Jain S, Shrivastava A, Kaur T, Soni A, Haseen J, Nimker V, Beotra A

# Detection of the misuse of testosterone gel in subjects with low basal Testosterone/Epitestosterone (T/E) value.

Ministry of youth Affairs and Sports, National Dope Testing Laboratory, India, New Delhi, India

## Abstract

Testosterone is one of the most abused endogenous anabolic androgenic steroids in sports. Testosterone Gel (T-gel) applied transdermally, metabolizes in a different way keeping the concentration of other metabolites low as compared to 5-alpha metabolites. The aim of the present work was to study the effect of T-gel application on the endogenous steroid profile and $\delta^{13}$C values in subjects with low basal T/E ratio (<0.3). Two healthy male volunteers with low T/E ratios (<0.5) were selected. Urine samples were collected before, during and after T-gel application for 10 days. All urine samples were analysed for the endogenous steroid profile and $\delta^{13}$C values of endogenous steroids. The results of the T-gel excretion study of both volunteers revealed that the endogenous profile and their respective ratios were within the World Anti Doping Agency (WADA) prescribed limits [1]. On the basis of the steroid profile, none of the sample qualified for GC-IRMS analysis as per existing and the proposed WADA technical document on endogenous steroids except one sample of volunteer-2 [1]. The most discriminating parameters of the steroid profile were the ratios Androsterone/Epitestosterone (Andro/E), 5α-androstane-3α,17β-diol/epitestosterone (5α-diol/E). For the GC-IRMS analysis, 5α–diol was the suitable target compound for long term detection of T-gel application. Individual reference ranges of the parameters of steroid profile were more suitable for the detection of misuse of T-gel application in subjects with low basal T/E values.

## Introduction

Availability of T in various forms e.g. tablets, Injections, transdermal patches, sprays and Gel-preparations makes detection of misuse a great challenge for doping control laboratories. Threshold for T/E of 4 was established by WADA in order to determine if a sample is suspicious for Testosterone misuse [1]. This threshold is based upon population statistics and encompasses big inter-individual variation. This single population based threshold is insensitive to ethnical and individual T/E variation. High frequency of UGT2B17 deletion in Asian population leads to low T excretion resulting in low basal T/E-ratio [2]. Several researchers have studied the effect of testosterone administration (oral and intramuscular) on steroid profile in Asian population but limited data is available on transdermal application of testosterone especially in low T/E subjects [2,3,4]. It has been proved that T-Gel enhances performance in athletes by increasing lean body mass, muscle strength & hemoglobin concentration [5]. It has different metabolism to the oral or intramuscular application due to high 5-alpha-reductase activity of skin converting testosterone to 5α-metabolites rather than 5β-metabolites [4]. Hence continuous absorption of T-Gel through transdermal application makes it a perfect weapon for Asian athletes to evade WADA criterion for adverse analytical findings (AAF) due to naturally low T/E ratio which might skip the WADA cut-off for IRMS analysis. The aim of the present work was to study the effect of T-gel application on the endogenous steroid profile and $\delta^{13}$C values in subjects with a low T/E ratio (<0.3) and explore for parameters with higher discriminating power.

## Experimental

### Reagents and Chemicals

Reference standards of endogenous steroids and deuterated internal standards were procured from Sigma-Aldrich (USA) and National Measurement Institute (Australia). C-18 sample preparation cartridges were procured from RFCL

 ISBN 978-3-86884-039-1

Ltd, β-glucuronidase enzyme (*E. coli*) was from Roche Diagnostics (USA). All other solvents and chemicals were of high performance liquid chromatography (HPLC) grade and analytical grade, respectively.

**Study design**

**Selection of Volunteer**

Selection of volunteer with low T/E ratio (< 0.5) was done by screening steroid profile of 20 healthy volunteers who declared not using any medication. Out of 20 volunteers, two healthy male volunteer with low T/E ratio (volunteer 1(V-1) with T/E ratio of 0.09 and Volunteer 2 (V-2) with T/E ratio of 0.27) were selected for the study. The excretion study of T-gel in human volunteers was approved by the Ethics Committee of the National Dope Testing Laboratory (NDTL), India.

