Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (11)

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(Editors)

Sport und Buch Strauß, Köln, 2003

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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (11). Sport und Buch Strauß, Köln, (2003) 183-193

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Preliminary results on the carbon isotope ratios of ketonic steroids in urine samples collected from different countries

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Introduction

Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) is a technique which has been adopted as a means of detecting and confirming the abuse of anabolic steroids which occur naturally in the body such as testosterone (Horning et al 1997). Whilst the administered steroid is chemically identical to that produced in the body, the ratio of ^{13}C to ^{12}C in the synthetic products is slightly different. Carbon isotope ratios are expressed in terms of $\delta^{13}C$ values (Craig 1957), with units in per mille (‰), where:

$$\delta^{13}C = [(r_{sample}/r_{std}) - 1] \times 1000$$

The differences arise because synthetic steroids are derived from stigmasterol obtained from soy (Coppen 1979), a C-3 plant that results in synthetic testosterone $\delta^{13}C$ values in the range -25.9 to -32.8‰ (de la Torre et al 2001). The carbon isotopic composition in humans reflects that in the diet which is normally based on a mixture of C-3 and C-4 plants (Smith and Epstein 1971). Because C-4 plants tend to predominate in the diet and are enriched in ^{13}C compared to C-3 plants the resulting gonadal steroids have less negative $\delta^{13}C$ values than their synthetic analogues. It is this difference which forms the basis of using isotopic composition for sports drug testing. It is prudent to compare the $\delta^{13}C$ value of the suspected synthetic steroid or its metabolites with the $\delta^{13}C$ value of a steroid precursor which would be unaffected by the administration of the synthetic steroid. Such precursors are called endogenous reference compounds (ERC's). Initial

studies (Shackleton et al 1997) used diol metabolites but this procedure requires extensive cleanup prior to GC-C-IRMS analysis. Since 1999 ASDTL has been measuring the $\delta^{13}C$ values of androsterone and etiocholanolone which are normally the dominant steroids present in high concentration in the urine and for which only a simple SPE extraction is needed prior to GC-C-IRMS analysis. In early 2000 as part of the EPO2000 project blood and urine samples were collected from some 1200 elite athletes around the world. The ethics approval (AIS 1999) for this project included the analysis of the urine samples for steroids. This project was designed to investigate the variability in $\delta^{13}C$ values throughout the world, which are presumed to be primarily related to diet. The results obtained for a subset of these samples are presented here.

Experimental

Urine samples (2 mL) were adjusted to pH 7.0 by the addition of phosphate buffer (0.2 M, 1.5 mL) before enzyme hydrolysis with β-glucuronidase from *E.Coli* K12 (50 μL) for 1.5 hours at 50°C. The hydrolysed steroid metabolites were then isolated from the urine matrix using a SPE method using BondElut® Certify (C8/SCX) columns and a vacuum manifold. The procedure was:

- Condition cartridge with MeOH (2 mL) and water (2 mL)
- Load urine hydrolysate
- Wash with water (2 mL) and MeOH/water (10:90, 2 mL)
- Vacuum dry 30 minutes
- Wash with hexane and dry 60 minutes
- Elute with ethyl acetate/MeOH (95:5, 2 mL)

17α-methyltestosterone (17-MeT) (120 μg/mL, 50 μL) was added to the steroid fraction before it was evaporated to dryness in a Turbovap (1.5 hours at 35°C and 20 psi.). The dry residue was stored in a desiccator under vacuum for 12 hours before being reconstituted with dichloromethane (100 μL) and transferred to a vial for GC-C-IRMS analysis.

The system used for GC-C-IRMS was a Finnigan-MAT Delta Plus with a Hewlett-Packard 6890 GC for separation of steroid metabolites. The carrier gas was helium with a constant flow rate of 3 mL min^{-1}. The injection volume was 2 µL in splitless mode at 280°C. The column (0.25 mm I.D x 30 m) was an Agilent HP-50+ cross-linked 50% phenyl-methyl siloxane (0.25 µm film thickness). The column temperature was programmed from 180°C for 1 min to 250°C at 12°C min^{-1}, to 280°C at 3°C min^{-1}, then finally to 300°C at 15°C min^{-1} and held for 4 min. The Thermo-Finnigan GC Combustion III interface was used with an oxidation reactor temperature of 940°C. High purity O_2 gas was flushed through the furnace for 3600 seconds prior to analysis of a sequence. The reduction reactor temperature was 620°C. The software operating the IRMS system was ISODAT 7.4.

