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Detection of testosterone misuse: comparison of two chromatographic sample preparation methods for gas chromatographic-combustion/isotope ratio mass spectrometric analysis

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Abstract

Two chromatographic methods, reversed-phase liquid chromatography (LC) and immunoaffinity chromatography (IAC), were compared in the preparation of purified testosterone extracts suitable for gas chromatography–combustion/isotope ratio mass spectrometry (GC–C-IRMS) analysis. We have shown previously that GC–C-IRMS is a promising means of detection of testosterone misuse in sport. The two clean-up procedures afford sufficient recovery and adequate purity of testosterone. LC presents several advantages over IAC: access to other urinary steroids, longer column life, no need for special equipment and no antibody preparation. For IAC, the antibodies to testosterone must be selected with care for high affinity and low cross-reactivity. Nevertheless, IAC is of some interest in our experiments, the recovery is slightly better for low concentrations of urinary testosterone and IAC does not induce isotopic discrimination even in overloading experiments. This is the first report on sample preparation by IAC prior to GC–C-IRMS and carbon isotope ratio values for urinary epitestosterone. The carbon isotope ratio test can identify users' urines missed by the testosterone to epitestosterone ratio (T/E>6) test.

Keywords: Testosterone; Epitestosterone

1. Introduction

Detecting the fact that synthetic testosterone has been misused to improve sport performance is still a challenge for analytical laboratories engaged in urine drug testing. The official method is based on the determination of the urinary testosterone to epitestosterone ratio (T/E) and a cutoff point of 6 was adopted [1]. In some rare cases, healthy athletes may have T/E ratios exceeding the threshold value without having taken exogenous testosterone. Some supplemental criteria have been suggested such as the urinary testosterone to luteinizing hormone ratio [2], the serum testosterone to 17α-hydroxyprogesterone ratio [3] and the urinary testosterone and epitestosterone glucuronides to 5-androsten-3β,17α glucuronides ratios [4]. These criteria are helpful but have some limitations [5]. For now, the International Olympic Committee recommends further tests in cases where T/E exceeds 6 before considering

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results as positive or negative [6]. The potential use of a ketoconazole test has been investigated [7].

Another approach to detecting testosterone abuse has been investigated: isotope ratio mass spectrometry. It has been shown previously [8], [9] that the ^{13}C content of endogenous testosterone differs from that of exogenous testosterone and that the difference is significant. The procedure consists of two main steps: first, clean-up of urine samples to obtain purified acetylated steroids amenable to gas chromatographic separation; second, isotope ratio measurements using a gas chromatograph connected to an isotope-ratio mass spectrometer via a combustion interface (GC–C-IRMS) [10], [11].

Semi-preparative reversed-phase liquid chromatography [8] was compared to immunoaffinity chromatography for urine clean-up. The latter technique is based on a highly specific binding between the drug molecule (testosterone) and an antibody raised against it. Immunoaffinity chromatography (IAC) has been applied to the preparation of crude extracts and combined with other analytical procedures for numerous compounds, especially for GC–MS [12–14] and LC–MS [15] analysis of steroids. This paper presents a preliminary evaluation of LC versus IAC for testosterone purification before GC–C-IRMS analysis.

2. Experimental

2.1. Chemicals, reagents and samples

All solvents and reagents were of analytical grade purity; 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 5-androstene-3β,17β-diol diacetate, androsterone, cholesterol, epitestosterone, etiocholanolone, dehydroepiandrosterone (=DHEA), dihydrotestosterone (=DHT), testosterone, [16,16,17-^{2}H]testosterone and β-glucuronidase from *Helix pomatia* were supplied by Sigma (Saint Quentin-Fallavier, France). Solid-phase extractions (SPE) were carried out on Bakerbond solid-phase extraction columns (SPE) filled with reversed-phase octadecyl (7020-07) or adsorption-phase silica gel (7086-03) (J.T. Baker, Noisy le Sec, France). [1,2,6,7-^{3}H]Testosterone (90 Ci/mol) was obtained from CEA (Saclay, France). CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech (Orsay, France). The characteristics of the urine samples under study are reported in Table 1. The protocol for obtaining the urines was approved by the relevant institutional review board. Healthy normal male volunteers received either oral testosterone undecanoate (2×40 mg per day) or intramuscular testosterone cypionate (600 mg) and urines were collected 4–10 h or one week after administration, respectively.

2.2. Overview of the analyses

Each sample underwent several procedures. First, an extraction, TMS derivatization and GC–MS anabolic steroid screen were carried out to estimate the T/E and the T concentration. Second, a different portion of each urine underwent the preparation of purified cholesterol and of a crude extract on which to compare further purification by LC or IAC. LC yielded testosterone and other steroids. IAC yielded testosterone and, in a few cases, epitestosterone. All steroid fractions were then acetylated, analyzed by GC–MS (to verify identity and purity) and submitted to GC–C-IRMS (to measure the carbon isotope ratio of testosterone and other steroids from LC and of testosterone and in a few cases, epitestosterone from

Table 1
Characteristics of urine samples

Urine sample	Type	Testosterone administration	[T] (ng/ml)	T/E	Volume used (ml)	
					LC	IAC
1	Blank	No	65	2.0	50	50
2	Blank	No	53	2.1	50	50
3	Blank	No	39	1.0	100	50
4	E.S.	p.o.	>150	43	25	10
5	E.S.	p.o.	>150	49	50	10
6	E.S.	p.o.	118	33	50	10
7	E.S.	p.o.	80	2.2	50	50
8	E.S.	p.o.	115	30	50	10
9	E.S.	p.o.	45	1.5	50	50
10	E.S.	i.m.	103	121	40	10

Blank = urine from drug-free male; E.S. = testosterone excretion study urine from male; p.o. = oral testosterone undecanoate, 2×40 mg/day; i.m. = intramuscular testosterone cypionate, 600 mg; [T] = testosterone concentration (estimated using internal standard [$^{2}H_{3}$]testosterone); T/E = testosterone/epitestosterone area ratio; [T] and T/E data came from steroid GC–MS screening.

