10

DISCUSSION: PERSPECTIVES OF STEROID PROFILING REVIEWED

STEROID PROFILING AND TESTOSTERONE

Initially, the abuse of steroids in sports was focussed on chemically modified derivatives of testosterone. These steroids were of exogenous origin, so detection of the respective substances or the metabolites was sufficient to prove administration. In order to detect the abuse of popular steroids as methandienone (Dianabol®), methenolone (Primobolan®) and stanazolol (Winstrol®) [1] "straightforward analysis" with gas chromatoghraphy-mass spectrometry (GC-MS) was considered to be adequate.

After the abuse of testosterone (T) was suspected during the Olympic Games of Moscow in 1980 more attention was paid to the abuse of steroids of endogenous origin. Therefore, endogenous steroid profiling techniques were developed for the detection of administration of T [2,3] and its metabolite with high anabolic potency 5α-dihydrotestosterone (5α-DHT) [4,5]. As described in Chapter 1 the ratio of testosterone to epitestosterone (T/E ratio) has been used until today for the detection of T abuse. Since 1982 the International Olympic Committee (IOC) has accepted the T/E ratio as method for the detection of T [3].

Besides T and E, usually GC-MS analysis of the main metabolites androsterone (AO), etiocholanolone (EO), stereoisomers of androstanediol, 5α-DHT and main biosynthetic precursors as dehydroepiandrosterone (DHEA), androst-5-ene-3β,17β-diol (Δ5-AEDIOL) and androst-4-ene-3,17-dione (Δ4-AEDIONE) is applied in routine procedures of doping analysis for the purpose of interpretation of an elevated T/E ratio.

Although the T/E ratio was generally and effectively applied in doping analysis, occasional critical comments appeared about the validity of the method by *e.g.*

lawyers involved in doping cases. In particular, the application of the same decision criteria for females as for males was attacked. As discussed in Chapter 1, several variables determine the intra- and intersubject reference values of the T/E ratio. Although amongst doping laboratories there is a general conviction of the validity of the T/E ratio, there is still insufficient published data about the influence of variables as race, menstrual cycle, use of contraceptives, age, exercise, etc. The controversy can therefore mostly be assigned to the availability of scientific data.

An example of one of the variables that effectively influences intra-individual values of the T/E ratio is the consumption of ethanol. As described in Chapter 3, the T/E ratio increases immediately after first alcohol administration. Although the effect is more significant for females compared to males, Chapter 2 shows that both sexes are at risk of an incidental T/E value above 6 after drinking a volume in the order of a bottle of wine (dose of 1.2 g ethanol per kg bodyweight, corresponding to a plasma concentration 1-2‰). The significance of the effect was dependent upon the mean basal intra-subject T/E ratio. Doping laboratories should therefore be equipped with a validated methodology to establish urine alcohol concentrations in case recent alcohol consumption is claimed.

STEROID PROFILING AND FOOD SUPPLEMENTS

Although already during the eighties experiments were performed with nasal administration of Δ4-AEDIONE in former Eastern Germany [6], this steroid did not become widely commercially available until the late nineties. Stimulated by accessibility via the Internet “food supplements” containing steroids became popular marketing products. Unlike Europe, food supplements containing testosterone precursors as DHEA and Δ4-AEDIONE are available as non-prescriptive substances in the United States.

DHEA and Δ4-AEDIONE were the first steroids that became widely available in food supplement formulations. The popularity of these products was illustrated by the case of the baseball player Mark McGwire [7], who publicly admitted and defended the use of Δ4-AEDIONE. In 1997 and 1998 respectively, DHEA and Δ4-AEDIONE were placed on the IOC list of forbidden substances. In 1999 these were followed by androst-5-ene-3β,17β-diol (Δ5-AEDIOL), androst-4-ene-3β,17β-diol (Δ4-AEDIOL), androst-5-ene-3,17-dione (Δ5-AEDIONE), 19-norandrost-4-ene-3,17-dione (19-nor-Δ4-AEDIONE), 19-norandrost-5-ene-3,17-dione (19-nor-Δ5-AEDIONE) and 19-norandrost-4-ene-3β,17β-diol (19-nor-Δ4-AEDIOL).

