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RECENT ADVANCES IN DOPING ANALYSIS (2)

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ANTIDOPING CONTROL LABORATORY AT THE GAMES OF THE XXV OLYMPIAD BARCELONA'92.
PART II. STRATEGIES AND RESULTS

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INTRODUCTION

The management of resources for preparing the antidoping laboratory for the Games of the XXV Olympiad have been presented in Part I of this report.

One of the major aims was to assure maximum reliability of results. All personal and technical means in order to assure this goal were made available. Aspects such as the education of personnel, the writing and validation of methods as Standard Operating Procedures, the use of state of the art analytical technology and the deep use of computer resources including in-house developed software packages, were some of the developed means.

As a logical consequences of the accurate preparation and planning of the project, the laboratory had a strict but smooth functioning during the Games. This fact was clearly shown by the development of about 40,000 analyses from 2,000 samples during 15 days by a team of 80 people working round the clock 24 hours a day, offering the results according to the pre-specified deadlines, offering maximum reliability of results and controls and without any additional noticeable incidence.

Details of the strategies used and of the results collected are presented below.

STRATEGIES FOR RELIABILITY

- Quality assurance

Quality assurance is one of the most important issues in an event such as antidoping control at Olympic Games. Some of the important features to have into account for Barcelona'92 were:

- The laboratory team was made up by analysts coming from different places. Except for the heads and deputy heads of screening procedures and other responsible staff, people joined the laboratory team just some weeks before the games. Training was one of the items in the quality assurance program to guarantee harmonization of work and so reproducibility of results.
- Standard operation procedures (SOP's) were written for all procedures to be done in the laboratory.
- Analyses were performed in batches containing control samples to check for the quality of the results. Batch definition was an important issue to ensure the validity and quality of batch to batch processes.

- Record keeping books

Different books were edited to annotate the information related with the different operations carried out in the laboratory. They were for: calibration and control books (balance, pHmeter, etc.), calibration book for dispensation material, chromatographic columns book, instrument use and maintenance book, reagents preparation book (batch number assignment), and reference material preparation book (batch number assignment).

- Archive of urine samples containing doping substances

A clinical trial protocol was written, submitted to a local Ethical Committee (Hospital del Mar, Barcelona) and approved by the Drug Regulation Authorities (DGFPS no. 87/71, no. 88/315, Ministry of Health, Madrid).

The drugs were administered to healthy volunteers usually as a single oral doses, except for anabolic steroids. Urine samples were collected as following: pre-dose (blank), 0-8 hours and 8-24 hours post-treatment. In some cases (anabolic steroids) urines were collected during 7-10 days after treatment. The pH and the density of the samples were measured, and they were analyzed for the administered compound. Subsequently samples were aliquoted and stored at -20ªC.

A total of 99 experiments were done to obtain urine samples for 90 different compounds. Other urines were kindly supplied by other accreditted laboratories.

- Analytical strategy

All "A" samples were analyzed using eigth screening procedures. Most of them (procedures 1A to 5A) were chromatography based methods; sample preparation and instrumental conditions used for those procedures are described in Table I. The specific substances and parameters analyzed by each of the analytical procedures were as follows:

Preanalysis: Measurement of pH and specific gravity.

Procedure 1A: Detection of volatile nitrogen containing compounds excreted free in urine, including most of the stimulants and some narcotics (extract 1A), and strichnine (extract 1B).

Procedure 2A: Detection of volatile and heavy volatile nitrogen containing compounds excreted free and conjugated in urine, including stimulants, narcotics and ß-blockers and metabolites.

Procedure 3A: Detection of pemoline and quantitation of caffeine and cortisol/cortisone ratio.

Procedure 4A: Detection of anabolic steroids and/or their metabolic products excreted free in urine

Procedure 4B: Detection of anabolic steroids and/or their metabolic products excreted free and conjugated in urine

Procedure 5A: Detection of diuretics, probenecid and mesocarb.

