	83
Sailing	3	77
Handball	2	71
Gymnastics[d]	2	69
Hockey	2	67
Tennis	2	59
Baseball	2	47
Badminton	2	44
Archery	1	39
Fencing	1	39
Taekwondo	1	39
Table tennis	1	38
Softball	1	27
Equestrian	1	20
Triathlon	1	21
Modern pentathlon	<1	13
Unspecified	4	107
Total	100	2926

[a] Aquatics comprises four Olympic disciplines: swimming, water polo, diving and synchronised swimming.
[b] Three cycling disciplines are included in the Olympic Games sports programme: mountain bike, road cycling, track cycling.
[c] Volleyball comprises two disciplines: beach volleyball and volleyball.
[d] Gymnastics disciplines are: artistic gymnastics, rhythmic gymnastics and trampoline.

3.2. Plasma expanders

From the 375 urine samples tested for plasma expander abuse, a percentage of 5% were subjected to the GC/MS screening procedure, as outliers to the cut-off curve (Fig. 3). Nevertheless, the mass spectrometric procedure showed that these suspected samples were false positive screening results.

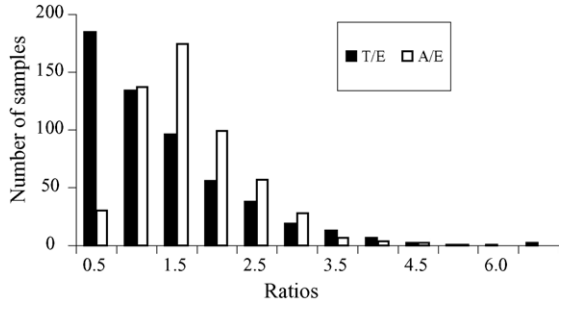

Fig. 7. Distribution of T/E and A/E ratios relevant to the number of samples analyzed. Indicative data taken from two GC/MS instruments.

3.3. EPO

The urinary EPO test run without any specific problems during the Olympic Games. From the 375 urine samples analyzed, there were no positive cases reported.

3.4. rGH

Samples analyzed for rGH (397 in total) were found all negative, while there was no significant difference between the male and female measurements (unpublished data).

The time elapsed from sample collection to analysis varied from 4 to 9 h per day. In general, no major problems were contacted in terms of time and quality of sample preparation, although from our experience consideration needed to be taken on some issues like pipetting, the water purity, possible europium contamination and the time of sample processing.

From previous experimentation the cut-off value for the Rec/Pit ratio, depending on two different screening assays, is equal either to 1 or 1.2 (M. Bidlingmeier, University Clinic, Munich, Germany, pers. commun.). The distribution analysis of the ratios obtained with the first Rec/Pit assay in 214 analyzed samples gave an average ratio of 0.36 with an SD value of 0.12, and a ratio of 0.47 with a SD equal to 0.2, in 183 samples tested with the second screening immunoassay (Fig. 8). The results obtained were well below the cut-off value, having calculated for each assay (1 and 2) an uncertainty value of 7.7 and 7.8%, respectively.

3.5. HBOCs

The results obtained using the HBOCs pre-screening procedure for the 397 serum samples analyzed, were below the cut-off level of 1.5 g/l. The HBOCs pre-screening method was considered to be a safe analytical tool for the detection of synthetic

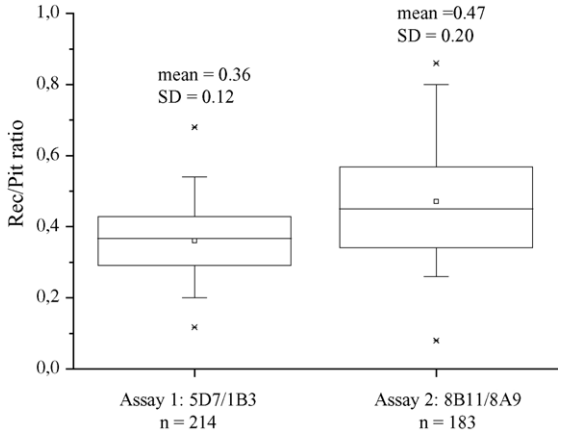

Fig. 8. Rec/Pit ratios for the samples analyzed for rGH in the Olympic Games period with two dual immunometric assays. The horizontal lines in the box denote the 25th, 50th and 75th percentile values. The error bars denote the 5th and 95th percentile values. The two symbols below the 5th percentile error bar denote the 0th and 1st percentile values. The two symbols above the 95th percentile denote the 99th and 100th percentiles. The square symbol in the box denotes the mean of the column of data.

blood abuse. Thus, no screening or confirmation procedure was applied.

