Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (3)

M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1996

E. Nolteernsting, W. Schänzer and M. Donike: 191
Experiences with D3Testosterone/D3 Epitestosterone as Internal Standards for Screening and Confirmation of T/E Ratios
In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (3). Sport und Buch Strauß, Köln, (1996) 191-199

E. Nolteernsting, W. Schänzer and M. Donike

Experiences with D3-Testosterone/D3-Epitestosterone as Internal Standards for Screening and Confirmation

Institut für Biochemie, Deutsche Sporthochschule Köln

1. Introduction

The measurement of the testosterone/epitestosterone ratios (T/E) in urine samples is used to confirm the misuse of testosterone. Calibrating the GC/MS-system by external standards is difficult due to matrix problems [1] and due to linearity problems in the GC/MS-system [1,2]. To reduce these matrix and instrumental factors d_3-testosterone (90 ng/ml urine) and d_3-epitestosterone (15 ng/ml urine) were introduced as internal standards to get standardised conditions in T/E-determination [3]. After one year of experience some results are given in this communication.

2. Results from the Screening Procedure for Anabolic Steroids - total fraction -

2.1 Sample Preparation

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 2.3.) 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 are added and hydrolysis is performed by heating 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 in vacuo.

2.2 Preparation of the calibration standard

20 µl of the internal standard solution (see 2.3.) and a methanolic solution of

androsterone	4000 ng (2000 ng/ml urine)
etiocholanolone	4000 ng (2000 ng/ml urine)
testosterone	80 ng (40 ng/ml urine)

epitestosterone	80 ng (40 ng/ml urine)
11ß-hydroxy-androsterone	800 ng (400 ng/ml urine)
11ß-hydroxy-etiocholanolone	400 ng (200 ng/ml urine)
dehydroepiandrosterone	80 ng (40 ng/ml urine)
5α-androstan-3α,17ß-diol	160 ng (80 ng/ml urine)
5ß-androstan-3α,17ß-diol	360 ng (180 ng/ml urine)
pregnandiol	2000 ng (1000 ng/ml urine)
tetrahydro-cortisol	2000 ng (1000 ng/ml urine)
DIPA-C_{14} till DIPA-C_{23}	10000 ng (5000ng/ml urine)

are evaporated to dryness in vacuo.

Note: DIPA = N,N-Diisopropyl-amino-alkane, chain length C_{14} - C_{23} [4,5]

2.3 Composition of internal standard solution

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)

^2H-labeled steroids were synthesised by W. Schänzer [6]

2.4 Derivatisation

The dry residue is derivatized with 100 µl of MSTFA/NH_4I/ethanethiol (1000:2:3 (v:w:w)) and heated for 15 min. at 60°C.

3 µl of the solution are injected.

2.5 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 D_3T and D_3E)	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)

2.6 Data evaluation

Computer: HP Apollo 425 t

Software: HP59944C ; Rev.C.00.00

Integrator: RTE

Smooth factor: 9

2.7. 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

2.8 Results

Additionally to routine samples and calibration standards, 2 quality control urines (QC-U1 and QC-U2) were prepared and analysed during the whole period (March.94 - May 95) .
There are only little differences between all 4 types of samples. The means of calibration standard and QC-U1 are nearly identical, the means of QC-U2 and the samples are slightly higher. There are interfering peaks in some urine samples especially with the d_3-testosterone peak but also with d_3-epitestosterone peak. The coefficient of variation for the samples is lower compared to the calibration mixtures in spite of the large variation of the urinary matrixes analysed.

	cal-std	QC-U1	QC-U2	samples
n	88	142	509	5217
mean	7.15	7.19	7.33	7.32
stdv	0.54	0.36	0.42	0.51
cv(%)	7.62	4.94	5.7	6.94

table 1: d_3-testosterone/d_3-epitestosterone ratios from screening analyses

cal-std:	calibration standard
QC-U1:	quality control with high T/E (about 6:1)
QC-U2:	quality control with normal T/E (about 1.4/1)
samples:	urine samples tested for anabolic steroids

3. Results from the confirmation of high T/E urines (about 6:1) with different concentrations

3.1. Sample preparation - confirmation of suspicious Testosterone/Epitestosterone samples

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

1. removal of the free steroids by extraction with diethyl ether or tert.-butylmethyl ether before hydrolysis and before adding the internal standards
2. n-pentane extraction after hydrolysis

3.2. Preparation of the T/E (6:1) calibration standard

20 µl of the internal standard solution (see 2.3.) and a methanolic solution of

testosterone/epitestosterone	6:1 (various amounts)
DIPA-C_{14} till DIPA-C_{23}	10000 ng (5000ng/ml urine)

are evaporated to dryness in vacuo.

