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The detection of the misuse of testosterone gel

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Abstract

To develop a method for the detection of the misuse of testosterone gel (T-gel) 18 healthy male volunteers were treated for six weeks continuously and intermittently with T-gel (100 mg testosterone per day). Blood and urine samples were collected before, during and after the application of T-gel. The following results were obtained. The treatment with T-gel leads to an increase of serum testosterone and a decrease of LH. The most discriminating parameters of the steroid profile are the ratios testosterone/epitestosterone, 5α-androstane-3α,17ß-diol / epitestosterone and androsterone/epitestosterone. Individual reference ranges of the parameters are more suitable for the detection of the misuse of T-gel than population based reference ranges. For the GC/C/IRMS analysis suitable target compounds are testosterone and 5α-androstane-3α,17ß-diol.

1 Introduction

According to the WADA-list of prohibited substances, the use of testosterone is prohibited in sports. In 2000 a new testosterone preparation, a testosterone gel (T-gel), was approved by the Food and Drug Administration of the USA for the testosterone replacement therapy in men for conditions associated with low testosterone. Since 2003 T-gel is also admitted to the European market. Several investigations have shown, that the application of T-gel leads to performance enhancing physiological effects like significant increase of the lean body mass, muscle strength and haemoglobin concentration (1).
Results of a pilot study with hypogonadal men have shown that it is difficult to detect the misuse of T-gel with the actual available methods of doping control (2).
The aim of the study is to develop a method to prove the misuse of testosterone gel i.e the search for a parameter or several parameters with the strongest discriminating power. These

parameters may be steroid profile parameters, pituitary gland hormones, carbon isotope ratios and hydrogen isotope ratios of steroids or combinations of these parameters.

2 Methods

Excretion studies with testosterone gel were performed with 18 healthy male volunteers (P1 – P18) between 20 and 30 years. The treatment with testosterone gel was approved by the Local Ethical Committee and the German Federal Institution of Drug Control and Drug Research (BfARM).

Application of testosterone gel

Group 1 (9 healthy male volunteers: P2, P3, P4, P5, P6, P10, P12, P16, P17): For six weeks daily transdermal application of 2 x 5 g Testogel® (Jenapharm, Jena Germany) corresponding to 100 mg testosterone. Application in the morning after the shower according to the recommendation.

Group 2 (9 healthy male volunteers: P1, P7, P8, P9, P11, P13, P14, P15, P18): For six weeks intermittent application of testosterone gel. One week daily transdermal application of 2 x 5 g Testogel® (Jenapharm, Jena Germany) corresponding to 100 mg testosterone. One week without testosterone gel etc. Application in the morning after the shower according to the recommendation.

Collection of urine samples (total number: 1300)

Urine samples were collected

- before the application of testosterone gel: each urine sample for five days
- during the application of testosterone gel: on the 6th day of each week collection of all urine samples
- after the application of testosterone gel: on the 6th and 15th day after the cessation of the application collection of all urine samples

Collection of blood samples

Blood samples were collected (one sample of 3 ml in the morning each)

- before the application of testosterone gel
- during the application of testosterone gel: on the 6th day of each week
- after the application of testosterone gel: on the 6th and 15th day after the cessation of the application

Analyses of the urine samples

All 1300 samples were analysed with GC/MS for endogenous steroid-glucuronides according to the previously described methods (3, 4). Concentrations and ratios were calculated for the following endogenous steroids: androsterone (And), etiocholanolone (Etio), testosterone (T), epitestosterone (E), 5α-androstane-3α,17ß-diol (Adiol) and 5ß-androstane-3α,17ß-diol (Bdiol). Additionally specific gravity and pH were measured.

Selected urine samples of 2 volunteers were analysed also with GC/C/IRMS. As target compounds were chosen the glucuronides of androsterone, etiocholanolone, testosterone and 5α-androstane-3α,17ß-diol and 5ß-androstane-3α,17ß-diol. For androsterone and etiocholanolone the GC/C/IRMS analyses were performed according to the standard operating procedures of the Cologne laboratory (5, 6). For the analyses of testosterone and 5α-androstane-3α,17ß-diol and 5ß-androstane-3α,17ß-diol new purification methods have been developed.