Procedure 6A: This procedure included all immunological techniques applied. Fluorescence polarization immunoassays (FPIA) was used to screen for the presence of amphetamine class compounds, opiates, cannabinoids and cocaine metabolite. Enzyme linked immunosorbent assays (ELISA) were used to test for clenbuterol, stanozolol, bronchodilators, buprenorphine and corticosteroids. Microparticle enzime immunoassays (MEIA) to detect human chorionic gonadotropin (ß-hCG) and luteinizing hormone (LH) were only performed in samples from male athletes.

	Procedure					
	1A	2A	3A	4A	4B	5A
Urine, mL	5	2.5	2.5	2.5	2.5	5
Internal standard, mg/L	diphenylamine, 5 pholcodine, 0.1	codeine-d_3, 1 MDMA-d_3, 1	7-ethyltheophylline, 10	stanozolol, 0.1	etiocholanolone-d_4, 0.5 testosterone-d_3, 0.02 11ß-hydroxyandrosterone-d_4, 0.24 methyltestosterone, 0.5	7-Propyltheophylline, 1
Hydrolysis enzime incubation	 - -	 ß-Gluc./aryls., *H. pomatia* pH 5.2, 2 h, 55°C	 - -	 - -	 ß-Gluc., *E. coli* pH 7, 1 h, 55°C	 - -
Purification step	-	-	-	-	Detect Abuse™	-
Extraction pH salt, g[a] solvent, mL[b]	liquid-liquid KOH 5M anh. Na_2SO_4, 3 diethyl ether, 2	solid-liquid pH 9, KOH 1M Bond-Elut Certify™ chloroform:^{i}propanol:NH_3, 2	liquid-liquid pH 9-10, carbonate buffer - chloroform/^{i}propanol, 7	liquid-liquid pH 7, phosphate buffer NaCl, 1 diethyl ether, 5	liquid-liquid pH 9-10, K_2CO_3 50 g/L - diethyl ether, 5	liquid-liquid pH 9.5, ammonium buffer NaCl, 1 ethyl acetate, 8
Derivatization reagents, µL incubation	 - -	 MSTFA 100/MBTFA 20 5 min, 60°C/10 min, 60°C	 - -	 MSHFB:TMSI (100:2) 40/MBHFB 10 5 min, 80°C/20 min, 80°C	 MSTFA:NH_4I:dithioerithritol (1000:2:4) 50 15 min, 60°C	 - -
Final vol, µL	300 MeOH + 700 ext (1A) 100 (1B)	120	100	50	50	100

[a] For solid-liquid extractions, columns

[b] For solid-liquid extractions, elution solvent

Table I-A. Sample preparation and instrumental conditions for procedures 1A to 5A.

	1A	2A	3A	4A	4B	5A
Injection vol, μL	3	1	10	2	2	20
Instrument	GC/NPD	GC/MSD	HPLC/DAD	GC/MSD	GC/MSD	HPLC/DAD
Column (length, i.d., particle size or film thickness)	Ultra-2 (12.5 m, 0.2 mm, 0.33 μm)	Ultra-2 (12.5 m, 0.2 mm, 0.33 μm)	Ultrasphere ODS (7.5 cm, 4.6 mm, 3 μm)	Ultra-1 (25 m, 0.2 mm, 0.11 μm)	Ultra-1 (25 m, 0.2 mm, 0.11 μm)	Ultrasphere ODS (7.5 cm, 4.6 mm, 3 μm)
Mobile phase	He	He	H_20/CH_3CN	He	He	ammonium acetate/CH_3CN
Flow rate, mL/min	0.75	0.7	1	0.65	0.7	1
Injector temp, °C	280 (1A), 320 (1B)	280	-	280	280	-
Injection mode	split (1/10)	splitless	-	split (1/10)	split (1/10)	-
Detector temp, °C	280	280	-	280	280	-
Program	100°C to 280°C (4 min), 20°C/min (1A) 300°C to 320°C (4 min), 20°C/min (1B)	100°C to 290°C (4.5 min), 20°C/min	90/10 to 70/30 (1 min) in 4.5 min	240°C (1 min) to 300°C (4.5 min), 40°C/min	200°C (1 min) to 242°C, 3°C/min; to 300°C (3.15 min), 15°C/min	90/10 to 85/15 in 2 min; to 55/45 in 3 min; to 40/60 (1 min) in 3 min
Acquisition mode	-	Scan (m/z 50-600)	216, 246, 280 nm	SIM	SIM	240, 270, 290, 300, 318, 350 nm

