Nirut Suknet, Sutheema Nimsoongnern, Prapin Wilairat, Thanit Kusamran and Tongtavuch Anukarahanonta

**Preliminary results on the carbon isotope ratios of endogenous steroids in urine collected from Asian countries**

National Doping Control Centre, Mahidol University, Bangkok, Thailand

**Introduction**

Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GC/C/IRMS) is a technique to detect and confirm the abuse of endogenous anabolic steroids. The administered steroids are chemically identical to that produced in the body, but the ratio of $^{13}$C to $^{12}$C of the synthetic products may be different due to the source of carbon. Carbon isotope ratio is expressed in term of $\delta^{13}$C values (1), with unit in per mil. Variation in the reference range of the $\delta^{13}$C value of the endogenous steroids is due to the effect of diet (2).

This work reports the variability in the $\delta^{13}$C values of Asian athletes, who took part in the 1st Asian Indoor Games held in Bangkok, Thailand, 12 – 19 November 2005.

**Experimental**

**Sample Preparation**

Urine samples (5 ml) were applied onto C-18 cartridge (Sep-Pak®), eluted with methanol and then dried. The residues were reconstituted in 1.0 ml buffer (pH 7.0) and free-form steroids in the aliquots extracted with $t$-butyl methyl ether. After addition of the ISTD (5α-androstan-3β-ol), the aqueous fractions were hydrolyzed by β-glucuronidase (E. coli) for 1 hr at 55°C. After extraction by n-pentane, the aliquots were evaporated to dryness, acetylated by acetic anhydride in the presence of pyridine, re-evaporated to dryness, and reconstituted in cyclohexane (50 µl).

The acetylated aliquots (2 µl) were injected for GC/C/IRMS analysis and another 2 µl aliquots were injected for GC/MS analysis in order to identify the steroids.
**Instrumentation**

GC/C/IRMS analyses were performed on an HP 6890 GC connected to Micromass Isoprime IRMS. The GC was equipped with CP-Sil 24 CB column (Chrompack, 30 m x 0.25 mm i.d. x 0.25 µm). Helium was the carrier gas at constant flow of 1.5 ml/min. The GC temperature program was initial at 160°C, then 20°C/min to 270°C, 2°C/min to 290°C, 5°C/min to 300°C and held for 8 min. GC/MS analyses were performed on an Agilent 6890N GC / 5973N MSD.

**Results and Discussion**

The $\delta^{13}$C values of the following endogenous steroids were measured: Etiocholanolone (Etio), Androsterone (Andro), 5β-androstan-3α,17β-diol (5β-diol), 5α-androstan-3α,17β-diol (5α-diol), 11-Ketoetiocholanolone (11-Keto), Pregandiol (P2) and Pregantriol (P3). 11-ketoetiocholanolone is the endogenous reference compound (ERC) for androsterone and etiocholanolone (one OH-group) and pregnandiol is ERC for 5β-androstandiol and 5α-androstandiol (two OH-groups). All the $\delta^{13}$C values were corrected for the derivatization using the equation of D.M. Johnes. (3)

The results obtained from the 1st Asian Indoor Games are shown in Table 1 and 2 and Figures 1 – 6.

**Table 1.** Summary of results obtained from the 1st Asian Indoor Games

<table>
<thead>
<tr>
<th>Delta value ($\delta^{13}$ (‰)) (n = 189)</th>
<th>Etio</th>
<th>Andro</th>
<th>5β-diol</th>
<th>5α-diol</th>
<th>11-Keto</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>1.09</td>
<td>0.96</td>
<td>1.43</td>
<td>1.02</td>
<td>1.15</td>
<td>1.21</td>
<td>1.14</td>
</tr>
</tbody>
</table>

**Table 2.** Summary of the difference obtained from the 1st Asian Indoor Games

<table>
<thead>
<tr>
<th>Difference ($\delta^{13}$ (‰))</th>
<th>Etio</th>
<th>Andro</th>
<th>5β-diol</th>
<th>5α-diol</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>1.06</td>
<td>2.19</td>
<td>0.41</td>
<td>-1.30</td>
</tr>
</tbody>
</table>
From Figure 1 and 2, the mode values of $\delta^{13}$C value for etiocholanolone, androsterone, 11-ketoetiocholanolone, 5$\beta$-androstandiol, 5$\alpha$-androstandiol and pregnandiol are -21 ‰, -21 ‰, -23 ‰, -22 ‰, -24 ‰ and -22 ‰, respectively. All $\delta^{13}$C value measured ($n = 189$) are less than -28.0 ‰$^{(2,4)}$, the cut-off value for a possible positive sample. The difference of $\delta^{13}$C value between endogenous steroid and ERC (Table 2) is less than 3 delta unit$^{(2,4)}$, the cut-off value for a possible positive sample.

All samples, from the 1st Asian Indoor Games are negative according to the WADA directive$^{(4)}$, whereby all criteria must be met for a sample to be declared positive.

**Conclusions**

In the 1st Asian Indoor Games Bangkok 2005, there were 45 participating nations. The data are representative of the $\delta^{13}$C values for athletes from Asia.

**References**

Figure 1. The distribution of δ\textsuperscript{13}C values of etiocholanolone, androsterone and 11-ketoetiocholanolone.

Figure 2. The distribution of δ\textsuperscript{13}C values of 5β-androstandiol, 5α-androstandiol and pregnandiol.