Results and Discussion

For each sample analysed the $\delta^{13}C$ values are calculated for the peaks corresponding to androsterone (A), etiocholanolone (Et) and 11-ketoetiocholanolone (Fig 1). 11-ketoetiocholanolone (11-keto) is a metabolite resulting from part of the corticosteroid pathway (Gower 1975), separate to that of the androgens and as such may be used as an ERC. It is present in all urine samples analysed but with widely varying concentrations ranging from barely detectable in a few samples to more than androsterone in others. Typical abundances range from 30 to 50% of the androsterone concentration using our extraction procedure. The $\delta^{13}C$ value of 11-keto has not been seen to change with administrations of androstenedione (Rogerson et al 1999) or dehydroepiandrosterone (DHEA) (Trout et al 2003). Under the chromatographic conditions 11-keto coelutes with pregnanediol, another ERC (Shackleton et al 1997), but the amount of 11-keto is always significantly higher in our procedure. The most significant effect of this coelution will be to increase the variability of the $\delta^{13}C$ values found for the 11-keto peak as 11-keto and pregnanediol may have slightly different $\delta^{13}C$ values but both reflect the natural $\delta^{13}C$ values. Work is currently underway to determine what, if any, difference exists between $\delta^{13}C$ values of 11-keto and pregnanediol.

The method was used from December 2000 for routine samples with all samples meeting any of the following criteria being analysed:

- Androsterone (A) or Etiocholanolone (Et) > 5000ng/mL
- T/E > 3
- DHEA > 100ng/mL
- epi-T > 200ng/mL
- suspicious for androstenedione

However since 2002 this practice has been discontinued owing to a lack of funding and now only samples with a T/E > 6 or those with gross abnormalities in steroid profile are subject to GC-C-IRMS analysis.

The criteria that have been used to establish if a sample is positive are those set out below. All must be met for a sample to be declared positive.

1. The difference between the average of δ^{13}C A and δ^{13}C Et values, and δ^{13}C 11-keto must be greater than 4.0‰.
2. The ratio must be greater than 1.15.
3. δ^{13}C A and δ^{13}C Et must be more negative than -27.0‰.

The results obtained for the samples collected from Australia, New Zealand, China and Kenya are presented here. From dietary patterns it could be expected that Australia and New Zealand would be similar, with China and Kenya being different to Australia and New Zealand, and to each other. The distribution of the δ^{13}C values found for androsterone in the New Zealand samples are shown in Figure 1 along with the distribution of values found for the internal standard 17-MeT. The tighter distribution found for the internal standard indicates that the variation in δ^{13}C values found for androsterone is due to the population and not merely to the instrument variability. The certified value for 17-MeT determined by combustion analysis was -32.80‰, the mean value found was -32.42‰ (n>700), with a SD of 0.90.

The δ^{13}C values found for the androsterone in the samples from China and Kenya are shown in Figures 2 and 3 respectively. These show a similar spread of values to that found for New Zealand. However the means for each country are different with Kenya

having the least negative values. This agrees with expectations relating to the high proportion of maize (a C-4 plant) in the diet.

The distributions of A and Et were similar to each other for each country whilst the 11-keto results were significantly less negative than either the A and Et results. These differences and similarities are shown in Figure 4 using the results from New Zealand. A summary of the findings for all countries is set out in Table 1.

Table 1. Summary of results obtained from each country.

