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## **4'hydroxy-androstenedione (4'OH-ADIONE): a confirmatory marker of androstenedione abuse in athletes**

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Androstenedione (ADIONE; androst-4-ene-3,17-dione) is an endogenous steroid often described as a “prohormone” since it can be converted to testosterone in the body [1]. Anecdotal evidence suggests that athletes use oral preparations of ADIONE to increase systemic levels of testosterone during training. Studies have shown that the administration of ADIONE significantly increases the urinary ratio of testosterone glucuronide to epitestosterone glucuronide (T/E) in subjects with a normal ( $\approx 1$ ) or naturally high ( $>1$ ) initial values [2,3]. Such screening results satisfy the WADA criteria for the detection of endogenous steroid administration, so long as the T/E value was greater than four [4]. Confirmation may then involve longitudinal T/E monitoring and/or carbon isotope ratio ( $\delta^{13}\text{C}$ ) analysis by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). The urinary T/E has been shown not to increase in subjects with naturally low initial values ( $<1$ ) following ADIONE administration, a situation that may lead to a false negative screening result [2,3]. However, an overload of the terminal urinary androgen metabolites, androsterone (A) and etiocholanolone (Et) can also provide evidence of ADIONE administration. Using any of the above indicators, the challenge for doping control laboratories lies in specific identification of ADIONE abuse, thereby distinguishing it from abuse of other prohormones such as dehydroepiandrosterone (DHEA). For this purpose, the detection of C-6 hydroxylated metabolites has been shown by Lévesque and Ayotte [5] to be indicative of ADIONE administration. Although the use of these compounds has been effective in characterising ADIONE administration in the routine GC-MS steroid profile, their relatively low

urinary concentrations limit the use of GC-C-IRMS to specifically confirm ADIONE administration based on depleted  $^{13}\text{C}$  content of these C-6 hydroxylated steroids.

## Experimental

ADIONE capsules (100 mg, batch No. 569) were obtained from One-Life Natural Foods (Santa Monica, CA, USA). The neutral fraction of two capsules was analysed by full scan GC-MS of underivatized and TMSI derivatized steroids to verify identity and purity of ADIONE. GC-C-IRMS analysis of the underivatized steroid fraction determined the  $\delta^{13}\text{C}$  value of ADIONE from the capsules to be  $-35.0 \pm 0.5\%$ . Subject 1 (30 year-old male) and subject 2 (21 year-old male) volunteered for multiple oral administrations of ADIONE with informed consent. The health ethics committee of Southern Cross University (Lismore, NSW, Australia) gave approval (ECN-05-99) for the study. Baseline urine samples were collected at 48 hours, 24 hours and immediately prior to an initial 100 mg oral administration of ADIONE. This was followed by a further five 100 mg oral administrations of ADIONE at regular 12-hour intervals. Urine samples were collected at regular 6-hour intervals for 72 hours post-administration and at 12-hour intervals for a further 72 hours. Isolation of urinary steroids from the glucuronide fraction for GC-MS analysis was conducted by automated solid phase extraction of hydrolysed urine samples prior to trimethylsilyl-enol-ether derivatization. Liquid-liquid extraction with HPLC purification was used to isolate urinary steroid glucuronides for underivatized GC-C-IRMS analysis.

## Results and discussion

A steroid of unknown structure but with a major ion at  $m/z$  518, was detected at  $R_t = 15.4$  min ( $RR_t = 1.13$  to 17-Met) in the routine GC-MS steroid screen. Its abundance was observed to increase following ADIONE administration (Figure 1). Identification was made by reference to a steroid standard (99% purity) of androst-4-ene-ol-3,17-dione (4'-OH-ADIONE) obtained from the National Measurement Institute (Sydney, NSW, Australia). The full scan mass spectra of underivatized and *tris*-TMS 4'-OH-ADIONE are shown by Figure 2. Van de Kerkhof [6] identified C-4 hydroxylation to be a major metabolic pathway following ADIONE administration, thereby resulting in urinary excretion of 4'-OH-ADIONE. A reference population study ( $n=200$ ) found a skewed distribution with a mean 4'-OH-ADIONE GC-MS screening concentration of 10 ng/mL and a standard deviation (sd) of 8 ng/mL. This allowed an upper limit