3.3. Derivatisation

The dry residue is derivatized with 100 µl of MSTFA/NH_4I/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

Data evaluation and correction of peak areas see 2.6. and 2.7. .

3.6. Results

Different aliquots from a high T/E-urine (about 12 ng of epitestosterone/ml urine) were analysed as well as aliquots spiked with a T/E (6:1) mixture covering in total a range from 3 ng up to 260 ng of epitestosterone/ml urine. T/E (6:1) calibration standards were prepared with the corresponding concentrations of epitestosterone. Additionally a T/E (6:1) calibration standard with 15 ng of epitestosterone/ml urine and 90 ng testosterone/ml urine was prepared containing the same amount of testosterone and epitestosterone as the corresponding deuterated internal standards are added. All samples and standards were analysed six times by GC/MS. Corrections of the T/E-ratios using the 15 ng of epitestosterone/ml urine calibration standard give significantly higher deviation from the 'ideal' value of 6 compared to the correction by the deuterated internal standards (table 2) . Internal and external corrections are more similar if the same amount of epitestosterone is used in the calibration mixture as in the urine sample. At low concentrations (3 to 6 ng of epitestosterone/ml urine) the internal correction seems to be more accurate but in the range from 45 to 130 ng epitestosterone/ml urine the external correction.

ng Epit/ml	internal	external	external
	corr	corr (15ng)	corr (var)
3	6.54	6.61	6.81
6	6.34	6.49	6.63
12	6.46	6.49	6.49
30	6.15	6.33	5.98
45	6.32	6.77	6.04
65	6.62	7.36	6.15
130	6.57	7.87	6.01
260	6.17	7.80	6.04

table 2: Comparison of internal and external corrected T/E-values from urines with high T/E (6:1) ratio

ng Epit/ml: Concentration of epitestosterone in the T/E(6:1) urine
internal corr: T/E-value calculated using the internal correction with d_3-T/d_3-Epit from the internal standards (means of 6 values)
external corr (15ng): T/E-value calculated using an external correction with a T/E(6:1) calibration mixture corresponding to 15 ng of epitestosterone/ml in a urine sample
external corr (var): T/E-value calculated using an external correction with a T/E(6:1) calibration mixture of nearly the same epitestosterone concentration as in the urine sample

4. Inter-laboratory results

Two different urine samples - MK I and MK II - (about 10 ng of epitestosterone/ml urine each) were analysed by nine IOC accredited laboratories. The methanolic solution with the internal standards were provided by the Cologne laboratory. The laboratories were asked to perform a confirmation procedure (see 3.1. to 3.4.) using an internal and external calibration. The results are listed in the tables 3 and 4. Three laboratories (lab 1, 5 ,7) reported relatively high deviations between both methods. The values reported by the other six laboratories correspond well for both methods.The values (internal method) reported for MK I differ from 5.36 to 6.47 and reported for MK II differ from 6.85 to 7.82.