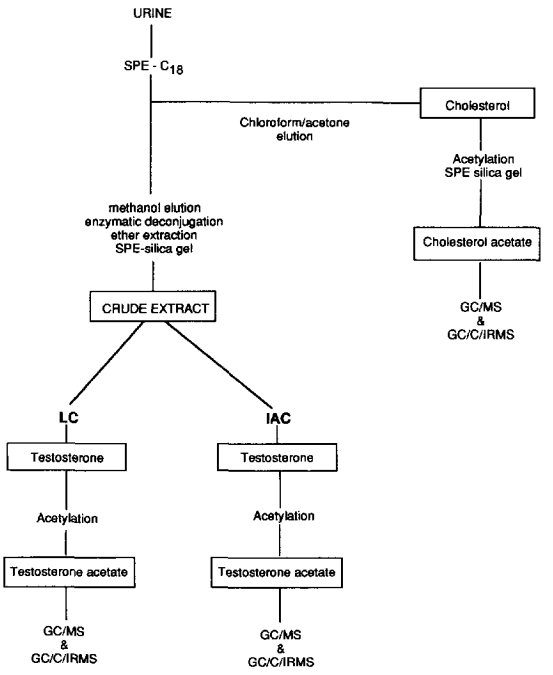

Fig. 1. Flow chart of sample preparation for comparison of LC to IAC.

IAC). The flow chart of steroid extraction and purification appears in Fig. 1.

2.3. Steroid screening

Each urine sample was submitted to an update of the steroid screening procedure previously described in Ref. [16] to estimate the testosterone concentration and the T/E ratio. In brief, it included addition of [16,16,17-^{2}H]testosterone, extraction on XAD-2, hydrolysis with β-glucuronidase from *Helix pomatia*, diethyl ether extraction, formation of trimethylsilyl derivatives and GC–MS analysis in the selected ion monitoring mode (*m/z* 432 for testosterone and epitestosterone bis-TMS and *m/z* 435 for [16,16,17-^{2}H] testosterone bis-TMS).

2.4. Sample preparation

The first step was extraction on a C_{18} column (1 g of reversed-phase packing material for 25 ml urine). The cholesterol fraction was eluted with a chloroform–acetone mixture (50:50, v/v, 6 ml). The eluate was dried under nitrogen and the residue acetylated overnight at room temperature with an acetic anhydride–pyridine mixture (0.3 ml of each). After evaporation to dryness the acetylated extract was redissolved in a dichloromethane–hexane mixture (5:95, v/v, 6 ml), then applied to a silica gel SPE column. The cholesterol acetate fraction was eluted with a dichloromethane–hexane mixture (25:75, v/v, 6 ml). This solution was analysed by GC–MS-EI to check the compound identity and purity then by GC–C-IRMS.

After elution of cholesterol from the above C_{18} column, the conjugated steroids were eluted with methanol (12 ml/column) and the solvent evaporated to dryness. To hydrolyze the conjugated steroids, the methanolic residue was redissolved in 10 ml of 0.5 *M* acetate buffer (pH 5.5) and 0.50 ml of β-glucuronidase suspension was added as supplied; incubation was carried out at 55°C for 3 h. After cooling, solid buffer (Na_2CO_3/$NaHCO_3$, 2:1, w/w, about 300 mg) was added. Extraction was performed with diethyl ether (2×50 ml) and the solvent layers combined and evaporated to dryness. The residue containing the hydrolyzed steroids was redissolved in an ethyl acetate–hexane mixture (5:95, v/v, 10 ml) and applied to a silica gel SPE column. After washing the SPE column with an ethyl acetate–hexane mixture (20:80, v/v, 6 ml) the steroids were eluted with 6 ml of the 50:50 (v/v) solvent mixture. The eluate was evaporated to dryness to yield the crude steroid extract.

