U. Sjöberg[1)], E. Palonek [1)], M. Garle[1)]

Investigation of variation of deuterated testosterone and epitestosterone internal standards in screening

[1)] Karolinska University Hospital, Department of Clinical Pharmacology, Stockholm, Sweden

Introduction

The administration of testosterone and its precursors is forbidden according to WADA Prohibited List (S1.1.b. Endogenous AAS when administered exogenously[1]). For determination of testosterone doping the ratio between testosterone (T) and epitestosterone (E) is used. The athlete sample will be deemed to contain testosterone as Prohibited Substance and an Atypical Finding will be reported if the ratio between testosterone and epitestosterone (T/E value) is higher than four. According to the WADA Technical Document - TD2004EAAS[2] there are specific requirements for GC/MS measurement of the T/E value, concentration of testosterone and concentration of epitestosterone.
As stated in the WADA document the T/E value is given by the peak area or peak height ratio of testosterone and epitestosterone (equivalent to the glucuronide) obtained by measuring the ion at m/z 432 by GC/MS analysis in a Single Ion Monitoring (SIM). The T/E value must be adjusted in the Screening Procedure or the Confirmation Procedure with help of appropriate standard. The deuterated internal standards are preferable and fulfill the criteria. As the reference compounds available have three deuterium atoms included in the molecules of both testosterone and epitestosterone, the ion at m/z 435 is used in GC/MS analysis in a SIM mode. We present here the statistical evaluation based on screening results of 4160 samples analyzed in our laboratory during 2008.

Materials and Methods

Internal standard mixture

The ingredients in internal standard mixture are 16,16,17-d3-Testosterone (NMIA D546), 16,16,17β-d3-Epitestosterone (NMIA D548) and 17α-Methyltestosterone (Sigma-Aldrich). Concentration of d3T is 6.0ng/µL and d3E 1.5ng/µL, which gives the d3T/d3E value 3.98±

0.05 (weight-to-weight). Concentration of methyltestosterone is 50ng/µL.

Sample Extraction Procedure

The samples were worked up according to the Screening Procedure for Anabolic Agents, described by Donike et al [3,4]. 2mL (4mL if sp.grav. ≤ 1.010 or 8mL if sp.grav. ≤ 1.005) of urine sample was mixed with 0.5mL 1M potassium phosphate buffer, pH 7.0, 20µL of internal standard mixture and 50µL β-glucuronidase from *E.coli*, ≥ 140U/mL. After hydrolysis (1h, 50°C), extraction with methyl t-butyl ether at pH 9.6 and derivatisation with MSTFA/NH4I/Ethanethiol at 60°C for 30 min. The samples were analyzed by GC/MS.

Instrumentation

The analysis was performed by Agilent GC 6890/MSD 5973N. For measurement of TMS-enol-TMS derivative of testosterone and epitestosterone the ion at m/z 432 was used. The deuterated internal standard was measured by ion at m/z 435.

Calculation of adjusted T/E value

The T/E value was adjusted according to the formula below

$$T/E_{adjusted} = (d3T/d3E_{StdMix}) \times (T_{area}/E_{area}) / (d3T_{area}/d3E_{area})$$

$d3T/d3E_{StdMix}$ = 3.98, concentration ratio
T_{area} = Area for ion at m/z 432 at RT for testosterone
E_{area} = Area for ion at m/z 432 at RT for epitestosterone
$d3T_{area}$ = Area for ion at m/z 435 at RT for deuterated testosterone
$d3E_{area}$ = Area for ion at m/z 435 at RT for deuterated epitestosterone

Integration of peaks was checked in all samples and adjusted if necessary.

Results and Discussion

There were 4160 samples analysed in our laboratory with Screening Procedure for Anabolic Agents during 2008 (Fig.1- 2). The average value for deuterated testosterone and deuterated epitestosterone ratio based on peak area was 4.72 with a standard deviation of 0.75 (Fig.3). For about 4% of all samples the d3T/d3E value differ more than 30% from the average value. The variation of the internal standard value is mainly due to the background interferences in the samples (Fig.4) especially in extreme outliers. The negative control urine sample which was analysed with every batch of the samples does not show such variation (Fig.5). The variation of the d3T/d3E value was lower for Confirmation Procedure for testosterone due to n-pentane extraction instead for methyl t-butyl ether. Five of the samples with the adjusted T/E value lower than four in screening were after confirmation found to have T/E value above four.

