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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

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U. FLENKER, S. HORNING, E. NOLTEERNSTING, H. GEYER,
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U. Flenker, S. Horning, E. Nolteernsting, H. Geyer, W. Schänzer

Measurement of $^{13}C/^{12}C$-Ratios to confirm Misuse of endogenous Steroids

Institute of Biochemistry, German Sports University Cologne

Abstract

The ratio of the two stable carbon isotopes ^{13}C and ^{12}C allows the differentiation of natural and synthetic steroids. The method to determine the isotopic composition of the relevant analytes comprises gas chromatography, subsequent combustion to CO_2 and finally mass spectrometric analysis of this gas in a special multi-collector mass spectrometer (gas-chromatography/combustion/isotope-ratio-mass-spectrometry, GC/C/IRMS). This method is sensitive to impurities of analytes, possible isotopic fractionation during sample preparation and chromatographic conditions. Possible factors influencing the measurements are investigated. Moreover we describe conditions which in our opinion allow reliable and valid measurements. Some cases in which suspicous steroid profiles could be clearly decided on by using GC/C/IRMS are presented.

1 Introduction

Misuse of steroids occurring naturally is still one of the most important problems in sports. This especially applies to substances which show anabolic/androgenic action. Testosterone, Dehydroepiandrosterone (DHEA), Dihydrotestosterone (DHT) and Androstenedion are substances with high potential of abuse. The analyst is therefore confronted with the challenge to find criteria which allow to discriminate endogenous from chemical identical but synthetic steroids in the urine from athletes.

A possible way to solve this problem is use of GC/C/IRMS (gas-chromatography/combustion/isotope-ratio-mass-spectrometry). Previous work shows the potential of this method

to unequivocally distinguish between steroid users and non-users [1, 2, 8]. The method benefits from the fact that synthetic substances usually show a different composition concerning the stable isotopes ^{13}C and ^{12}C than material of exclusive biological origin. It first makes use of gas chromatographic seperation, then all the effluent from the chromatographic column is combusted on-line to CO_2 and H_2O preserving the chromatographic resolution as far as necessary. Finally the resulting CO_2 is analyzed mass spectrometrically. Ion abundances are measured at $m/z = 44$, 45 and 46. Masses 44 and 45 for the most part represent ^{12}C and ^{13}C. Mass 46 is recorderd to perform corrections for the isotopes of Oxygen (^{17}O,^{18}O) [4, 12].

Results refering to isotopic composition usually are expressed using the δ-notation [4].

$$\delta = \frac{R_{SPL} - R_{STD}}{R_{STD}} \cdot 10^3 \tag{1}$$

R generally refers to the isotope ratio ($^{13}C/^{12}C$ in case of carbon) and SPL and STD refer to "sample" and "standard" respectively. δ-values are expressed in per mil ($^0/_{00}$). The international standard for carbon is derived from $CaCO_3$ from Pee Dee Belemnite (PDB) with $R_{PDB} = 0.0112372$ [4].

In contrast to its unique capabilities, this method faces one with several problems. First of all only approximately 1.11% of all carbon (on the surface of the earth) consits of ^{13}C and the difference between natural and synthetic material reaches only several per mil related to these 1.11%. Therefore relative large ammounts of substance are required to achieve sufficiently precise results. Due to the same facts is the sensitivity to contamination of the analytes. Already small ammounts of impurities can render the measurement useless when the isotopic composition is significantly different from the measured substance.

Visualizing the fact that gas-chromatographic seperation preceeds isotopic analysis this means that one is in need of

- complete baseline seperation of the peaks [9],
- sufficiently large signal-to-noise ratio,
- low and –more important, as will be outlined– constant background conditions.

The latter point refers especially to the isotopic composition of the background. After combustion of the material to CO_2 it is no longer possible to determine its origin. Therefore the apparent $^{13}C/^{12}C$-ratio of the observed peak is always a mixture of that of the background and that of the analyte. The "true" value can only be calculated if the backgrounds proportion of ^{13}C and ^{12}C is known and can be assumed to keep constant over time within only small limits. Moreover also the absolute intensity of the background has to fulfill these requirements, especially when its $^{13}C/^{12}C$-proportion is relatively different from that of the analytes.