2.5. Immunoaffinity procedures

The hemiglutaramide derivatives of 3β-amino-androst-4-en-17β-ol (3β-HG-T), 17β-amino-androst-4-en-3-one (17β-HG-T) and 17α-methyl amino-17-hydroxy-androst-4-en-3 one (17α-HGM-T) were synthesized from testosterone according to a previously described procedure [17] and the corresponding activated *N*-hydroxy-succinimide esters were coupled to bovine serum albumin (BSA). The antisera were raised in female New Zealand rabbits by repeated injections of 0.5 mg of the 3β-HG-T-BSA and 17β-HG-T-BSA antigens dissolved in complete Freund's adjuvant. Titers were defined as the dilution of antibodies that bound 50% of the added [1,2,6,7-^{3}H]testosterone tracer. Titers were

measured as follows. Dilutions of the antibodies were prepared in 0.1 *M* sodium phosphate buffer containing 0.15 *M* NaCl, 0.1% gelatin and 0.1% sodium azide (PBS-gelatin). Aliquots (700 μl) of these dilutions were incubated with [1,2,6,7-^{3}H]testosterone (15 000 dpm) in 100 μl of PBS-gelatin for 2 h at 37°C followed by 15 min at 4°C. Next, to adsorb the unbound tritiated testosterone, a cold suspension (1 ml) of dextran-coated charcoal (DCC: 0.05% dextran and 0.5% charcoal in phosphate buffer) was added. Following centrifugation (8000 *g* for 15 min at 4°C) the supernatants containing the bound fractions were counted after addition of 4 ml of scintillation fluid.

The specificities of the antibodies were estimated by competitive binding experiments. Each antibody was diluted in PBS–gelatin at the titer dilution. Next, 200 μl were incubated with 600 μl of PBS–gelatin containing tritiated testosterone, testosterone and each of the competitors. Testosterone and the competitors were added at seven concentrations (ranging from 0.7×10^{-10} to 35×10^{-10} mol). Five competitors were assayed: androsterone, etiocholanolone, DHEA, DHT and 5β-androstane-3α,17β-diol. Incubation was for 2 h at 37°C, followed by 15 min at 4°C. Free and bound steroids were separated with DCC as described above.

The antibodies were purified by affinity chromatography using steroid antigens immobilized on Sepharose as reported [18]. The antibodies obtained from 3β-HG-T-BSA were purified on a 17α-HG-T-BSA affinity column, and the anti-17β-HG-T antibodies were purified on the homologous 17β-HG-T-BSA affinity column. The antibodies retained on the affinity columns were eluted by 1 *M* NH_4OH, and immediately dialyzed against a solution containing 0.1 *M* $NaHCO_3$ and 0.5 *M* NaCl. The purified antibodies were coupled to CNBr-activated Sepharose 4B according to the instructions from Pharmacia. The overall yield estimated by titration of the purified fractions after dialysis was 40% for anti-3β-HG-T antibodies and 70% for anti-17β-HG-T antibodies.

The maximal capacity of the immunoaffinity columns was determined by adding different amounts of blank urine (1, 5 and 10 ml containing about 65 ng/ml of testosterone) to 1 ml of gel-antibody. The testosterone recovery was determined in the wash and eluate fractions by GC–MS analysis of the acetylated derivatives.

2.6. Immunoaffinity chromatography

The two immunoaffinity gels were first tested in analytical experiments with 1 ml of gel. Testosterone purification for isotopic measurements must be done on a larger scale than that used for GC–MS analysis because of the difference in sensitivity. Therefore solvent volumes and gel quantity were 15-times greater for the semi-preparative procedure than for the analytical procedure. The 17β-HG-T-BSA immunoaffinity gel (15.2 ml) was poured into a glass column (17×2 cm I.D.) and washed with 50 ml of water. The crude steroid extract was dissolved in 0.5 ml of ethanol and diluted with 9.5 ml of water. This solution was transferred to the column. Unbound material was washed away with 6×5 ml of water. The antibody-bound testosterone was eluted with 25 ml of 80% ethanol in water. The eluate was dried under a stream of nitrogen at 50°C. Before use or re-use, the immunoaffinity gel column was washed with large volumes of water and stored at 4°C in 0.01 *M* phosphate buffer containing 0.01% sodium azide.

2.7. Liquid chromatography

The crude steroid extract was redissolved in 50 μl of methanol and injected on a semi-preparative C_{18} column: 25×1 cm I.D., 5 μm, Spherisorb ODS 2 (Interchim, Montluçon, France). The mobile phase, water–acetonitrile (54:46, v/v), was delivered by a Merck L-600 pump at a flow-rate of 4 ml/min. Elution was isocratic and it was monitored by UV detection at 240 nm (Kratos Spectroflow, Manchester, UK). Testosterone eluted between 13 and 15 min.

2.8. GC–MS analysis

Acetylation of the purified extracts was carried out by overnight room temperature incubation with a mixture of pyridine and acetic anhydride (0.5 ml of each). GC–MS analyses were performed on a Hewlett-Packard (HP) 5970 B mass selective detector linked to an HP 5890 gas chromatograph equipped

with an SGE BPX 5 fused-silica capillary column (25 m×0.22 mm I.D.; 0.25 μm film thickness). The injections were made in the splitless mode (1 μl). The carrier gas was helium.

For acetylated steroid derivatives, the oven temperature started at 200°C (1 min) and increased at 2°C/min to 250°C, then at 20°C/min to 300°C and stayed at the final temperature for 4 min. For cholesterol acetate, the temperature increased from 200 to 300°C by 15°C/min.

2.9. Isotopic analysis

GC–C-IRMS analyses were performed on an HP 5890 gas chromatograph connected to a home-made combustion furnace (Centre National de la Recherche Scientifique-Service Central d'Analyse) linked to a Finnigan MAT 252 isotope ratio mass spectrometer (Bremen, Germany). The GC was equipped with a BPX 5 column (SGE, 25 m×0.22 mm I.D., 0.25 μm film thickness). Helium was the carrier gas (135 kPa). Samples were injected in the splitless mode at 285°C. The GC separation began at 80°C during 10 min then the column temperature was programmed from 80°C to 260°C at 10°C/min, and then at 5°C/min from 260 to 315°C which was held for 5 min.