Table I-B. Sample preparation and instrumental conditions for procedures 1A to 5A.

RESULTS

- Samples

A total of 1871 samples were received in the laboratory during the whole Olympic Games. Among them, 1317 (70.4%) were from males and 554 (29.6%) were from females. The number of daily samples ranged from 55 to 192, with the maximum on August 1st 1992. (see Figure 1). Samples arrived at the laboratory between mid morning (11 a.m.) and very late at night (5 a.m.), according with the schedule of finishing the events. In some cases it was necessary to collect samples at the Polyclinics. The time of arrival of samples is presented in Figure 2.

31 Sport generated samples for the laboratory. Largest number of samples was received from Athletics (n:248), from Volleyball (n:144), from Swimming (n:119) and from Handball (n:116). Samples received from other sports were less than n:100 for each of them (see Table II).

As previously described, a full battery of screening methods were applied to all samples (except for a few samples that were not analysed because of insufficient volume), with additional confirmatory analysis in several samples. Taken all together, a total of about 40,000 analysis were carried out during the 15 days.

- Samples containing banned products

The Laboratory reported to the IOC Medical Commission all these samples containing some substance included in the list of banned or restricted substances. As some of the products found could arise from common medication for common cold or flu, the IOC asked for the quantitative values in the urine and for explanations from the team physicians involved. After deep discussions of the full Commission and Hearings with the Competitor and the Delegation involved, some cases were not considered true doping cases by the IOC Medical Commission. In general, they were related to drugs present in over the counter products used for minor diseases and where the concentration in the urine indicated just a therapeutic use. The substances found in theses samples were the following : 1 sample with a mixture of ephedrine

derivatives and dihydrocodeine; 1 sample with dihydrocodeine; 2 samples with low codeine and 1 sample with low caffeine. In addition, a case with relatively abnormal hCG value tested twice and was recommended accurate medical follow up.

The number of definitive positive cases was five, corresponding to the substances Strychnine, Norephedrine, Mesocarb and Clenbuterol (n:2) (see Table III). Three additional cases of T/epiT ratio higher than 6 and lower than 10 were reported to the Chairman of the Medical Commission, who ordered to the respective National Olympic Committees additional information and follow up as it was stated in the corresponding rules.

An additional mechanism was implemented by the Medical Commission in order to verify the reliability of the work at the laboratory. It was the introduction of blind control samples which contained banned products as well. All these controls were adequately detected by the laboratory. The products present in these samples were methyltestosterone (anabolic steroid), atenolol (beta blocker), metandienone (anabolic steroid) and etacrynic acid (diuretic).

- Other biochemical parameters measured

According to plans previously agreed with the Sub Commission Doping and Biochemistry, the laboratory also measured several important parameters related to the different classes of banned substances and that could be useful for the interpretation of results. These data are also useful as a large population study not previously developed for some of these parameters. They corresponded to the following issues:

1. pH values
2. Specific Gravity (density)
3. Caffeine concentrations

4. Cortisol concentration in males
5. Cortisone concentrations in males
6. Cortisol/Cortisone ratio in males
7. Tetrahydrocortisol concentrations in males

8. Cortisol concentrations in females
9. Cortisone concentrations in females

10.Cortisol/cortisones ratio in females
11. Tetrahydrocortisol concentration in females

12. Testosterone concentrations in males
13. Epitestosterone concentrations in males
14. Testosterone/Epitestosterone ratios in males