Country	Number	Mean Etio	Mean Andro	Mean 11-keto	Difference	Ratio
Australia	59	-23.0 SD 0.97 Min -25.3	-22.5 SD 1.16 Min -24.6	-21.1 SD 1.29 Min -24.1	1.7	1.08
New Zealand	108	-23.3 SD 1.07 Min -25.8	-23.0 SD 1.16 Min -25.1	-20.9 SD 1.21 Min -23.8	2.2	1.11
China	157	-22.5 SD 1.29 Min -25.0	-21.6 SD 1.53 Min -25.0	-20.1 SD 1.59 Min -24.0	2.0	1.10
Kenya	127	-20.5 SD 1.53 Min -24.0	-20.3 SD 1.38 Min -23.0	-18.6 SD 1.81 Min -24.0	1.8	1.10

The data can be briefly summarised as follows:

- The $\delta^{13}C$ values obtained for A, Et and 11-keto were found by ANOVA analysis to be significantly different ($p<0.0001$) for Kenya, China, and Australia/New Zealand.
- The distributions are close to normal with some differences in standard deviations for each country.
- There is a consistent difference of approximately 2.0‰ between the average of A and Et, and 11-keto.
- The most negative $\delta^{13}C$ value found for A is -25.1‰ and for Et is -25.8‰.

The results have a number of implications for doping control. Firstly, an absolute cut-off of -27.0‰ is supported both by the lowest value found being -25.8‰ and by the mean and standard deviation results. The lowest calculated $\delta^{13}C$ value (mean minus 3σ) is -26.5‰ for New Zealand. The $\delta^{13}C$ values for Australia, China and Kenya are ^{13}C enriched in comparison.

Secondly, the use of ratios of $\delta^{13}C$ values is not appropriate as the ratio found varies with the $\delta^{13}C$ value of the ERC. Table 2 shows how subjects with the same difference in $\delta^{13}C$ value of 4.0‰ between metabolites and endogenous reference compound give different ratios. In fact a ratio of 1.15 can be obtained with a difference of only 3.0‰ for a subject with a marker more enriched in ^{13}C. The table also shows that using a ratio of 1.15 in combination with a difference of 4.0‰ adds no additional information, as any sample with a difference of 4.0‰ will by the nature of the numbers involved automatically have a ratio greater than 1.15.

Table 2. Effect of marker $\delta^{13}C$ value on calculated ratios.

Steroid metabolite	Marker	Diff.	Ratio
-30	-26	4	1.154
-28	-24	4	1.167
-26	-22	4	1.182
-24	-20	4	1.200
-22	-18	4	1.222
-23	-20	3	1.150

There is also the consideration of whether a difference of 4.0‰ between the average $\delta^{13}C$ value of A and Et and $\delta^{13}C$ 11-keto is adequate proof of doping by itself. For this to apply there would be no samples in our study that had averaged differences of 4.0‰ or greater. However in the 451 samples there were 25 occasions when the difference was 4.0‰ or greater. The large differences occurred when $\delta^{13}C$ values of 11-keto were substantially ^{13}C enriched and were more common in the samples from China and Kenya. None of these samples were declared positive by our criteria as the $\delta^{13}C$ A and $\delta^{13}C$ Et values were less negative than -27.0‰. From the data so far no false positives would result from applying an averaged difference of 6.0‰.

The requirement for $\delta^{13}C$ values of A and Et to be more negative than -27.0‰ has been shown to be appropriate for the Australian and New Zealand groups but is not appropriate for Kenya. In the Kenyan group the most negative (ie. ^{13}C depleted) $\delta^{13}C$ value of androsterone found was -23.0‰ and mean minus 3σ was -24.5‰. Thus a difference of 4.0‰ coupled with a requirement for the $\delta^{13}C$ A value to be more negative than -25.0‰ would be more appropriate for this population. The results from Kenya indicate that there will be effects on the detection of doping with synthetic steroids relating to the less negative $\delta^{13}C$ values. It would be expected to find bigger differences in $\delta^{13}C$ values between metabolites and ERC's for those with naturally less negative $\delta^{13}C$ values. The $\delta^{13}C$ values expected from the administration of a synthetic analogue of an endogenous steroid are shown in Table 3. The assumptions made in the calculations are:

- A steroid with a $\delta^{13}C$ value of -30.0‰ has been ingested by subjects with natural $\delta^{13}C$ values of androsterone of -24.0‰, -21.0‰, and -18.0‰.
- A linear relationship exists between the $\delta^{13}C$ value of the excreted androsterone and the proportion coming from the ingested steroid.