Another important point is that any isotopic fractionation during the sample preparation must be excluded [3]. One of the most problematic fields when using IRMS-techniques is to guarantee complete conversion of the analytes as any non-quantitative procedure is a possible source of isotopic fractionation [3]. At least one has to make sure that the system is given enough time to get as close to equilibrium conditions as possible because kinetic isotope effects usually are much larger than equilibrium isotope effects [14].

It has to be stressed that the problem of non-quantitative conversion does not only refer to chemical processes: For instance the differing diffusion rates of $^{13}CO_2$ and $^{12}CO_2$ in air bring about a fractionation of stable carbon isotopes exceeding 4 ‰ [11]. Although from a theoretical point of view the fractionating effects will be smaller in larger molecules, one should always keep in mind that this is principally possible, e.g. when using liquid/liquid extraction or when analytes are vaporized.

In this paper some possible sources of isotopic fractionation concerning analysis of endogenous steroids by GC/C/IRMS are investigated. Furthermore results from suspicous samples that could be confirmed by GC/C/IRMS are presented.

2 Material and Methods

All analyses of isotope ratios were performed on a delta-C isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to a 5890 gas chromatograph (Hewlett Packard, Palo Alto, USA) by a Combustion Interface II (Finnigan MAT). The combustion interface roughly comprises

- an oxidation furnace, consisting of a 0.5 mm ID ceramic tube which is filled with three small wires (Cu,Ni,Pt),
- an reduction furnace, similar to oxidation device, but filled with Cu only,
- a Nafion^{TM} water trap,
- and finally an open split construction to connect the gas stream to mass spectrometer.

The temperature of the combustion oven was kept at 940 °C during analysis, that of the reduction device at 600 °C.

Unless indicated GC-parameters were kept as follows:

- Mode: Splitless.
- Injection volume 0.6 μl.
- Column: DB-5ms, 17 m, 0.25 mm ID, 0.25 μm film (J&W Scientific, Folsom, USA).
- Headpressure: 29 psi.
- Temperature: Contsant at 60 °C for 1.5 min, 40 K/min to 213 °C, keep constant for 10 min, 40 K/min to 310 °C, keep constant for 4 min.

The solvent was 2-Propanol. In case of biological samples the concentration was set to at least 15 ppm when possible. Higher concentrated substances were diluted to 20 – 40 ppm.

Preparation of biological samples proceeded according to the usual method "Screening IV " described elsewhere [5, 13], with the exceptions that no internal standards were added and no derivatization was performed. Additional cleaning of biolgical samples was performed by HPLC. The device used was a LC 1090 (Hewlett Packard). LC conditions were set to the following values:

- Injection volume: 50 μl.
- Flow: 1ml/min.

- Column: HP ODS Hypersil 5 μm, 250×4 mm.
- Solvent: Acetonitril/Water, starting at a ratio of 30:70 and ending at a ratio of 100:0 changed linearly over the time of analysis (20 min).

Fractions of analysed substances were collected manually, neglecting a possible time lag between detector and end of capillary. Collection times were set according to the table 1 Standards were measured before each run to correct possible changes of retention times, followed by injection of a Methanol blank.

The targeted steroids were:

- Pregnanediol (PD) and Pregnanetriol (PT) because these are not involved in metabolism of androgenes and thus serve as internal reference,
- Testosterone (T) as the substance of major interest,
- Androsterone (A) and Etiocholanolone (E) as these represent quantitatively the most important metabolites of androgenes,
- 5α-Androstane-3α, 17β-diol (AD) and 5β-Androstane-3α, 17β-diol (BD) probably giving the best insight into the state of androgene metabolism and
- Dehdroepiandrosterone (DHEA) and 5α-Dehydrotestosterone (DHT) in case of a sample suspicous with these substances.

To investigate some factors possibly influencing the measurements, the following experiments were performed:

Table 1: Collection times for HPLC cleaning proceedure

Fraction	Time [min]	Substance
LC0	7.9–8.3	Control
LC1	8.3–9.1	T
LC2	9.1–9.6	Control
LC3	9.6–10.5	PT, AD, BD
LC4	10.5–10.9	Control
LC5	10.9–12.2	A, E
LC6	12.2–13.0	PD

1. **Baseline separation**

   Three standard solutions were prepared. The first contained E, the second A and the third solution contained A and E together. Concentrations were set to 100 ppm for all substances. Ten repeated measurements were performed. Resulting δ^{13}C-values were compared using t-statistics.

