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Automated sample preparation and gas chromatographic–mass spectrometric analysis of urinary androgenic anabolic steroids

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Abstract

This paper presents an automated method for extracting anabolic agents from urine samples for their GC–MS analysis by selected-ion monitoring. The sample preparation was carried out in a Hewlett-Packard 7686 SPE PrepStation system. Each 0.6-ml aliquot was hydrolyzed, extracted, dried and trimethylsilyl (TMS) derivatized in a 2-ml vial without any hands-on labor. When sample preparation was finished 2 μl of the extract was injected into the gas chromatograph by split (1:10) mode. Due to the small amount of free space in the 2-ml vials for handling the sample, parameters like time of hydrolysis, type of shaking, number of extractions and some TMS derivatization parameters had to be adjusted to achieve the best recovery for all of the compounds in the screening. Manual and automated sample preparation schemes were compared in terms of linearity, precision, accuracy, limit of detection and recovery data. When large concentrations were analyzed using the automated method no carry-over effect was observed.

Keywords: Anabolic steroids

1. Introduction

Since 1976 the International Olympic Committee (IOC) has included the androgenic anabolic steroids (AASs) in the list of banned substances in sports [1]. Their control is guaranteed by the 25 IOC accredited laboratories responsible for detecting anabolic agent misuse, all over the world. Due to the large number of samples to be analyzed, the interests involved and the huge repercussions in the media of any positive case, a quick, consistent, sensitive and reliable methodology for doping control analysis has to be applied.

The AASs or their metabolites are excreted in urine together with a large variety of compounds like salts, endogenous steroids, and metabolites of medicine if any is taken, the concentration of which, like that of the anabolic agents in the urine sample, can vary a great deal, from a few μg/ml to pg/ml. Therefore, it is imperative to have analytical methods with enough selectivity to make the detection of any banned substance possible, in spite of the presence of any interfering compounds at high concentrations.

Before gas chromatography–mass spectrometry (GC–MS) analysis of the sample, a sample preparation process is required. In most of the cases it consists of cleaning the matrix before the enzymatic hydrolysis, a liquid–liquid extraction of the free

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anabolic agents with an organic solvent at alkaline pH, then evaporation of the organic solvent and finally a trimethylsilyl (TMS) derivatization [2,3]. In this way an average recovery for most of the anabolic agents of approximately 90% has been reached [4].

For screening purposes GC–MS analysis takes place in the selected ion monitoring (SIM) mode, each anabolic agent is monitored with three diagnostic ions. This acquisition mode allows us to achieve limits of detection (LODs) of around a few nanograms. Once a suspicious signal is detected a second GC–MS analysis has to be done. Due to the low anabolic metabolite concentration and the high level of physiological background, further purification steps are involved in confirming the sample preparation, thanks to which a higher degree of certainty in confirmation analyses is achieved. The additional purification methods can be based on immunoaffinity, high-performance liquid chromatographic (HPLC) purification and/or extinction with mixtures of organic solvents with different degrees of polarity [5–8].

In most of the cases the sample preparation is a tedious and time-consuming process that requires the full time attention of the analyst and provides a multitude of opportunities for errors. An ideal situation would provide less hands-on time labor for the operator, thus freeing him or her for other laboratory tasks.

In many different fields instrumental techniques are used in which full or partial automation is achieved. Automated sample preparation based on the use of a PrepStation system has been used for preparation of barbiturates in human urine and serum samples by solid-phase extraction (SPE) for further GC–MS analyses [9,10]. In doping control this system was applied to the screening of free excreted nitrogen-containing compounds in urine [11]. Some attempts have been made to automate several steps of anabolic agent extraction [12] but none of them permit total integration of the whole analytical process.

The aim of this paper is to introduce an automated method of sample preparation for screening purposes of anabolic steroids in urine whilst maintaining the same detection capacity achieved with the traditional manual liquid–liquid extraction method.

2. Experimental

2.1. Chemicals and reagents

tert.-Butyl methyl ether (TBME), cyclohexane, acetonitrile and methanol were of analytical grade and obtained from Scharlau (Barcelona, Spain).

The enzyme β-glucoronidase (*Escherichia coli*) was obtained from Boehringer (Mannheim, Germany), disodium hydrogenphosphate dihydrate, potassium dihydrogenphosphate, potassium carbonate, sodium hydrogencarbonate and ammonium iodide (NH_4J) were from Merck (Darmstadt, Germany), dithioerythritol (DTE) from Sigma (St. Louis, MO, USA) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Machery-Nagel (Düren, Germany).