The temperature of the combustion oven was 800°C and the oxidative catalyst was a wire of copper oxide (0.25 mm in a quartz tubing of 0.5 mm).

The δ standard notation for expressing carbon isotope ratios is defined as the relative difference in isotope ratio between the sample and an international standard (PDB or Pee Dee Belemnite), calculated as:

$$\delta^{13}C\,‰ = \left(\frac{R_{sample} - R_{PDB}}{R_{PDB}}\right) \times 10^3$$

Where R_x refers to the $^{13}C/^{12}C$ of the sample or international standard. Each sample was submitted to two or three replicate measurements which were averaged. A typical standard deviation associated with these GC–C-IRMS determinations is 0.4‰ [9].

3. Results

The goal was to compare two methods of purification of testosterone from a crude urinary steroid extract, reversed-phase LC and IAC. During the preparation of the crude extract, free cholesterol was isolated from urine. This cholesterol purification procedure differed from the one described in our previous work [9]. The urine underwent one solid-phase extraction on C_{18} column which afforded free cholesterol upon elution with a chloroform–acetone mixture and the crude extract (deconjugated steroid fraction) upon subsequent elution with methanol.

3.1. Comparison of the two antibodies

Two types of polyclonal antibodies were produced against testosterone, one from antigen 17β-HG-T-BSA in which the steroid was attached to BSA at the D ring so that the specificity was directed toward the A and B rings, the other from antigen 3β-HG-T-BSA in which the steroid was attached to BSA at the A ring so that the specificity was directed toward the D ring. Binding measurements were carried out and typical curves were obtained (Fig. 2 and Fig. 3). As expected, minor cross-reactivity was observed with 17β-HG-BSA antibodies because the 4-ene-3-one function distinguished testosterone from all five competitors. The cross-reactivity of 3β-HG-T-BSA antibodies for DHT and 5β-androstane-3α,17β-diol was expected since both of these steroids have the same 17β-hydroxylated D ring as testosterone. The lack of separation between testosterone and these two steroids was not considered to be a problem, even though the GC step of the GC–C-IRMS analysis did not resolve them. This is because the ultimate goal was to detect a measurable change in δ‰ when exogenous testosterone has been taken, and because both DHT and 5β-androstane-3α,17β-diol are metabolites of testosterone, so that the δ‰ of the mixture of the three compounds should reflect the administration of exogenous testosterone.

Therefore, the testing of the two types of testosterone antibodies on a crude extract from a blank urine proceeded (urine 1, Table 1). The capacity of each immunoaffinity column (1 ml) was determined to be approximately 500 ng and 350 ng of testosterone for the antibodies to 17β-HG-T-BSA and 3β-HG-T-BSA, respectively. The resulting steroid extracts were dried, acetylated and subjected to GC–MS analysis. Although the chromatograms of the testosterone fractions (Fig. 4 and Fig. 5) seemed to