2. **Fractionation by HPLC**

   A standard solution containing A and E at a concentration of 1000 ppm each was prepared. 50 μl of this solution underwent HPLC analysis. HPLC-fractions were collected every six seconds over a time interval covering the complete elution times of both substances. Every resulting fraction was analyzed for $^{13}C/^{12}C$-ratio.

3. **Injection speed**

   A standard solution containing A, E, T and PD at a concentration of 20 ppm each was prepared. δ^{13}C-values were determined for each substance at injection speeds of 2, 10, 20 and 40 μl/s. Five replicate measurements were performed at each level of injection speed. Results were evaluated by two-factor ANOVA.

3 Results and Discussion

As was pointed out in section 1 one has to take into account an effect on apparent $^{13}C/^{12}C$-ratios when complete chromatographic baseline seperation of investigated substances is not given. Figure 1 depicts the result of the corresponding experiment. Whereas the $^{13}C/^{12}C$-ratio of E is not influenced by the presence of its 5α-isomer ($p > 0.1$), A shows a small but statistically significant difference in its ^{13}C-content when E is present ($p < 0.01$, t-Test using BONFERRONI-adjustment). The two substances investigated in this experiment differ approximately by 30 s in their retention times. The chromatograms (not shown) give only weak evidence for lacking baseline seperation. But the influence of common analysis on δ^{13}C-values of A indicates that seperation is not given completely. It is likely that the tail of the E-peak changes the background for the calculation of δ^{13}C-values of A. The software used (Isodat 5.3) calculates the background from a certain time before the peak starts and assumes the background to be constant afterwards. It has to be emphasized that ^{13}C-content of GC-peaks changes over time. Heavy isotopomers show

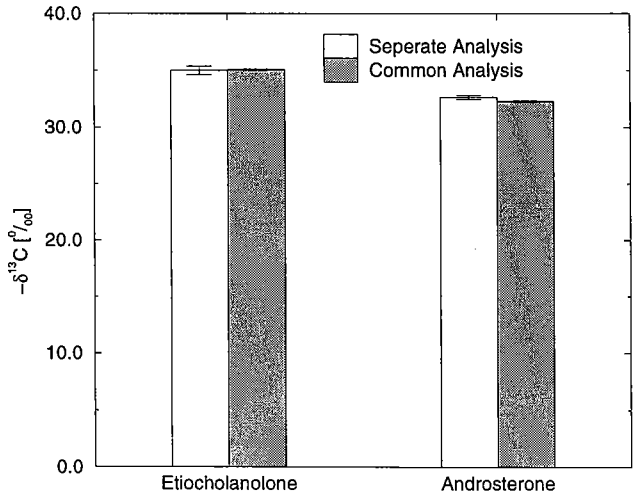

Figure 1: Influence of common vs. seperate analysis on δ^{13}C-values of E and A ($\bar{x} \pm s$). Retention times differ by $\approx$ 30s. Difference is significant in case of A ($p < 0.01$) whereas no significant difference occurs in case of E ($p > 0.1$).

fewer retention as is well known from deuterated standards and as will be shown for LC in the following section. Thus the tail of the E-peak contains relative light substance causing the $^{13}C/^{12}C$-ratio of A being calculated too heavy. Furthermore no correct $^{13}C/^{12}C$-ratios can be calculated from GC-peaks that are integrated partially. This fact and the results shown above clearly support the demand for extraordinary good GC-separation.

The influence of isotopic fractionation caused by HPLC is depicted by figure 2. The difference of $^{13}C/^{12}C$-ratio between the first and the last collected fraction reaches approximately $10^0/_{00}$ with respect to both analysed substances. Although no further evaluation of the data was performed this result clearly illutrates the high risc of producing doubtful results when HPLC is used to clean the samples. When this technique is used in spite of its riscs, it has to be made sure under any circumstances that the whole peak of interest is collected. It also has to be taken into account that substances might coelute on the GC/C/IRMS-device, whereas they might show different retention times under LC-conditions. As was outlined before no statement is possible concerning the source of measured CO_2. Therefore also substances that are not targeted and show only small concentrations might heavily influence the results.