2.2. Standards

Methyltestosterone (internal standard) was obtained from Steraloids (Wilton, NH, USA), 3′-hydroxystanozolol, 19-norandrosterone, 19-noretiocholanolone, 1-methylene-5α-androstan-3α-ol-17α-one (methenolone metabolite), 1α-methyl-5α-androstan-3α-ol-17-one (mesterolone metabolite), epimetendiol, drostanolone, boldenone, norethandrolone, 4-clortestosterone, 7α,17α-dimethyl-androst-4-3en-17β-ol-3-one, 7α,17α-dimethyl-5β-androstane-3α,17β-diol (bolasterone metabolites), furazabol PC, fomebolone, oxymesterone, *cis*-androsterone, etiocolanolone, 5α-androstan-3α,17β-diol, 5β-androstan-3α, 17β-diol, dehydroepiandrosterone, epitestosterone, dihydrotestosterone, testosterone, 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, pregnantriol, pregnandiol and tetrahydrocortisol were obtained from the Cologne IOC accredited Laboratory (Cologne, Germany).

2.3. Stock solutions

Stock solutions of all steroids were prepared in methanol in 5-ml capped vials and were stored at 4°C until used.

For measuring the recovery of steroids the mixture of the authentic standards was added to the negative urine. The calibration standards containing 19-noran-

drosterone were prepared by adding the authentic standard of this compound to the negative urine.

3. Sample preparation

3.1. Automated preparation

The 0.6-ml urine samples were pipetted into 2-ml capped ALS vials. All reagents for hydrolysis: 40 μl of 0.4 *M* phosphate buffer (pH 7), 15 μl (10 μg/ml methanol stock solution) of methyltestosterone (internal standard) and 15 μl of β-glucuronidase from *E. coli* (Boehringer Mannheim) were aspirated through the needle to the sample loop, and then dispensed to the vial. Next, the vial was placed in the heating position, 25 min at 55°C. Then, the vial was moved to position 1 for dispensing 90 μl of K_2CO_2 buffer (pH 11) and 0.5 ml of TBME. Next, it was transferred to the mixer, where the extraction started. After 20-step agitation (mix and stop cycles) the vial was returned to position 1 where the top layer of the organic solvent was aspirated and dispensed in a high-recovery vial (La-Pha-Pack, Langerwehe, Germany). Evaporation was completed in position 3 of the tower. When evaporation was finished a second extraction with 0.5 ml TBME of the same sample was run. The organic phase was aspirated and dispensed in the same high-recovery vial. After evaporation the dried residue was derivatized with 20 μl of MSTFA–NH_4I–DTE (1000:2:4, v/w/w) for 5 min at 65°C. The high-recovery vial with TMS extract was ready for analysis by GC–MS.

3.2. Manual sample preparation

To aliquots of 2 ml, 50 μl (10 μg/ml methanol stock solution) of methyltestosterone (internal standard), 100 μl of phosphate buffer (pH 7) and 25 μl of β-glucoronidase from *E. coli* were added. The hydrolysis was completed in 1 h at 55°C. After cooling at room temperature, 300 μl of the carbonate buffer (pH 11) was added and the extraction was done with 5 ml of TBME. After 5 min shaking, the organic layer was evaporated and derivatized with 50 ml of MSTFA–NH_4I–DTE (1000:2:4, v/w/w) for 30 min at 65°C. The TMS extract was transferred to a vial and placed on the gas chromatograph tray.

4. Instrumental

4.1. PrepStation system

The whole system was controlled with the Bench Supervisor software (Version A.04.02). this package coordinates the activities of the PrepStation tower, the 100-vial position tray and 10-position heater module. The control of each instrumental part involved in sample preparation is achieved by specific programs that are coordinated by the Bench Supervisor.

The samples were processed in a standalone SPE PrepStation 7686 (Hewlett-Packard; Fig. 1). The tower contains two needles, one over the processing position for aspirating and dispensing the reagents and another one over the heating position for evaporation purposes. Through a 2.5-ml standard syringe pump it is possible to dispense liquid reagents from reservoirs. The agitation is done in a rotatory mixer, extraction takes place repeating cycles of ''mix'' and ''stop''. The sample vials to be processed as well as some reagent vials are located in a tray with a capacity for 100 vials. The automatic arm transports the vials through the Station (see scheme in Fig. 1).

