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Steroid Profiling in Human Blood

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Introduction

High resolution mass spectrometry coupled with gas chromatography (GC/HRMS) was used to determine endogenous steroid concentrations in human blood. As part of a trial project, blood was collected from male and female athletes following competition at IAAF Track and Field Meets held in Europe during the summers of 1993 and 1994. Steroid concentrations were determined for a several unconjugated and conjugated steroids and some results from samples collected in 1993 were presented at the 12th Cologne Workshop [1,2]. Parallel to this study, plasma steroid concentrations were determined using radio immunoassay at the Oslo Doping Laboratory, Aker Hospital, Oslo, Norway.

A goal of this study was to determine if endogenous plasma steroid profiling can be used to detect steroid misuse. It has been proposed that blood analyses could supplement urinary analyses as individual variations in plasma steroid concentrations, for example, testosterone, have been shown to small, even during periods of strenuous training [3]. Also, the plasma level ratio of testosterone to 17α-hydroxyprogesterone (T/17OHP) has been found to be a good indicator for testosterone doping [4], and it has been suggested that this ratio be determined in cases of suspected testosterone doping where the urinary testosterone to epitestosterone (T/E) ratio is greater than 6.

In this report plasma testosterone and 17α-hydroxyprogesterone levels are reported for male and female athletes. Values obtained by GC/HRMS are compared to those determined by radio immunoassay. Additionally, plasma levels have been determined by GC/HRMS for male and female individuals (general population) and from subjects participating in endocrinological studies. Endogenous steroid concentrations have also been determined in two male individuals following oral administration of 5α-dihydrotestosterone (25 mg) and 4-chlorodehydromethyltestosterone (40 mg) and preliminary results are presented.

Experimental

Sample Preparation

A large number of endogenous steroids can be identified in human blood (plasma). Some of these are listed in Table 1. These steroids are normally bound to proteins which act as carriers; however, this binding is weak and the bound steroid can be freed, for example, by treatment in an alkaline solution. Plasma steroids can also be conjugated with glucuronic acid or with sulphate, in which case the steroid is hydrolyzed, either by treatment with an enzyme or by chemical solvolysis, prior to GC/MS analysis.

Table 1

Endogenous plasma steroids, exact masses of their per-TMS derivatives (molecular ion or abundant fragment ion), and temperature programmed Kovat indices. Also indicated (G) are steroids which are found as glucuronic acid conjugates.

STEROID	FORM	MASS MONITORED	KOVAT INDEX
androsterone bis-TMS	G	434.3036	2516
etiocholanolone bis-TMS	G	434.3036	2523
5α-androstan-3α,17β-diol bis-TMS	G	421.2958	2537
5β-androstan-3α,17β-diol bis-TMS	G	421.2958	2542
5α-androstan-3β,17β-diol bis-TMS	G	421.2958	
dehydroepiandrosterone bis-TMS		432.2880	2589
5α-androstane-3,17-dion bis-TMS		432.2880	2606
androst-4-ene-3,17-dion bis-TMS		430.2723	2637
5α-dihydrotestosterone bis-TMS		434.3036	2624
testosterone bis-TMS		432.2880	2660
11β-hydroxyandrosterone tris-TMS	G	522.3880	2672
11β-hydroxyetiocholanolone tris-TMS	G	522.3880	2683
5β-pregnane-3α,17α,20α-triol tris-TMS		435.3114	2807
17a-hydroxypregnenolone tris-TMS		548.3537	2941
17α-hydroxyprogesterone tris-TMS		546.3380	3007

A simple procedure was used to extract steroids from plasma. First 20 µl of a mixture of deuterated steroids (Table 2) was added to 1 ml plasma. Unconjugated steroids were extracted

after adjusting to pH 12 (ca. 50 µl 5 M KOH) with 8 ml *tert.*-butylmethylether. After centrifuging, the organic extract was transferred, dried and the residue derivatized with 50 µl MSTFA/NH_4I/ethanethiol (100:0.2:0.3 v:w:v), which is equivalent to a MSTFA/trimethyliodosilane (1000:2 v:v) solution. To obtain the conjugated and unconjugated steroids (total combined fraction), 1 ml 0.2 M phosphate buffer (pH 7) and 50 µl β-glucuronidase from *Escherichia coli* (Boehringer, Mannheim, Germany) were added to 1 ml plasma and the sample incubated at 50°C for 1 h. The free steroids were extracted (pH 12) with ether and derivatized.

