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# RECENT ADVANCES IN DOPING ANALYSIS (9)

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# Differentiation of endogenous and exogenous steroids in urine by means of isotope ratio mass spectrometry

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## Introduction

Evidence obtained by isotope ratio analysis is used to draw definitive conclusions on doping with naturally occurring synthetic steroids.[1] As no criteria for this test was set in anti-doping codes, the decision may be made in accordance with in-house reporting criteria in laboratories[2)-6)]. In the geo-science field, carbon isotope ratio(CIR) database in the literature is used to speculate on the origin of the compounds. Commercial steroids are synthesized from higher plants such as stigmasterol from ***soy*** or ***calabar beans***, diosgenin and solasodine from ***dioscorea***, hecogenin from ***sisal***. Synthesis of androstenedione from sitosterol from ***soy*** and ***calabar beans*** or ***rice embryos***, androstadienedione from cholesterol, direct synthesis of 11-oxo and 11-hydroxy pregnenes from 11-deoxycortisol came into reality in 1952 by introducing microbiological processes.[7] Cholesterol is even obtained from sauces such as wool grease. In all the processes, the resultant steroids retain C18, C19 or C21 carbon skeleton from the source materials. C3-plants are largely of temperate zone, which fix $CO_2$ directly into the three-carbon 3-phosphoglycerate.[8),9)] Carbon source of photosynthetic land plants is atmospheric $CO_2$, which have fairly constant CIRs particularly in rural areas.[10),11)] Carbon fixation step in C3-plant leads to an isotopic fractionation to reduce $^{13}C$-content of the products.[9),11),12)]. One possible influencing factor on CIR in humans is diet. As breath gas $CO_2$ reflects three macronutrients, the CIR for breath $CO_2$ predicts a diet-induced variation of CIRs of steroids.[12),13)] A ring test for isotopic analysis was performed in order to demonstrate the inter-laboratory traceability. This report deals with the difference of steroidal CIR depending on the origin and the ring test results. Several hints to improve performance of GC combustion isotope ratio mass spectrometer (GC/C/IRMS) are given.

## Experimental

Active ingredients of 26 different synthetic steroids in dosage forms were extracted with analytical grade ethanol. The extract was analyzed by GC/C/IRMS without any chemical modification[4]. Some confirmed positive urines were selected and screened from 2,120 athlete's urine samples by our isotope methodology based on CIRs of androsterone(Andro) and etiocholanolone(Etio) in the total fraction. The steroid fraction was extracted by our known procedure for steroid screening[14] and analyzed by GC/C/IRMS after TMS derivatization without enolization. Samples in question were further analyzed for glucuronides of diol-steroids and dehydroepiandrosterone(DHEA) sulfate as their acetate derivatives by our published optimized procedure[4]. The excess derivatization reagent was removed carefully, and the residues were re-dissolved in 30 µl of cyclohexane. The CIRs were expressed as δ-value, which mean a part per thousand difference(‰) of isotope abundance from the international isotope standard[15], and the raw values were corrected for derivatization if applicable. GC/C/IRMS instrument was Isoprime from Micromass (Manchester, UK) equipped with cryo-trap for removal of water and uncombusted compounds. Other detailed instrumental conditions are in our previous report[4]. Expired breath gas samples were randomly collected from 150 Japanese female and male adults under uncontrolled diet without increased energy expenditure. The breath gas samples were measured by a stable isotope gas analysis system type ABCA-G from PDZ Europa limited (Cheshire, UK). CIR of the reference $CO_2$ was verified in-house using certified reference material: NBS-19 limestone from NIST (Gaithersburg, MD, USA)[15] before being placed in service and measured bimonthly. The GC/C/IRMS instrument performs a calibration both in the front and in the end of each chromatographic run against the reference $CO_2$.

## Results and Discussion

CIRs of etiocholanolone and DHEA in the sulfate fraction, and glucuronides of 5α- and 5β-androstan-3α,17β-diols(5a-diol, 5b-diol) and pregnanediol, have been measured on 443 urine samples of winter athletes who had participated in the XVII$^{th}$ Olympic winter games in Nagano 1998 (Figure-1). The mean values for steroids in urine of this multiracial

uncontrolled population were around -20‰, but the highest frequency was located at -21 or -22‰ for most of the individual steroids. The detailed results are given as table in our previous report.[4] Constituents of the population, chronic difference of athlete's diet in the pre-testing period, potential positive results with undetected steroids, and large variations of concentrations of the target steroids etc. probably cause the skewness of the histograms. Such a tendency was notable especially in the 5a-diol distributions.