For increased productivity there is an external 10-position heater controlled by a 19265 B Controller coupled to the HP 7673 tray. The heater allows a significant improvement to the throughput of the PrepStation system by making it possible for the long heating steps to take place out of the processing tower, which frees the PrepStation to begin processing subsequent samples.

4.2. GC–MS

GC–MS analysis was carried out on a Hewlett-Packard 5973 mass-selective detector with direct coupling to a Model 6890 Hewlett-Packard gas chromatograph. The separation was done on a Hewlett-Packard Ultra-1 capillary column (25 m×0.22 mm I.D., 0.11 μm film thickness).

For the GC–MS analysis of anabolic agents, 2 μl of the TMS enol derivatization mixture was injected in the split mode (1:10) into a split–splitless injection port with an open glass insert. Helium was used as the carrier gas and the oven temperature program was as follows: starting at 190°C and

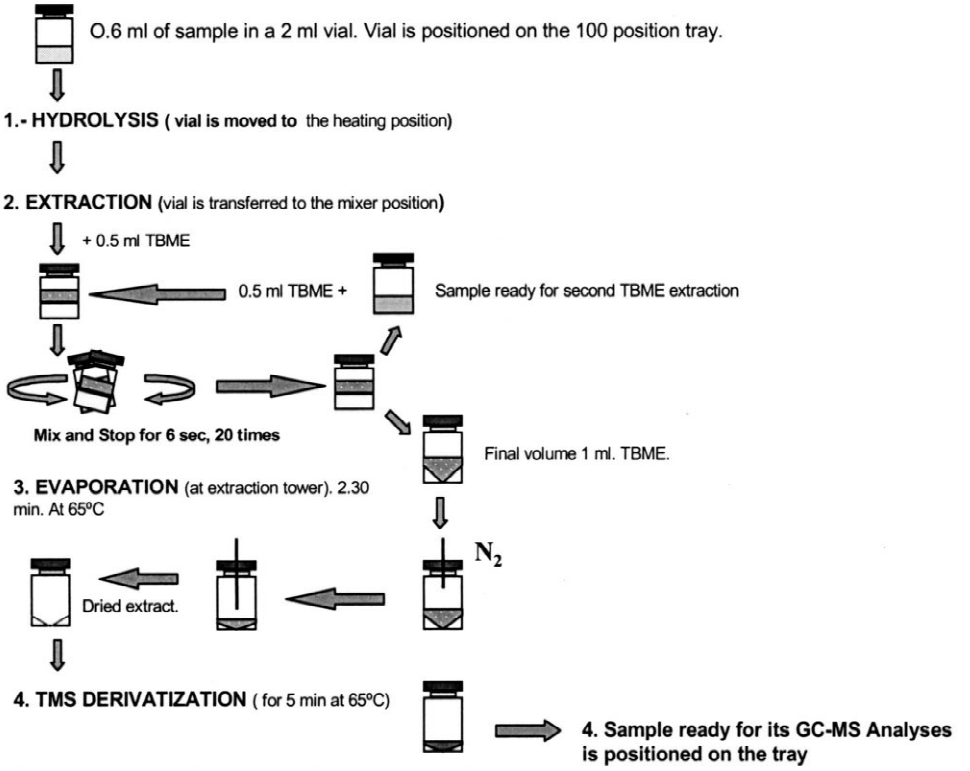

Fig. 1. The automated sample preparation scheme.

increasing the temperature by 2°C/min to 235°C then by 15°C/min to 320°C and maintaining this temperature for 5 min. The temperature of the injection port was kept at 270°C and the transfer line at 300°C. The electron multiplier was fixed during the analysis at 200 eV higher than the voltage obtained from the automatic tuning with electron impact ionization at 70 eV. Mass spectra were obtained by scanning from m/z 50 to 600.

Under standard operating conditions, each sequence of samples analyzed included the analysis of a standard mixture of exogenous anabolic agents in order to verify the analytical performance of the GC–MS system. This mixture contained pemoline, salbutamol, clenbuterol, boldenone, mesterolone, bolasterone, estanozolol, fluoxymesterone, oxandrolone (8 ng/μl injected) and methyltestosterone (internal standard) (10 ng/μl injected). For the quantitation of endogenous steroids in urine samples, 2 μl of a calibrating standard mixture: androsterone, etiocolanolone (80 ng/μl injected), 5α-androstan-3,17-diol (2 ng/μl injected), 5β-androstan-3,17-diol, dehydroepiandrosterone (4 ng/μl injected), epitestosterone (1 ng/μl injected), dehydroepitestosterone (4 ng/μl injected), testosterone (1 ng/μl injected), 11-OH-androsterone (10 ng/μl injected), 11-OH-etiocolanolone (5 ng/μl injected), pregnandiol (8 ng/μl injected), pregnantriol, tetrahydrocortisol (40 ng/μl injected) and methyltestosterone (internal standard) (10 ng/μl injected) were injected.