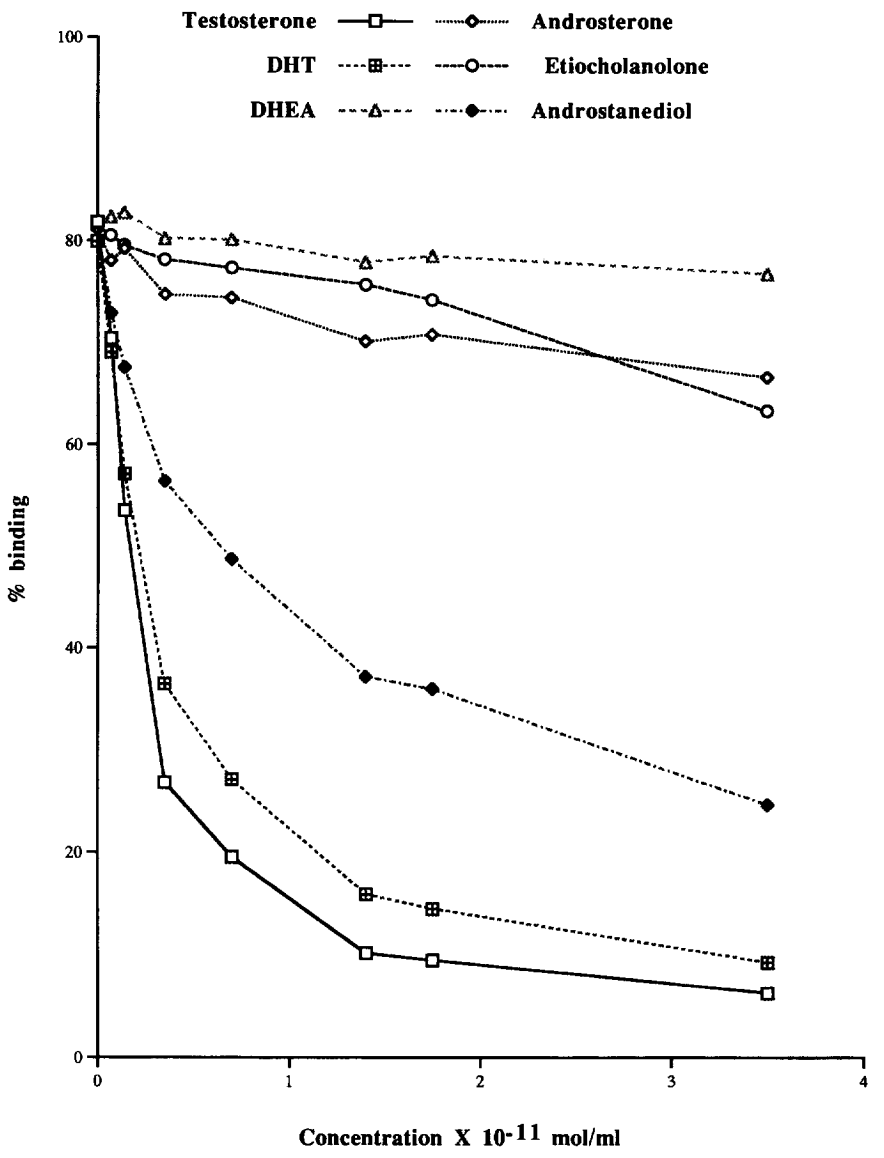

Fig. 2. Cross-reactivity of antibodies to 3β-hemiglutaramide-androst-4-en-17β-ol-bovine serum albumin antibodies (3β-HG-T-BSA antibodies).

indicate that the extracts were relatively clean, the mass spectral data revealed an important difference. As expected, the mass spectrum of the product obtained using antibodies to 17β-HG-T-BSA matched that of a testosterone acetate reference standard (Fig. 4). In contrast, the product obtained using antibodies to 3β-HG-T-BSA had a different mass spectrum (Fig. 5) with the ions characteristic of testosterone acetate and an extraneous ion at m/z 314 from a coeluting compound. The latter was identified as 5-androsten-3α,17β-diol diacetate by comparison with authentic reference standard. Since 5-androsten-3α,17β-diol is a precursor of testosterone, its δ‰ should not be affected by exogenous testosterone

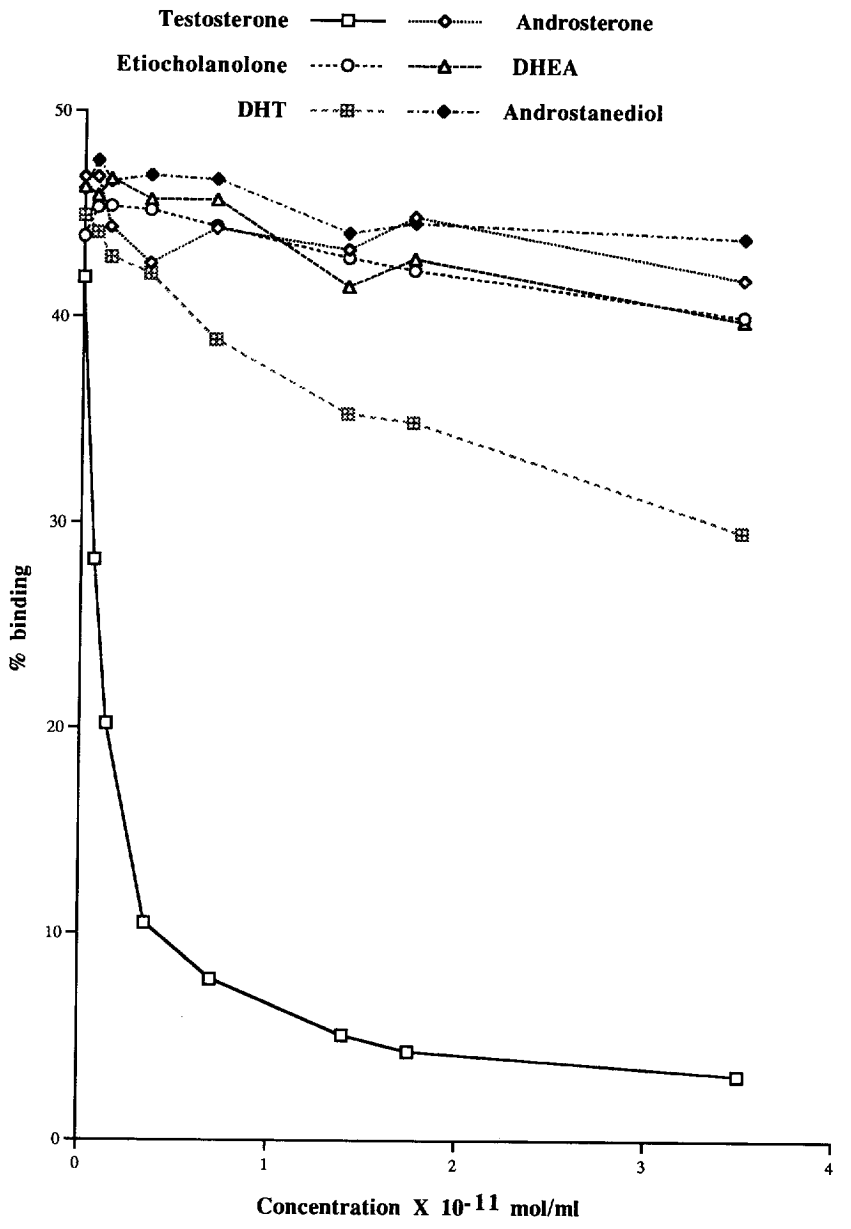

Fig. 3. Cross-reactivity of antibodies to 17β-hemiglutaramide-androst-4-en-3-one-bovine serum albumine antibodies (17β-HG-T-BSA antibodies).