Table 3. Calculated effects of synthetic steroid administration on delta values.

A $\delta^{13}C$	11-keto δ13C	Exogenous proportion	Calculated delta value	Difference
-24	-22	0.8	-28.8	6.8
		0.6	-27.6	5.6
		0.4	-26.4	4.4
		0.2	-25.2	3.2
-21	-19	0.8	-28.2	9.2
		0.6	-26.4	7.4
		0.4	-24.6	5.6
		0.2	-22.8	3.8
-18	-16	0.8	-27.6	11.6
		0.6	-25.2	9.2
		0.4	-22.8	6.8
		0.2	-20.4	4.4

It is apparent that a subject with a natural androsterone $\delta^{13}C$ value of -24.0‰ who takes a synthetic steroid will be detected by our criteria (averaged difference > 4.0‰ and $\delta^{13}C$ A < -27.0‰) when approximately 50% of the excreted androsterone is from the synthetic steroid. However should the same proportion be excreted by a subject with a natural androsterone $\delta^{13}C$ value of -18.0‰ the resultant values (averaged difference = 8.0‰ and $\delta^{13}C$ A value of -24.0‰) would not result in a positive finding. This is despite the difference being equal to the maximum theoretically achievable by a subject with a natural $\delta^{13}C$ A value of -24.0‰. For a subject with a natural $\delta^{13}C$ A value of -18.0‰ to achieve an excreted androsterone value of -27.0‰ almost 80% of the androsterone would be required to come from the synthetic steroid. The corresponding averaged difference is 11.0‰, which is double that of any observed difference in the population studies. It can be concluded that whilst an absolute cutoff of -27.0‰ may be appropriate for the Australian and New Zealand populations it is not appropriate for subjects from Kenya with less negative natural $\delta^{13}C$ values. If such subjects were to inject testosterone then the change in $\delta^{13}C$ values for a given dose will be much more pronounced than for an Australian subject but the resultant $\delta^{13}C$ value will obviously not be as negative. A more general rule to define doping could be a difference of 6.0‰ or greater, or a difference of 4.0‰ or greater provided the $\delta^{13}C$ values of A and Et are more negative than -27.0‰.

Conclusions

The results clearly show that country of residence and hence presumably diet has a major impact on the measured isotope ratios. The data supports the application of an absolute cutoff value of -27.0‰ or perhaps -26.0‰. Current criteria applied in ASDTL to define endogenous steroid doping should be reviewed. For instance, the ratio measurement gives no additional information and should not be used. The requirement for an averaged difference of 4.0‰ and androgen metabolite $\delta^{13}C$ values more negative than -27.0‰ is not appropriate for those with natural $\delta^{13}C$ values less negative than -22.0‰. An averaged difference of greater than 6.0‰ should be considered as sufficient proof of doping in such cases. It is envisaged that a statistical analysis of the complete data set

will provide a more comprehensive set of criteria involving both differences and absolute $\delta^{13}C$ values.

Acknowledgments

Thanks go to all the athletes who participated in this study and to the sporting organisations around the world whose assistance was invaluable. The funds to carry out this project were provided by the Australian Government as part of their Backing Australia's Sporting Ability program.