To detect food supplement steroids in doping analysis, profiling techniques are mostly applied as were originally developed for detection of T administration [2,3], with the T/E ratio as one of the main parameters. However, these profiling techniques were developed in times that T precursors as DHEA and Δ4-AEDIONE were not generally available.

The increased number of abused endogenous steroids has complicated the interpretation of steroid profiles. As shown in Chapters 4-7 both oral DHEA and Δ4-AEDIONE administration result in an increased T/E ratio. As oral administration of Δ5-AEDIOL, Δ4-AEDIOL and Δ5-AEDIONE has a comparable effect [7,8] it is a very unspecific parameter for all these steroids. The same conclusion can be drawn for other previously mentioned parameters as AO, EO, stereoisomers of androstanediol and 5α-DHT, which are main metabolites of all these food supplement steroids. Efficient identification of the abused steroids by the present methodology is therefore not possible.

Regarding 19-norsteroids found in food supplements, applied analytical techniques are limited to the screening of 19-norandrosterone (19-nor-AO) and 19-noretiocholanolone (19-nor-EO). As these are the main metabolites of 19-nor-Δ4-AEDIONE, 19-nor-Δ5-AEDIONE, 19-nor-Δ4-AEDIOL as well as 19-nortestosterone (nandrolone) [1,7,8], also no identification of the administered steroid can be performed by this methodology.

Providing this specific information on the identity can be of particular relevance in case of unintentional steroid administration by polluted food supplement products, as has frequently been claimed in recent years [13,14]. Furthermore, there is increasing scientific acceptance for the concept of natural presence of 19-nor-AO and 19-nor-EO in low concentrations in urine [9,10], as well as the concept of natural excretion of these steroids after consumption of meat [11,12]. To determine the, either endogenous or exogenous, source of 19C- or 19-norsteroids, more specificity of the analytical method is required as is available today.

As explained in Chapter 3, profiling of oxygenated metabolites of food supplement steroids could provide essential information that can aid the identification of the abused substances. To obtain a more specific steroid profile for the detection of food supplement steroids, the metabolism was studied of the model compounds DHEA and Δ4-AEDIONE with a main focus on oxygenated metabolites.

To compare the studied metabolites as parameters in steroid profiling, they were described by means of sensitivity and specificity. For the metabolites, sensitivity was defined as the increase of excretion rate, compared to the mean basal excretion rate (see Table 1, Chapter 6). AO and EO are mostly considered as sensitive parameters for the detection of DHEA and/or Δ4-AEDIONE [7,8]. The steroids that showed a

larger sensitivity for DHEA than AO or EO were (see Chapter 4 and 5): DHEA, 7β-hydroxy-DHEA, Δ5-AEDIOL, 7-keto-AO, 6α/β-OH-Δ4-AEDIONE, 16α-OH-AO and 16α-OH-EO. Higher sensitivity for detection of Δ4-AEDIONE was observed for 4-OH-Δ4-AEDIONE and 6α/β-OH-Δ4-AEDIONE.

To quantitatively describe specificity in this experimental setup (regarding only the administration of two food supplement steroids), the sensitivity of a metabolite for administration of DHEA can divided by the sensitivity for the administration of Δ4-ADIONE (or vice versa). Table 10.1 summarizes quantitative values for specificity of each parameter for respectively DHEA and Δ4-AEDIONE. The best specificity for DHEA administration is supplied by 7β-OH-DHEA, DHEA, 7-keto-AO, Δ5-AEDIOL, 16α-OH-DHEA and 7-keto-DHEA. The best specificity for Δ4-AEDIONE was supplied by 4-OH-Δ4-AEDIONE, T, E and 6α/β-OH-T.