15. Testosterone concentrations in females
16. Epitestosterone concentrations in females
17. Testosterone/Epitestosterone ratios in females

18. Androsterone concentrations in males
19. Eticholoanolone concentrations in males
20. Androsterone/Etiocholanolone ratios in males

21. Androsterone concentrations in females
22. Eticholoanolone concentrations in females
23. Androsterone/Etiocholanolone ratios in females

24. 11-OH-Androsterone concentrations in males
25. 11-OH-Eticholoanolone concentrations in males
26. 11-OH-Androsterone/11-OH-Etiocholanolone ratios in males

27. 11-OH-Androsterone concentrations in females
28. 11-OH-Eticholoanolone concentrations in females
29. 11-OH-Androsterone/11-OH-Etiocholanolone ratios in females

30. Luteinizing hormone LH concentrations in males
31. Testosterone/LH ratios in males
32. hCG concentrations in males

Specific data for any of these analytes may be obtained upon request

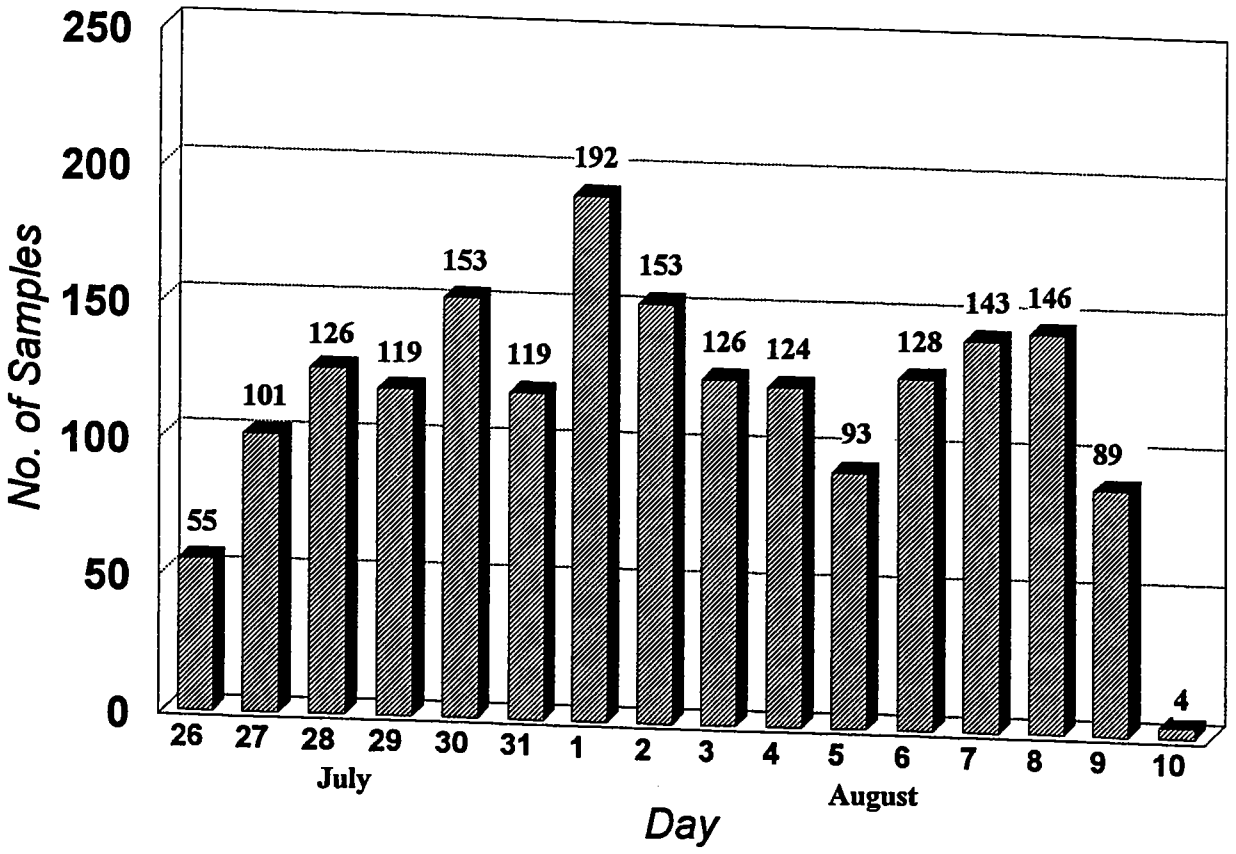

Figure 1. Daily distribution of samples received during '92 Olympic Games

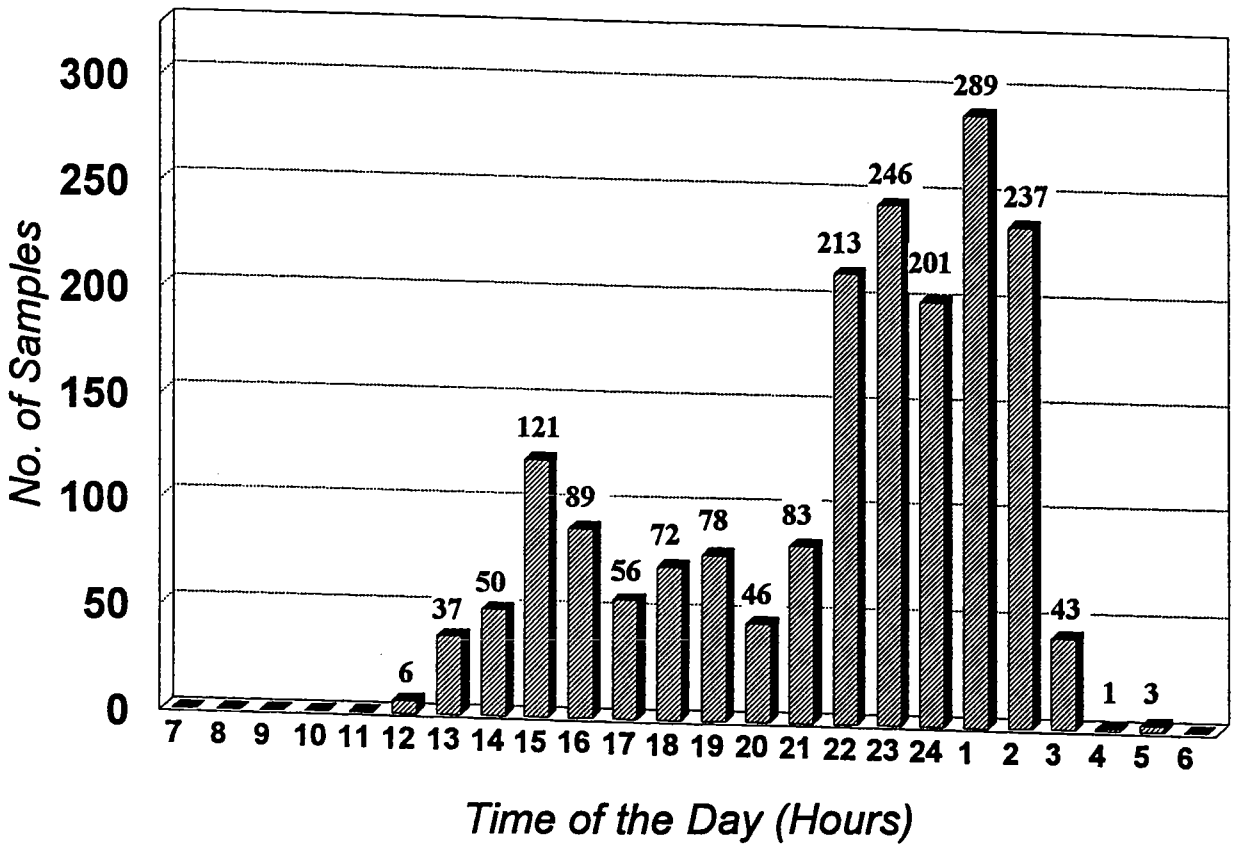

Figure 2. Distribution of samples received during the 92'Olympic Games according to the time of the day

Table II. Samples per sport received during the Olympic Games.