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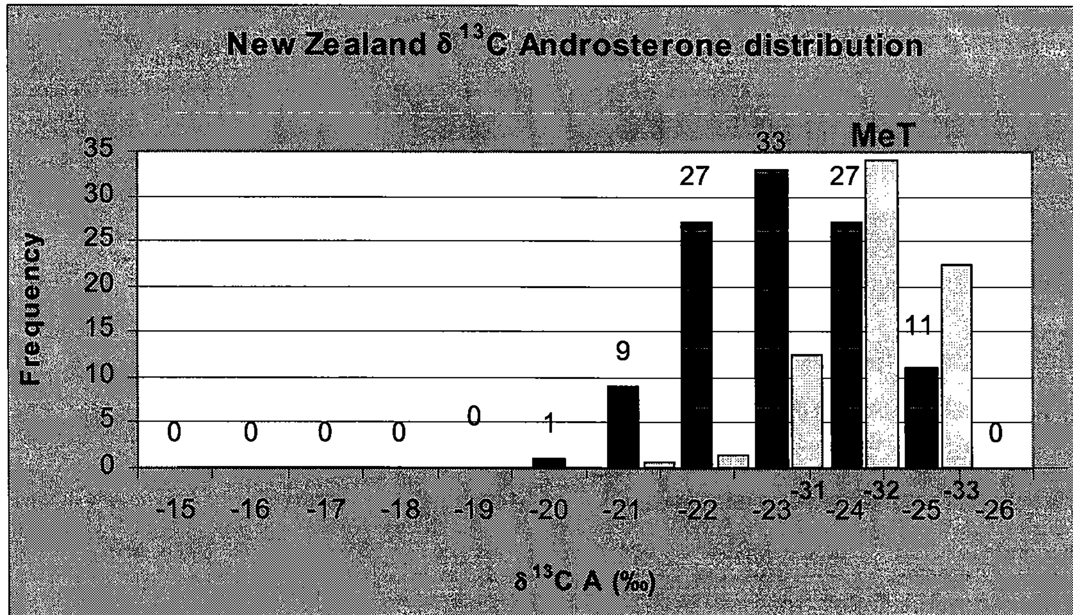

Figure 1. $\delta^{13}C$ values of Androsterone in samples collected in New Zealand.

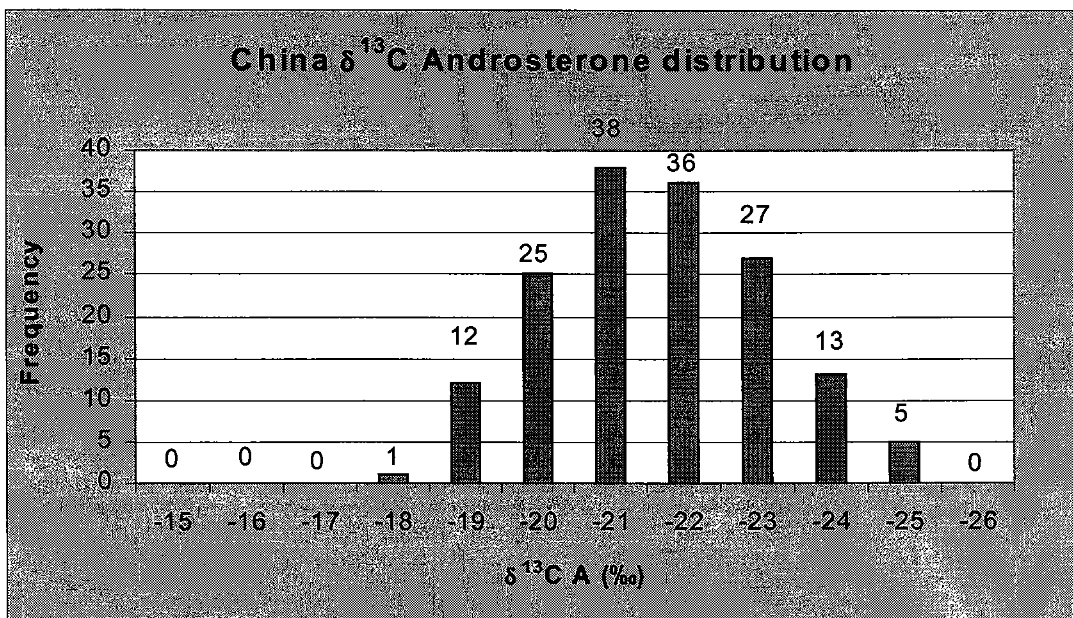

Figure 2. $\delta^{13}C$ values of Androsterone in samples collected in China.