Figure 3 shows the effect of injection speed on measured $^{13}C/^{12}C$-ratios of four different

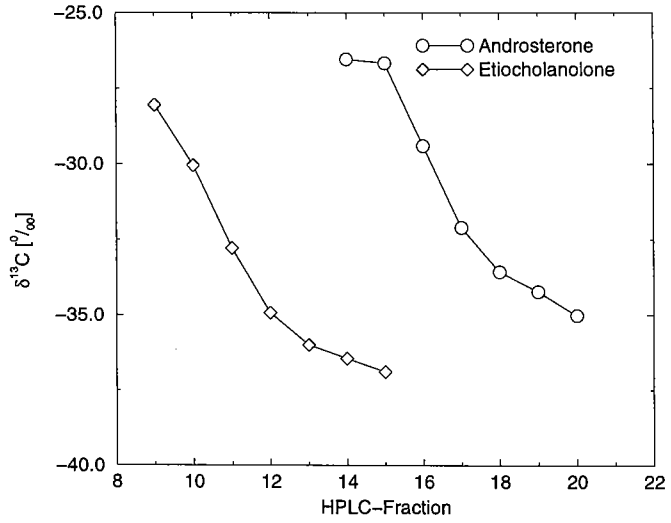

Figure 2: Effect of HPLC-fractionation on δ^{13}C-values on a standard solution of Androsterone and Etiocholanolone.

analytes. Table 2 contains the results of the corresponding two factor ANOVA. There seems to be a tendency towards lighter isotopic composition of the analytes with increasing injection speed. The values are approximately 0.76 $^0/_{00}$ lighter at 40μl/s than at 2μl/s. As can be concluded from table 2 the effect of injection speed is highly significant ($p < 0.001$). There seems to be no difference in the consequences of this phenomenon with respect to the different analytes as the interaction effect is not significant ($p > 0.1$). Therefore the effect of injection speed is not too serious as long this factor is kept constant for the different substances of interest. To make sure compareability of the measurements it also should be kept constant over time.

The reason for the dependence of measured δ^{13}C-values from injection speed is not fully clear, because the processes in the injector are difficault to judge at. On the one hand the diffusion rate of vaporous substances is $\sim 1/\sqrt{M_r}$, where M_r indicates the relative atomic mass [7]. On the other hand heavy isotopomers move much faster when chromatographic seperation occurs. Thus there are two antagonistic processes in the injector/column-head which may cause isotopic fractionation. One possible explanation would be that increasing injection speed causes a situation where the injector part of the GC is overloaded in a sense that the volume of the vaporized solvent exceeds that of the insert liner. One has to keep in mind that injection is performed in splitless mode. The lighter isotopomers of the

analytes now will diffuse faster to the column than the heavier species. The latter will be blown out of the injector as the rapid increasing pressure may cause the caulkings to be leaky for a certain time. On the contrary when injections are performed slowly there will be isotopic fractionation caused by differing diffusion rates as well. But now the heavy isotopomers are given enough time to condense on the column head because there is less overloading of the insert liner.

If the explanation given above holds true the recommendation would be to keep the injection speed as slow as possible and to use solvents showing small increase of vapour pressure with temperature. In practice a compromise has to be found as slow injection speed usually worsens chromatographic conditions. A solution avoiding any possible isotopic fractionation in the injector would be to generally use on-column injection.