Analyses of blood samples

The blood samples were analysed with standardized commercial electro chemiluminescence immunoassays (Roche Diagnostics) for total testosterone, free testosterone and LH.

3 Results and Discussion

3.1 Blood samples

The results of the analyses of the blood samples are presented in the tables 1-4.
After continuous application of T-gel the serum testosterone levels exceeded the upper limit of the normal range in four volunteers (P4, P6, P10, P12). For the volunteers P2, P3 and P5 increases of the serum testosterone concentrations, but not above the limit of the normal range, could be observed. The volunteers P16 and P17 showed no change of the serum testosterone levels. During the intermittent application five volunteers reached serum testosterone values above the normal range, three volunteers showed an increase within the normal range and one volunteer showed no change.

week	P2	P3	P4	P5	P6	P10	P12	P16	P17
1	16.5	9.5	28.7	12.9	17.1	21.9	23.3	20.6	18.5
2	**21.9**	**40.0**	**52.1**	**22.9**	**32.6**	**39.1**	**29.2**	**14.4**	**15.1**
3	**21.0**	**24.3**	**52.1**	**18.8**	**52.1**	**39.6**	**28.8**	**17.3**	**14.0**
4	**20.8**	**23.0**	**23.9**	**18.1**	**52.1**	**52.1**	**31.2**	**15.6**	**17.4**
5	**24.4**	**22.3**	**32.0**	**20.5**	**52.1**	**52.1**	**42.0**	**13.8**	**19.8**
6	**27.6**	**32.5**	**52.1**	**33.8**	**21.3**	**52.1**	**52.1**	**20.3**	**18.9**
7	**15.8**	**27.7**	**38.7**	**24.3**	**52.1**	**52.1**	**46.2**	**14.5**	**12.6**
8	15.3	11.8	24.3	15.5	23.4	17.0	14.7	19.1	14.0
9	15.8	11.0	27.3	15.9	16.2	19.6	25.9	18.6	16.0

Tab. 1: Total serum testosterone concentrations [nmol/l] of 9 volunteers before, during and after a 6 week continuous application (week 2-7) of testosterone gel (grey area: values during application). Normal range: 12-40 nmol/l

week	P1	P7	P8	P9	P11	P13	P14	P15	P18
1	22.1	29.4	15.4	13.6	22.0	18.4	28.3	24.3	26.3
2	**32.6**	**52.1**	**28.3**	**38.2**	**50.4**	**22.1**	**52.1**	**15.3**	**31.4**
3	25.0	27.7	16.3	14.6	27.5	18.3	29.7	19.6	23.6
4	**29.2**	**32.6**	**0.0**	**19.5**	**31.3**	**20.9**	**52.1**	**18.5**	**19.4**
5	24.3	23.5	17.8	13.9	28.2	18.1	33.1	19.8	28.1
6	**43.2**	**52.1**	**-**	**23.2**	**40.4**	**36.8**	**52.1**	**16.5**	**41.9**
7	23.7	17.9	0.0	14.5	26.4	17.2	28.1	-	27.2
8	21.7	22.2	0.0	-	29.2	18.5	37.8	22.6	24.1
9	19.0	23.4	0.0	14.0	22.6	21.1	31.9	17.9	28.8

Tab 2: Total serum testosterone concentrations [nmol/l] of 9 volunteers before, during and after an intermittent application (week 2, 4, 6) of testosterone gel (grey area: values during application). Normal range: 12-40 nmol/l

The serum LH-concentrations decreased in five volunteers after continuous application of T-gel below the lower limit of the normal range, two volunteers showed a suppression within

the normal range and 2 volunteers showed no change. After intermittent application of T-gel the LH concentrations decreased during application in six volunteers (P7, P8, P11, P13, P14, P15). In three volunteers no clear decrease could be observed (P1, P9, P18).
These results show that testosterone has penetrated through the skin into the circulation and has caused physiological effects. There is no clear explanation, why three volunteers showed no change of the serum testosterone concentrations.