3.6. TUEs

The prohibited substances that may be permitted for use by IOC through a certain formal process, named as the therapeutic use exemption (TUE), were strictly limited in the inhaled beta2-agonists and in glucocorticosteroids taken by non-systematic routes (inhalation, intra-articular, topic, etc.), according to the "International Standard for Therapeutic Use Exemptions" [20]. During the games, all urine samples were evaluated for glucocorticosteroids and beta2-agonists. In total, there were 79 positive cases for glucocorticosteroids and 28 positive cases for beta2-agonists, all of those being cross-examined by the IOC TUE Committee (TUEC) to have had undertaken through TUE process. The substances detected as well as the estimated concentration ranges are given in Table 5. Glucocorticosteroids estimated concentrations were between 1 and 30 ng/ml (namely below the MRPL set within the DCL), except from the 16α-hydroxyprednisolone data where the concentration values were more scattered. In the beta2-agonists, data for salbutamol varied between 100 and 1000 ng/ml. In six samples a combined use of a glucocorticosteroid and a beta2-agonist was found. More specifically, in three cases there was a combined detection of betamethasone and/or its metabolite with formoterol, in one case betamethasone with formoterol were used, in one case salbutamol with budesonide metabolite were used and finally one case salbutamol with methyl-prednisolone were encountered. The administration of synthetic glucorticosteroids and beta2-agonists were detected more frequently in sports like aquatics and athletics.

3.7. Positive cases

In less than 1% of the analyzed urine samples, adverse analytical findings were reported. While in Sydney 2000 Olympic Games, 11 positive cases were reported [21], in the Athens Olympic Games, the number of samples with prohibited substances was surprisingly increased up to 23 (Table 6). In 15 of these, "B" sample confirmation was requested. The prohibited substances found were: 16 anabolic steroid substances and their metabolites (mainly stanozolol, methyltestosterone, methandienone, testosterone and oxandrolone), two diuretics, three stimulants and two beta2-agonists (clenbuterol). Regarding the confirmatory analytical instruments used, the GC/HRMS was applied in 14 cases, while the GC/MS in 11 cases, the GC/C/IRMS in 2 cases and the LC/MS (ion trap) in 1 case.

An unusual case was that of an athlete, who was administered with a medication containing the substance isometheptene as a mucate salt, which has structure similarities with heptaminol; and thus the screening GC/MS analysis showed a screening result for heptaminol. Isometheptene metabolism in man is not reported in the literature, so an excretion study was carried out in DCL, in order to investigate whether heptaminol was a produced artifact of the acidic hydrolysis of the screening and confirmation procedures used. The results of the study are presented elsewhere [22].

The IOC introduced four random quality control (QC) samples to enquiry the quality of tests by DCL. The first one, which was negative, was submitted in the DCL halfway of the games. Another two QC samples with nandrolone and benzoylecgonine (cocaine metabolite) were received the day before the end of the games. The last QC sample with furosemide and triamterene was received on the last day of the games. The accurate identification of the QC samples was demonstrated.

In addition to the 23 positive cases reported, confirmation procedures were performed for another 7 samples after evaluating the screening results. The four out of seven cases were related to anabolic steroids (two for stanozolol, one for methandienone and one for high T/E ratio), two out of seven were related to EPO abuse, and one to blood transfusion. The confirmation procedures did not meet the positivity criteria for six of the seven samples. Only the blood sample, collected from a cyclist, showed indications of possible positivity, but finally this was reported negative with, an annotation stating that the ath-

Table 5
Glucocorticosteroids and beta2-agonists detected

Glucocorticosteroids	Number of substances for TUEs	Concentration range (ng/ml)			
		1–30[a]	31–100	100–300	301–600
Triamcinolone acetonide	29	22	5	2	–
Budesonide	11	11	–	–	–
16α-Hydroxy prednisolone	18	3	7	6	2
Dexamethasone	5	5	–	–	–
Betamethasone	5	4	–	–	1
Methyl prednisolone	5	5	–	–	–
Prednisolone	5	5	–	–	–
Prednisone	1	1	–	–	–
Beta2-agonists		1–10	10–100	100–1000	>1000
Formoterol	8	8	–	–	–
Salbutamol	14	–	–	14	–
Terbutaline	5	1	–	3	1
Salmeterol	1	–	1	–	–

[a] MRPL set by WADA for glucocorticosteroids.