**Excretion study (Collection of urine samples)**

One day prior to T-gel application, five blank urine samples (Drug free urine- DFU) were collected throughout the day. From the next day T-gel (Cernos Gel-one sachet having 50 mg testosterone with $\delta^{13}C$ values of testosterone: -29.6 ‰) was applied for 5 days after bath twice a day (morning and evening) over the shoulders, abdomen and thighs. The delta value of T-Gel was determined by extracting the drug from gel by liquid liquid extraction with tert butyl methyl ether (TBME) and injecting on to IRMS after acetylation. The collection of urine samples was started after three days of T-gel application (4$^{th}$ day). Urine samples were collected at similar regular intervals as blank urine samples for six days (upto 9 days) as described in Figure 1.

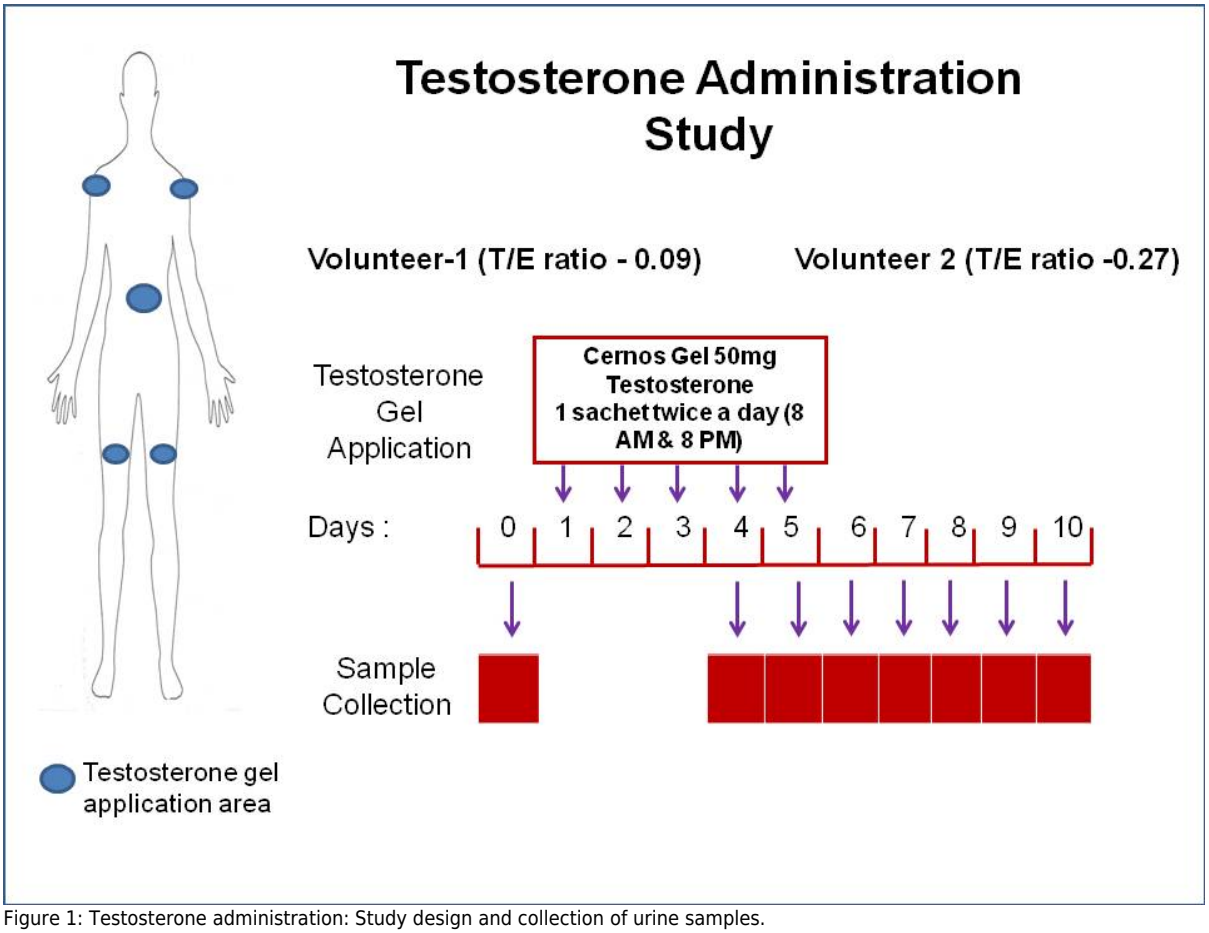


Figure 1: Testosterone administration: Study design and collection of urine samples.