administration, on the contrary it should reflect the subject's baseline carbon isotope ratio. To detect an altered carbon isotope ratio due to the administration of exogenous testosterone, separating precursors and metabolites of testosterone prior to measuring δ‰ is essential. Antibodies to 3β-HG-T-BSA which yield a mixture of testosterone and a precursor that can not be resolved by GC cannot be used to achieve our goal. Therefore, to process the crude steroid extracts from all ten urine samples by IAC, only antibodies to

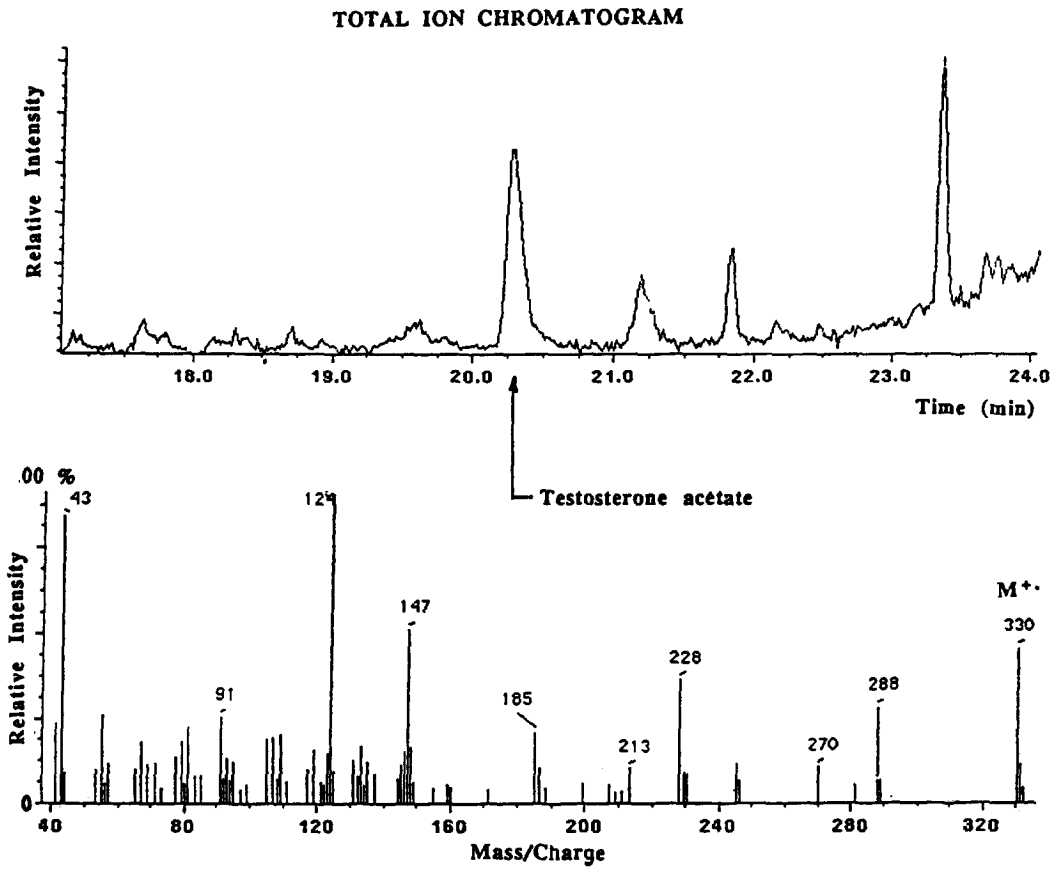

Fig. 4. GC–MS analysis of acetylated testosterone fraction from IAC with antibodies to 17β-HG-T-BSA.

17β-HG-T-BSA were used: a single column was used for the ten urine samples.

3.2. Comparison of LC and IAC for steroids purification

IAC was compared to LC for steroid purification. As shown in Table 1, in a few cases different urine volumes were used for LC or IAC clean-up. For relatively high testosterone concentrations (>100 ng/ml) only 10 ml of urine was needed for IAC. Although 50 ml was used for LC, 10 ml would probably have been sufficient to prepare enough purified testosterone for the GC–C-IRMS experiment.

GC–MS analysis of the acetylated steroids obtained after the two types of clean-up showed that for blank urine or urine with relatively low testosterone concentration (<50 ng/ml) the IAC yields are 15–20% higher than those from LC. The testosterone acetate concentration was estimated by GC–MS using the response factor of a reference testosterone acetate solution. 200 ng/50 μl to 1500 ng/50μl of testosterone acetate were found in the final extracts used for GC–C-IRMS analysis.

This GC–MS analysis also provided information about the purity of the steroid extracts: there were no noticeable differences between the products of the two clean-up methods.

