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Steroid Profiling in Human and Horse Blood: Some Results

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Introduction

High resolution (high performance) mass spectrometry coupled with capillary column gas chromatography (GC/HRMS) is used to determine endogenous steroid concentrations in horse and human blood. Due to their low concentrations, blood or serum steroids levels have largely been determined by radio immunoassay (RIA), and a large number of highly sensitive immunoassays are commercially available. With immunoassays, however, each steroid of interest must be determined separately and high specificity is required to limit any occurrence of cross reactivity, thus a large number of assays must be performed in order to derive a steroid profile. With GC/HRMS, on the other hand, a single analysis serves to identify and quantify several steroids and comprehensive endogenous steroid profiles can readily be obtained. Moreover, the GC/MS method is not subject to cross reactivity, which in the RIA assay may lead to erroneous results.

One of the goals of this study is to determine whether steroid profiling in blood can be used to detect steroid misuse. As noted by Garle and Palonek [1,2], the serum level ratio of testosterone to 17α-hydroxyprogesterone (T/17OHP) is a good marker for testosterone doping. These authors also suggested that this ratio be measured in all suspected cases of testosterone doping where the urinary testosterone to epitestosterone (T/E) ratio is found to be greater than 6. Individual variations in serum testosterone levels of top male athletes have been shown to be small [3], even during periods of heavy training. Thus it is reasonable that blood analyses, as a supplement to urine analyses, may provide an opportunity to follow several endocrine parameters in individual athletes and thereby lessen the chance that doping misuse occurs.

Here we report our first results for blood steroid profiles of top male and female athletes taken after competition at major IAAF Track and Field Events in Europe during the summer of 1993. After describing the analytical procedure, and the use of internal standards for quantification, measurements of blood steroids made by GC/HRMS will be compared to those obtained by RIA [4]. Some preliminary results for the determination of testosterone and

nortestosterone in horses follows. Steroid profiles are given for male and female volunteers from this laboratory and finally profiles are presented for male and female athletes competing at IAAF meets. The question of testosterone and 17α-hydroxyprogesterone and its significance is the topic of a second paper in this proceeding..

Experimental

Sample Preparation

A large number of endogenous steroids can be identified in human and horse blood (plasma or serum). Many of these are listed in Table 1. Plasma steroids are normally bound to proteins which act as carriers; however, this binding is weak and the bound steroid can easily be set free, e.g., by treatment in an alkaline solution. The plasma steroids can also be conjugated with glucuronic acid or with sulphate, in which case the steroid must be hydrolyzed, either by treatment with an enzyme or by chemical solvolysis, prior to analysis by GC/MS. Here the focus is on the detection of unconjugated plasma steroids, although some reference will be made to experiments performed in order to detect conjugated plasma steroids.

ENDOGENOUS STEROIDS	
testosterone	androsterone
17α-hydroxyprogesterone	etiocholanolone
dehydrotestosterone	11β-hydroxyandrosterone
androst-4-en-3,17-dion	11β-hydroxyetiocholanolone
5α-androstane-3,17-dion	cortisol
dehydroepiandrosterone	cortisone
pregnanetriol	norandrosterone

Table 1. Sampling of endogenous steroids which can be identified and quantified in human and equine blood.

A simple and rapid procedure for extracting steroids in serum and preparing them for GC/HRMS analysis is summarized in Figure 1. For humans, 1 ml of serum or plasma is used for analysis, for horses 2 ml. A mixture of deuterated steroids (Table 2) is added to the sample and used for quantification [5]. The internal standard mixture also contains methyltestosterone and oxymesterone, the later of which is used for the quantification of 17a-hydroxyprogesterone. An additional sample clean up step, to remove nonpolar biological material (Figure 1), can also be introduced, although it has no influence on the determination of steroid concentrations and is only performed when the dried sample extract is visibly dirty, e.g., cloudy or containing an oily residue.

Internal Standard Mixture Added to 1 ml of Serum	Amount
D_3-testosterone	2 ng
oxymesterone	2 ng
D_4-11β-hydroxyandrosterone	4.8 ng
D_4-etiocholanolone,	10 ng
methyltestosterone	10 ng

Table 2. Internal standards added to 1 ml of serum.