**Analysis of the urine samples**

All the urine samples collected before, during and after T-gel applications were analysed with GC/MS by routine screening procedure for the endogenous steroid profile [6]. Concentrations and ratios were calculated for the following endogenous


ISBN 978-3-86884-039-1

steroids: androsterone (Andro), etiocholanolone (Etio), testosterone (T), epitestosterone (E), 5α-androstane-3α,17ß-diol (5α-diol) and 5ß-androstane-3α,17ß-diol (5ß-diol). Additionally specific gravity (SG) and pH were also measured. For GC-IRMS analysis, selected urine samples were processed by Solid phase extraction (SPE) using enzymatic hydrolysis. Prior to GC-IRMS analysis two step High Performance Liquid Chromatography (HPLC) fractionation was performed in accordance with the routine sample preparation procedures and thereafter injected onto GC-IRMS after acetylation [6]. Delta $^{13}C$ values of metabolites of testosterone: Andro, Etio, 5α-diol, 5β-diol along with T and E were measured. 11 keto-etiocholanolone (11 keto) and pregnanediol (PD) were used as endogenous reference compound (ERC).

**LH Analysis:**
Urinary luteinizing hormone (LH) hormone was determined by microparticle enzyme immunoassay using Immulite system (model-1000).

## Results and Discussion

**Steroid Profile parameters**
As the specific gravity (SG) of all the urine samples ranged from 1.007-1.019, correction for the SG for steroid profile was not applied. The results of the excretion study of the two volunteers revealed that endogenous profile and their respective ratios were within the WADA prescribed limits [1](Fig. 2-5). Figure-2 depicts the concentration of Andro, Etio, 5α-diol and 5β-diol in urine samples collected before, during and after T-gel application in both the volunteers which never crossed the WADA limit of 10,000 ng/mL for Andro & Etio and concentrations achieved for 5α and 5β-diols were also not very high throughout the excretion study. The conversion of testosterone to the 5α-metabolites( Andro and 5α -diol) was not higher than the conversion to 5β-metabolites (Etio and 5β-diol) in volunteer-1 which is in conformity with the findings of Geyer et al [7]. However, results of volunteer-2 showed slight increase in 5-alpha-metabolites.