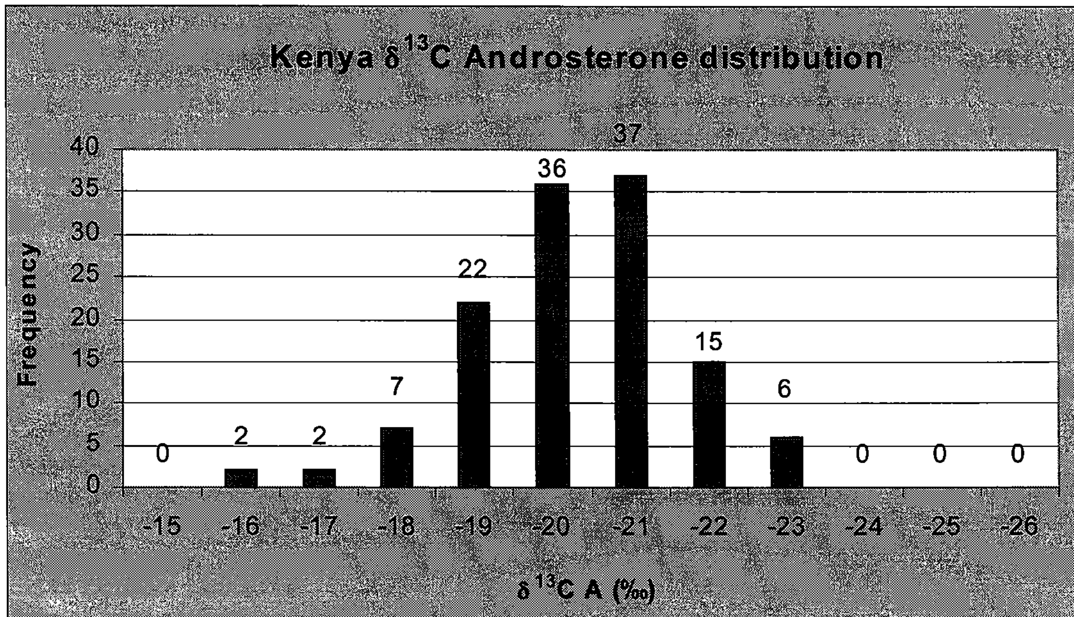

Figure 3. $\delta^{13}C$ values of Androsterone in samples collected in Kenya.

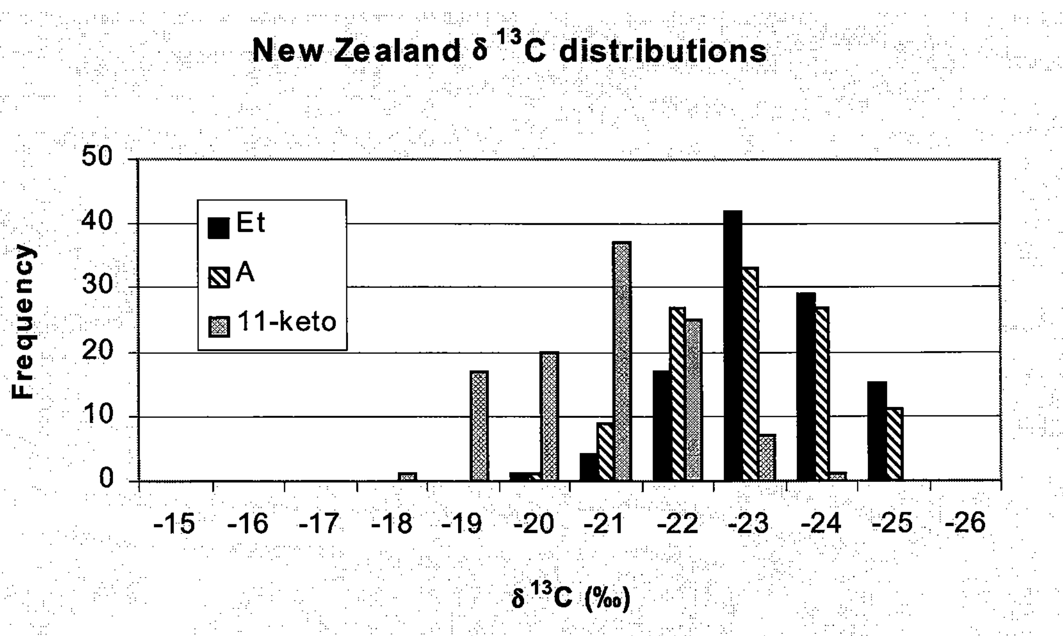

Figure 4. $\delta^{13}C$ values of Androsterone, Etiocholanolone and 11-ketoetiocholanolone in samples collected in New Zealand.