SAMPLE PREPARATION	
Unconjugated Steroids	1 ml Plasma or Serum (2 ml horse) + 20 µl ISTD mix (40 µl horse) + ca. 750 µl 0.5 M KOH (pH 12) + 8 ml t-butylmethylether Shake, centrifuge, remove organic phase and dry Derivatize with 50 µl MSTFA/NH_4I/ethanethiol (100:0.2:0.3 v:w:v) and heat 1 hour at 60° C
Conjugated Steroids	1 ml Plasma or Serum (2 ml Equine) + 1 ml 0.2 M Phosphate Buffer (pH 7) + 50 µl (β-glucuronidase or *Helixa Pomatsia*) continue as described above for the unconjugated steroids starting with the addition of 20 µl ISTD
Additional Clean Up (to remove nonpolar biological material)	to Dry Organic Phase + 2 ml MeOH/H_2O (100:5) + 5 ml n-Pentane Shake, Centrifuge, Discard Organic Phase Dry Aqueous Phase derivatize as described above

Figure 1. Rapid procedure for extraction and derivitization of endogenous steroids in human and horse serum for GC/HRMS analysis.

Gas Chromatography / Mass Spectrometry

GC/HRMS analyses were performed using a Finnigan MAT 95 double focussing mass spectrometer interfaced with a Hewlett Packard 5890 gas chromatograph. The derivatized samples were analyzed using a Hewlett Packard Ultra 1 (OV1) fused silica capillary column

(17 m length, 0.2 mm i.d., 0.11 μm film thickness). Helium was used as the carrier gas and the column flow was 1 ml/min at 185° C. 2 μl of sample was injected in the split mode at a split flow of 20 ml/min. The injection port and transfer line temperature was 300° C. The oven temperature was 185° C upon injection, then heated at a rate of 5° C/min to 320° C and held at the final temperature for 3 min.

The steroid TMS derivatives were ionized via electron impact ionization at an electron energy of 65 eV and an emission current of 1 mA. The ion source and source probe temperature were held at 250° C. A mass resolution of 3,000 was employed since this allowed good sensitivity and good background discrimination. The operation of the MAT 95 mass spectrometer has been described in the Proceedings of the 11th Cologne Workshop on Dope Analysis [6]. Mass analysis was performed in the selected ion monitoring (SIM) mode using an electric field scan, i.e., the magnetic field is held fixed at a given (reference) mass and the acceleration and electric sector voltages are set accordingly to pass the ion mass of interest. Calibration of the electric fields was continuously performed using perfluoronapthlene (FC-5311) as the reference compound. In Table 3 the exact masses of the ions used to monitor the derivatized steroids are listed. For most steroids the molecular ion is used since it is most abundant.

STEROID	MASS MONITORED	KOVAT INDEX
androsterone bis-TMS	434.3036	2516
D_4-etiocholanolone bis-TMS	438.3287	2519
etiocholanolone bis-TMS	434.3036	2523
dehydroepiandrosterone bis-TMS	432.2880	2589
5α-androstane-3,17-dion bis-TMS	432.2880	2606
nortestosterone bis-TMS	418.2723	2612
5α-dehydrotestosterone bis-TMS	434.3036	2624
D_3-testosterone bis-TMS	435.3068	2658
testosterone bis-TMS	432.2880	2660
methyltestosterone bis-TMS	446.3036	2753
5β-Pregnane-3α,17α,20α-triol tris-TMS	435.3114	2807
oxymesterone tris-TMS	534.3381	2950
17α-hydroxyprogesterone tris-TMS	546.3380	3007

Table 3. Endogenous steroids and exact ion masses of their per-TMS derivatives (molecular ion) recorded via high-resolution selective ion monitoring (GC/HRSIM). Also included are temperature programmed Kovat indices.

A scan cycletime of 0.5 s was chosen for the SIM analysis 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 order of elution from the GC column. The ion dwell times ranged from 30 ms to 150 ms, depending on the number of ions in the group.