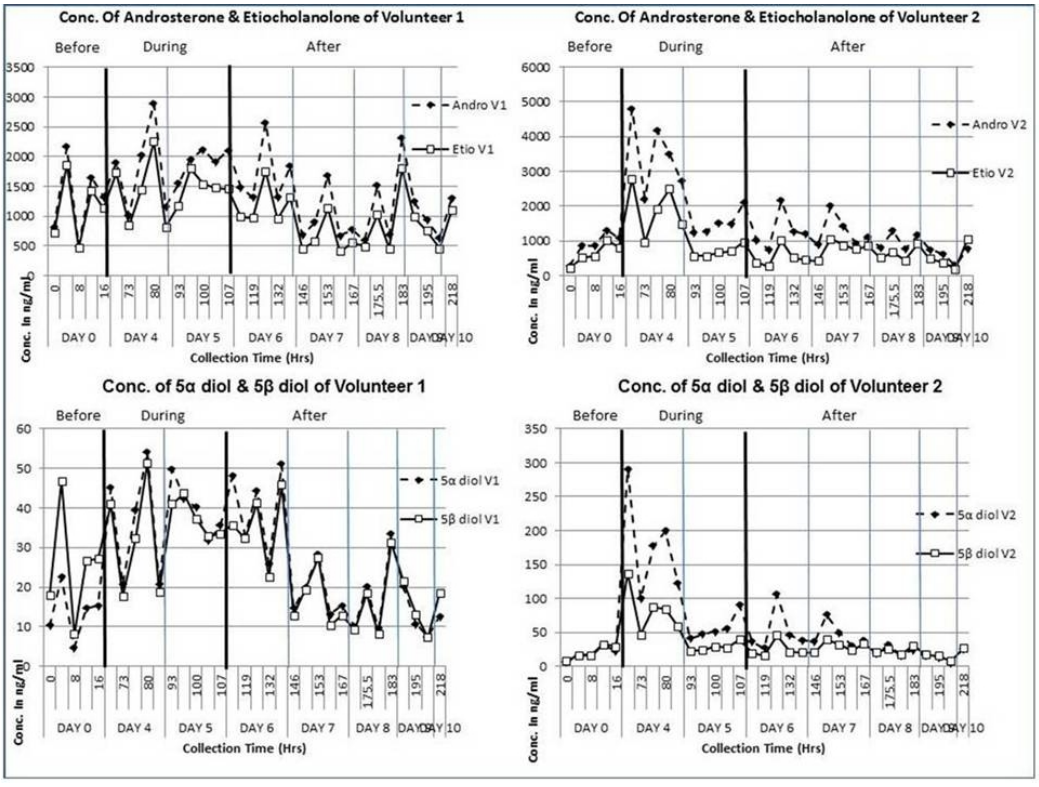

Figure 2: Concentration of Andro, Etio, 5a-diol and 5b-diol in volunteer-1 & 2 before, during and after T-gel application.

 ISBN 978-3-86884-039-1

It was observed that during and after T-gel application, the concentration of testosterone showed 2-10 times increase from the basal threshold in volunteer-1 as compared to 2-40 times in volunteer-2 whereas T/E increased 2-15 times above basal threshold in both volunteers. However on the basis of concentrations of T and E, Andro/Etio and T/E ratio, none of the samples qualified for GC-IRMS analysis (Fig. 3) except for one sample (7$^{th}$ day) in volunteer-2, which had T/E ratio slightly above 4.0. In all other samples till 8$^{th}$ day, T/E values were far below the cut-off limit of 4 but all the values were above the basal value of volunteer-1 which is in conformity to the findings reported by Geyer et al [7]. As proposed in WADA technical document (TD2013EAAS) for EAAS, urine sample having Andro/Etio ratio greater than 4.0 in male is to be treated as suspicious for abuse of endogenous steroid, in the present study none of the samples from both the volunteers showed Andro/Etio ratios > 4 . Thus as per criteria laid down in existing as well as proposed WADA Technical document, athletes with low T/E value when using T-gel application may evade detection.

The other ratios of endogenous steroids i.e. 5α -diol/5β-diol, Andro/T, Andro/E & 5α -diol/T ratios in both the volunteers are shown in Fig.-4. The 5α- diol/5β-diol ratio was found to be elevated from 0.5 to 1.4 in volunteer-1 and from 0.7 to 2.4 in volunteer-2 and resumed basal value on 7$^{th}$ day i.e. 2 days after cessation of T-gel application. The ratio Andro/T showed a continuous steep decline upto day 9 in volunteer 1 whereas Andro/E ratio showed significant increase from 50 to 270 till day 9. It is evident form figure- 5 that the 5α -diol/E ratio also increased from a value of nearly a unit up to 13 on day 7 and remained high till day 8 in volunteer 2 which makes this ratio a potential marker to be considered for T-Gel abuse. The ratio T/LH didn't contribute towards any conclusive outcome for the study as the ratio remained in the reference ranges of the laboratory's data (Fig.5).

In brief, none of the sample qualified for GC-IRMS analysis in volunteer-1 (basal T/E ratio 0.09), as per both existing and draft version of WADA technical document on EAAS. However in volunteer-2 (basal T/E ratio 0.27), only one sample qualified for GC-IRMS analysis. The most obvious changes of the steroid profile after T-gel application to subjects with low T/E ratio were the changes of the ratio of Andro/T, Andro/Epit, 5α-diol/Epit and T/E from their individual basal values (Fig.3-5).