of 40 ng/mL (mean+3sd) to be proposed as a criterion to select samples for GC-C-IRMS analysis. Samples collected from the two subjects administered with ADIONE displayed urinary concentrations of 4'OH-ADIONE greater than 40 ng/mL for 12 to 24 hours post-administration (Figure 3). Confirmation was provided by stable carbon isotope ratio ( $\delta^{13}\text{C}$ ) values for 4'OH-ADIONE (-32.8‰ to -35.0‰) reflecting the  $^{13}\text{C}$  content of administered ADIONE (-35.0‰), including a 12-hour post-administration period (Figure 4).

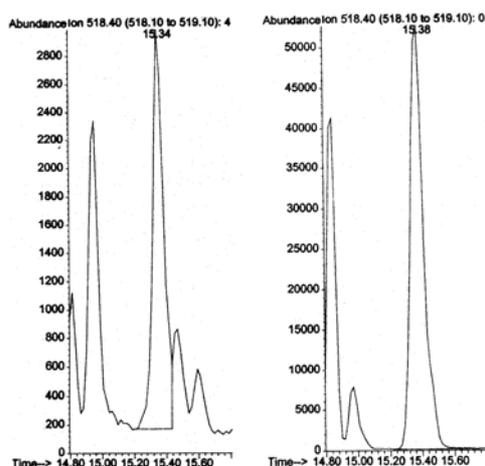


Figure 1: SIM response of  $m/z$  518 pre- (left) and post-ADIONE administration (right)

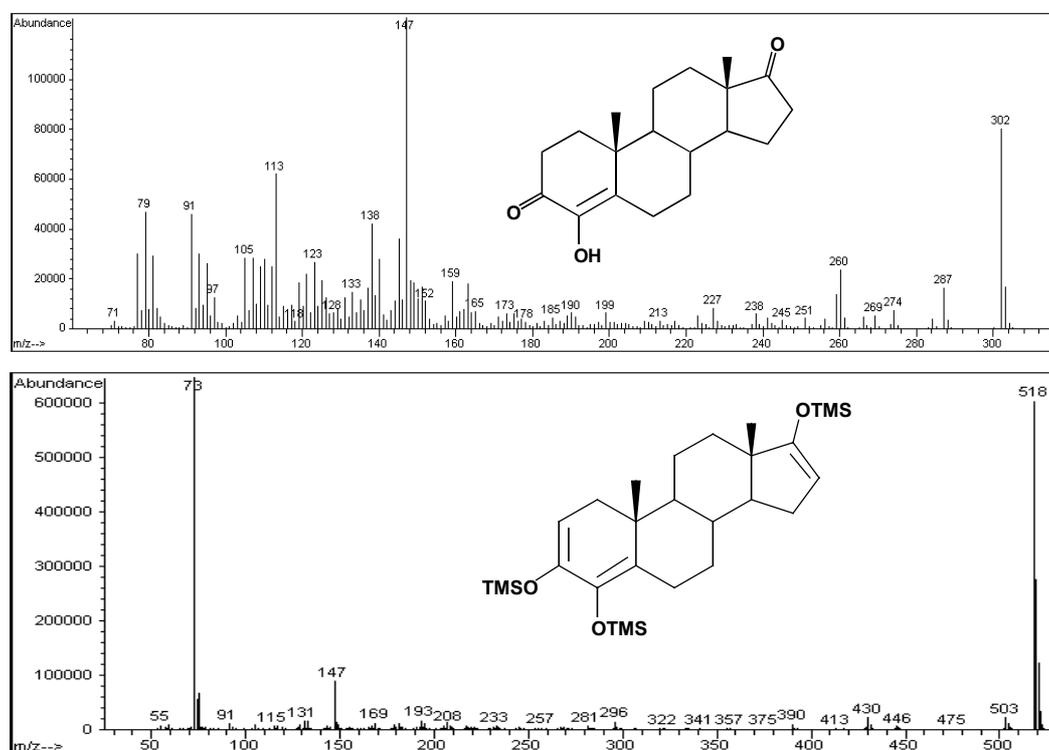


Figure 2: EI mass spectra of 4'OH-ADIONE (top) and *tris*-TMS 4'OH-ADIONE (bottom)