3.3. IRMS measurements

The results of carbon isotope ratio mass spectrometry are summarized in Table 2. IAC and LC clean-up lead to similar δ‰ values for testosterone. For cholesterol the results were identical to those

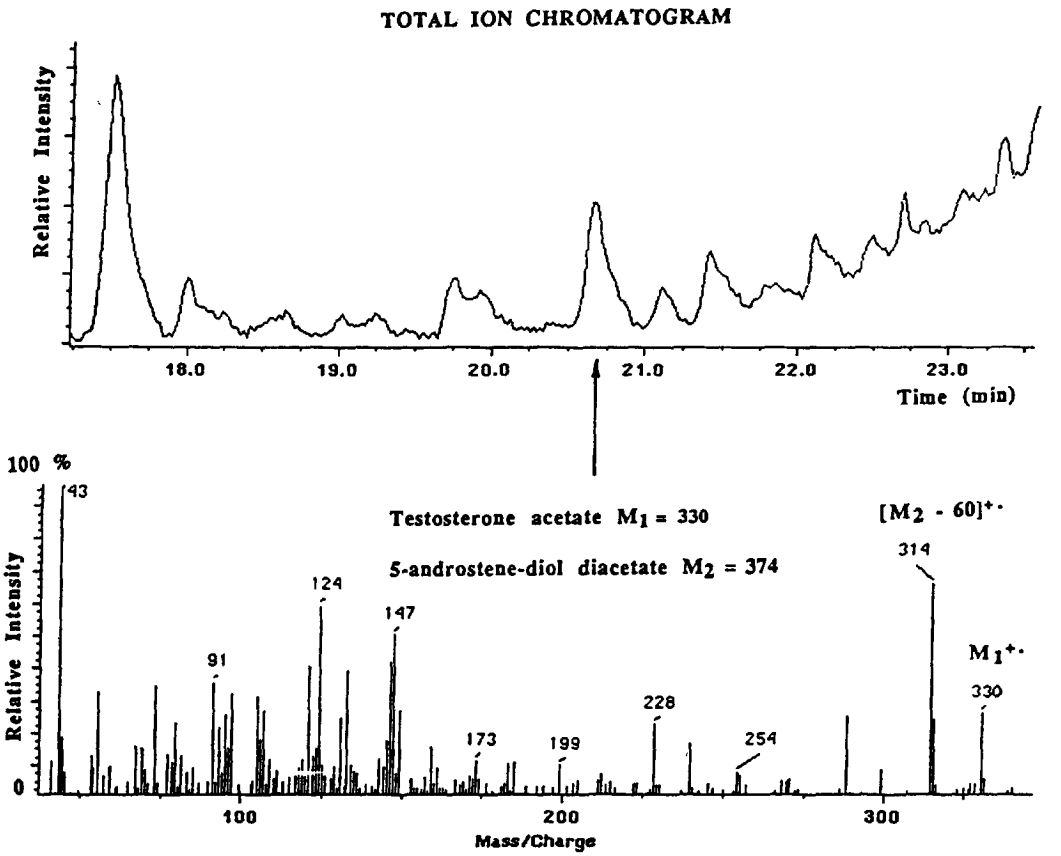

Fig. 5. GC–MS analysis of acetylated testosterone fraction from IAC with antibodies to 3β-HG-T-BSA (the difference in retention time between Fig. 4 and Fig. 5 is due to a change in GC column).

Table 2
Results of δ‰ IRMS measurements on urine samples

Urine samples	Steroids δ‰ with LC clean-up			Steroids δ‰ with IAC clean-up			
	Cholesterol (C)	Testosterone (T)	Differences in δ‰ C−T	Cholesterol (C)	Epitestosterone	Testosterone (T)	Differences in δ‰ C−T
1	−24.1	−24.0	−0.1	−24.6	n.a.	−25.0	0.4
2	−24.5	−26.0	1.5	−25.0	−26.5	−26.3	1.3
3	−25.3	−26.3	1.0	−25.5	n.a.	−25.7	0.2
4	−24.0	−30.8	6.8	−24.5	n.a.	−31.6	6.1
5	−24.5	−29.4	4.9	−24.6	−26.5	−30.5	5.9
6	−25.4	−30.8	5.4	−25.0	n.a.	−31.2	6.2
7	−24.6	−28.3	3.7	−25.3	−25.9	−29.6	4.3
8	−24.5	−28.6	4.1	−24.8	n.a.	−30.9	6.1
9	−24.3	n.a.	–	−24.0	−26.9	−31.9	7.9
10	−21.3	−32.2	10.9	−21.9	n.a.	−32.7	10.8

n.a. = not available because there was not sufficient material to measure δ‰.

obtained by liquid–liquid extraction with diethyl ether [9]. There were characteristic differences in carbon isotope values between blank urines corresponding to endogenous natural testosterone and excretion study urines corresponding to exogenous synthetic testosterone. A new variable was calculated, namely the difference between the δ‰ of precursor cholesterol and the δ‰ of testosterone (δ‰C − δ‰T in Table 2). According to the δ‰C − δ‰T variable the difference between blank urines (δ‰C − δ‰T < 1.5) and excretion study urines (δ‰C − δ‰T > 3.7) was obvious. To evaluate the agreement between the LC and IAC clean-up methods, the values of (δ‰C − δ‰T) by LC and (δ‰C − δ‰T) by IAC were compared according to Lee et al. [19]. An analysis of variance included t-test, evaluation of the method factor, and correlation test. The method factor was not significant ($F = 1.34$, $P = 0.27$), i.e., the two methods were interchangeable. In the correlation test, the interclass correlation ($r_i = 0.96$) measured the agreement between the two methods, which is considered good if r_i is between 0.75 and 1.0.