Table 2

Internal standards added to 1 ml plasma and exact masses of their per-TMS derivatives (molecular ion).

Internal Standard	Amount [ng]	Mass Monitored
D_5-androsterone-glucuronide	5.8	439.3321
D_4-etiocholanolone	10.0	438.3287
D_3-epitestosterone	0.3	435.3068
D_3-testosterone	1.8	435.3068
D_4-11β-hydroxyandrosterone	4.8	526.3631
methyltestosterone	10.0	446.3036
oxymesterone	2.0	534.3381

Gas Chromatography / Mass Spectrometry

GC/HRMS analyses were performed with a reverse geometry, double focussing mass spectrometer (Finnigan MAT 95) interfaced with a Hewlett Packard 5890 gas chromatograph (Hewlett Packard Ultra 1 (OV1) fused silica capillary column, 17 m length, 0.2 mm i.d., 0.11 µm film thickness with helium carrier gas at 1 ml/min flow). 2 µl of the derivatized sample was injected in the split mode (split 20 ml/min). The injection port and transfer line were held at 300° C. The initial oven temperature was 185° C and was heated at 5° C/min to 320° C.

The steroid TMS-derivatives were ionized via 65 eV EI ionization (1 mA emission current). The ion source and source probe temperature were held at 220° C and 250° C, respectively. A mass resolution of 3,000 was employed since this allowed for good sensitivity and good background discrimination. Mass analysis was performed in the selected ion monitoring (SIM) mode using an electric field scan, i.e., the magnetic field was held fixed at a given (reference) mass and the acceleration and electric sector voltages were set accordingly to pass the

ion mass of interest. Calibration of the electric fields was continuously performed using perfluorophenanthrene as the reference compound. Exact masses of the ions used to monitor the derivatized steroids are listed in Tables 1 and 2. A scan cycletime 0.5 s was selected to assure that an adequate number of sample points were obtained over a GC peak. The registered ions were put into four different groups, based on the GC elution order. The ion dwell times ranged from 30 to 150 ms, depending on the number of ions in the group.

Results and Discussion

Preparation of Standards

To quantify the endogenous steroids standard mixtures of the steroids at increasing concentrations of 0.1, 0.2, 0.5, 2, 5 and 10 ng/ml were prepared together with 20 µl of the internal standard mixture. Response factors for each steroid were determined using the appropriate internal standard. The response factor of testosterone to D_3-testosterone was constant over the range of concentrations (0.1 ng/ml to 10 ng/ml), except at the lowest levels, where there was an increase in the response of testosterone which is attributed to coelution with the internal standard.

Steroid Profile Following Application of 5α-Dihydrotestosterone

Plasma concentrations of several unconjugated and conjugated endogenous steroids were determined in a male individual following ingestion of 25 mg 5α-dihydrotestosterone. The unconjugated steroids (free fraction) are listed in Table 3. The unconjugated and conjugated steroids (total combined fraction, hydrolysis using β-glucuronidase from *E. coli*) are listed in Table 4.

There are several interesting features illustrated by these data. Foremost is the large change in the endogenous steroid profile following ingestion of 5α-dihydrotestosterone. As in the urinary excretion [5], there are significant increases in the levels of the hydroxylated metabolites 5α-androstan-3α,17β-diol, 5α-androstan-3β,17β-diol and androsterone. The level of the possible androsterone intermediate, 5α-androstane-3,17-dione, did not vary, indicating that the dione might be rapidly metabolized. It is of particular interest that 5α-dihydrotestosterone and its metabolites are predominantly found in plasma as β-glucuronic acid conjugates. Testosterone and 17α-hydroxyprogesterone are present in the free fraction and their levels are similar, but not identical, to those determined in the plasma of the individual shown in Table 5. The concentration of plasma testosterone did not increase following administration of 5α-dihydrotestosterone.

