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Correlation of steroid profile data on GC-MSD with delta values on GC-IRMS after administration of Testosterone preparations to healthy volunteers with low and high T/E ratio

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Introduction:

Detection of abuse of endogenous steroids is a challenge faced by Doping Control Laboratories Specific markers allow detection of administration of these steroids using GC-IRMS. As per WADA guidelines if the testosterone/epitestosterone (T/E) ratio is greater than 4:1 or if the concentration of androsterone (andro.) and etiocholanolone (etio.) exceeds 10,000 ng/ml, the sample should be subjected to GC-IRMS (1). However, there is a wide variation in natural T/E ratio between individuals (2, 3) and it is observed that in some cases the T/E ratio may be above 4 even though the individual has not taken steroids exogenously whereas in others, the T/E ratio may remain below 4 despite steroid abuse**.** In view of low T/E ratio in Indian population, the present study is aimed to co-relate steroid profile data on GC-MSD with data on GC-IRMS after administration of testosterone preparations (tablet and injection) to healthy volunteers with low and high T/E ratio (4).

Material & Methods

Testosterone undecanoate tablets (40 mg) were administered to two healthy male individuals (aged 25-27 year, body weight 50 kg & 55 kg) one with basal value of low T/E ratio(0.12) and the other with high T/E ratio (2.2). After a gap of one month of administration of oral tablet, testosterone propionate (50 mg) intra muscular injection (I/M) was administered to the same healthy volunteer with low T/E ratio. Urine samples were collected at different intervals. Approval of the ethics committee of Sports Authority Of India was taken for this project.

Sample Preparation procedure

GC-MSD: Urine samples were processed according to screening procedure IV (using XAD2 columns) for anabolic agents and subjected to GC-MSD for Steroid profiling (5).

GC-IRMS: 5 ml of urine sample was applied onto C-18 column (Samprep-Rankem), eluted with methanol and then dried. The residue was reconstituted in 1 ml of 0.2 M sodium phosphate buffer and the free fraction discarded after extraction with diethyl ether. Then the steroid conjugates were enzymaticaly hydrolyzed by adding 50 µl of β-glucuronidase (E.Coli) for 1 hour at 60º C. Thereafter, the extract was alkalized and extracted in 5 ml of TBME. The organic aliquot was evaporated to dryness, derivatised with 50 µl of acetic anhydride + 50 µl pyridine at 60º C for 1hr, re-evaporated, and reconstituted in cyclohexane (50 µl) and 2 µl injected on GC-IRMS for analysis. It is expected that in fact acetylation introduces some shifts for the delta ^{13}C values of andro., etio. & 11-oxo-etiocholanone, but the ratio of them could only be affected feebly. Hence ^{13}C delta values were not corrected for acetylation.

Instrumentation

GC/C/IRMS analyses were performed on HP 6890 GC connected to Isoprime IRMS (GV instruments). The GC was equipped with HP 50+ column (30 m x 0.25 mm id x 0.25 µm). Helium was the carrier gas at constant flow of 1 ml/min. The GC temperature program was: 3 min 100º C, + 40º C/min, 0 min. 245º C, +5º C/min, 0 min 280º C, +2º C/min, 3 min. 300º C. GC/MS analyses were performed on an Agilent 6890GC/5975N MSD. Split mode was used with a ratio of 1:10. The value for ^{13}C of the standard carbon dioxide used as a reference gas was calibrated against n- alkane mixture (C_{17}-C_{25}) obtained from Indiana University, USA.

RESULTS & DISCUSSION

After administration of 50 mg of testosterone propionate (I/M) to the volunteer with low basal T/E ratio, the T/E ratio did not increase beyond 2.0 (range 0.11 to 1.93) up to 10th day (Fig 1A), The concentrations of testosterone (T) and epitestosterone (E) were below 100 ng/ml and 200 ng/ml respectively (Fig.1A). Andro. and etio. levels increased above 8000 ng/ml from 6 hrs post administrations and remained high till 52 hours (Fig.1A).

When to the same volunteer with low T/E ratio was given 40 mg of testosterone undecanoate tablet orally, the T/E ratio did not increase above 0.20 (Fig.1B). The levels of T and E also remained within permissible range. Interestingly after post administration the increase was observed in levels of E instead of T. However, andro. and etio. levels increased above 8000

ng/ml only from 1.3 hours post administration till 7 hours, after which the normal steroid profile was observed.