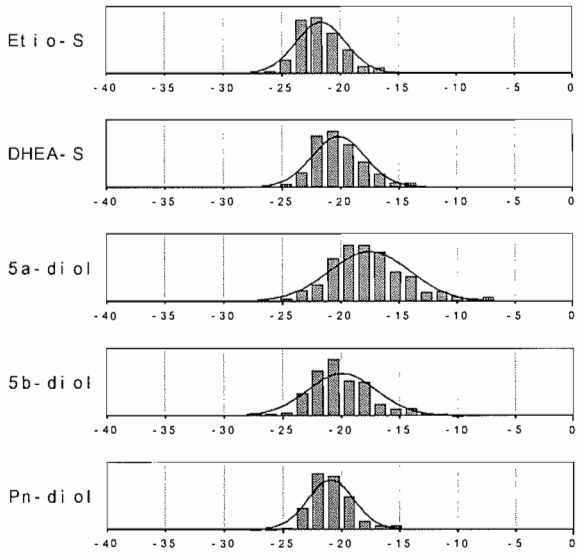


Figure 1 Distributions of CIRs of diol glucuronides and sulfated keto steroids.

Isotope abundance of steroids has been measured on 443 urine samples from Olympic athletes. No sex difference was found. Histograms are drawn for each steroid. Samples with any sign of drug use were rejected from the population.

As shown in Figure-1, the best stable parameter was a CIR for pregnanediol(Pn-diol), which could not be converted from androgens, and this observation supported usefulness of Pn-diol as an endogenous reference steroid (ERS).

Distributions of CIR for the major classes of plant groups[9),11),12)], synthetic steroids from pharmaceutical products[4)], $CO_2$ from expired breath gases, and Andro and Etio in a total fraction of human urine samples were shown as the histograms in Figure-2. Reported CIRs for C4-plants and algae are far more enriched than C3-plants. Mean CIRs for free synthetic steroids and esters were -30.2 and -29.9‰ respectively, and the whole group was -30.1±2.6‰.[4)] For breath gas samples, the mean CIR for 154 Japanese adults was -23.3±0.8‰ and appeared 1 to 2 units more negative and far less-scattered than that for steroids. Schöller et al. reported that CIR for plasma lipids of North American subjects was -21.6±1.0‰.[16)] They have not seen any difference of CIRs for lipid fraction in the diet or in plasma. It is because ***in vivo*** isotopic fractionation of carbon in humans is small. CIR for breath gas reflects an average CIR of three macronutrients taken, since the carbon source is

fuel being oxidized. In other words, the CIR can be used as a marker to indicate inter-population difference of CIR caused by diet. Lacroix et al. reported that it would take weeks or months to establish isotopic equilibrium under controlled feeding[17]. When considering the small diet-induced variations of CIR for the breath gas, the deviation of steroidal CIR, depending on diet in a single population, is expected to be small. The acute change of CIR of steroids is probably occurring as a result of unusual diet such as heavy supplementation, medication, doping etc. by individuals.