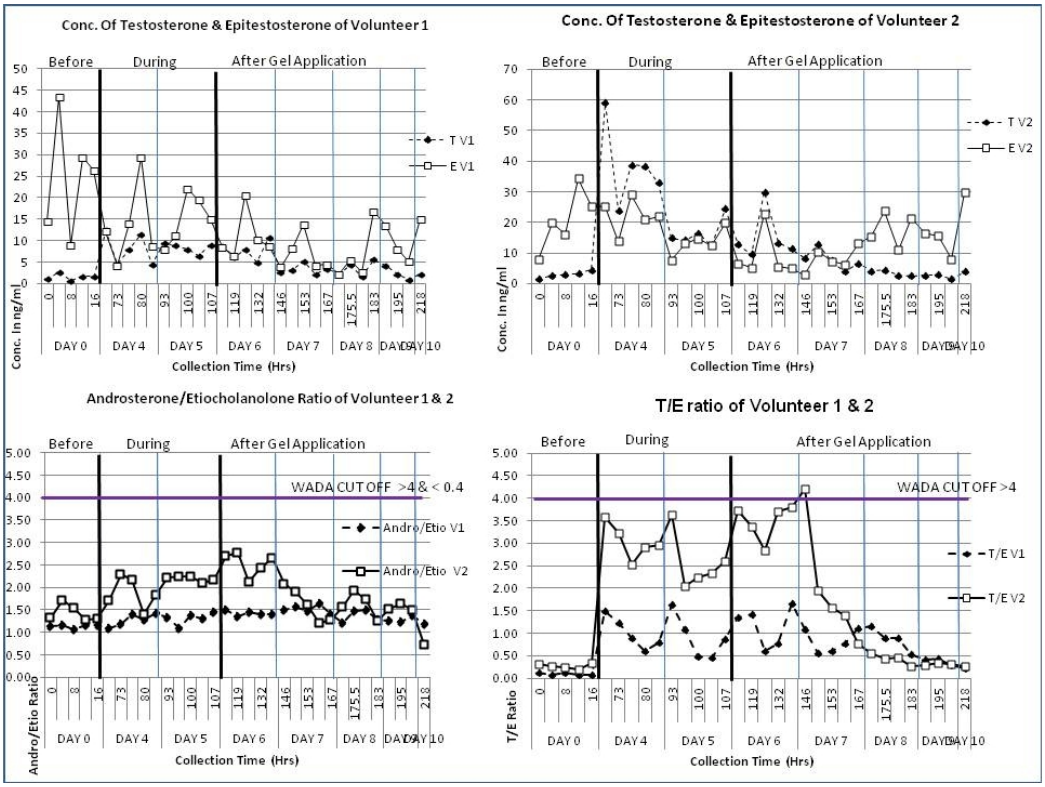

Figure 3: Concentrations of T, E along with T/E & Andro/Etio ratios for Volunteer 1 & 2 before, during and after T-gel application.