week	P3	P4	P5	P6	P10	P12	P16	P17
1	6.35	2.49	4.39	4.43	3.39	4.76	4.87	3.80
2	**3.52**	**1.82**	**3.19**	**1.64**	**1.79**	**1.53**	**2.05**	**5.08**
3	**2.67**	**0.11**	**3.06**	**2.63**	**0.44**	**1.49**	**3.99**	**2.64**
4	**3.33**	**1.08**	**1.66**	**1.01**	**0.13**	**1.27**	**5.55**	**3.16**
5	**2.55**	**0.10**	**2.09**	**2.03**	**0.10**	**0.84**	**3.59**	**2.98**
6	**2.25**	**0.10**	**1.75**	**1.93**	**0.20**	**0.19**	**4.13**	**2.39**
7	**2.07**	**1.00**	**3.05**	**1.84**	**0.65**	**0.21**	**5.34**	**3.39**
8	7.12	6.09	6.16	4.02	3.60	3.03	4.20	4.81
9	7.57	5.72	4.23	4.71	6.30	2.00	2.96	4.37

Tab. 3: Serum LH concentrations [IU/l] of 9 volunteers before, during and after a 6 week continuous application (week 2-7) of testosterone gel (grey area: values during application). Normal range: 1.7-8.6 IU/l

week	P1	P7	P8	P9	P11	P13	P14	P15	P18
1	2.99	5.50	3.93	4.98	6.63	4.84	5.18	4.49	4.32
2	**2.50**	**2.31**	**1.65**		**4.16**	**4.08**	**3.39**	**2.23**	**4.09**
3	3.04	6.40	5.25	5.43	6.46	4.06	4.91	4.32	3.45
4	**3.11**	**1.96**		**6.49**	**4.89**	**2.51**	**3.02**	**1.73**	**2.02**
5	3.28	3.81	5.00	3.47	6.28	4.94	4.17	3.26	3.53
6	**2.83**	**1.41**		**5.12**	**4.05**	**3.37**	**2.98**	**2.09**	**3.23**
7	3.16	6.7		5.75	6.14	5.35	6.27		3.56
8	2.30	4.77			6.06	4.02	7.00	3.81	2.53
9	4.72	4.59		5.44	6.55	5.34	6.05	3.24	1.77

Tab 4: Serum LH concentrations [IU/l] of 9 volunteers before, during and after an intermittent application (week 2, 4, 6) of testosterone gel (grey area: values during application). Normal range: 1.7-8.6 IU/l

3.2 Steroid profile parameters

3.2.1 Discriminating parameters

The most obvious changes of the steroid profile after application of testosterone gel were the changes of the ratios Adiol/E, And/E and T/E. These changes were based on the decrease of the E concentrations and the increase of the T, And and Adiol concentrations. This is shown as examples for volunteer P5 and P11 in Figures 3 and 5. These results in young healthy volunteers confirm the observations obtained from middle-aged hypogonadal men (2).

The results also indicate that after transdermal application the conversion of testosterone to the 5-alpha-metabolites And and Adiol is stronger than to 5-beta-metabolites Etio and Bdiol. The conversion of testosterone to 5-alpha-steroids after transdermal application is probably due to a high 5-alpha-reductase activity of the skin.

3.2.2 The discriminating power of individual reference ranges

To compare the discriminating power of the limits of individual reference ranges versus limits of population based reference ranges, the T/E ratio was chosen as parameter. The individual reference range for a volunteer was calculated from the T/E values obtained before the administration of T-Gel. The limits of the individual reference range were defined as mean of the pretest values (about 20-40 values) ± 3 x standard deviation. As limit of the population based reference range was chosen the WADA cut off limit T/E = 4.

As presented in Figure 1 nearly all samples of volunteers P3 reach T/E ratios both above the cut off limit of 4 and far above the upper limit of the individual reference range. This was not the case for volunteer P5. During the T-Gel application more than 50 % of the T/E ratios didn`t exceed the cut off limit of 4, but nearly all T/E ratios exceeded the upper limit of the individual reference range of 1.86 (Fig. 2). Also individual reference ranges of other ratios, especially the ratios Adiol/E and And/E, show a strong discriminating power (Fig. 3).