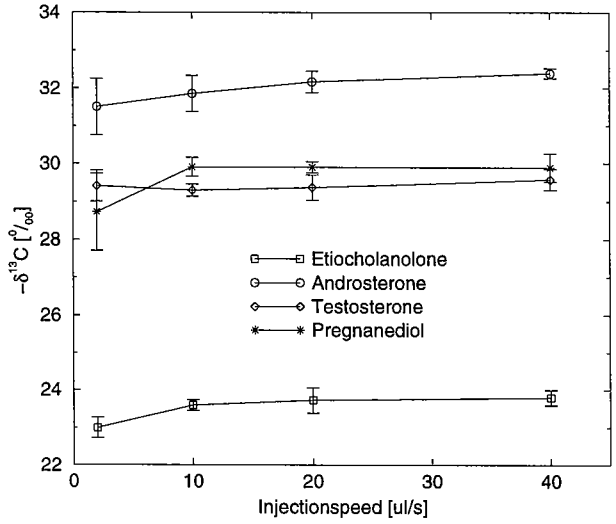

Figure 3: Influence of injection speed on δ^{13}C-values of standard solutions from four different analytes ($\bar{x} \pm s$).

Table 2: Two-factor analysis of variance corresponding to figure 3. IS: injection speed [μl/s]; ALT: analyte (E, A, T, PD); IS×ALT: interaction effect.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)	Signif
IS	3	6.65	2.216	10.24	1.344e-05	***
ALT	3	776.49	258.829	1196.55	< 2.2e-16	***
IS×ALT	9	3.05	0.339	1.57	0.1440	n.s.
Residuals	64	13.84	0.216			

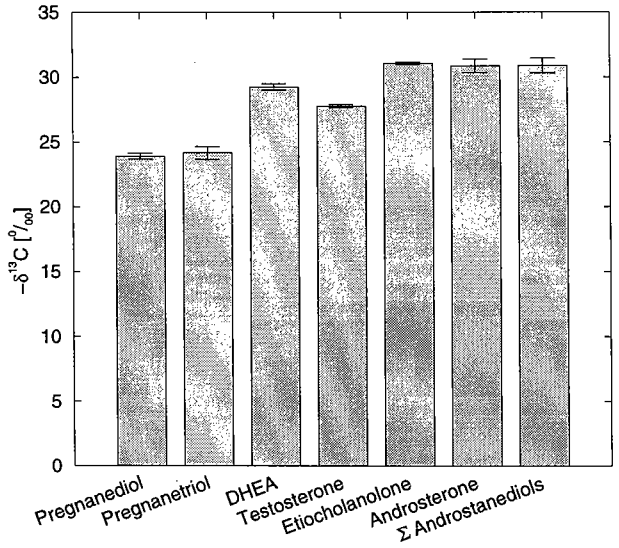

Figure 4: Sample positive with DHEA. Androstanediols (5α-Androstane-3α, 17β-diol and 5β-Androstane-3α, 17β-diol) were integrated together.

4 Examples of Analysed Samples

All figures presented show $\bar{x} \pm s$. In all cases five replicate measurements were performed on each LC-fraction.

Figure 4 shows the δ^{13}C-values of a sample positive with DHEA. The δ^{13}C-values of PD and PT are around -24 ‰, showing only few variation. DHEA, T and the metabolites of T are sifgnificantly lighter in their isotopic composition, showing values around -30 ‰. Due to lack of chromatographic seperation there is only one value for the two isomers of Androstane-3α, 17β-diol. The fact the δ^{13}C-values of A (-30.88 ± 0.52 ‰) and E (-31.08 ± 0.10 ‰) are identical to that of the AD-isomers (-30.90 ± 0.56 ‰) indicates that common integration of metabolic related substances will still lead to valid results. When complete baseline separation is not given, this kind of data evaluation always will perform better than "cutting" relevant peaks. Cutting will result in wrong $^{13}C/^{12}C$-ratios for reasons described above.

GC/C/IRMS also bears the capability to avoid false positive results. Figure 5 shows the δ^{13}C-values from a sample which showed a suspicous steroid profile, the ratio of Testosterone and Epitestosterone reaching $\approx$ 8. There are no hints for application of synthetic androgenes as all targeted steroids show similar isotopic composition. The