5. Results and discussion

5.1. Method development

An important difference between the automated and the manual preparation is related to the volume of sample to be processed. The PrepStation can only work with vials of 2 ml capacity, thus in order to

ensure enough free space for adequate mixing of the phases only 0.6 ml of urine was taken. This scale factor has an important effect on each of the steps involved in sample preparation, so that parameters like time of hydrolysis, way of shaking, time of derivatization and volume of derivatizing agent had to be studied to guarantee an optimal performance by the PrepStation system.

A standard procedure for extraction of steroid compounds from urine is divided into three steps: hydrolysis, liquid–liquid extraction and derivatization.

(1) Hydrolysis: most of the steroid compounds are excreted as glucuronide–sulfate conjugates, therefore a hydrolysis process is required in order to make GC–MS analyses possible. Percentage of deconjugation for anabolic agents changes when the time of hydrolysis varies. From all the anabolic agents assayed in this study, androsterone, etiocolanolone, 11-OH-androsterone and 11-OH-etiocolanolone were the anabolic agents that were shown to be the most difficult to deconjugate from glucuronic acid. Fig. 2 shows deconjugation for androsterone and etiocolanolone glucuronides with reference to the internal standard, after hydrolysis for different times of the same urine sample. Androsterone and

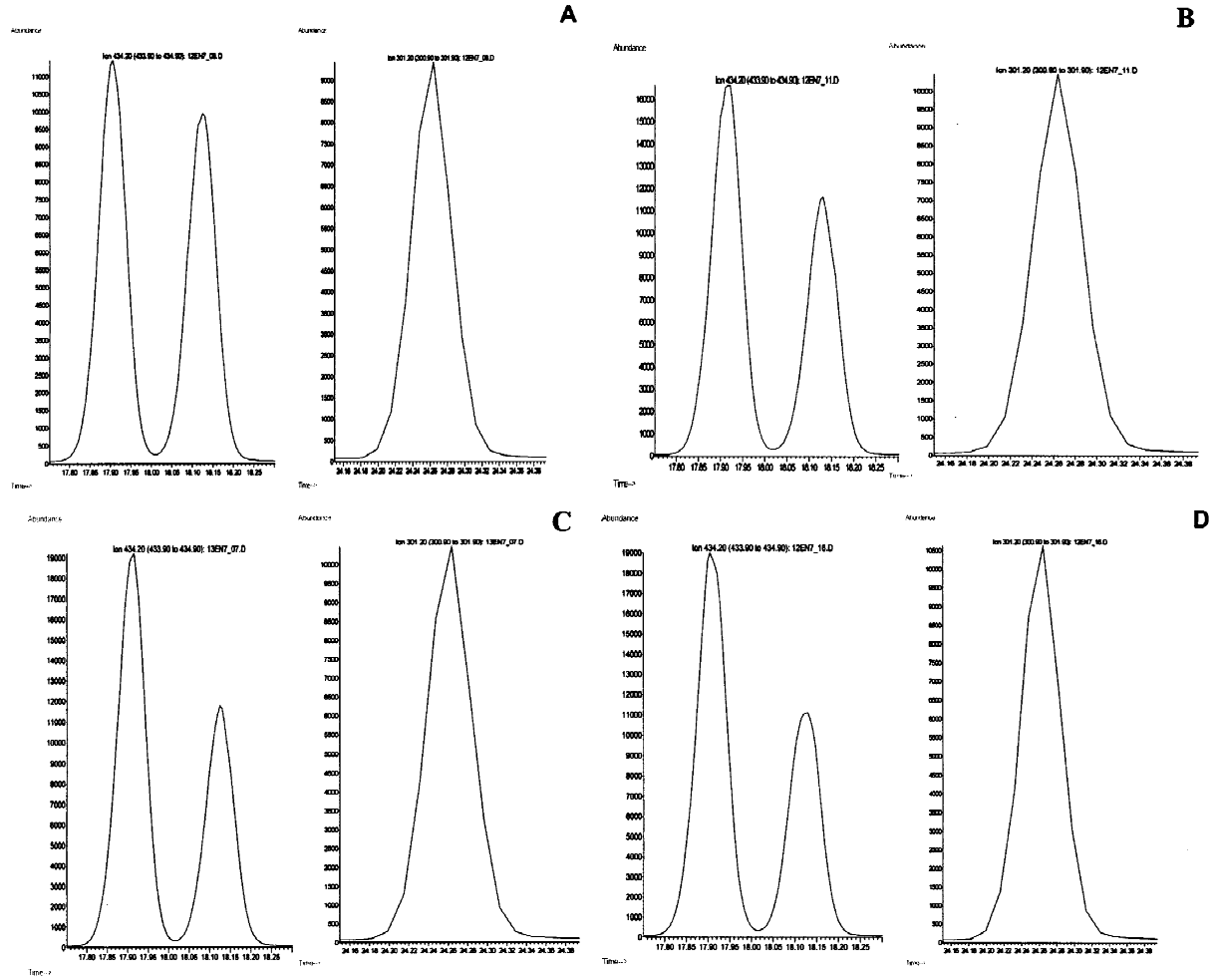

Fig. 2. Effect of the time of hydrolysis over androsterone and etiocholanolone glucuronic deconjugation. (A) A 5-min hydrolysis was applied, (B) after 10 min of hydrolysis, (C) after 20 min of hydrolysis and (D) after 25 min of hydrolysis.

etiocolanolone were monitored at 434 m/z and the internal standard at 301 m/z. It can be observed that a longer hydrolysis time is required in the case of androsterone for total deconjugation. The abundance ratio of free androsterone/etiocholanolone goes up when the hydrolysis time is longer. Hence, to guarantee a total deconjugation of all endogenous anabolic compounds of screening a hydrolysis of 25 min at 55°C was selected.

(2) Extraction: an important improvement in terms of signal level was observed when multiple TBME extractions were carried out. An average increase of 20% in the recovery of most compounds was observed after a second extraction. After the third extraction the values increased by only 5%. As each new extraction involved an increase of 20 min in the time spent processing and taking into account the improvement of recovery a double extraction was used in this method.