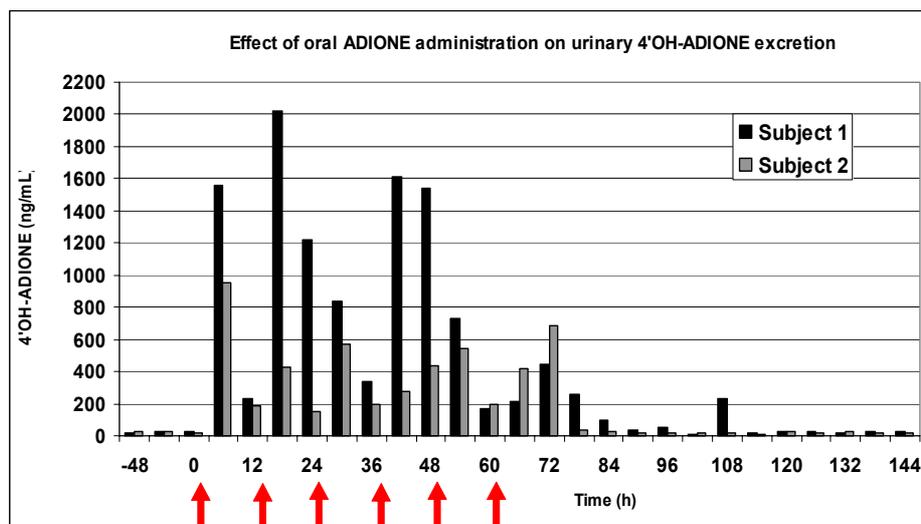


Figure 3: Urinary 4'OH-ADIONE concentration (↑ indicates oral 100 mg ADIONE dose)

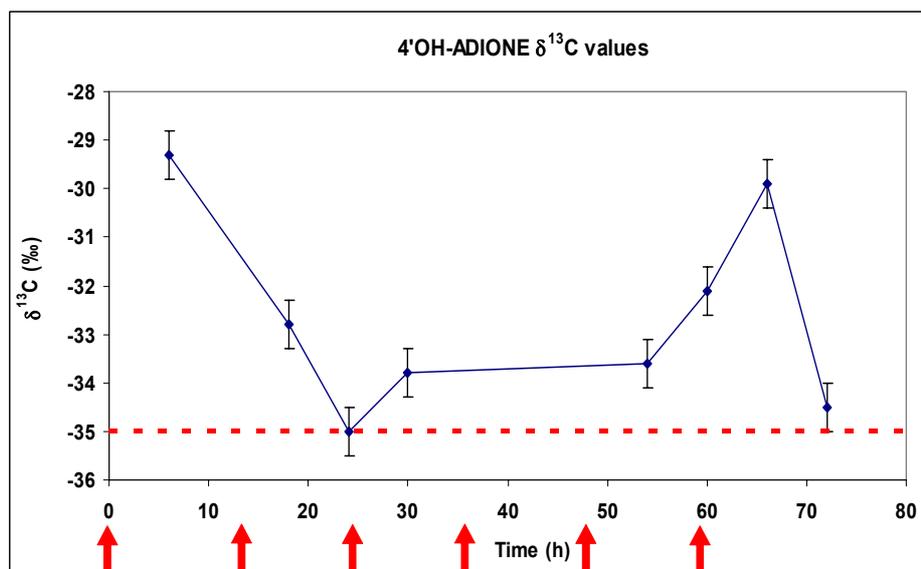


Figure 4: 4'OH-ADIONE  $\delta^{13}\text{C}$  values for subject 1 (↑ indicates oral 100 mg ADIONE dose)

## References

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