SPORT	SAMPLES
Athletics	248
Archery	26
Baseball	30
Badminton	42
Basketball	88
Boxing	72
Canoe	70
Cycling	86
Diving	10
Equestrian	17
Fencing	32
Football	32
Gymnastics	30
Handball	116
Hockey	46
Judo	70
Modern pentathlon	14
Pelota	20
Roller hockey	26
Rowing	70
Shooting	76
Swimming	119
Synchronised swimming	6
Table tennis	56
Taekwondo	26
Tennis	53
Volleyball	144
Water polo	34
Weightlifting	61
Wrestling	80
Yachting	71

Table III. Summary of samples declared positive by IOC-MC

Detected Substance	Sport	Sex of the competitor
Strychnine	Voleyball	Female
Norephedrine	Athletics (Marathon)	Female
Clenbuterol	Athletics (Hammer thrower)	Male
Clenbuterol	Athletics (Shot put)	Female
Mesocarb	Athletics (Long jump)	Female

- Drugs declared by the athletes

During the collection of doping control samples, 654 athletes declared they had taken any drug(-s) in the last three days. None of the declarations contained forbidden drugs according to I.O.C. doping lists. The most relevant information about the drugs declared during doping control in the Olympic Games are resumed, according to their classification in therapeutic classes:

VITAMINS, MINERALS and FOOD SUPPLEMENTS

Multivitamins products	574
Ascorbic acid (vitamin C)	380
Minerals	97
Iron preparations	87
Ginseng	85
Tocopherol (vitamin E)	50
Other products	340

RESPIRATORY SYSTEM

Salbutamol	31
Terfenadine	14
Sodium cromoglycate	9
Xylometazoline	6
Other products	49

ALIMENTARY TRACT

Loperamide	12
Antipeptic ulcer	8
Other products	29

ANTIINFLAMMATORY AND ANALGESIC DRUGS	
Diclofenac	106
Acetylsalicylic acid	79
Ibuprofen	75
Paracetamol	47
Piroxicam	41
Naproxen	41
Other products	57
ANTIINFECTIVE DRUGS	
Penicillin derivatives	23
Quinolones	38
Trimethoprim	11
Other products	29
CARDIOVASCULAR SYSTEM	
Enalapril	1
Verapamil	1
Other products	2
CENTRAL NERVOUS SYSTEM AGENTS	
Temazepam	7
Hypnotic drugs (not defined)	6
Piracetam	6
Tetrazepam	5
Other products	12
HORMONES	
Progestogens and estrogens	73
Glucocorticoids	49
Thyroid hormones	4
LOCAL ANESTHESICS	
Lidocaine	12
Bupivacaine	8
Mepivacaine	7
Other products	7

- Analytical Quality Control

As described previously, each batch of samples contained blank urines and control urines so that inter-batch reproducibility could be followed and potential defective batches rejected and re-extracted. The quantitative response of the respective internal standards in each sample was also used to check intra-batch reproducibility, reject defective analysis and follow individual instruments performance.

In some screening procedures, calibration samples were used to calculate the response factors of the compounds with quantitative cutoff values. The quantitation was checked in each case using a urine spiked with a know concentration of the compound. An example for the quality control of a reference urine for T/E ratio is presented in Figure 3.

- Scientific activity

The continued scientific activity was carried out in parallel with the preparation of the more practical aspects of the antidoping project and mainly involved presentations in different symposia and in the publication of papers in relevant journals of the field. Some of the publications are listed below under "References".