Results and Discussion

Preparation of Standards

To identify and quantify the endogenous steroids listed in Table 3, 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 the internal standard mixture listed in Table 2. Response factors for each steroid were determined using the appropriate internal standard. The response factor of testosterone bis-TMS to the internal standard D_3-testosterone bis-TMS was constant over the range of concentrations (0.1 ng/ml to 10 ng/ml), except at the lowest level, where there was an increase in the response of testosterone which is attributed to coelution with the internal standard. In Figure 2, an ion chromatogram is depicted showing testosterone bis-TMS and D_3-testosterone bis-TMS at concentrations of 0.5 ng/ml and 2 ng/ml, respectively.

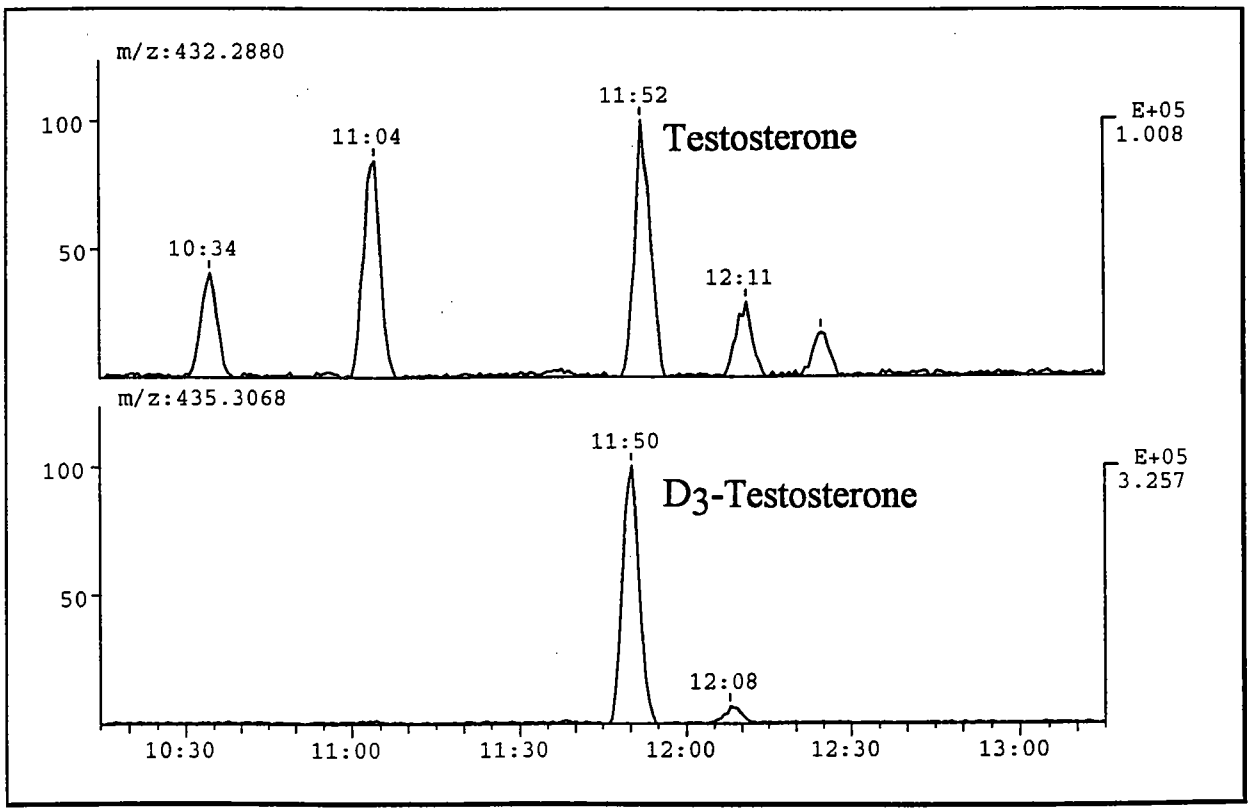

Figure 2. High mass resolution ion chromatograms of a standard solution containing testosterone bis-TMS (upper trace, m/z 432.2880 amu) and D_3-testosterone bis-TMS (lower trace, m/z 435.3068 amu) at concentrations of 0.5 ng/ml and 2 ng/ml, respectively.

