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

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### **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  $^{13}\text{C}$  to  $^{12}\text{C}$  of the synthetic products may be different due to the source of carbon. Carbon isotope ratio is expressed in term of  $\delta^{13}\text{C}$  values <sup>(1)</sup>, with unit in per mil. Variation in the reference range of the  $\delta^{13}\text{C}$  value of the endogenous steroids is due to the effect of diet <sup>(2)</sup>.

This work reports the variability in the  $\delta^{13}\text{C}$  values of Asian athletes, who took part in the 1<sup>st</sup> 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<sup>®</sup>), 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 (5 $\alpha$ -androstan-3 $\beta$ -ol), the aqueous fractions were hydrolyzed by  $\beta$ -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  $\mu\text{l}$ ).

The acetylated aliquots (2  $\mu\text{l}$ ) were injected for GC/C/IRMS analysis and another 2  $\mu\text{l}$  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  $\mu$ m). 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  $\delta^{13}\text{C}$  values of the following endogenous steroids were measured: Etiocholanolone (Etio), Androsterone (Andro), 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (5 $\beta$ -diol), 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (5 $\alpha$ -diol), 11-Ketoetiocholanolone (11-Keto), Pregnandiol (P2) and Pregnantriol (P3). 11-ketoetiocholanolone is the endogenous reference compound (ERC) for androsterone and etiocholanolone (one OH-group) and pregnandiol is ERC for 5 $\beta$ -androstadiol and 5 $\alpha$ -androstadiol (two OH-groups). All the  $\delta^{13}\text{C}$  values were corrected for the derivatization using the equation of D.M. Johnes.<sup>(3)</sup>

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

**Table 1.** Summary of results obtained from the 1<sup>st</sup> Asian Indoor Games

	Delta value (‰) (n = 189)						
	Etio	Andro	5 $\beta$ -diol	5 $\alpha$ -diol	11-Keto	P2	P3
MEAN	-21.04	-21.44	-22.04	-23.75	-23.23	-22.45	-21.00
SD	1.09	0.96	1.43	1.02	1.15	1.21	1.14

**Table 2.** Summary of the difference obtained from the 1<sup>st</sup> Asian Indoor Games

	Difference ( $\delta^{13}\text{steroid} - \delta^{13}_{11\text{-Keto}}$ )		Difference ( $\delta^{13}\text{steroid} - \delta^{13}_{\text{P2}}$ )	
	Etio	Andro	5 $\beta$ -diol	5 $\alpha$ -diol
MEAN	1.06	2.19	0.41	-1.30

From Figure 1 and 2, the mode values of  $\delta^{13}\text{C}$  value for etiocholanolone, androsterone, 11-ketoetiocholanolone,  $5\beta$ -androstandiol,  $5\alpha$ -androstandiol and pregnandiol are -21 ‰, -21 ‰, -23 ‰, -22 ‰, -24 ‰ and -22 ‰, respectively. All  $\delta^{13}\text{C}$  value measured (n = 189) are less than -28.0 ‰<sup>(2, 4)</sup>, the cut-off value for a possible positive sample.

The difference of  $\delta^{13}\text{C}$  value between endogenous steroid and ERC (Table 2) is less than 3 delta unit<sup>(2, 4)</sup>, the cut-off value for a possible positive sample.