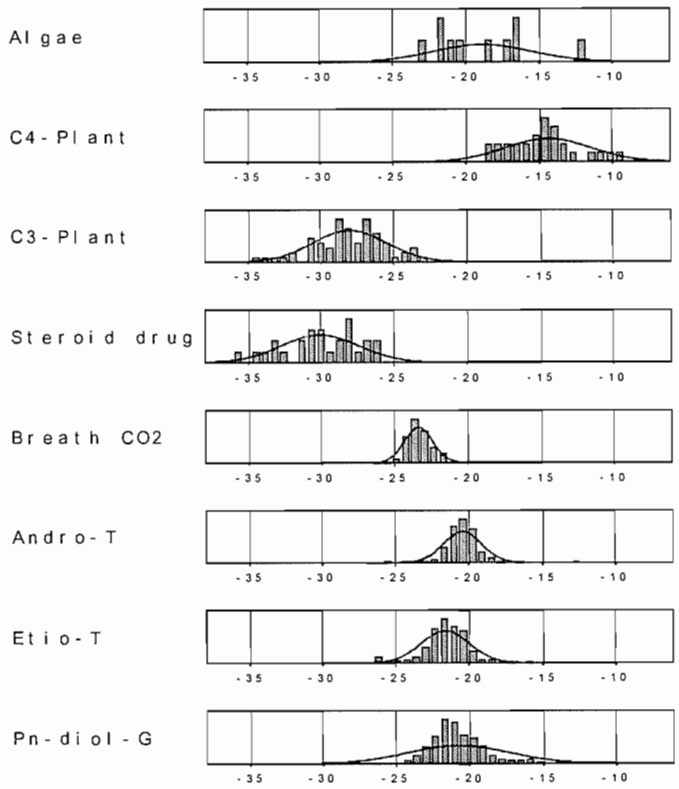


Figure-2 Isotope abundance of various carbon sources

Typical plant classes have their diagnostic isotope abundance. Certain C3-plants produce phytosterols, which are used as a source for synthetic steroids, thus isotope abundance of pharmaceutical steroid is identical to the plant sources.
Suffix T:Total, S:Sulfate, G:Glucuronide.
The values were corrected for derivatization when applicable.

The distributions of CIR for urinary Andro and Etio of total fractions (Andr-T, Etio-T) of normal Japanese adults which include 77 females and 90 males are also shown in Figure-2. The reference values obtained by a parametric calculation are –20.4±0.85‰ and –21.5±1.29‰ respectively. The mean CIR for Etio-T was slightly more scattered due to the presence of a small co-eluted peak in front of Etio on the ion traces under screening conditions. However, the influence of this interference is practically negligible. Although distributions of CIR in the international populations were more scattered than for the Japanese as shown in Figure-1 and -2, they are not significantly different from each other, regardless of whether the values were obtained after derivatization, by the use of different conjugate fractions, or for steroids with hydroxyl- or keto group, both in females and in males.

**Ring Test Samples**

The results of the inter-laboratory ring test on isotopic measurement are summarized in Table-1. A calcium carbonate packed in dry helium for calibration checks, and a hydrolyzed extract of DHEA sulfate for comparison of overall performance, were provided to 11 international laboratories. Five of the laboratories did not return results since their

**Table-1 Summary of inter-laboratory ring test on isotopic measurements**

| Lab. No. | Referencing | $CaCO_3$ | DHEA-Acetate | Acetate | DHEA | DHEA corr. |
|---|---|---|---|---|---|---|
| 1 | Alkanes | -5.50 | none | none | -23.94 | -23.67 |
| 2 | NIST | -4.90 | none | none | -20.50 | -20.83 |
| 3 *) | NBS-22 | -5.77 | none | none | -26.46 | -25.92 |
| 4 | NBS-19 | -5.23 | -24.10 | -35.33 | -22.90 | -22.90 |
| 5 | NBS-19/-18 | -5.15 | -24.41 | -48.17 | -21.90 | -21.98 |
| 6 | unknown | N/A | -23.70 | -50.10 | -20.90 | N/A |
| | Mean | -5.31 | -24.07 | -44.53 | -22.77 | -23.06 |
| | | | | | -22.03 | -22.35 |
| | SDn-1 | 0.33 | | | 2.21 | 1.92 |
| | | | | | 1.42 | 1.22 |
| | CV(%) | 6.3 | | | 9.7 | 8.3 |
| | | | | | 6.4 | 5.5 |

Acetate: Acetic anhydride *) DHEA: Overlapped peak N/A:Not applicable

facility was not equipped with a gas analysis system, but five of the other laboratories have reported satisfactory results on the calcium carbonate. Three of the participated laboratories do not focus on DHEA under a routine basis so that one laboratory detected DHEA as an overlapped peak. The results for DHEA were therefore evaluated before and after rejection of an outlying data point. Three laboratories measured DHEA as the acetate. CIR of the acetylating reagent was -35.33 to -50.10‰. In these cases, the negative bias caused by introduced foreign carbon from acetylating reagent was 1 to 3 units. According to the reports by Aguilera et al., the mean CIR of androstanediol-bis-acetates[5] and that of androstane-mono-ol-acetates[18] in the urine of American residents were about -26.0 and -22.5‰ respectively. Matsumoto et al. reported on sterols in sediments[19] that a large variation of isotope abundances of acetylating reagents exists, and the values range between -35.3 and -161.8‰ (-40.0±5.6‰‰). They could see a significant negative bias caused by acetylation and the bias was estimated at -2‰ per derivatizable function. The other three laboratories measured DHEA without any chemical transformation, but the reported results