Comparison of GC/HRMS to Radio Immunoassay

To examine the effectiveness of the GC/HRMS steroid analysis, a series of plasma standards (freeze dried extracts), obtained from the Aker Hospital Hormone Laboratory, Oslo, Norway [4], were prepared and analyzed. The plasma standards are used routinely in the Oslo laboratory as calibrants for their radio immunoassay of 17α-hydroxyprogesterone and testosterone in plasma. In Table 4 the results obtained by GC/MS are shown together with the values provided by the Oslo laboratory. The GC/MS results are average values based on multiple sample preparations (n=3) and multiple sample analyses (n=5). There is good agreement between the methods, except in one case, where much higher levels of 17α-hydroxyprogesterone (9.24 ng/ml) were determined by radio immunoassay than by GC/MS (5.30 ng/ml). It is worth noting that in the GC/HRMS experiment several endogenous steroids, in addition to testosterone and 17α-hydroxyprogesterone, were determined in the analysis. In the radio immunoassay, testosterone and 17α-hydroxyprogesterone levels were determined by separate assays.

HUMAN PLASMA STANDARDS			
Testosterone [ng/ml]		17α-hydroxyprogesterone [ng/ml]	
GC/MS	RIA	GC/MS	RIA
4.45	4.98	1.93	2.06
2.10	2.57	5.30	9.24
0.38	0.38	0.76	1.19

Table 4. Plasma concentration of testosterone and 17α-hydroxyprogesterone determined by GC/HRMS and radio immunoassay (RIA). The values reported are averages based on several measurements.

Endogenous Steroids in Horse Blood

GC/HRMS is also used for the determination of endogenous steroids in horse blood. Unconjugated testosterone and nortestosterone blood concentrations are listed in Table 5 for 13 stallions of unknown age. Testosterone and nortestosterone are both present in the stallion, with testosterone levels being somewhat higher, approximately 0.5 ng/ml. In the mare, nortestosterone is not detected and the testosterone level is much lower, ca. 0.1 ng/ml (data not shown). Examination of the enzymatically hydrolyzable conjugate glucuronic acid and sulphate fractions revealed that nearly all nortestosterone and testosterone in equine blood is unconjugated. Other steroids detected in equine blood include 5α-androstane-3,17-dion, androsterone, etiocholanolone, cortison and cortisol. Not detected in either the conjugated or

unconjugated fractions are dehydrotestosterone, estranediol, estrenediol, norandrosterone and epitestosterone.

TESTOSTERONE AND NORTESTOSTERONE CONCENTRATION IN STALLION BLOOD		
Sample	**Testosterone [ng/ml]**	**Nortestosterone [ng/ml]**
1	0.60	0.10
2	0.42	0.15
3	0.47	0.13
4	0.87	0.41
5	0.24	0.23
6	0.65	0.33
7	0.25	0.52
8	0.48	0.20
9	0.62	0.34
10	0.52	0.46
11	0.37	0.47
12	0.52	0.29
13	0.53	0.38
	avg. 0.503	avg. 0.308

Table 5. Blood concentration of unconjugated testosterone and nortestosterone in stallions determined by GC/HRMS.

Blood Steroid Profiles in Humans

Concentrations of several unconjugated endogenous steroids have been determined in human serum (male and female volunteers from this laboratory) and some of these are listed in Table 6. In males, the serum testosterone concentration lies around 4 ng/ml, for females the testosterone levels are much lower. It is of interest that epitestosterone was not detected in either the male or female serum. Androsterone and etiocholanolone and their 11β-hydroxy forms were detected in the unconjugated fraction, but in low amounts compared to the conjugated fraction.