Fig.1 Scatterplot of d3T/d3E Area Ratio

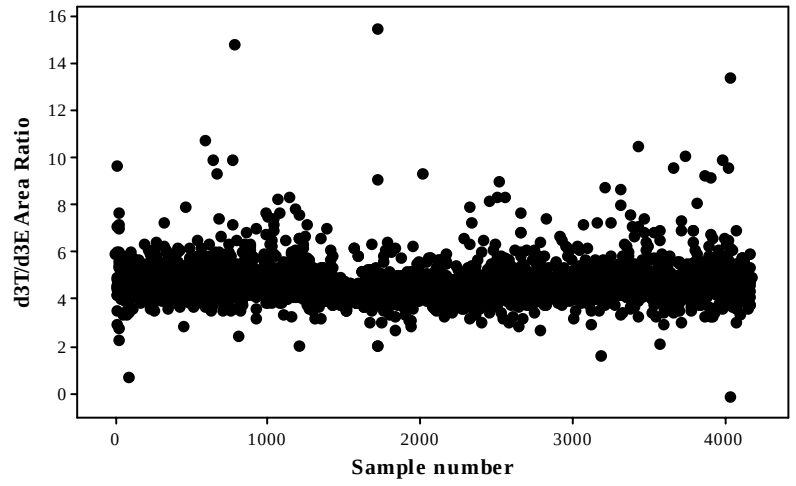


Fig.2 Histogram of the adjusted T/E area values

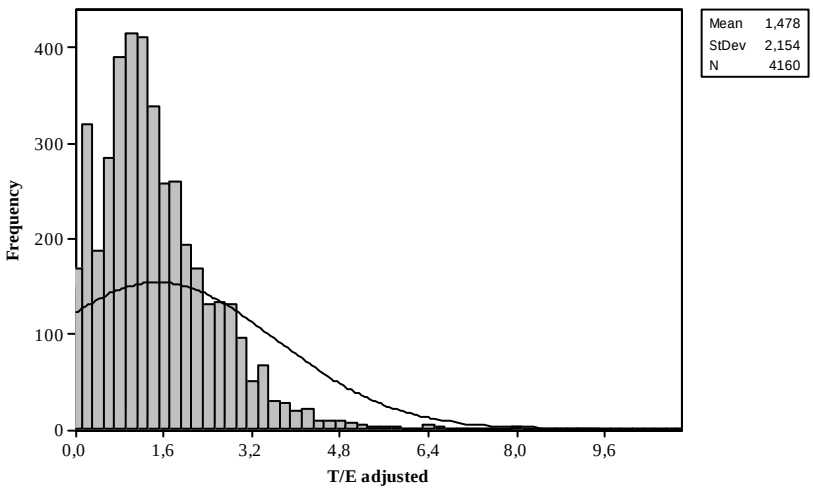
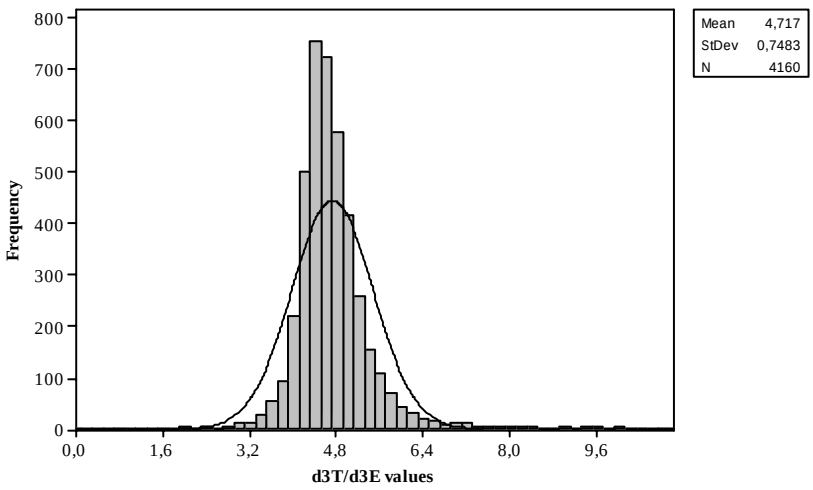