Fig. 1: T/E ratios of volunteer P3 before and during the application of T-Gel.
dashed line: T/E=4; solid line: upper limit of individual reference range

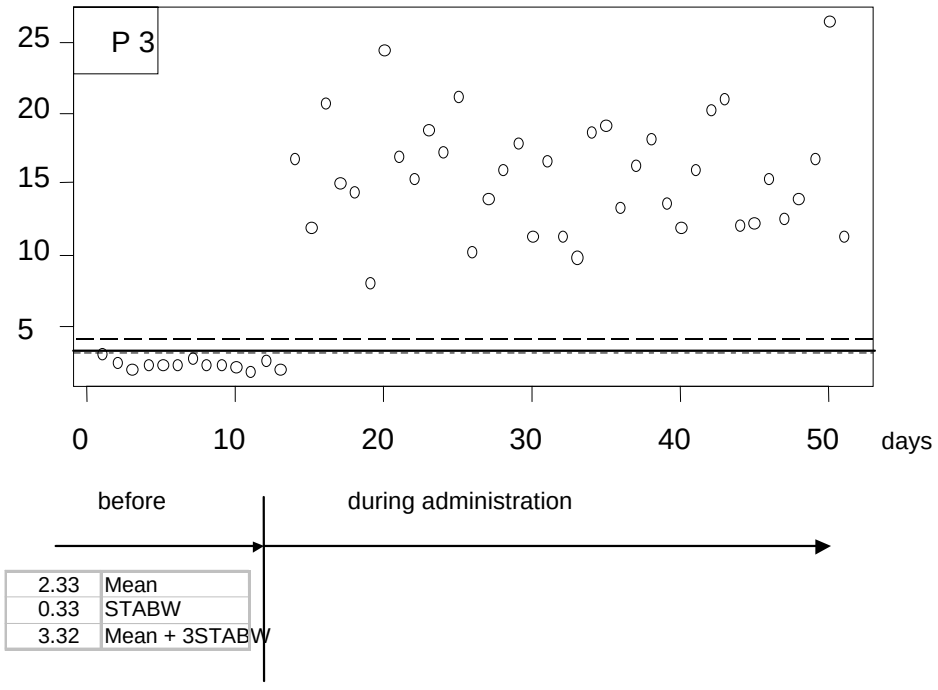


Fig. 2: T/E ratios of volunteer P5 before and during the application of T-Gel. dashed line: T/E=4; solid line: upper limit of individual reference range