Table 3

Unconjugated (free fraction) plasma steroid concentrations (ng/ml) following sublingual application of 25 mg 5α-dihydrotestosterone (DHT). Testosterone (T), 17α-hydroxy-progresterone (17αP), 5α-androstan-3,17-dion (5αD), androsterone (AND), etio-cholanolone (ETIO), 5α-androstan-3α,17β-diol (3α5αA), 5β-androstan-3α,17β-diol (3α5βA), 5α-androstan-3β,17β-diol (3β5αA).

	T	17αP	DHT	5α-D	AND	ETIO	3α5αA	3α5βA	3β5αA
24h prior	7.2	0.9	0.7	0.95	0.60	0.46	-	-	-
1 hr after	6.3	0.8	12.9	0.44	1.18	0.43	5.2	-	0.8
4 hr after	4.3	0.3	1.3	0.23	1.08	0.91	0.7	-	-

Table 4

Unconjugated and conjugated (total combined fraction) plasma steroid concentrations (ng/ml) following sublingual application of 25 mg 5α-dihydrotestosterone.

	T	17αP	DHT	5α-D	AND	ETIO	3α5αA	3α5βA	3β5αA
24h prior	5.3	0.6	0.7	0.71	41.9	18.2	2.6	1.0	0.6
1 hr after	5.5	0.8	26.0	1.23	243.3	20.7	48.4	1.6	6.9
2 hr after	4.7	0.5	20.9	0.74	288.0	16.3	42.2	1.2	7.4
4 hr after	4.9	0.4	7.0	1.01	159.8	17.5	23.3	1.2	2.9

Steroid Profile Following Application of 4-Chlorodehydromethyltestosterone

Plasma concentrations of several unconjugated and conjugated endogenous steroids (total combined fraction, hydrolysis using β-glucuronidase from *E. coli*) were determined in a male individual (Table 5) following ingestion of 40 mg 4-chlorodehydromethyltestosterone. During the first 6 hours after ingestion there is little variation in the plasma levels of testosterone and 17α-hydroxyprogesterone. Other plasma steroids show some small variation, but this does not appear to be influenced by the administered anabolic steroid. Over the 8 day period there is little variation in the steroid concentrations, supporting other findings [3], but not aiding the suggestion that endogenous profiling can be used to detect anabolic steroid misuse.

Table 5

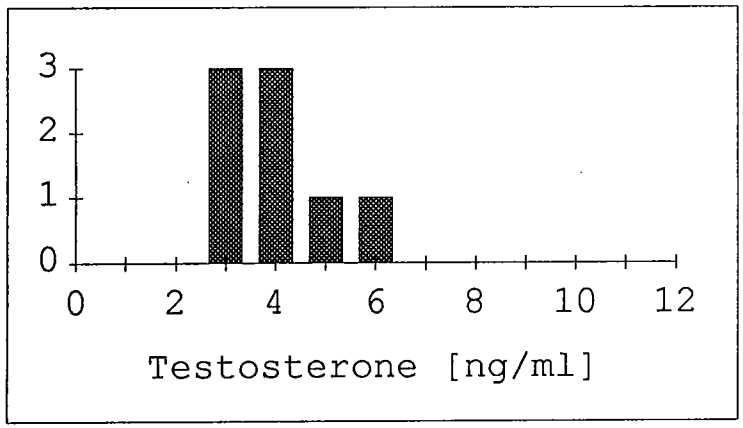
Plasma steroid concentrations (ng/ml) for the total combined fraction following ingestion of 40 mg 4-chlorodehydromethyltestosterone. Testosterone (T), 17α-hydroxyprogesterone (17αP), dehydroepiandrosterone (DHEA), androsterone (AND), etiocholanolone (ETIO), 5α-androstan-3α,17β-diol (3α5αA), 5β-androstan-3α,17β-diol (3α5βA), 11β-hydroxyandrosterone (11βA), 11β-hydroxyetiocholanolone (11βE).