4. Discussion

During steroid purifications recovery was maximized at the expense of purity. This was because the ^{12}C and ^{13}C homologs of a given compound might get partially resolved so that excessive fractionation might lead to artefactual enrichment in one or the other isotope (isotopic discrimination). The results of GC–C-IRMS obtained after IAC clean-up (Table 2) indicated that there was no isotopic discrimination when the immunoaffinity column was not overloaded, or slightly overloaded. An experiment in which the IAC column was overloaded with a crude steroid extract from 20 ml of urine 6 ([testosterone] ~118 ng/ml) was also conducted. In this case, testosterone was not totally retained on the column: testosterone was found in the wash phase mixed with all other steroids. The bound testosterone was eluted, dried and acetylated. The carbon isotopic measurement was identical to the one obtained without overloading. Thus we concluded that even with overloading, IAC did not induce isotopic discrimination.

With the two clean-up methods, LC and IAC, we focused on testosterone and its precursor cholesterol. Getting more information by measuring the carbon isotope ratio of other steroids is of interest. Considering the biosynthetic and metabolic pathways of androgens, we expect the administration of exogenous testosterone to affect the carbon isotope ratio of testosterone but not that of precursors (progesterone, DHEA, androstenediol, etc.). One of the advantages of LC is the ability to purify other steroids such as DHEA and androstanediols [9]. In this respect IAC is more limited. However, in a few cases IAC yielded sufficient amounts of epitestosterone to measure its δ‰. This was possible only for urine samples 2, 5, 7 and 9 (Table 2). For these samples the T/E ratio observed after IAC, acetylation and GC–MS analysis did not correspond to the T/E ratio calculated from the steroid screening data obtained on TMS derivatives. In all cases the acetylated epitestosterone was in lower concentration than predicted by the T/E ratio of the TMS derivatives. A plausible explanation is an incomplete binding of epitestosterone on the antibodies. Testosterone administration has no influence on the epitestosterone δ‰ as similar values were obtained for blank urines and for excretion study urines (Table 2). This confirms that there is no direct metabolic transformation of testosterone into epitestosterone. This is the first report of carbon isotope ratio measurement on epitestosterone.

With the IAC clean-up all other androgenic steroids were recovered in the wash fraction. The GC–MS analysis of this fraction after acetylation showed a complex chromatogram indicating that the mixture was not suitable for GC–C-IRMS (Fig. 6).

Another advantage of LC compared to IAC was a shorter sample preparation time. Using several IAC columns in parallel would reduce this time but increase the cost. Moreover IAC involves time-consuming preparatory work, use of special radio-immunoassay equipment and the life of the Sepharose-coupled antiserum is shorter than that of the LC column.

Urines 7 and 9 were remarkable because they were collected after exogenous testosterone administration but have T/E ratios not greater than 6 (2.2 and 1.5, respectively). According to the current cutoff they were falsely negative however the carbon isotope ratio test indicated that exogenous testosterone was present in the urine (δ‰T values for urine 7: −28.3

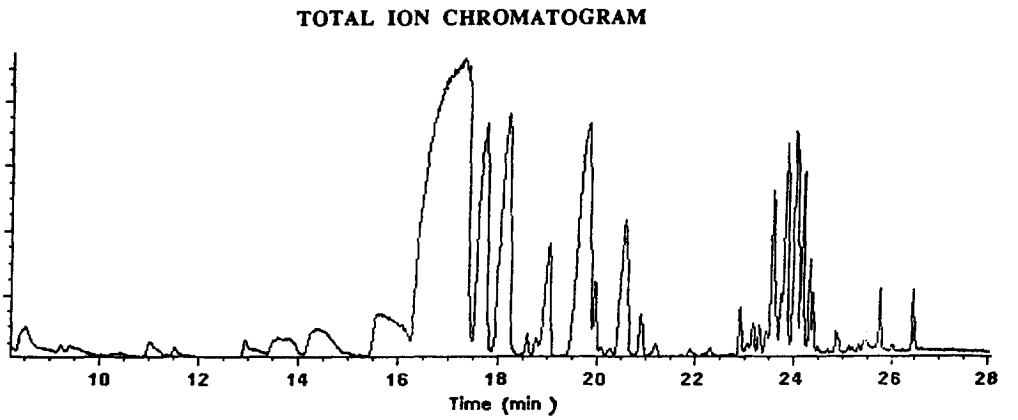

Fig. 6. GC–MS analysis of acetylated wash fraction from IAC.

after LC, −29.6 after IAC, urine 9: not enough testosterone for measurement after LC, −31.9 after IAC).

5. Conclusion

Both LC and IAC are useful methods of purification of urinary testosterone for IRMS analysis. LC presents several advantages over IAC, shorter sample preparation time, access to other urinary steroids, longer column life, no need for special equipment and no antibody preparation. For IAC, the antibodies to testosterone must be selected with care for high affinity and low cross-reactivity. Nevertheless, IAC is of some interest in our experiments, the recovery is slightly better for low concentrations of urinary testosterone and IAC does not induce isotopic discrimination. Finally, the carbon isotope ratio test can identify users' urines missed by the T/E>6 test.

Acknowledgments

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