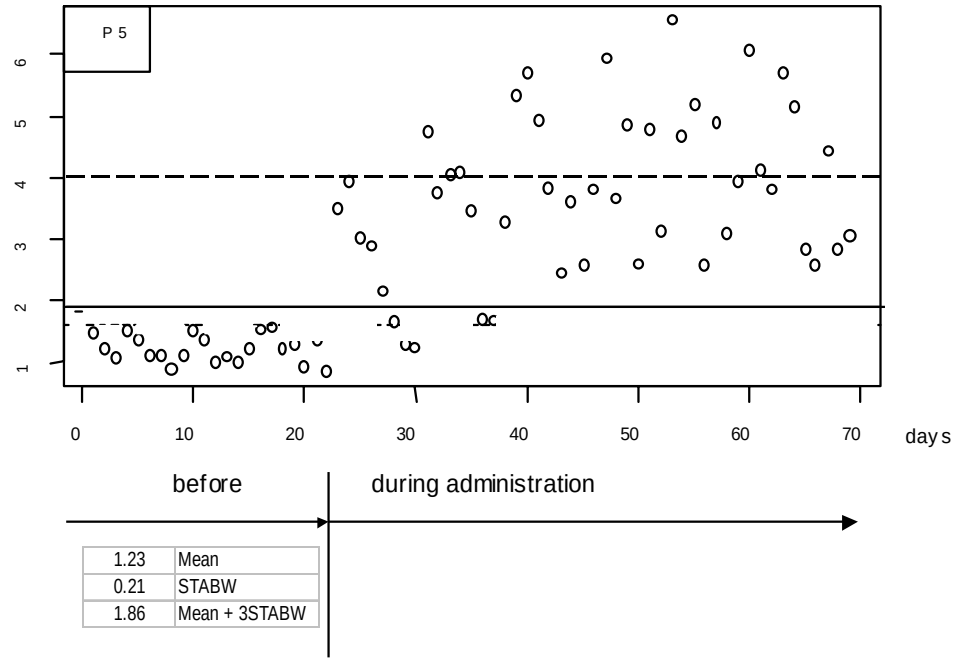
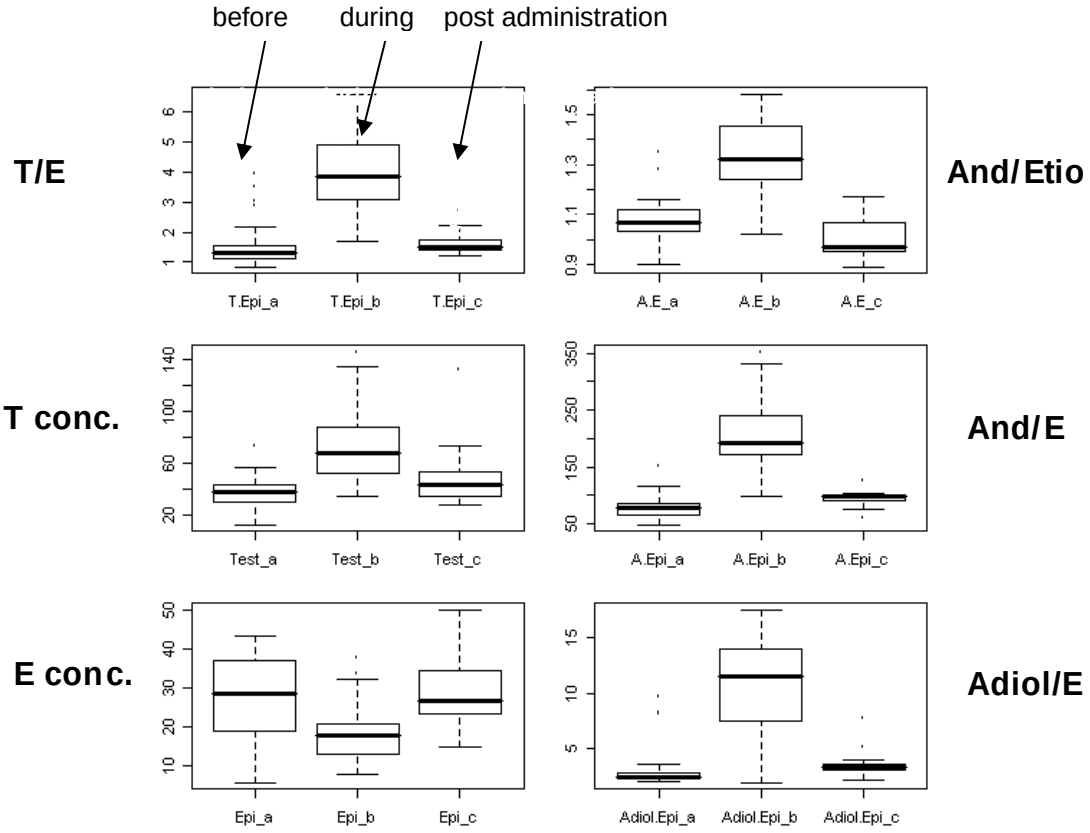


Fig. 3: Changes of steroid profile parameters before during and after administration of T-Gel (volunteer P5)



In Figure 4 are presented data of volunteer P11, who performed an intermittent application of T-Gel. The mean of his pretest T/E ratios was 0.44. During the T-Gel application all T/E-values were far below the cut-off limit of 4, but all values were above the upper limit of the individual reference range of 0.79. These results show that it is necessary to implement individual reference ranges for the detection of the misuse of T-Gel and that the cut off limit of 4 is not sufficient for this purpose. In Figure 5 the other steroid profile parameters of volunteer P11 are shown. Once again the discriminating power of the ratios Adiol/E and And/E is obvious.

Fig. 4: T/E ratios of volunteer P11 before and during an intermittent application of T-Gel. solid line: upper limit of individual reference range