lab	6:1 corr		D3 corr		delta
	mean	stdv	mean	stdv	
1	6.07	0.28	6.47	0.20	-0.40
2	6.25	0.14	6.27	0.04	-0.02
3	5.74	0.21	5.65	0.25	0.09
4	5.76	0.27	5.70	0.20	0.06
5	5.40	0.12	6.00	0.05	-0.60
6	5.30	0.09	5.36	0.02	-0.06
7	5.52	0.08	6.29	0.14	-0.77
8	5.96	0.07	5.90	0.15	0.06
9	6.03	0.10	5.98	0.05	0.05
mean	5.78		5.96		
stdv	0.33		0.35		
cv (%)	5.63		5.91		

table 3: MK I

6:1 corr : external correction using a T/E(6:1) calibration mixture
D3 corr : internal correction using D3-T/D3-Epit-ratios (from internal standards)
delta: external corrected ratio minus internal corrected ratio

lab	6:1 corr		D3 corr		delta
	mean	stdv	mean	stdv	
1	7.22	0.18	7.82	0.10	-0.60
2	7.56	0.06	7.72	0.03	-0.15
3	6.87	0.04	6.85	0.13	0.02
4	6.58	0.30	6.84	0.14	-0.26
5	6.49	0.22	7.09	0.20	-0.60
6	7.04	0.06	7.36	0.17	-0.32
7	6.82	0.09	7.77	0.19	-0.95
8	7.11	0.14	7.34	0.24	-0.23
9	7.59	0.18	7.43	0.11	0.16
mean	7.03		7.36		
stdv	0.39		0.37		
cv (%)	5.52		5.07		

table 4: MK II

6:1 corr : external correction using a T/E(6:1) calibration mixture
D3 corr : internal correction using D3-T/D3-Epit-ratios (from internal standards)
delta: external corrected ratio minus internal corrected ratio

5. Conclusion

The addition of d_3-testosterone (90 ng/ml urine) and d_3-epitestosterone (15 ng/ml urine) to urine sample as internal standards reduces matrix and instrumental variations during T/E-ratio analyses. There are in some cases peaks interfering with the signals of d_3-testosterone and/or d_3-epitestosterone. This number can be reduced by the removal of the free steroids before hydrolysis and by a n-pentane extraction (confirmation procedure). The accuracy in the determination of the T/E-ratio 6:1 in a range of 3 ng to 260 ng epitestosteron per ml urine can be accepted. An inter laboratory comparison of internal and external method shows the same standard deviation but a little bit higher values using the internal method. Less deviation in the inter laboratory comparison might be available by harmonisation of integration parameters and gas chromatographic parameters.

6. References

1. GEYER, H., MARECK-ENGELKE, U., NOLTEERNSTING, E., OPFERMANN, G. AND DONIKE, M.: The Matrix Problem in Calibration of the GC/MS for Endogenous Steroids.The Search for an Artificial Urine. *In: Recent advances in doping analysis. Proceedings of the 12th Cologne Workshop on Dope Analysis 1994. M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.),* Sport und Buch Strauß, Cologne (1995).
2. VOBEJDA, C.: Zur Frage der Linerarität von Eichkurven für gaschromatographisch-massenspektrometrische Untersuchungen. Diplomarbeit. Deutsche Sporthochschule Köln, 1990.
3. NOLTEERNSTING, E, GEYER, H., MARECK-ENGELKE, U., SCHÄNZER, W. AND DONIKE, M.: Standardisation of the T/E-determination by deuterated internal standards. *In: Recent advances in doping analysis. Proceedings of the 12th Cologne Workshop on Dope Analysis 1994. M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.),* Sport und Buch Strauß, Cologne (1995).
4. NOLTEERNSTING, E.: 1-(N,N-Diisopropylamino)-alkane. Ein neues Referenzsystem für gas-chromatographische und gas-chromatographisch/massenspektrometrische Untersuchungen. *Examensarbeit.* Deutsche Sporthochschule Köln, 1984.
5. NOLTEERNSTING, E., *Opfermann,G. and Donike, M.:* 1-(N,N-Diisopropylamino)-alkanes: A new reference system for systematical identification of nitrogen containing substances by gas-chromatography and nitrogen specific or mass spectrometrical detection *In: Recent advances in doping analysis. Proceedings of the 13th Cologne Workshop on Dope Analysis 1995. M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.),* Sport und Buch Strauß, Cologne (1996).
6. SCHÄNZER, W.; DONIKE, M.: Synthesis of deuterated steroids for GC/MS quantification of endogenous steroids. *In: Recent advances in doping analysis. Proceedings of the 12th Cologne Workshop on Dope Analysis 1994. M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.),* Sport und Buch Strauß, Cologne (1995).