 ISBN 978-3-86884-039-1

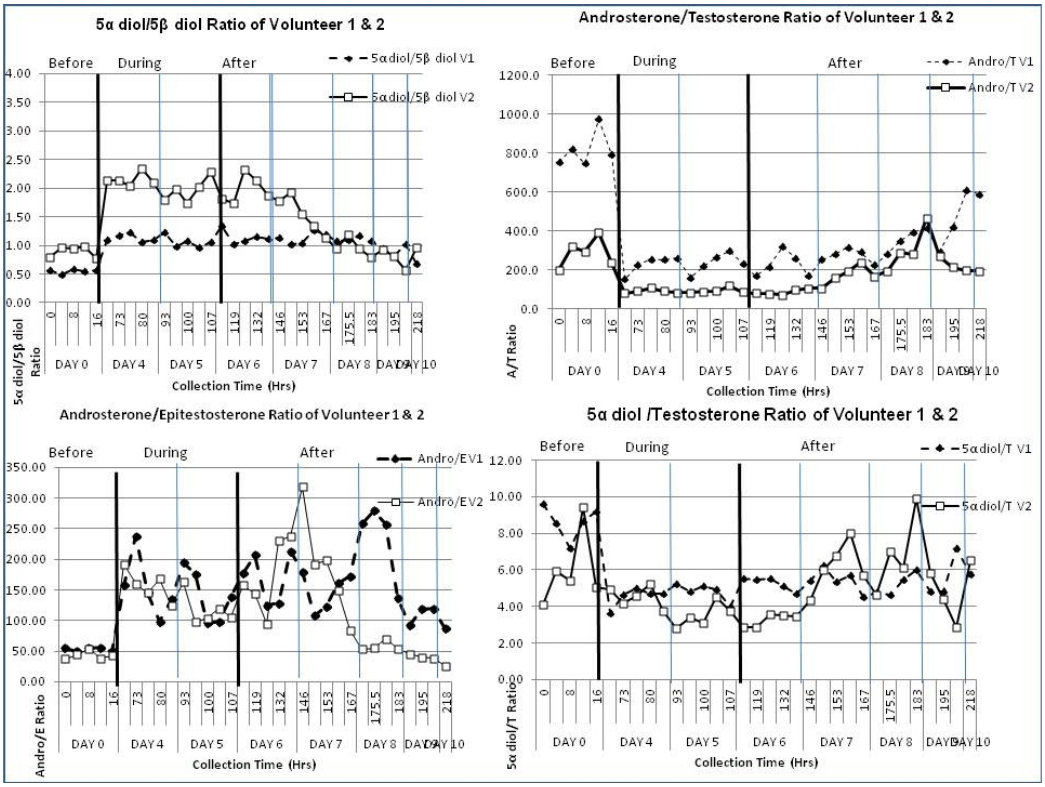


Figure 4: The ratios of endogenous steroids i.e. 5a-diol/5b-diol, Andro/T, Andro/E & 5a-diol/T ratios in volunteer 1 & 2 before, during and after T-gel application.

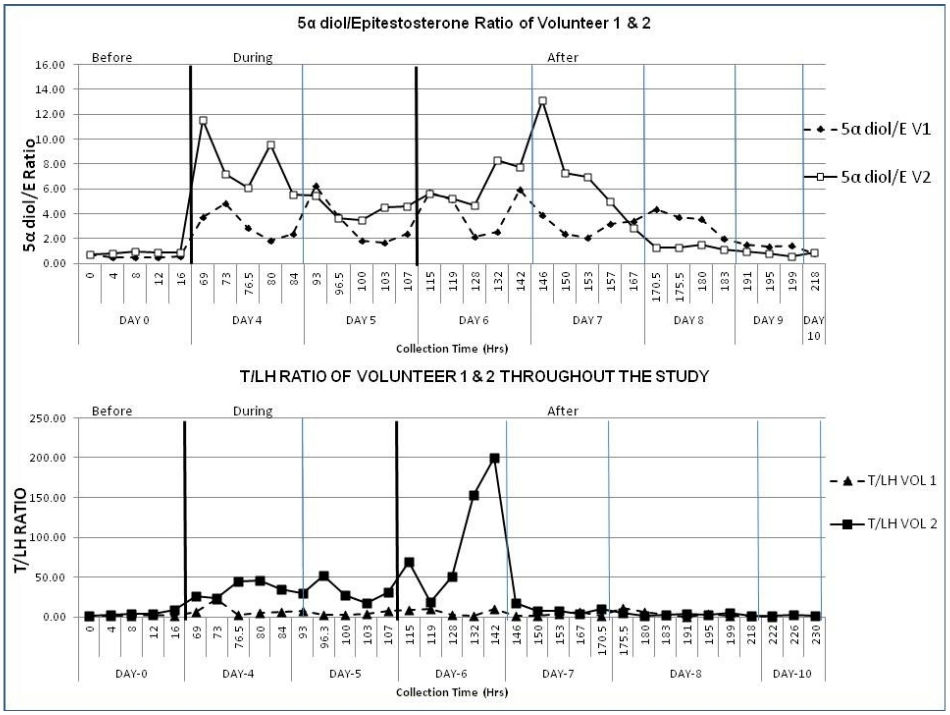


Figure 5: The ratios of 5a-diol/E & T/LH in volunteer 1 & 2 before, during and after T-gel application.