Table 6
Adverse analytical findings reported by the DCL during the Olympic Period

a/a	Substances identified	Sport	Sex	Instruments of screening procedure	Instruments of confirmation procedure	B-sample request
1	3′-OH-stanozolol/16β-OH-stanozolol	Athletics	Female	GC/HRMS (Proc IV-SOP106)	GC/HRMS	Yes
2	3′-OH-stanozolol/4β-OH-stanozolol 16β-OH-stanozolol	Athletics	Male	GC/HRMS (Proc IV-SOP106)	GC/HRMS	Yes
3	3′-OH-stanozolol/4β-OH-stanozolol 16β-OH-stanozolol	Baseball	Male	GC/HRMS (Proc IV-SOP106)	GC/HRMS	Yes
4	3′-OH-stanozolol/4β-OH-stanozolol 16β-OH-stanozolol	Weightlifting	Male	GC/HRMS (Proc IV-SOP106)	GC/HRMS	No
5	3′-OH-stanozolol/4β-OH-stanozolol 16β-OH-stanozolol	Wrestling	Female	GC/HRMS (Proc IV-SOP106)	GC/HRMS	Yes
6	3′-OH-stanozolol	Weightlifting	Female	GC/HRMS (Proc IV-SOP106)	GC/HRMS	Yes
7	3′OH-Stanozolol, 18-nor-17, 17-dimethyl-5β-androst-1,13-diene-3α-ol[a]	Weightlifting	Male	GC/HRMS (Proc IV-SOP106)	GC/HRMS	No
8	17α-methyl-5β-androstane-3α,17β-diol, $\delta^{13}C_{5\alpha\text{-androstan-}3\alpha,17\beta\text{-diol}} - \delta^{13}C_{pregnanediol} > 3$, $\delta^{13}C_{5\beta\text{-androstan-}3\alpha,17\beta\text{-diol}} - \delta^{13}C_{pregnanediol} > 3$	Weightlifting	Female	GC/HRMS (Proc IV-SOP106)	GC/HRMS, GC/MSD, GC/C/IRMS	No
9	17α-methyl-5β-androstane-3α,17β-diol	Weightlifting	Female	GC/HRMS (Proc IV-SOP106)	GC/MSD, GC/HRMS	No
10	18-nor-17,17-dimethyl-5β-androst-1,13-diene-3α-ol	Weightlifting	Female	GC/HRMS (Proc IV-SOP106)	GC/HRMS	Yes
11	Oxandrolone	Weightlifting	Male	GC/HRMS (Proc IV-SOP106)	GC/HRMS	Yes
12	Oxandrolone	Weightlifting	Male	GC/HRMS (Proc IV-SOP106)	GC/HRMS	No
13	19-Norandrosterone/19-noretiocholanolone[b]	Weightlifting	Female	GC/HRMS (Proc IV-SOP106)	GC/MSD	No
14	T/E > 6	Weightlifting	Female	GC/MSD (Proc IV-SOP106)	GC/MSD	Yes
15	T/E > 6	Weightlifting	Male	GC/MSD (Proc IV-SOP106)	GC/MSD	No
16	T/E > 6, $\delta^{13}C_{androsterone} - \delta^{13}C_{11\text{-keto-etiocholanolone}} > 3$, $\delta^{13}C_{etiocholanolone} - \delta^{13}C_{11\text{-keto-etiocholanolone}} > 3$, $\delta^{13}C_{5\beta\text{-androstan-}3\alpha,17\beta\text{-diol}} - \delta^{13}C_{pregnanediol} > 3$	Weightlifting	Male	GC/MSD (Proc IV-SOP106)	GC/MSD, GC/C/IRMS	Yes
17	Norpseudoephedrine	Boxing	Male	GC/MSD (Proc II-SOP103)	LC/MSD	Yes
18	Heptaminol	Cycling	Female	GC/MSD (Proc II-SOP103)	GC/MSD	Yes
19	Ethamivan	Rowing	Female	GC/MSD (Proc II-SOP103)	GC/MSD	Yes
20	Furosemide	Weightlifting	Female	GC/MSD (Proc V-SOP107)	GC/MSD	Yes
21	Hydrochlorothiazide	Baseball	Male	GC/MSD (Proc V-SOP107)	GC/MSD	Yes
22	Clenbuterol	Athletics	Female	GC/HRMS (Proc IV-SOP106)	GC/HRMS	Yes
23	Clenbuterol	Athletics	Male	GC/HRMS (Proc IV-SOP106)	GC/HRMS	No

[a] Methandienone metabolite.
[b] Nandrolone metabolites.

lete was suspicious for homologous blood transfusion due to the lack of method accreditation. The case was further investigated according to the IOC instructions.