	T	17αP	DHEA	AND	ETIO	3α5αA	3α5βA	11βA	11βE
0 hr	3.0	0.33	3.1	11.3	14.5	2.9	6.9	12.2	2.0
1 hr	1.9	0.42	5.0	6.0	7.7	3.0	5.5	8.9	1.5
2 hr	2.3	0.48	2.7	7.5	8.2	2.4	4.2	10.5	1.4
3 hr	2.2	0.49	2.4	6.8	6.6	2.0	2.9	7.9	1.2
4 hr	2.5	0.36	2.6	8.2	10.2	2.1	3.7	7.5	1.4
5 hr	2.2	0.26	4.5	8.8	9.0	2.2	4.1	8.1	1.4
6 hr	1.7	0.23	2.9	8.8	8.1	2.8	4.2	10.1	1.9
24 hr	2.5	0.28	5.2	10.3	13.2	2.8	6.8	10.5	2.5
48 hr	2.4	0.27	3.1	9.2	9.9	2.5	6.0	11.4	1.9
72 hr	2.1	0.25	2.1	8.5	8.3	2.2	5.1	10.5	1.7
6 d	2.3	0.40	2.2	12.8	11.8	2.6	8.2	14.7	2.3
8 d	3.0	0.27	2.0	10.6	11.9	2.4	8.0	11.8	3.3

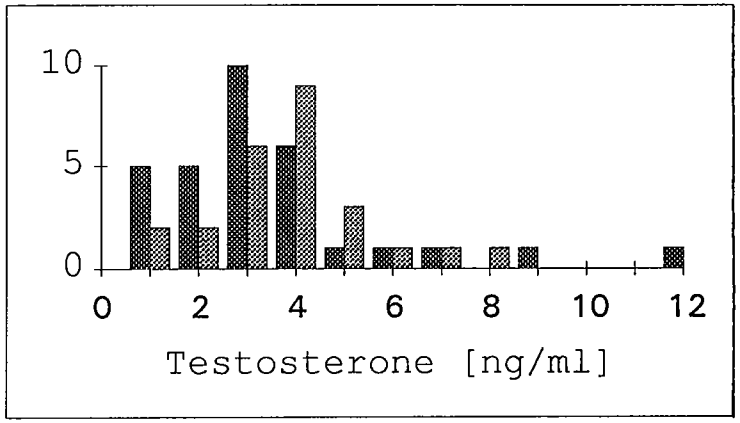
Plasma Testosterone and 17α-hydroxyprogesterone Levels in Males

Testosterone and 17α-hydroxyprogesterone plasma levels were determined for males from three different reference groups. The data, summarized as frequency distributions, are shown in Figures 1 and 2. In the samples obtained from the general population (n=8) testosterone plasma levels ranged from 3 ng/ml to 6 ng/ml, while for IAAF athletes sampled after competition the testosterone levels ranged from 1 ng/ml to 12 ng/ml. The average testosterone level was similar for the two groups. Plasma testosterone levels for subjects participating in an endocrinology study (individuals with high urinary T/E ratios) were normal. 17α-hydroxyprogesterone plasma levels are much lower than testosterone and there are nearly no differences between males in the general population and IAAF athletes. Interestingly, abnormally high 17α-hydroxyprogesterone plasma levels were found for two individuals participating in the endocrinology study.