When 40 mg of testosterone undeconate tablets were given to the healthy volunteer with natural high T/E ratio, increased T/E ratio was observed from 2 hours till 20 hours. (Fig.1C). Interestingly, concentration of T increased to 1515 ng/ml (3.4 hrs) whereas E was not much affected which provides evidence that there exists inter–individual variation in metabolizing testosterone. Andro. & etio. levels increased above 8000ng/ml from 3.4 hrs till 20 hrs. and after that the normal steroid profile was observed.

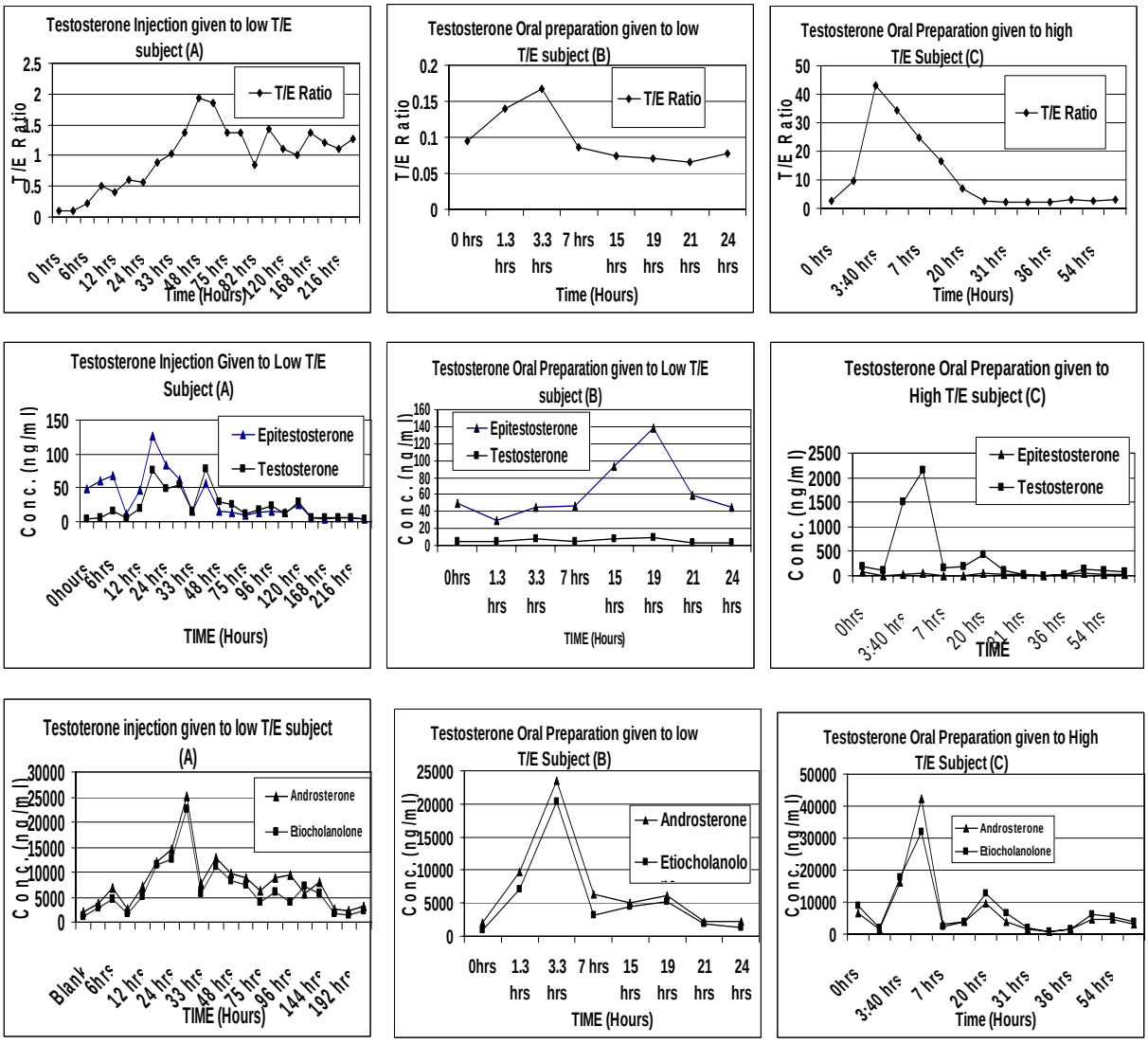

Fig. :1 *Effect of Testosterone injection (A) and oral preparation(B) in subject(low basal T/E ratio), and in subject(high T/E ratio)(C) on T/E ratio, conc. of testosterone, epitestosterone, andro. and etio.*