 ISBN 978-3-86884-039-1

**GC-IRMS Analysis**

The GC-IRMS analysis was done on selected samples. The target compounds chosen for GC-IRMS analysis were Andro, Etio, 5α-diol, 5β-diol, T and 11-keto & PD as ERC. WADA criterion for a sample to be consistent with exogenous administration of endogenous steroid is when the $\delta^{13}C$ value measured for the metabolite(s) differs significantly i.e. by 3 delta units or more from that of the urinary reference steroid chosen. The differences of delta delta $^{13}C$ values of Andro & Etio from ERC (11 keto) and delta delta 13C values of 5α–diol, 5β-diol and T from ERC (PD) of the urine samples collected before, during and after T-gel application are shown in figure-6. The differences of delta delta 13C values of testosterone from PD (ERC) was more than 3 till 6$^{th}$ day in volunteer-2 only. In the samples collected after 7$^{th}$ day, due to very low concentration of testosterone the peak height of testosterone was less than 0.1 nA which failed the identification criteria. In volunteer-1, it was observed that none of the samples had peak height of testosterone more than 0.2 nA due to very low concentration of testosterone. Therefore the GC-IRMS analysis in volunteer-1 did not give conclusive results for testosterone application. However, in both the volunteers, the differences of delta delta $^{13}C$ values of 5α-diol from PD (ERC) was more than 3 till 8$^{th}$ day indicating abuse of testosterone or its prohormone.

However, for other parameters only few samples intermittently showed delta/delta difference above 3. The results of GC-IRMS analysis indicate that 5α-diol may be more suitable target compound as compared to Andro, Etio, 5β-diol and T for the detection of the misuse of T-gel in subjects with low T/E.

