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RECENT ADVANCES IN DOPING ANALYSIS (2)

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Standardisation of the T/E-determination by deuterated internal standards

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1. Introduction

It is a fact that the measurement of the testosterone to epitestosterone ratio (T/E) in an urine sample may be influenced by several instrumental factors e.g. :

1. the injection port, which may cause chemical or physical discrimination
2. the ion source
3. the electron multiplier-voltage
4. the integration software and parameter settings

To compensate for the above mentioned instrumental factors a mixture of deuterated testosterone (90 ng/ml urine) and deuterated epitestosterone (15 ng/ml urine) is added to the urine extract before enzymatic hydrolysis. It is obvious that the addition of deuterated testosterone and deuterated epitestosterone will compensate for instrumental variations in the screening procedure and in the confirmation. First results are given in this communication.

2. Screening Procedure for Anabolic Steroids - total fraction - and determination of the steroid profile

2.1 Sample Preparation (1)

2 ml of urine are added to a Amberlite XAD-2 column. The column (pasteur pipette, closed with glass pearl, bed height 2 cm) is washed with 2 ml of bidistilled water and the absorbed fraction is eluted with 2 ml of methanol.

To the methanolic eluate, 20 µl of the internal standard solution (see 2.4.) is added.

The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0.2 M sodium phosphate buffer pH 7.

To the buffer solution, 50 µl of beta-glucuronidase from E.coli is added and hydrolysis is performed for 1 h at 50° C. The buffered solution is alkalized with 250 µl of 7% potassium carbonate solution to pH 9-10 and the steroids are extracted with 5 ml of diethyl ether or

tert.-butylmethyl ether on a mechanical shaker for 5 minutes. After centrifugation the etheral layer is transferred and evaporated to dryness under vacuo.

2.2 Derivatisation

The dry residue is derivatized with 100 µl of MSTFA/NH_4J/ethanethiol (1000:2:3 (v:w:w)) and heated for 15 min. at 60°C.

3 µl of the solution are injected.

2.3 GC/MS parameters

GC/MS:	HP 5890 II/ HP 5971A
carrier gas:	1 ml helium at 180°C; split ca 1:10;
column:	17 m OV-1, 0.2 mm i.d., 0.11 µm film thickness
temperature program:	181°C, 3°C/min - 230°C, 30°C/min - 310°C

dwell times :

432 m/z (for T and E)	40 msec
435 m/z (for D3T and D3E)	40 msec
for all other endogenous steroids	20 msec

cycles/sec: 1.8 hz

The instrument is tuned manually for optimal response on m/z 502 of PFTBA (peak width 0.5-0.55 amu)

2.4 Composition of internal standard solution (2)

17α-methyltestosterone	50 ppm (500 ng/ml urine)
[2,2,4,4-$^2H_4$]-etiocholanolone	50 ppm (500 ng/ml urine)
[16,16,17-$^2H_3$]-testosterone	9 ppm (90 ng/ml urine)
[16,16,17-$^2H_3$]-epitestosterone	1.5 ppm (15 ng/ml urine)
[2,2,4,4-$^2H_3$]-11ß-hydroxyandrosterone	24 ppm (240 ng/ml urine)

2.5 Data evaluation

Computer: HP Apollo 425 t

Software: HP59944C ; Rev.C.00.00

Integrator: RTE

Smooth factor: 9

3. Confirmation of suspicious Testosterone/Epitestosterone samples

Note: To exclude or minimize the effect of interfering peaks, compared to the screening procedure described above the following modifications are made:

1. removal of the free steroids
2. n-pentane extraction after hydrolysis

3.1 Sample preparation (3 aliquots)

2 ml of urine are added to a Amberlite XAD-2 column. The column (pasteur pipette, closed with glass pearl, bed height 2 cm) is washed with 2 ml of bidistilled water and the absorbed fraction is eluted with 2 ml of methanol.

The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0.2 M sodium phosphate buffer pH 7. 5 ml of t-butyl methyl ether are added to the aquaous layer.

After shaking for 5 minutes and centrifugation the organic layer is discarded and the residual organic solvent is removed by vacuum rotation.

To the buffer solution, 20 µl of the internal standard solution (see 2.4.) and 50 µl of beta-glucuronidase from E.coli are added and hydrolysis is performed for 1 h at 50° C. The buffered solution is alkalized with 250 µl of 7% potassium carbonate solution to pH 9-10 and the steroids are extracted with 5 ml of **n-pentane** on a mechanical shaker for 5 minutes. After centrifugation the etheral layer is transferred and evaporated to dryness under vacuo.

3.2 Preparation of a calibration standard T/E 6:1

The epitestosterone concentration should correspond to the epitestosterone concentration of the suspicious sample.

To overcome problems of the missing biological matrix, a solution of DIPA's * (20 µl of Dipa C_{14}-C_{23} , 500 ppm in methanol each) added to the standard.

Note: DIPA = N,N-Diisopropyl-amino-alkane, chain length C_{14}-C_{23}

DIPA's were synthesized according to E. Nolteernsting (3):

DIPA C_{14}-$C_{18,20,22}$ were prepared by reaction of alcyl-bromides and N,N-disopropylamine in an autoclave

DIPA $C_{19,21,23}$ were prepared by reducing N,N-disopropylamides (prepared from fatty acid chlorides and N,N-disopropylamine) with $LAlH_4$

The same internal standard as for the sample should be added to the standard.