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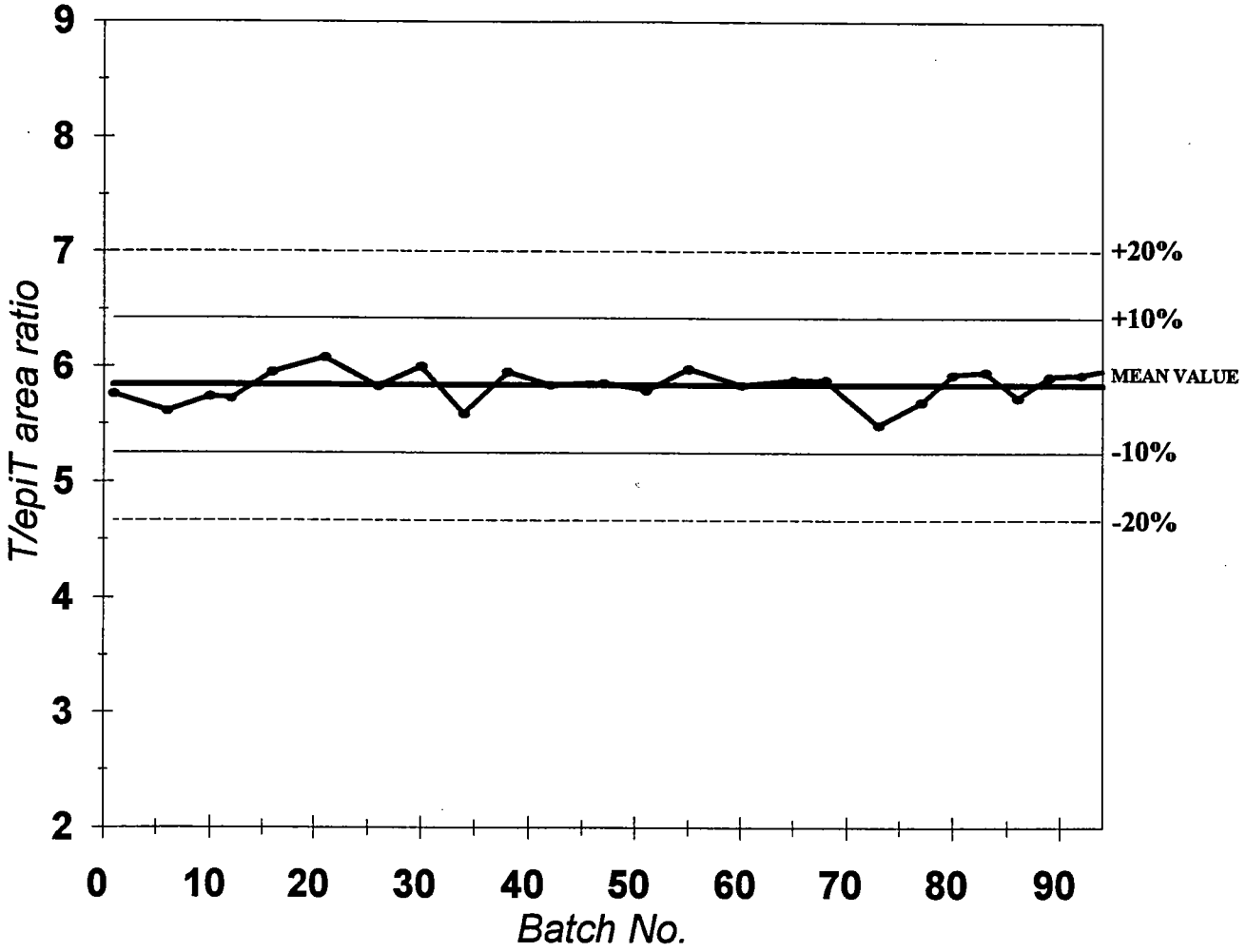

Figure 3. Example of quality control chart: Testosterone/epitestosterone ratio obtained after analysis by Procedure 4B of a urine with epitestosterone concentration of 20 ng/mL and a testosterone concentration adjusted to 120 ng/mL with testosterone glucuronide. Instrument: hpux10.