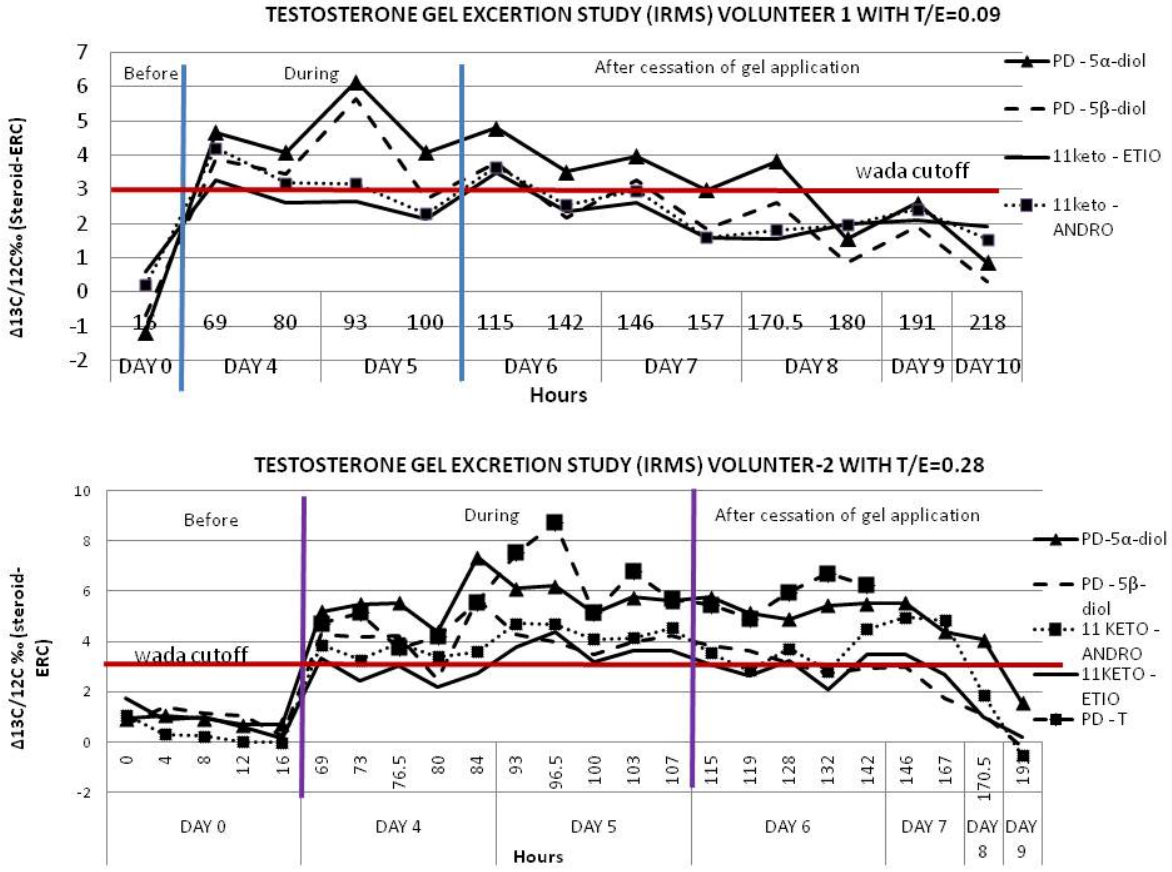


Figure 6: GC-IRMS data (delta/delta-differences) of urine samples of volunteer 1 & 2 before, during and after T-gel application.

Hence the findings of volunteer-2 which were in conformity with findings of low T/E subjects reported by Geyer et al could not be confirmed in volunteer-1. This may be due to very low basal T/E value (0.09) in volunteer-1 indicative of pure UGT2B17 deletion (del/del) case [5,8].

## Conclusions

The present study confirms that the cut off values of endogenous steroids as mentioned in the present (TD2004EAAS) and the proposed WADA technical document (TD2013EAAS) on EAAS are not sufficient to detect a misuse of T-gel in low T/E subjects thereby increasing the risk of a false negative result by a laboratory. The suitable target compound to detect misuse of T-gel application in low T/E subjects on GC-IRMS is 5a-diol. It may be necessary to implement individual reference ranges for, 5a-diol/E, Andro/E, Andro/T, T/E for the detection of the misuse of T-gel in individuals with low T/E basal value. Further work is in progress with more number of volunteers.

## References

[1] World Anti-Doping Agency. WADA Technical Document-TD2004EAAS. Reporting and evaluation guidelines for Testosterone, Epitestosterone, T/E and other endogenous steroids (2004) http://www.wada-ama.org/rtecontent/document/end_steroids_ aug_04.pdf (last accessed on 10thMarch 2013)
[2] Jakobsson J, Ekstrom L, Inotsume N, Garle M, Lorentzon M, Ohlsson C, Roh hk, Carlstrom K, Rane A. (2006). Large differences in testosterone excretion in Korean and Swedish men are strongly associated with a UDP-glucuronosyl transferase 2B17 polymorphism. J. Clin. Endocri. And Metab., 91, 687
[3] Fabregat A, Pozo O J, Yang s, Xing Y, Wu M, Xu Y, Marcos J, Segura J, Ventura R. (2012). Measurement of testosterone metabolites released after basic treatment in subjects with low T/E basal values. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) Recent Advances in Doping Analysis (20), Köln, pp193-196
[4] Mareck U, Geyer H, Opfermann G, Thevis M and Schänzer W. (2008). Factors influencing the steroid profile in doping control analysis. J.Mass Spectrom, 43,877-891.
[5] Swerdloff R S, Wang C, Cunningham G, Dobs A, Iranmanesh A, Matsumoto A M, Synder P J, Weber T, Longstreath J, Berman N (2000) Long term pharmacokinetics of transdermal testosterone gel in hypogonadal men. J Clin Endpcrinol Metab, 85, 4500-10.
[6] Jain S, Kaur T, Garg T, Shrivastava A, Soni A, Beotra A. (2012) Effect of storage conditions on carbon isotope ratios of endogenous steroids. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) Recent Advances in Doping Analysis (20), Köln, pp 217-220.
[7] Geyer H, Flenker U, Mareck U, Platen P, Piper T, Schmechel A, Schrader Y, Thevis M, Schänzer W. (2007) The detection of the misuse of Testosterone gel. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) Recent Advances in Doping Analysis (15), Köln, pp133-144
[8] Geyer H, Flenker U, Mareck U, Sommer F, Schänzer W. (2004) Preliminary results regarding the detection of misuse of testosterone gel. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds) Recent Advances in Doping Analysis (12), Köln, pp121-127.

## Acknowledgements

We acknowledge the financial support extended by Ministry of Youth Affairs and Sports, Government of India.