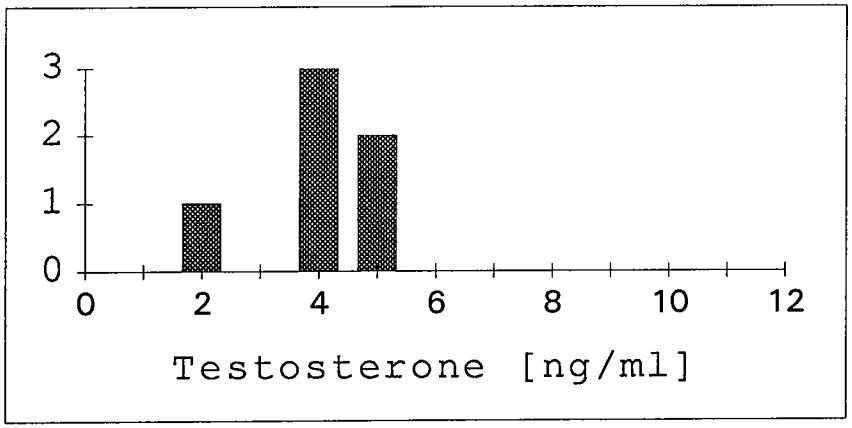
The samples obtained from the IAAF athletes were also screened for the presence of anabolic steroids, parent compounds and major urinary metabolites, both in the unconjugated and conjugated forms. In no sample were anabolic steroids detected, which is consistent with the results from the analysis of the urine samples.



Males General Population

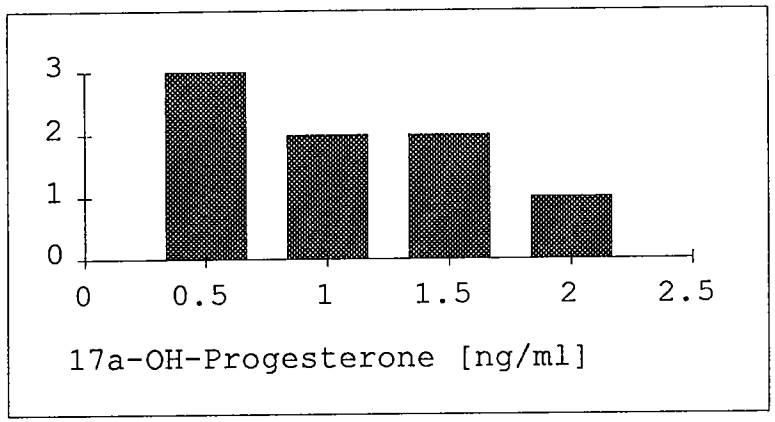


Males IAAF 1993 1994



Endocrinology Study

Figure 1. Frequency distributions of testosterone plasma (free fraction) concentrations in males.



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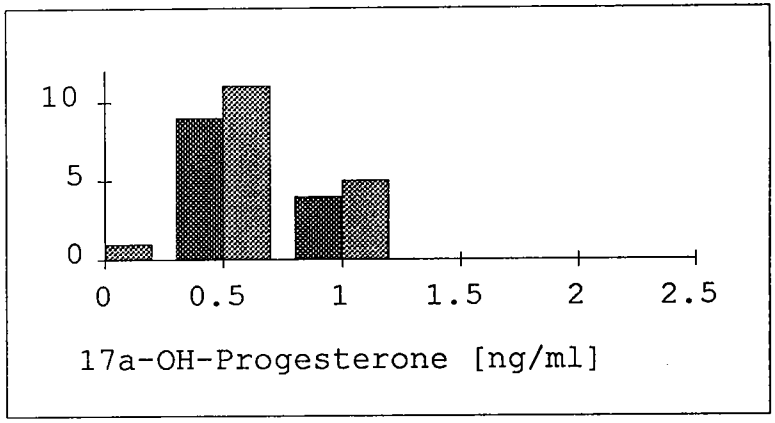
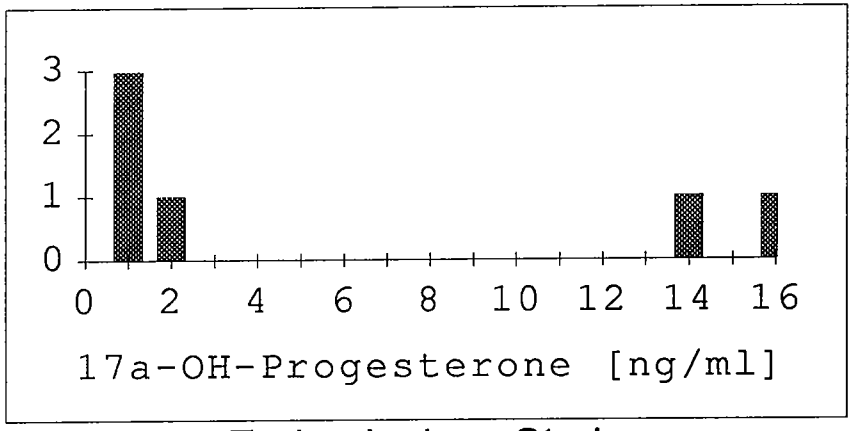
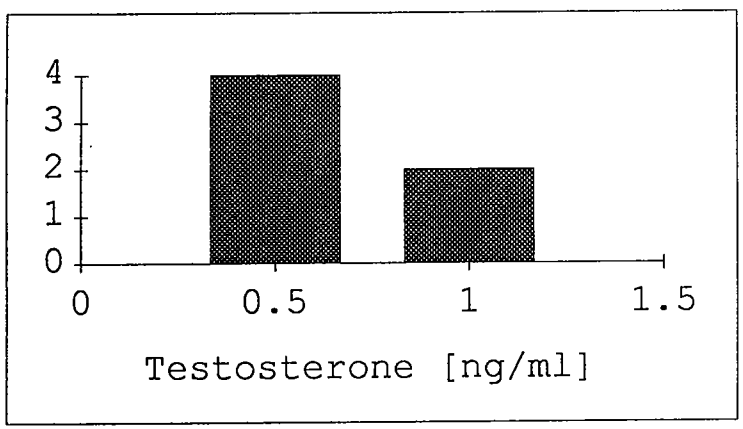