ENDOGENOUS STEROID CONCENTRATIONS IN HUMAN SERUM					
GENDER	**T**	**17αP**	**5αAD**	**DHEA**	**PT**
F	0.4	1.1	0.8	7.6	59
F	0.3	0.4	1.0	2.0	37
F	0.5	0.6	1.1	6.9	50
M	3.4	0.6	9.8	2.4	30
M	4.3	0.6	3.3	3.8	72
M	3.3	0.8	3.2	7.8	25

Table 6. Serum concentrations of some unconjugated endogenous steroids in male and females [ng/ml] determined by GC/HRMS. Testosterone (T), 17α-hydroxyprogesterone (17αP), 5α-androstane-3α,17β-diol (5αAD), dehydroepiandrosterone (DHEA), pregnanetriol (PT).

Blood steroid analyses have also been performed on samples taken from top male and female athletes during four IAAF meets in Europe in the summer of 1993. The blood samples were analyzed using radio immunoassay in the Doping Laboratory at the Aker Hospital, Oslo, Norway and by GC/HRMS in this laboratory. The GC/HRMS results are summarized in Tables 7 and 8. In addition, these blood samples 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.

The unconjugated testosterone serum levels in the female athletes was near the levels determined for the female volunteers from this laboratory, in most cases. It is not known if the samples with high testosterone levels (1.57 ng/ml and 2.10 ng/ml) were confirmed by immunoassay or by urinary analysis. At the time of the Oslo Games, 17α-hydroxyprogesterone was not determined by GC/HRMS and only immunoassay results are available.

ENDOGENOUS STEROID CONCENTRATIONS IN FEMALE SERUM SAMPLES TAKEN AT IAAF MEETINGS IN 1993							
EVENT	**Nr.**	**T**	**17αP**	**AND**	**ETIO**	**DHEA**	**PT**
Brussels	A75	0.20	0.19	12.9	11.1	10.5	3.5
Zurich	23	0.28	0.33	21.6	43.4	11.9	26.1
Brussels	A81	0.36	0.09	12.3	27.0	20.2	1.8
Brussels	A80	0.37	0.23	26.3	23.4	6.1	14.3
Brussels	A87	0.39	0.41	41.2	35.0	9.4	14.9
Zurich	14	0.40	1.27	15.6	15.7	3.1	31.3
Berlin	33	0.41	0.38	23.0	23.1	2.0	36.8
Berlin	31	0.43	1.11	18.5	28.4	7.7	59.0
Berlin	38	0.56	0.63	28.5	25.0	7.0	50.0
Oslo	48	0.60		17.1	25.4	5.3	
Zurich	15	0.63	0.95	17.9	24.9	14.5	14.6
Berlin	32	0.72	1.92	16.4	26.2	8.4	78.4
Oslo	42	0.85		12.7	13.9	4.8	
Oslo	30	0.90		18.8	18.4	5.6	
Oslo	36	1.11		12.4	10.9	3.7	
Berlin	28	1.23	0.67	36.8	56.3	7.0	48.0
Oslo	31	1.34		11.5	8.7	3.1	
Brussels	A84	1.57		7.5	25.7	6.9	1.9
Oslo	28	2.10		40.0	33.4	5.8	

Table 7. Serum concentrations of some unconjugated endogenous steroids [ng/ml] in female athletes determined using GC/HRMS. Testosterone (T), 17α-hydroxyprogesterone (17αP), androsterone (AND), etiocholanolone (ETIO), dehydroepiandrosterone (DHEA), pregnanetriol (PT). Note that at the Oslo event, 17α-hydroxyprogesterone was not determined by GC/HRMS.

Other endogenous steroids detected in the female blood include androsterone, etiocholanolone, dehydroepiandrosterone and pregnanetriol. At this time, no profile analyses have been performed using these data. The ratio of testosterone to 17α-hydroxyprogesterone and its significance is the subject of a second paper in this proceeding.