Fig.3 Histogram of the d3T/d3E values



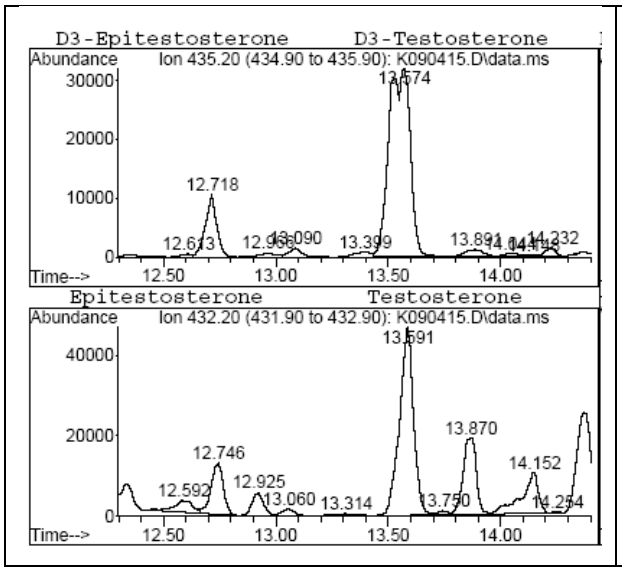


Fig.4 Athlete sample – d3T peak influenced by Background

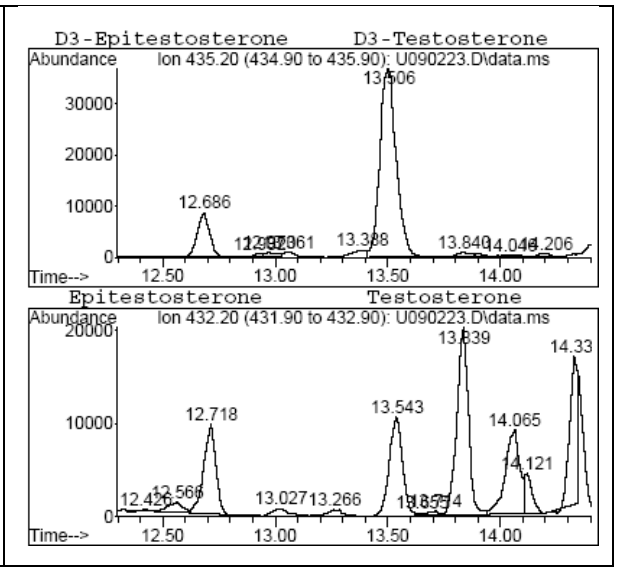


Fig.5 Negative Quality Control sample

Conclusion

The use of deuterated internal standard d3T/d3E for adjustment of the T/E value is satisfactory for most of the samples. If there is an obvious background influence and d3T/d3E value is higher than expected caution is necessary. In such cases the unadjusted T/E value could be better to make decision if to proceed with confirmation. An alternative sample preparation method as Solid Phase Extraction, n-pentane extraction could help to get rid of the background. One way to reduce variation could be by using d4-Epitestosterone (available on the market) instead of d3-Epitestosterone. Since the Steroid Profile would be registered in Athlete Passport soon, the composition and concentration of internal standard could be harmonized to achieve more equalised results.

References

1. World Anti-Doping Agency. The 2008 Prohibited List. International Standard, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2008_EN.pdf (accsess date 26.11.2010)

2. WADA Technical Document – TD2004EAAS

http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2004EAAS_EN.pdf (accsess date 26.11.2010)

3. Donike M,Geyer H, Gotzmann A, Kraft M, Mandel F,Nolteernsting E,Opfermann G,Sigmund G,Schänzer W,Zimmermann J. Dope analysis. In *official proceedings of the International Athletic Foundation World Symposium on Doping in Sport*,Belloti P,Benzi G,Ljungqvist A(eds). IAAF:Florenc, 1988;53-87

4. Geyer H, Schänzer W,Mareck-Engelke U, Nolternsting E, Opfermann G, Screening procedure for anabolic steroids – the control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In *Recent Advances in Doping Analysis*, vol.5 Scänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds) Sport und Buch Strauss: Cologne, 1998; 99-101.