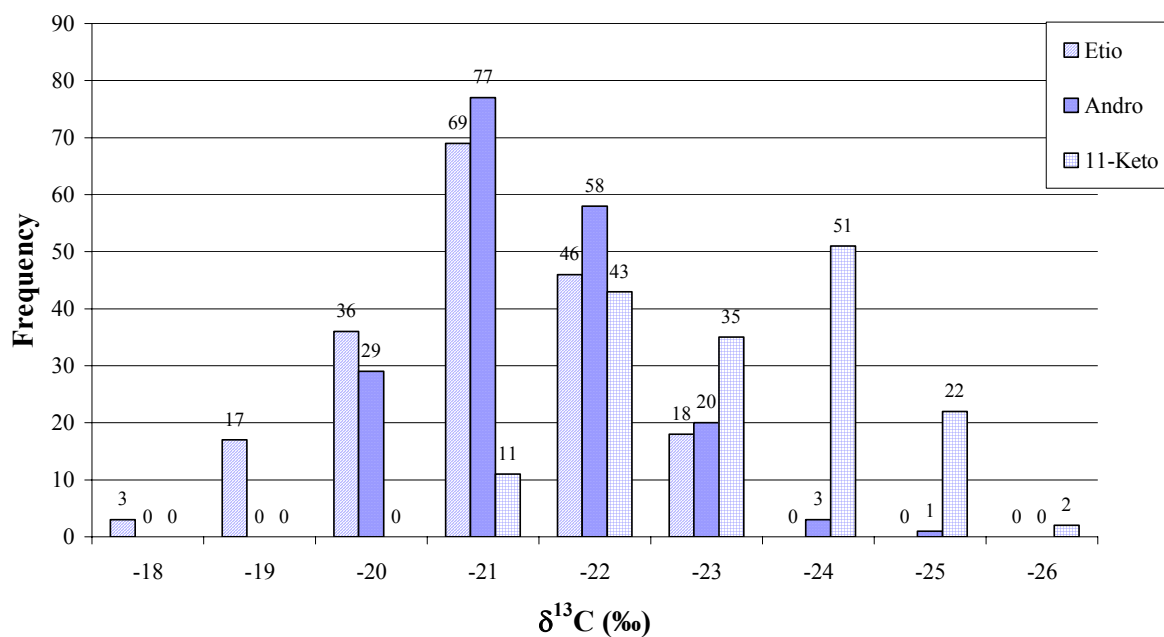
All samples, from the 1<sup>st</sup> Asian Indoor Games are negative according to the WADA directive<sup>(4)</sup>, whereby all criteria must be met for a sample to be declared positive.

## Conclusions

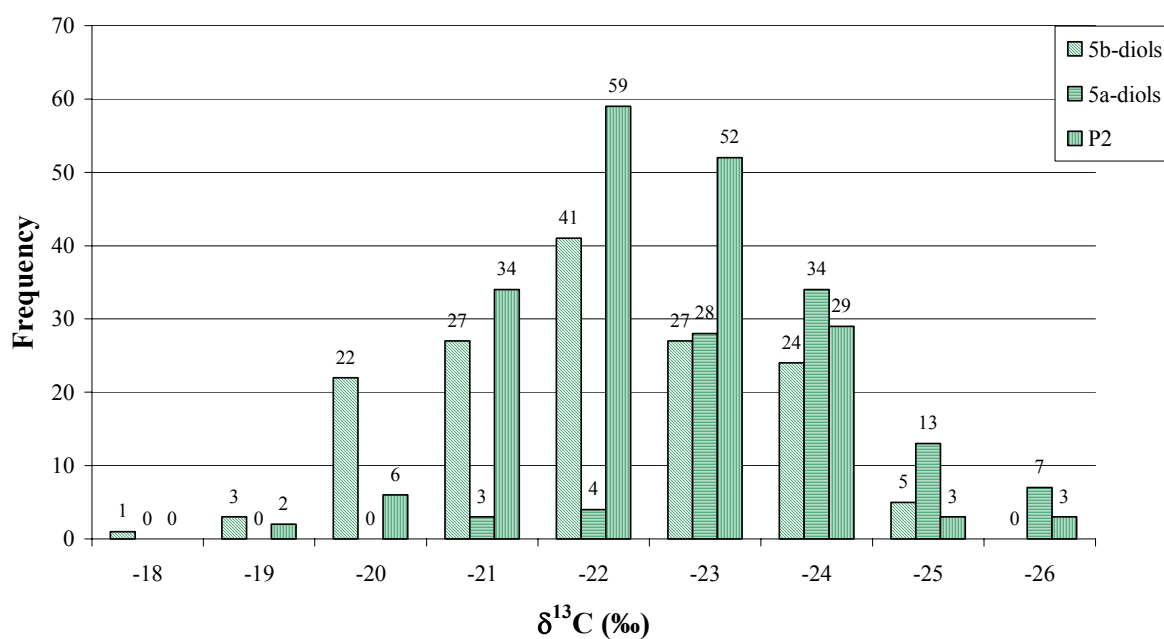
In the 1<sup>st</sup> Asian Indoor Games Bangkok 2005, there were 45 participating nations. The data are representative of the  $\delta^{13}\text{C}$  values for athletes from Asia.

## References

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**Figure 1.** The distribution of  $\delta^{13}\text{C}$  values of etiocholanolone, androsterone and 11-ketoetiocholanolone.



**Figure 2.** The distribution of  $\delta^{13}\text{C}$  values of 5β-androstandiol, 5α-androstandiol and pregnandiol.