except one outlier were in good agreement with that for the acetate. When the reported CIR for DHEA was further corrected for referencing methods by the reported CIR for calcium carbonate as a correction factor, the variation was slightly improved from -22.03±1.42‰ with CV=6.4% to -22.35±1.22‰ with CV=5.5%.

The overall results of the ring test suggest that uncertainty of the measurement is mainly caused by a peak purity, bias from introduced foreign carbon, and the referencing methods[20]. The use of common reference materials for GC/C/IRMS, such as a steroid with certified isotope abundance, and proper correction for derivatization may improve the traceability toward the use of absolute CIR. Even though the reported results on ring test samples seemed to be quite reasonable and they supported a possible use of absolute CIR as a threshold within the participating laboratories, inter-laboratory differences of the measurement still seemed to be far greater than intra-laboratory uncertainty, when considering the reference values that have been published in several recent reports.[2)-6)] Thus, the use of absolute CIR threshold, before harmonization of the procedure, could disable the sensitive detection of natural hormone doping. Furthermore, most of the laboratories measure a few testosterone metabolites only and do not target the precursor steroids. Under these instances, concentrations of steroids, namely, steroid profiles should be evaluated together with CIRs of the steroids of interest.

Table-2 shows nine confirmed or suspected cases of doping with naturally occurring steroids. They have been selected from 2,120 measurements based on CIRs of the urinary Andro-T and Etio-T. The initial cut-offs for further considerations were CIR<-24 or relative CIR: target/ERS>1.13. For case-1 listed in Table-2, the concentration of Etio-T was suppressed and 5a-diol was slightly elevated. However, the steroid profile was not suspicious. On the other hand, CIR for Etio-T was significantly negative, and ratios to ERS for DHEA-S, 5a- and 5b-diols were above our reference range. The results on this case were supported by the declaration of DHEA use on the form. Cases 2 to 7, except case-5, were also finally considered positive for DHEA because CIR for DHEA and the target compounds were under the reference ranges. In steroid profiles of these cases, concentrations of Andro-T, Etio-T, etc. were partially but not extensively elevated, so that no conclusive

Table-2 CIR for athletes with abnormal steroid profiles screened from 2,120 samples (Concentration: Total)

| Category | SEX | T | ET | Andro | Etio | 5a-diol | 5b-diol | EA | DHEA | T/ET | Andro/ Etio | 5a-/5b-diols |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.Keirin | M | 5.1 | 21.6 | 2,104 | 990 | 336.0 | 184.4 | 4.1 | 202 | 0.2 | 2.1 | 1.8 |
| 2.Bodybuilding | M | 3.5 | 33.2 | 10,860 | 17,100 | 550.4 | 389.1 | 52.1 | 1,635 | 0.1 | 0.6 | 1.4 |
| 3.Athletics | F | 111.0 | 45.3 | 19,040 | 4,442 | 76.3 | 80.5 | 5.5 | 193 | 2.5 | 4.3 | 0.9 |
| 4.Bodybuilding | M | 4.8 | 16.8 | 7,522 | 8,858 | 258.4 | 359.0 | 471.8 | 4,037 | 0.3 | 0.8 | 0.7 |
| 5.Bodybuilding | M | 11.3 | 74.3 | 2,101 | 12,098 | 137.8 | 449.7 | 195.7 | 493 | 0.2 | 0.2 | 0.3 |
| 6.Bodybuilding | M | 28.7 | 21.3 | 2,007 | 3,754 | 34.1 | 146.4 | 42.8 | 2,419 | 1.3 | 0.5 | 0.2 |
| 7.Bodybuilding | M | 63.8 | 47.7 | 7,189 | 24,170 | 150.2 | 1739.0 | 147.1 | 7,477 | 1.3 | 0.3 | 0.1 |
| 8.IOC Test 200( | - | 661.4 | 28.5 | 6,111 | 5,377 | 268.2 | 644.0 | 58.2 | 840 | 23.2 | 1.1 | 0.4 |
| 9.Volleyball | M | 155.4 | 301.4 | 6,175 | 4,367 | 161.7 | 795.7 | 61.6 | 782 | 0.5 | 1.4 | 0.2 |