Males IAAF 1993 1994

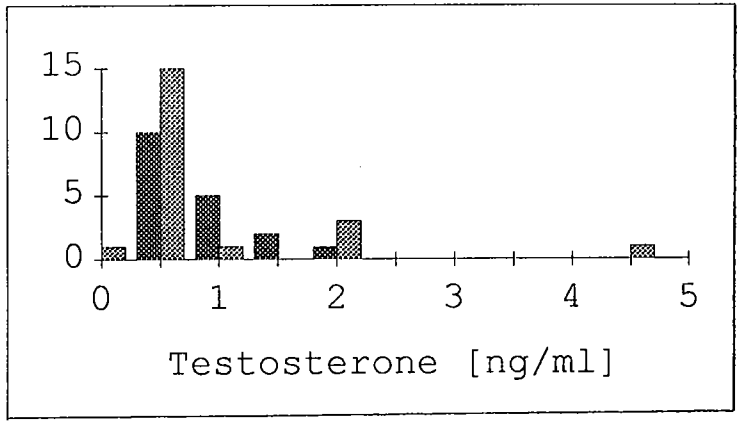


Endocrinology Study

Figure 2. Frequency distributions of 17α-hydroxyprogesterone plasma (free fraction) concentrations in males.



Females General Population



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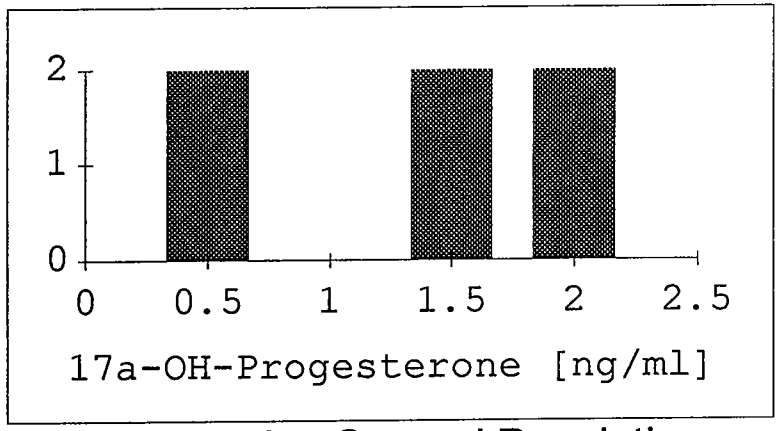