4. Conclusions

During the Athens Olympic Games, a total of 3617 samples were tested at the DCL, for the WADA prohibited substances and accredited methods. In 23 samples, prohibited substances were found, although quite common in origin (anabolic substances like stanozolol metabolites, T/E high ratios, oxandrolone, etc.) and a controversary case of BT. However, the high number of positives found in the present Olympic Games comparatively to the previous ones, shows that doping issues have to be taken under scrutiny.

The WADA Independent Observers characterized the overall performance of the DCL “as better that can be revealed from the underpinning reference documents”.

Acknowledgements

The general feeling amongst the staff at DCL was that the Olympic Games were a very enjoyable and fruitful experience. Special thanks are to the 50 volunteers (University and technical graduates), the 16 colleagues from WADA Accredited Laboratories, Professor Rainer Stephany, WADA Laboratory Independent Observer, Professors Martin Bidlingmeier and Christian Strasburger for the development of the rhGH assay, the IOC support group consisted by Dr. Patrick Schamasch, IOC Medical Director, Professors Jordi Segura, Don Catlin, Peter Hemmersbach and Dr. Moutian Wu, Dr. Olivier Rabin, WADA Scientific Director and Ann-Muriel Steff, WADA Research Manager for the excellent collaboration. Hellamco S.A. and the engineers that serviced our equipment need to be acknowledged. We finally extend our gratitude to IOC, GSS and ATHOC for funding this project.

References

[1] World Anti-Doping Agency, Independent Observers Report, Olympic Summer Games 2004. www.wada-ama.org.
[2] C.G. Georgakopoulos, C. Tsitsimpikou, M.-H.E. Spyridaki, E. Lyris, E.G. Cookeas, D. Thieme, Trends Anal. Chem. 18 (1999) 1.
[3] World Anti-Doping Agency, The World Anti-Doping Code, www.wada-ama.org.
[4] World Anti-Doping Agency, The World Anti-Doping Code, International Standard for Laboratories, version 4.0, August 2004. www.wada-ama.org.

[5] A.G. Fragkaki, I.-P. Leontiou, N. Kioukia-Fougia, M. Tsivou, M.-H.E. Spyridaki, C.G. Georgakopoulos, Technovation, in press.
[6] World Anti-Doping Agency, The World Anti-Doping Code, The 2004 Prohibited List International Standard, 2003. www.wada-ama.org.
[7] World Anti-Doping Agency, The World Anti-Doping Code, WADA Technical Document, TD2004MRPL, Version 1.0, 2004. www.wada-ama.org.
[8] M. Thevis, G. Opfermann, W. Schanzer, J. Chromatogr. B 744 (2000) 345.
[9] M. Thevis, G. Opfermann, W.Schanzer, J. Mass Spectrom. 35 (2000) 77.
[10] F. Lasne, J. de Ceaurriz, Nature 405 (2000) 635.
[11] F. Lasne, J. Immunol. Meth. 253 (2001) 125.
[12] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, Anal. Biochem. 311 (2002) 119–126.
[13] World Anti-Doping Agency, WADA Technical Document, TD2004EPO Version 1.0, 2004. www.wada-ama.org.
[14] Z. Wu, M. Bidlingmaier, R. Dall, C.J. Strasburger, Lancet 353 (1999) 895.
[15] M. Thevis, R.R. Ogorzalek Loo, J.A. Loo, W. Schanzer, Anal. Chem. 75 (2003) 3287.
[16] F. Lasne, N. Crepin, M. Ashenden, M. Audran, J. de Ceaurriz, Clin. Chem. 50 (2004) 410.
[17] E. Varlet-Marie, M. Ashenden, F. Lasne, M.-T. Sicart, B. Marion, J. de Ceaurriz, M. Audran, Clin. Chem. 50 (2004) 4723.
[18] M. Nelson, H. Popp, K. Sharpe, M. Ashenden, Transfus. Med. 88 (11) (2003) 1284.
[19] L. Xin, Z. Yinong, W. Jingzhu, W. Moutian, Recent Adv. Doping Anal. 11 (2003) 341.
[20] World Anti-Doping Agency, International Standard for Therapeutic Use Exemptions 3.0, 2003. www.wada-ama.org.
[21] R. Kazlauskas, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, vol. 9, 2001, p. 167.
[22] E.M. Lyris, G. Tsiakatouras, M-H.E. Spyridaki, Y. Aggelis, M. Koupparis, C.G. Georgakopoulos, J. Chromatogr. B, in press.