3.3 Derivatisation

The dry residues are derivatized with 100 µl of MSTFA/NH_4J/ethanethiol (1000:2:3 (v:w:w)) and heated for 15 min. at 60°C.

3 µl of the solution are injected.

3.4 GC/MS parameters

GC/MS:	HP 5890 II/ HP 5971A
carrier gas:	1 ml helium at 180°C; split ca 1:10;
column:	17 m OV-1, 0.2 mm i.d., 0.11 µm film thickness
temperature program:	181°C, 3°C/min - 230°C, 30°C/min - 310°C
cycles/sec:	1.8 hz

Dwell times and registered ions:

430 m/z (for coeluting substances)	10 msec
431 m/z (for coeluting substances)	10 msec
446 m/z (for methyltestosterone)	10 msec
432 m/z (for T and E)	200 msec
435 m/z (for D3T and D3E)	200 msec

3.5 Data evaluation

Computer: HP Apollo 425 t

Software: HP HP59944C ; Rev.C.00.00

Integrator: RTE

Smooth factor: 9

3.6 Sequence of injection for external calibration

1. At least two urine samples to equilibrate the system
2. Two injections of standard T/E 6:1
3. Two injections of 1. replicate of sample
4. One injection of standard T/E 6:1
5. Two injections of 2. replicate of sample
6. One injection of standard T/E 6:1
7. Two injections of 3. replicate of sample
8. Two injections of standard T/E 6:1

3.7 Sequence of injection for internal calibration

1. At least two urine samples to equilibrate the system
2 Two injections of 1. replicate of sample
3. Two injections of 2. replicate of sample
4. Two injections of 3. replicate of sample

4. Correction of peak areas for isotopical interference

The raw areas measured for testosterone, epitestosterone, d_3-testosterone and d_3-epitestosterone are corrected using the following equations:

$$(1)\ a\_T\ (corr) = \frac{a\_T(raw) - a\_D3T\ (raw)*0.013}{0.999649}$$

$$(2)\ a\_D3T\ (corr) = \frac{a\_D3T\ (raw) - a\_T(raw)*0.027}{0.999649}$$

$$(3)\ a\_E(corr) = \frac{a\_E(raw) - a\_D3E\ (raw)*0.013}{0.999649}$$

$$(4)\ a\_D3E\ (corr) = \frac{a\_D3E\ (raw) - a\_E(raw)*0.027}{0.999649}$$

symbols:

a_T(raw) : area of testosterone detected
a_E(raw) : area of epitestosterone detected
a_D3T(raw) : area of d_3-testosterone detected
a_D3E(raw) : area of d_3-epitestosterone detected
a_T(corr) : corrected area of testosterone
a_E(corr) : corrected area of epitestosterone
a_D3T(corr) : corrected area of d_3-testosterone
a_D3E(corr) : corrected area of d_3-epitestosterone

5. Results and Discussion

5.1 Results from screening procedure

The screening procedure was tested with n=397 routine samples (prepared as described in 2.) and 10 routine calibration standards (4) over a time period of 21 days. All samples were analysed as described for the screening procedure.

The means of the d_3-testosterone/d_3-epitestosterone ratios differ slightly. For the analytical 6:1 d_3-testosterone/d_3-epitestosterone mixture we find a mean of 7.55 in the biological matrix and of 7.24 in the calibration mixture. The higher ratio observed is due to all the above mentioned instrumental factors.

There is a small tendency to higher values in biological samples than in standards and the standard deviation is also higher in the biological samples (Table 1). The reasons for the higher values and higher standard deviation can be explained by interferences of coeluting substances with d_3-testosterone and d_3-epitestosterone. Strong interferences can be recognized by inspection of the chromatograms and can be eliminated in most cases by manual reintegration of the d_3-testosterone and/or d_3-epitestosterone. In Table 1 the values of the measurements are not manually reintegrated in order to show the "worst case" situation.

Table 1: d_3-testosterone / d_3-epitestosterone ratios (areas) in routine samples

	routine samples (n=397)	calibration standards (n=11)
mean	7.55	7.24
stdv	0.54	0.36
cv (%)	7.17	4.94

5.2 Results from confirmation procedure

The confirmation procedure was tested with four different samples all containing 5-10 ng epitestosterone/ml urine. External correction and internal correction of of the T/E-ratio give slightly, but not significantly different means for the four samples (Table 2).

Table 2: d_3-testosterone / d_3-epitestosterone ratios (areas) in routine samples

sample	external corr		internal corr	
	mean	stdv	mean	stdv
I	5.90	0.08	5.86	0.11
II	4.71	0.03	4.80	0.07
III	6.70	0.08	6.87	0.18
IV	8.08	0.15	7.91	0.10

external corr: external correction using a 6:1 (T/E) calibration mixture
internal corr: internal correction using d_3-testosterone / d_3-epitestosterone ratios from internal standards

6. Summary

To compensate for instrumental variations and losses during sample preparation, the use of isotopically labelled internal standards is a well accepted technique. In the case of steroid profiling and especially the determination of the testosterone/epitestosterone ratio in the total or conjugated urinary steroid fraction, the addition of 90 ng of d_3-testosterone and 15 ng of d_3-epitestosterone per ml urine to be processed is a useful tool to control accuracy and decrease imprecision.

7. References

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