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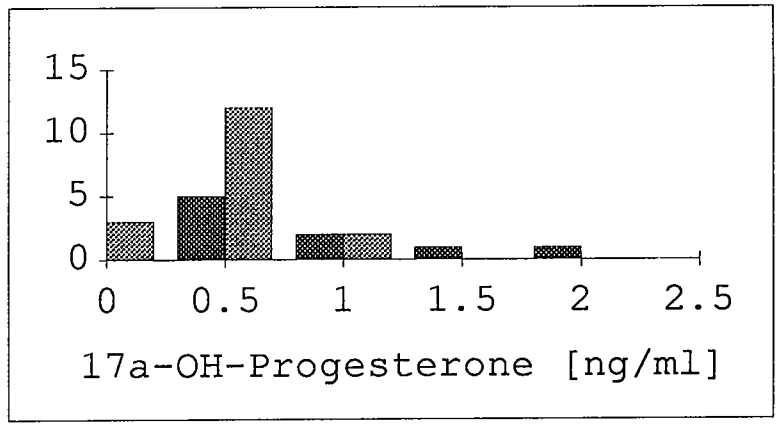
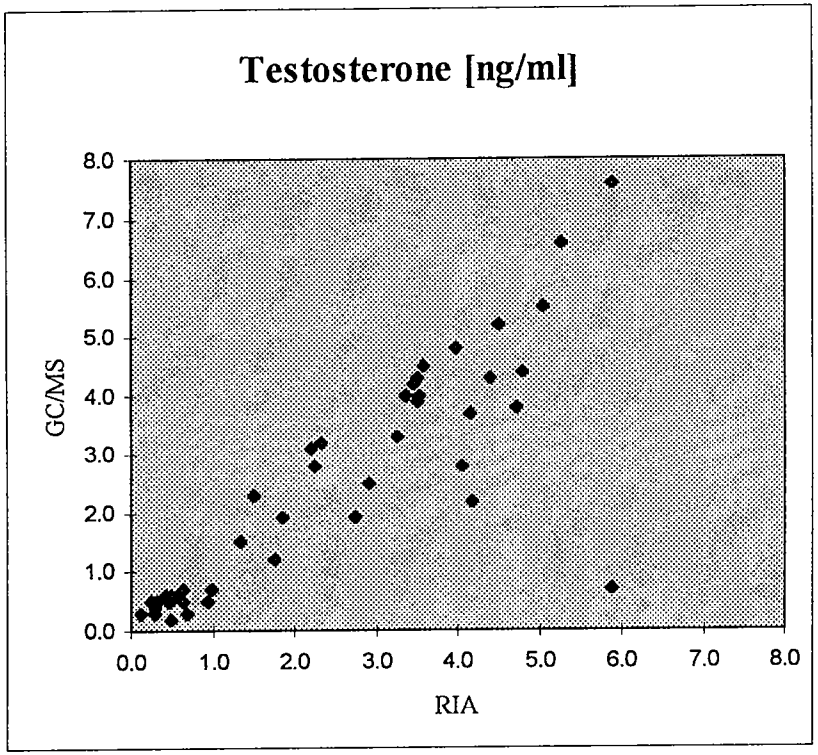


Females IAAF 1993 1994

Figure 3. Frequency distributions of testosterone and 17α-hydroxyprogesterone plasma (free fraction) concentrations in females.