The unconjugated testosterone serum levels in the male athletes varied over a very wide range, 0.5 ng/ml to 12.5 ng/ml, with the average value being around 4 ng/ml. As with the female serum samples, androsterone, etiocholanolone, dehydroepiandrosterone and pregnanetriol were determined only in their unconjugated form. Also detected, but not quantified, in both the male and female serum, were unconjugated androst-4-en-3,17-dion, 5α-androstane-3,17-dion, 5α-dehydrotestosterone, cortisone and cortisol

ENDOGENOUS STEROID CONCENTRATIONS IN MALE SERUM SAMPLES TAKEN AT IAAF MEETINGS IN 1993							
EVENT	**Nr.**	**T**	**17αP**	**AND**	**ETIO**	**DHEA**	**PT**
Zurich	13	0.53	0.95	24.3	17.6	1.97	13.6
Brussels	A73	0.55	0.13	10.4	9.3	4.6	11.0
Zurich	22	1.12	0.20	22.2	16.9	6.6	44.6
Zurich	20	1.36	0.29	21.7	14.5	8.8	26.4
Brussels	A85	1.48	0.03	18.8	12.4	4.0	1.4
Zurich	16	1.50	0.20	26.4	11.9	4.8	14.5
Brussels	A78	1.59	0.04	18.1	11.4	5.5	1.8
Zurich	27	1.69	0.34	17.5	14.5	10.8	10.6
Brussels	A92	1.74	0.28	13.9	7.3	4.6	15.0
Oslo	49	2.31		12.4	8.8	1.5	
Zurich	17	2.52		15.1	13.3	2.8	28.6
Zurich	26	2.72		28.5	17.8	2.7	47.6
Berlin	39	2.77	0.82	46.5	18.6	10.3	35.6
Oslo	40	2.91		9.1	7.4	2.0	
Zurich	21	2.99		8.9	12.5	5.4	34.8
Berlin	35	3.00	0.51	14.2	7.4	2.0	21.2
Berlin	34	3.32	0.76	42.5	18.9	7.7	25.5
Brussels	A91	3.34	0.22	14.6	12.8	10.0	6.1
Oslo	34	3.35		23.8	10.0	2.7	
Berlin	29	3.42	0.60	20.0	10.9	2.4	30.0
Berlin	37	3.66	0.80	34.5	37.7	3.8	95.0
Berlin	36	3.98	0.64	28.3	13.5	4.1	48.8
Zurich	24	4.02		42.7	52.4	2.7	72.5
Berlin	30	4.34	0.47	53.8	50.5	3.8	72.0
Oslo	46	4.44		16.3	11.7	2.2	
Zurich	19	4.49	0.22	24.2	14.1	4.2	33.4
Oslo	44	4.59		18.2	12.0	1.7	
Brussels	A72	6.42	0.16	20.7	15.1	20.6	10.1
Zurich	18	6.79	0.38	18.0	22.9	7.5	36.0
Oslo	32	8.74		29.4	16.5	3.1	
Zurich	25	12.49	0.66	39.4	20.7	5.6	23.8

Table 8. Serum concentrations of some unconjugated endogenous steroids [ng/ml] in male athletes determined using GC/HRMS. Testosterone (T), 17α-hydroxyprogesterone (17αP), androsterone (AND), etiocholanolone (ETIO), dehydroepiandrosterone (DHEA), pregnanetriol (PT). Note that at the Oslo event, 17α-hydroxyprogesterone was not determined by GC/HRMS.

An correlation was made between the blood testosterone levels determined by GC/HRMS and by radio immunoassay performed in the Doping Laboratory at the Aker Hospital, Oslo, Norway. This is shown in Figure 3.