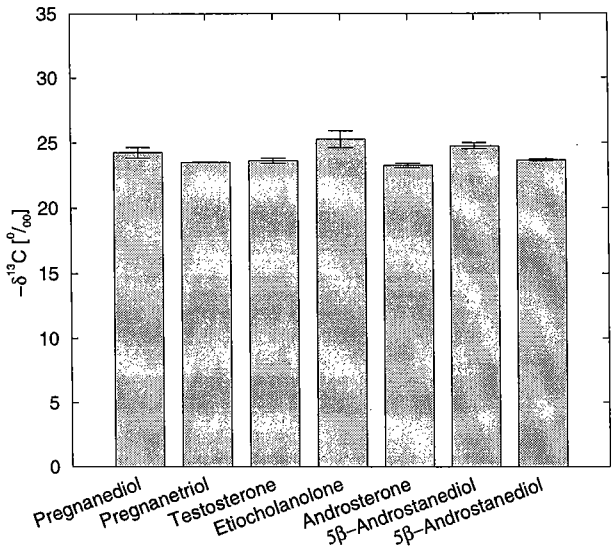

Figure 5: Negative sample.

values are within a range of -24 to -25 ‰. Interestingly the 5β-steroids E and BD are a bit lighter concerning their $^{13}C/^{12}C$-ratios. With respect to the results presented in section 3 this is possibly an artifact, and may be due to lacking baseline seperation concerning the 5α/5β-isomers.

Figure 6 may give an impression of the capabilities of GC/C/IRMS to track metabolic pathways. The sample was sent to the Cologne laboratory as part of IOC's reaccreditation proceedure. It showed extraordinary high DHT concentrations when being screened by GC/MS. It can be seen clearly that only 5α-steroids are influenced, showing $\delta^{13}C$-values reaching from -27 to -30 ‰. It may be interesting that DHT itself and AD show similar $^{13}C/^{12}C$-ratios whereas A is a bit closer to the values of the endogenous references. This possibly indicates that not only the metabolism of DHT leads to the formation of A, the heavier fraction resulting from different pathways. Another interesting point is illustrated by this sample: The absolute $\delta^{13}C$-values of PD, BD and E are close to -20 ‰. This is extraordinary heavy compared to the samples discussed before. This observation is explained by the american origin of the sample. Most of food on the american continent is derived from C_4-plants, which show less isotopic fractionation towards ^{13}C than C_3-plants, which are predominant in Europe [6, 10].

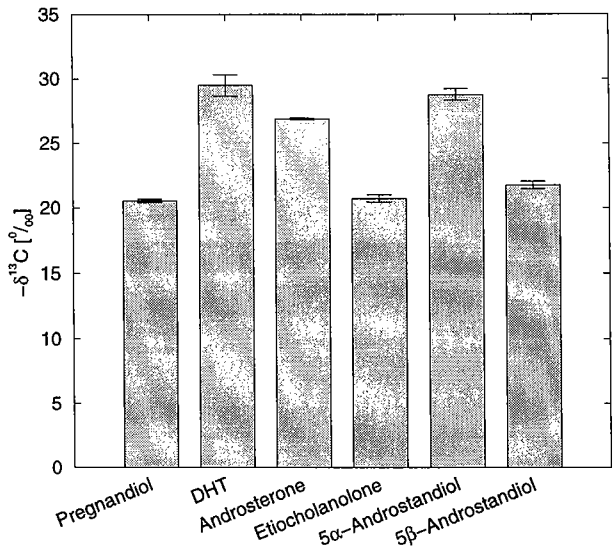

Figure 6: Sample positive with DHT (IOC-reaccreditation 1997).

5 Conclusions

GC/C/IRMS can be a very powerful method to unequivocally decide on the question whether an athlete has abused synthetic endogenous steroids. To confirm a suspicous sample it has to be treated much more carefully than in case of GC/MS techniques. One possible source of isotopic fractionation results from overloading the insert liner. In order to obtain most reliable δ^{13}C-values, chromatographic parameters should be optimized for isothermic conditions, because intensity and $^{13}C/^{12}C$-ratio of column bleeding changes with temperature. To calculate δ^{13}C-values from GC-peaks, background must be assumed to keep constant over relevant time of elution. For the same reasons complete baseline seperation of targeted analytes is required. In case of lacking seperation, common integration of peaks will give more elucidating results, because chromatography generally causes isotopic fractionation. Usage of HPLC to clean the samples is affected with a high risc concerning adulteration of $^{13}C/^{12}C$ for the latter reason. Under any circumstances complete collection of LC-fractions has to be made sure. More generally quantitative treatment of samples is required in any step of sample preparation.

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