(3) TMS derivatization: at 65°C, the TMS forms of the anabolic agents were already prepared in only 5 min. To maintain proportions between manual and automated preparation only 15 μl of derivatizing mixture should be added. Due to the design of the high-recovery vials, the viscosity of extract and its small volume, it was difficult to reproduce autosampler intake, which can compromise the results. The RSD of the signal ($n=10$) was checked when

Table 1
The recovery and the limit of detection for the anabolic agents in manual and automated sample preparation by PrepStation

Anabolic agent	Urinary metabolite	PrepStation			Manual preparation	
		LOD	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Nandrolone[a]	19-Norandrosterone	0.4	76.5	5.8	82.5	9.0
	19-Noretiocholanoleone	2.3	77.8	6.9	80.6	16.6
Epimetendiol[a]	Epimetendiol	3.2	65.3	8.1	77.2	8.0
3′-Hydroxystanozolol[a]	3′-Hydroxystanozolol	14.3	35.3	2.4	63.0	10.7
Methenolone[a]	1α-Methy1ene-5α-androstane-3α-ol-17-one	2.6	62.8	9.8	81.0	10.1
Mesterolone[a]	1α-Methyl-5α-androstan-3α-ol-17-one	2.0	45.4	17.6	70.0	5.6
Boldenone[a]	5β-Androst-1-en-17β-ol-3-one	1.4	75.8	5.8	88.2	21.1
Norethandrolone[a]	17α-Ethyl-5β-estrane-3α,17β-diol	0.2	73.1	6.1	81.6	8.6
Methyltestosterone[a]	17α-Methylandrost-4-en-17β-ol-3-one	1.8	67.4	8.8	76.2	7.7
Drostanolone[a]	2α-Methyl-5α-androstan-3α-ol-17-one	1.3	65.8	8.1	80.7	8.5
Clortestosterone[a]	4-Chloro-androst-4-en-17β-ol-17-one	1.5	79.3	4.9	83.8	10.2
Bolasterone[a]	7α,17α-Dimethyl-androst-4-3-en-17β-ol-3-one	2.7	80.9	7.3	88.9	6.7
	7α,17α-Dimethyl-androst-4-3-en-17β-diol	0.5	68.3	7.0	79.3	8.3
Furazabol[a]	Furazbol	2.6	76.9	5.0	85.2	17.0
Oxymesterone[a]	17α-Methyl-4-androsten-4,17β-diol-3-one	6.3	76.2	32.4	82.0	25.4
Formebolone[a]	2-Hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one	2.1	89.5	11.9	70.4	16.1
cis-Androsterone[b]	3α-Hydroxy-5α-androsten-17-one		81.0	4.7	84.0	7.9
Etiocolanolone[b]	3α-Hydroxy-5β-androsten-17-one		87.44	6.5	89.8	3.7
5α-Androstandiol[b]	5α-Androstan-3α17β-diol		108.8	12.8	91.4	5.7
5β-Androstandiol[b]	5α-Androstan-3α17β-diol		88.8	7.3	80.0	8.0
DHEA[b]	3α-hydroxy-5-androsten-17-one		86.9	6.1	89.7	4.4
Epitestosterone[b]	17α-Hydroxy-4-androsten-3-one		86.3	7.3	95.4	7.1
DHT[b]	17β-Hydroxy-5α-androstan-3-one		82.5	5.6	92.1	3.6
Testosterone[b]	17β-Hydroxy-4-androsten-3-one		84.2	8.5	92.8	5.7
11β-Hydroxyandrosterone[b]	11β,3α-Dihydroxy-5β-androstan-17-one		78.7	8.2	79.9	10.4
11β-Hydroxyeticolanolone[b]	11β,3α-Dihydroxy-5β-androstan-17-one		84.8	6.9	87.7	2.3
Pregnandiol[b]	5β-Pregnane-3α,20α-diol		80.1	6.1	81.2	4.3
Pregnantriol[b]	5β-Pregnane-3α,17α,20α-triol		78.2	6.7	55.6	14.0
Tetrahydrocortisol[b]	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one		59.2	6.0	102.8	13.3