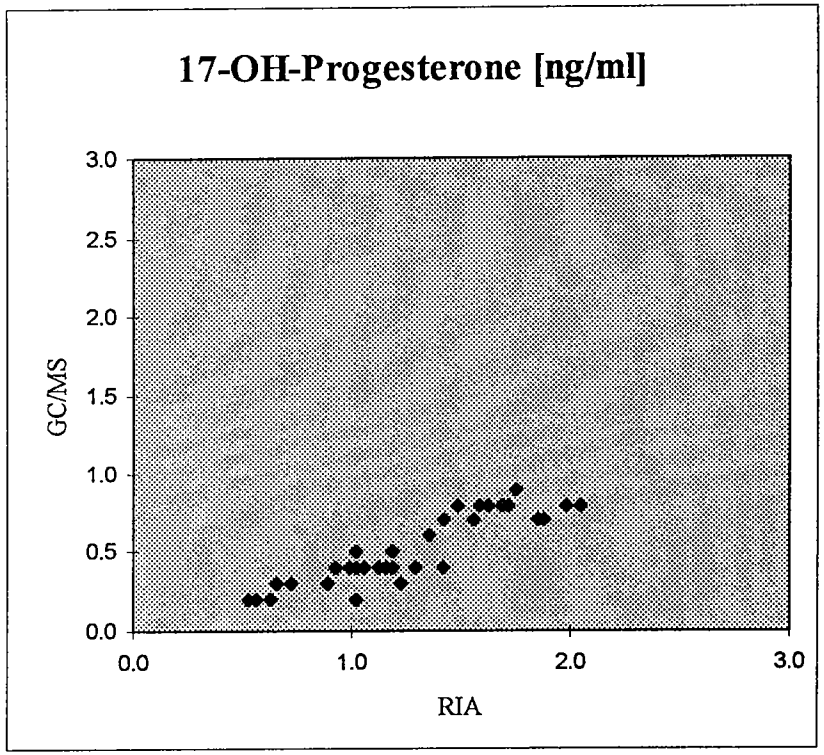


Figure 4. Correlation between GC/HRMS and RIA measurements of steroids in blood taken during IAAF-Meetings 1994

Plasma Testosterone and 17α-hydroxyprogesterone Levels in Females

Testosterone and 17α-hydroxyprogesterone plasma levels in females are shown in Figure 3. In the general population (n=6) testosterone levels ranged from 0.5 ng/ml to 1 ng/ml, while for IAAF athletes sampled after competition the levels ranged from 0.5 ng/ml to 4.5 ng/ml. The testosterone levels were much lower in the female population than in the male. 17α-hydroxy-progesterone plasma levels are nearly the same in the male and female populations.

Correlation between Radio Immunoassay and GC/HRMS Measurements for Samples Measured in 1994

As illustrated in Figure 4 there is good agreement between the radio immunoassay and GC/HRMS methods. The slope and correlation (r=0.85) for plasma testosterone levels are much improved as compared to the values determined in 1993 (r=0.70, y=0.78 x + 2.3) [1]. Note that the GC/HRMS method was not altered between 1993 and 1994 so the improved correlation must be attributed to changes in the radio immunoassay procedure. The agreement for 17α-hydroxy-progesterone was not as good, although there was a high correlation (r=0.93). The levels determined by GC/HRMS were only half as large as those determined by radio immunoassay, suggesting that interference due to cross reactivity may lead to erroneously high values in the immunoassay analysis.

Conclusion

These results supplement the preliminary study presented at the 12th Cologne Workshop [1,2] and show that steroid profiling in human blood by gas chromatography combined with high resolution mass spectrometry (GC/HRMS) is a viable analytical technique. One useful aspect of the GC/HRMS method is that a large number of steroids, or related substances, can be determined in a single analysis. With the use of appropriate internal standards, in this case deuterated analogues of the steroids, it is possible to quantify steroids in plasma at levels as low as 100 pg/ml. Comparison of GC/HRMS data with that obtained by radio immunoassay shows that the two methods yield similar results for testosterone but not for 17a-hydroxyprogesterone

As has been suggested, blood analyses can be used as a supplement to urinary analyses. Upon ingestion of a single dose of 5a-dihydrotestosterone, there were large changes in the plasma concentration of several endogenous steroids, all metabolites of the applied substance. Ingestion of the anabolic steroid, 4-chlorodehydromethyltestosterone did not result in any alteration of the endogenous profile. Extensions of these studies are being made towards the detection of steroids and their metabolites in equine plasma as here plasma, rather than urine, is available for analysis.

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