**Abbreviations**

T: testosterone
ET: epitestosterone
others: see text

**Units**

Concentration: ng/ml
CIR: $\delta^{13}C$ in ‰

(Absolute and relative CIR)

| Pn-diol | Andro-T | Etio-T | 5a-diol | 5b-diol | DHEA-S | DHEA/ Pn-diol | 5a-diol/ Pn-diol | 5b-diol/ Pn-diol |
|---|---|---|---|---|---|---|---|---|
| -19.9 | -22.7 | -26.5 | -22.7 | -23.2 | -23.8 | 1.20 | 1.14 | 1.17 |
| -20.1 | -30.0 | -29.8 | -30.6 | -31.0 | -27.9 | 1.39 | 1.52 | 1.54 |
| -18.6 | -29.8 | -30.1 | -29.8 | -30.6 | -24.6 | 1.32 | 1.60 | 1.65 |
| -19.2 | -29.0 | -29.4 | -29.2 | -29.1 | -25.0 | 1.30 | 1.52 | 1.52 |
| -18.0 | -29.4 | -30.6 | -30.0 | -29.5 | -14.7 | 0.82 | 1.67 | 1.64 |
| - | -25.8 | -27.0 | -23.8 | -22.7 | -25.4 | - | - | - |
| -15.7 | -28.1 | -29.5 | -26.7 | -28.6 | -30.3 | 1.93 | 1.70 | 1.82 |
| -22.1 | -25.7 | -26.0 | -25.4 | -26.8 | -21.8 | 0.99 | 1.15 | 1.21 |
| -21.5 | -22.4 | -21.9 | -22.1 | -21.3 | -20.2 | 0.94 | 1.03 | 0.99 |

For abusolute CIR, steroids with suffix:"T": total fraction (screening), "S":sulfate (confirmation), others: glucuronide (confirmation).

evidence that can identify the parent compound was obtained from the concentrations. High concentrations of Etio and EA were the signs in case-5, and identification of the administered compound was only possible by the measurement of CIR. According to our unpublished data, administration of androstenedione moderately raises DHEA concentration, but the CIR of DHEA in the same sample does not significantly move to negative since the metabolism of androstenedione back to DHEA is minor. Abnormal CIRs on case-5 were therefore considered as the results of androstenedione administration. Elevated T/ET with a high testosterone concentration in an IOC test sample apparently confirms doping according to the presented non-isotopic criteria, but the differentiation of T from any of the precursors comes into reality by means of GC/C/IRMS. The last case with a high ET concentration was initially suspected for epitestosterone administration, but no sign of doping was found by isotopic analysis, thus the case was considered as naturally elevated epitestosterone.

**Summary**

CIR of 26 synthetic steroids, urinary steroids, and $CO_2$ in breath gases has been measured. A clear and significant difference between the average CIRs of pharmaceutical and endogenous steroids was confirmed. The mean value for synthetic steroids agreed well with the reported CIRs for C3-plants, and they are supposed to be of plant origin. The variability of a usual diet probably does not result in an acute change of the base CIR level in an individual, because the distribution of isotope abundance of $CO_2$ in the breath gas samples was stable, and appeared less scattered than that for urinary steroids. Reported results on a ring test sample were found to produce a larger inter-laboratory scatter than expected. The cause of one outlying data point is attributed not to the referencing method of MS but clearly to the peak separation of the chromatography. In conclusion, our study showed a goal of isotope analysis using absolute and relative isotope abundances for better detectability of doping. However, validated in-house criteria may be used in each laboratory for the time being since anti-doping laboratories are individually adopting different referencing methods. Practically, the relative isotopic parameters, i.e. ratios target/precursor or target/ERS of CIRs, may be used till the methods have been harmonized, since relative values absorb the

difference among laboratories, thus enabling inter-laboratory comparison of the test results.

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