GC/C/IRMS analysis

The δ ^{13}C values of synthetic Etio., Andro. and 11-oxo-etiocholanone (11-oxo-etio.; used as Endogenous Reference Compound, ERC) ranged from -29.83 to -30.26 ‰, -32.2 to -33.34 ‰ and -15.97 to -17 ‰ respectively at different concentrations. In drug free urines (DFU), δ ^{13}C values of the natural endogenous etio., andro. and 11-oxo-etio. ranged from -21.38 to -26.38, -21.68 to -26.76 and -23.0 to -27.0 ‰ respectively. After injection of testosterone to the subject with low basal T/E value, δ ^{13}C values of andro. and etio. were significantly lower than the basal value (more than 4.0) from 3 hours onwards up to 10th day (Fig. 1A) but could not detect 11-oxo-etio.(ERC) in any of the urine sample. Other ERC's like pregnanediol (P2) and pregnanetriol (P3) were also not detectable.

When to the same subject was administered the tablet, δ ^{13}C values of andro. and etio. were significantly lower than the basal value (more than 4.0) from 1.3 hours onwards up to 20 hours post administration. (Fig 1B). Again could not detect 11-oxo-etio.(ERC) or pregnanediol (P2) and pregnanetriol (P3) in any of the urine samples.

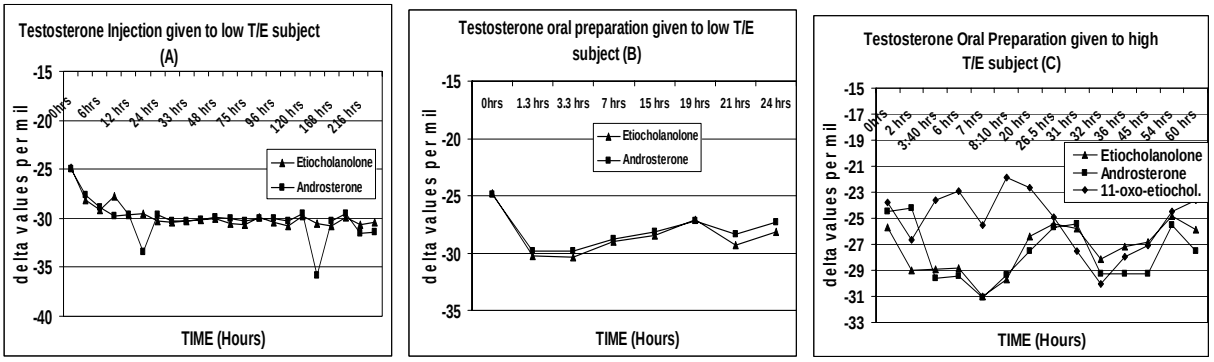

Fig:2 *Effect of Testosterone injection on δ ^{13}C values in low T/E subject (A). Effect of Testosterone tablet on δ ^{13}C values (per mil) in low T/E subject (B) and in high T/E subject (C)*

Similarly, δ ^{13}C values of andro. and etio. were significantly lower (more than 4.0 ‰) from basal value till 20 hrs for subject with high basal T/E value (Fig 2C). In this case, we could detect ERC (11-oxo-etio). The difference between the average of δ ^{13}C values of andro. and etio., and ERC was more than 4.0 ‰ till 20 hours indicating abuse of exogenous steroid.

CONCLUSION

The unexpected finding of increased levels of E after oral administration of T to the volunteer with low T/E needs further investigation to study the metabolism of testosterone. The result of preliminary study suggests that the results of steroid profile on GC-MSD may not be enough to suspect the abuse of natural endogenous steroid in sportspersons having low basal T/E ratio. However, further study is required to conclude these findings.

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