[a] Exogenous anabolic agents.
[b] Endogenous anabolic agents.

Table 2
Reproducibility – the between-run variation for $n=8$

Anabolic agent	Urinary metabolite	PrepStation		Manual preparation	
		Average mean	RSD (%)	Average mean	RSD (%)
Nandrolone[a]	19-Norandrosterone	0.231	4.4	0.22	6.4
	19-Noretiocholanolone	0.063	3.8	0.016	7.9
Epimetendiol[a]	Epimetendiol	0.038	13.2	0.016	6.0
3′-Hydroxystanozolol[a]	3′-Hydroxystanazolol	0.012	7.2	0.071	10.1
Methenolone[a]	1α-Methylene-5α-androstane-3α-ol-17-one	0.078	16.3	0.130	6.5
Mesterolone[a]	1α-Methyl-5α-androstan-3α-ol-17-one	0.113	41.3	0.100	4.2
Boldenone[a]	5β-Androst-1-en-17β-ol-3-one	0.304	3.7	0.093	6.1
Norethandrolone[a]	17α-Ethyl-5β-estrane-3α,17β-diol	0.371	8.4	0.104	6.3
Methyltestosterone[a]	17α-Methylandrost-4-en-17β-ol-3-one	0.530	14.0	0.082	6.1
Drostanolone[a]	2α-Methyl-5α-androstan-3α-ol-17-one	0.178	13.1	0.273	5.7
Clortestosterone[a]	4-Chloro-androst-4-en-17β-o1-17-one	0.084	3.9	0.090	7.5
Bolasterone[a]	7α,17α-Dimethyl-androst-4-3en-17β-ol-3-one	0.0192	4.2	0.010	9.1
	7α,17α-Dimethyl-5β-androstane-3α,17β-diol	0.0147	11.5	0.052	6.0
Furazabol[a]	Furazabol	0.025	2.5	0.001	10.8
Oxymesterone[a]	17α-Methyl-4-androsten-4,17β-diol-3-one	0.031	29.6		
Formebolone[a]	2-Hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one	0.476	12.2	0.931	8.1
cis-Androsterone[b]	3α-Hydroxy-5α-androstan-17-one	7.45	6.6	5.16	5.7
Etiocolanolone[b]	3α-Hydroxy-5β-androstan-17-one	0.456	6.1	3.69	3.1
5α-Androstandiol[b]	3α-Androstan-3α17β-dio1	0.122	5.7	0.097	1.9
5β-Androstandiol[b]	5β-Androstan-3α17β-diol	0.217	6.4	0.156	2.5
DHEA[b]	3α-Hydroxy-5-androsten-17-one	0.072	6.0	0.070	3.5
Epitestosterone[b]	17α-Hydroxy-4-androsten-3-one	0.255	4.1	0.207	2.5
DHT[b]	17β-Hydroxy-5α-androstan-3-one	0.052	8.2	0.031	5.0
Testosterone[b]	17β-Hydroxy-4-androsten-3-one	0.274	3.6	0.239	1.8
11β-Hydroxyandrosterone[b]	11β,3α-Dihydroxy-5α-androstan-17-one	1.278	9.3	0.71	12.1
11β-Hydroxyetiocolanolone[b]	11β,3α-Dihydroxy-5β-androstan-17-one	0.145	8.2	0.086	17.6
Pregnandiol[b]	5β-Pregnane-3α,20α-diol	2.044	6.9	1.569	2.0
Pregnantriol[b]	5β-Pregnane-3α,17α,20α-triol	1.867	11.2	1.473	8.8
Tetrahydrocortisol[b]	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one	8.757	6.5	10.174	4.1