Table 1: *Quantitative values for specificity of the studied parameters for the administration of DHEA and Δ4-AEDIONE. Specificity for Δ4-AEDIONE is the reciprocal value of the specificity for DHEA.*

Parameter	Specificity for DHEA	Specificity for Δ4-AEDIONE
7β-OH-DHEA	36.3	<0.1
DHEA	17.5	0.1
7-keto-AO	15.2	0.1
Δ5-AEDIOL	10.3	0.1
16α-OH-DHEA	8.4	0.1
16α-OH-AO	0.9	1.1
7-keto-DHEA	5.3	0.2
16α-OH-EO	1.7	0.6
AO	0.5	2.0
7α-OH-DHEA	0.5	1.9
EO	0.4	2.7
6α/β-OH-Δ4-AEDIONE	0.3	3.6
16α-OH-Δ4-AEDIONE	1.2	0.8
T	0.2	5.4
E	0.2	5.0
6α/β-OH-T	0.2	5.0
4-OH-Δ4-AEDIONE	<0.1	62.8

As described in the Appendix, administration of one of the most recently available supplement steroids 7-keto-DHEA, or its analogue 3-acetyl-7-keto-DHEA [16], can

also lead to production of oxygenated DHEA metabolites as 7α-OH-DHEA, 7β-OH-DHEA, 7-keto-DHEA and 7-keto-AO. As 7-keto-DHEA is not on the IOC list of forbidden substances, excluding the administration of this steroid is necessary for the analysis of DHEA abuse by means of oxygenated metabolites. As shown in the case study, no non-oxygenated metabolites are formed after oral administration of 7-keto-DHEA-acetate. Therefore, profiling non-oxygenated metabolites still remains necessary, as those show no sensitivity for 7-keto-DHEA.

The described experiments show that measurement of oxygenated and non-oxygenated steroids can provide sensitive and specific information that is needed for identification of the administered steroid.

More research is needed to study the specific metabolism of other mentioned food supplement steroids, leading to additional parameters to evaluate. To facilitate the interpretation of the larger number of parameters in doping analysis, multivariate statistical analysis could be applied as was done previously for profiling non-specific T metabolites [17].

ISOTOPE RATIO MASS SPECTROMETRY AND STEROID PROFILING

Since the Olympic Games of Nagano, Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) is applied as confirmation method for abuse of “endogenous” steroids. This technique is able to distinguish between endogenous and exogenous steroids by quantitative analysis of the $^{13}C/^{12}C$ isotope ratio after combustion to CO_2, as explained in Chapter 1.

Analytical procedures for GC-C-IRMS analysis are reported for the analysis of T [18-21], E, DHEA, 5α-DHT [21,22], hydrocortisone and cortisone [24]. The analysis is mostly performed on metabolites of the steroids under investigation as Δ5-AEDIOL [22], AO, EO [25], stereoisomers of androstanediols [19,21,25], tetrahydrocortisol and tetrahydrocortisone [24]. GC-C-IRMS can therefore be considered as a similar technique as regular steroid profiling, with the extra ability to differentiate between metabolites of endogenous and metabolites of exogenous origin. The development of this technique resulted in major advancement in discriminative power of doping analysis regarding steroids of endogenous vs. exogenous origin. However, when specificity is regarded no real innovation was established.

GC-C-IRMS is still mostly used for confirmation purposes, as extensive and laborious sample cleanup is required. For T analysis some methodologies have been reported that are suitable for fast and efficient screening purposes [24,26]. However, the main

contribution of GC-C-IRMS is still the confirmation of T abuse after detecting a high T/E ratio, *e.g.* in cases of an increased T/E ratio caused by alcohol consumption.

Furthermore, could this technique be used to eliminate the ketoconazole test (Chapter 1, Appendix A), in which an athlete is required to self-administer a relatively large dose of ketoconazole. This should be avoided as based on ethics and potential side effects.

GC-C-IRMS can form an excellent combination with profiling of suggested steroid parameters to combine specificity and sensitivity with discrimination between endogenous and exogenous steroids, for the detection of food supplement steroids.

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