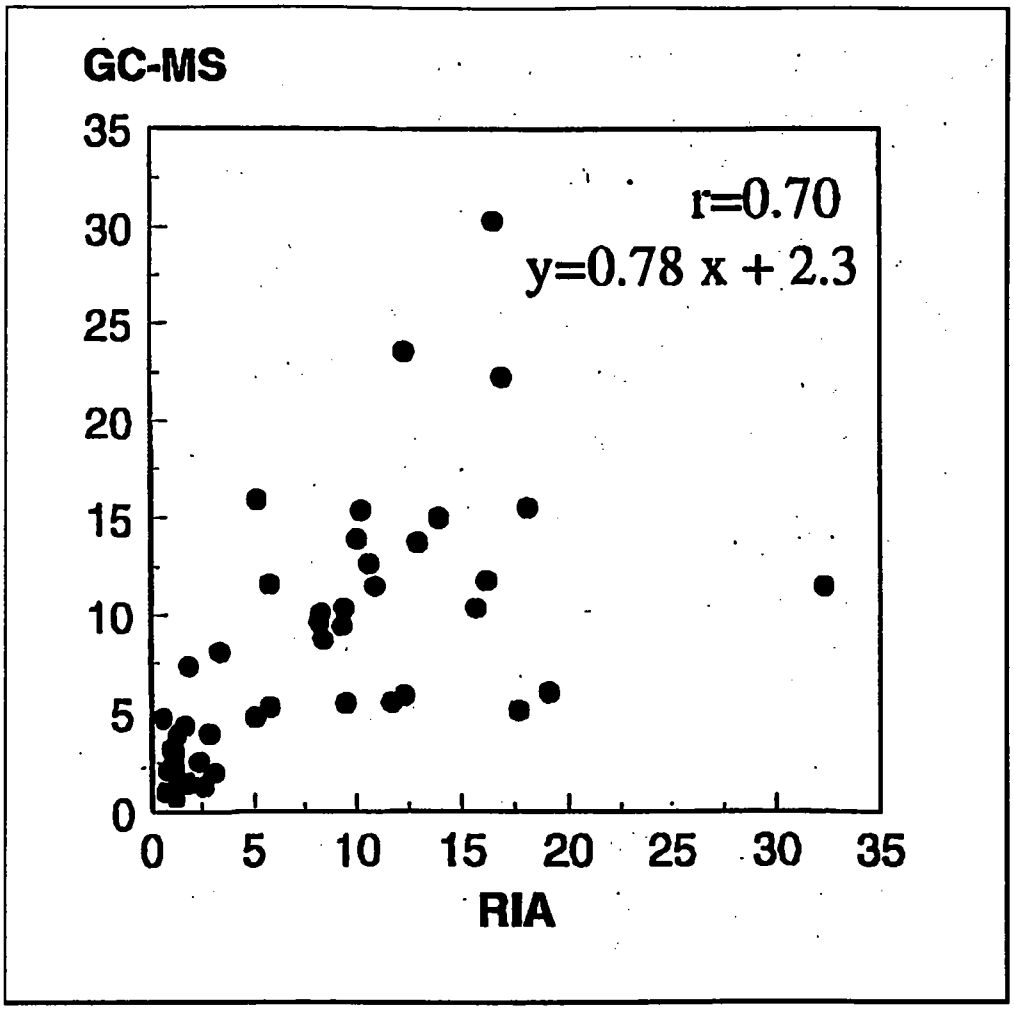

Figure 3. Comparison of unconjugated testosterone serum determinations made by radio immunoassay (RIA) and GC/HRMS for samples taken at IAAF meets during the summer of 1993. Figure kindly provided by Dr. Birkeland from the Doping Laboratory at the Aker Hospital, Oslo, Norway.

As seen in Figure 3 there is good overall agreement between the two methods, but there are a number of cases where large differences are found. In the GC/HRMS analysis an internal standard was employed for the quantification of testosterone. The steroids of interest were well separated chromatographically under the conditions employed and full scan mass spectra data obtained at the elution time of testosterone bis-TSM did not indicate the presence of any coeluting species. The agreement between the two methods for the freeze dried plasma standards was quite good, as shown in Table 4. Thus it is not clear for what reasons the testosterone values determined by the two methods largely differ in some cases.

Conclusion

These results have shown that steroid profiling in human and horse blood by gas chromatography combined with high resolution mass spectrometry (GC/HRMS) is a viable analytical method. One highly useful aspect of GC/HRMS analysis 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 the steroids in plasma. Comparison of GC/HRMS data with that obtained by radio immunoassay shows that the two methods yield similar results, although there are some differences which remain to be uncovered.

A large number of endogenous steroids can be determined in human and horse blood. In this study only a limited number of steroids are presented. Further work, which is in progress, includes a wider spectrum of steroids, which in turn provides a more complete steroid profile. In addition, extensions of these studies are being made towards the detection of anabolic steroids and their metabolites in equine and human plasma and some of these results have recently been published [7].

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