[a] Exogenous anabolic agents.
[b] Endogenous anabolic agents.

different volumes of derivative mixture was added. A 20-μl volume of the extract final volume makes it possible to obtain a relative standard deviation (RSD) ranging from 5 to 8%.

5.2. Method evaluation

In the following experiments the performance of the automated method was tested and compared with results obtained with the manual process.

To estimate the recovery of both procedures, two batches of 10 samples were fortified with 10 ng of each anabolic agent, the first group before processing and the second just before derivatization. The signal level of each compound that corresponds to three times the standard deviation of the noise was defined as the LOD. For its anabolic agents three of the most characteristic fragments were monitored. To fix the

Table 3
The comparison of linearity for automated and manual preparation obtained for 19-norandrosterone

Statistical parameters	PrepStation	Manual
Equation	$0.0028x+0.0005$	$0.003x-0.0011$
S_m	0.000019	0.000031
C_m	99.31%	98.94%
S_b	0.001002	0.000881
R	0.99995	0.99988

noise level at the retention time of each anabolic agent 10 negative samples were processed. Table 1 shows the calculated recovery of the manual and automated extraction for each anabolic agent. In the case of automated preparation, the LOD was measured. The use of a PrepStation allows automated sample preparation with a recovery ranging from 80 to 90% for most of the anabolic agents tested. Similar recovery values were measured for the routine sample preparation. When some exogenous anabolic agents were tested it seems that the difference between the manual and automated recoveries became bigger in favor of the manual preparation. Regarding the error, no remarkable difference was observed between the two extraction methods. LODs after an automated preparation for some exogenous anabolic agents are shown in Table 1. Except for 3′-hydroxystanozolol and oxymesterone, in most of

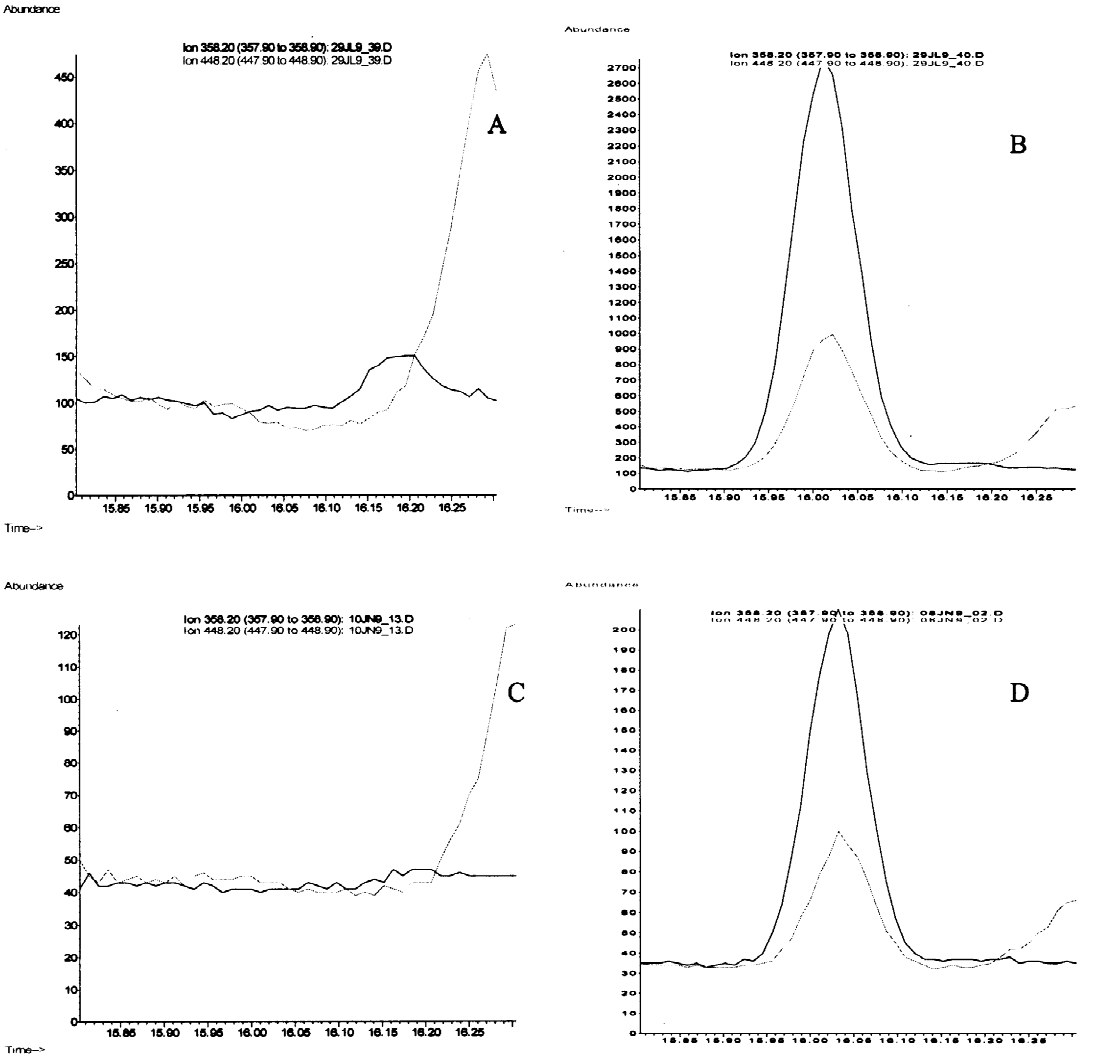

Fig. 3. Chromatographic signal of m/z ion traces 358 and 448 of epimetendiol. Results were obtained after automatic extraction and GC–MS (SIM mode) analyses of next samples. (A) Blank sample, (B) real positive case to be confirmed, (C) negative control urine and (D) control reference urine.

the cases the values were below 3 ng/ml, which is an acceptable value for screening purposes.

To compare the precision of both methods the same spiked urine sample was prepared manually and automatically. The between-run variation ($n=8$) in relative signal to the internal standard of each anabolic compound is shown in Table 2. In general data show a slightly better reproducibility for the manual preparation.

To evaluate the linearity of both methods blank urine was fortified with 19-norandrosterone at concentrations of 1, 2.5, 5, 25, 50 and 100 ng/ml, internal standard (methyltestosterone) concentration 250 ng/ml. Each point of the plot was the average of three aliquots.

The data fitted to a linear empirical relationship. For statistical evaluation of the results, the standard deviation of the slope (S_m) and the intercept (S_b), the correlation coefficient (R) and the linearity coefficient (C_m) were estimated. As shown in Table 3, automated and manual sample preparation provide quite similar values.

A potential contamination of the system when samples with large compound concentration are processed was tested. Between each 19-norandrosterone-spiked sample in a concentration range from 2400 to 400 ng, two negative samples were prepared. No carry-over effect was observed.

Automation of sample preparation allows preparing batch samples for detection or confirmation purposes. Once a suspicious signal has been detected in the first sample analyses, a confirmation batch have to be analyzed. Fig. 3 shows a GC–MS (SIM mode) analysis of a confirmatory pool for a real positive finding with epimetendiol. Water blank (A), sample to be confirmed (B), negative control urine (C) and finally positive control urine (D) were automatically extracted and GC–MS analyzed.

Regarding reagent and labor time consumption the comparison between manual and automated preparation seems to be quite difficult. A detailed analyses of reagents and solvents consumption in each sample preparation step, automated or manual, indicates an important saving in favor of automated preparation. Nevertheless, this saving is counterbalanced by a need to wash the flow paths of the system with cyclohexane (5 ml), methanol (5 ml), TBME (9 ml) and acetonitrile (5 ml) between each sample.

In relation to time consumption, the preparation of one sample with a PrepStation takes about 1 h and 40 min, 15 samples can be prepared during the night.

6. Conclusions

The automated method developed for the liquid–liquid extraction of urine samples permits the analysis of anabolic steroids for doping control purposes with a detection capacity similar to that obtained with the routine manual method. Manual or automated sample preparation can be considered as similar in terms of linearity, reproducibility, sensitivity and recovery. Saving of some reagents enzyme, internal standard, and buffers), matrix and time of the employee's exposure to hazardous materia1s makes its sample preparation interesting in doping analyses.

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