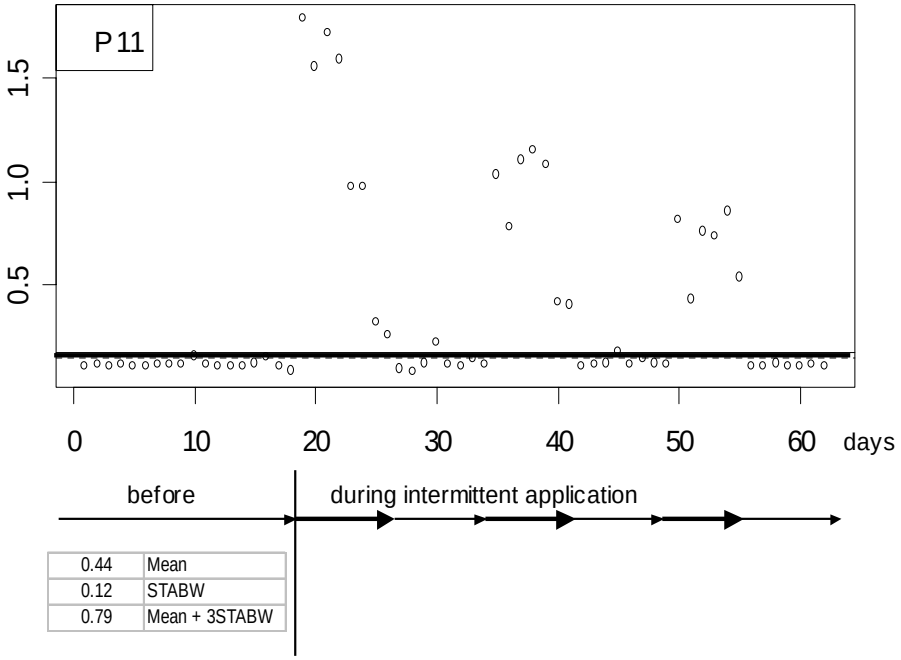
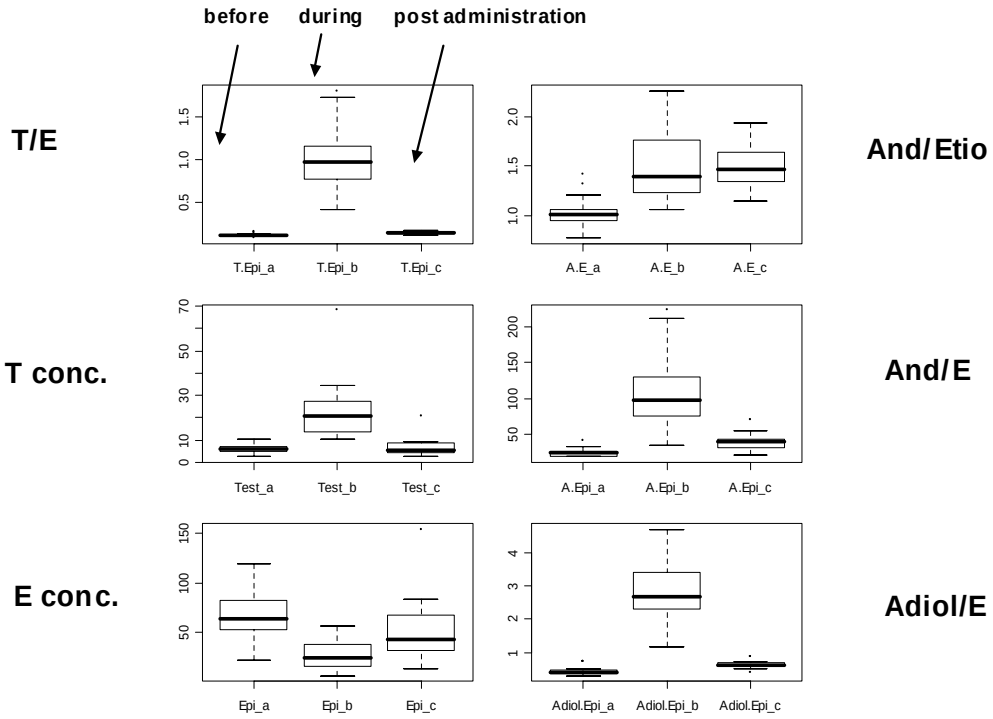


Fig. 5: Changes of steroid profile parameters before during and after administration of T-Gel (volunteer P11)



3.3 GC/C/IRMS results

At the moment only urine samples of 2 volunteers (P10 and P9) have been analysed with GC/C/IRMS. As target compounds were choosen And, Etio and T for volunteer P10 and additionally Adiol and Bdiol for volunteer P9. As internal reference compounds 11-hydroxy-androsterone (11OHA) and pregnanediol were analysed.

In Table 5 T/E values and the corresponding GC/C/IRMS results (difference of delta values of the target compounds and the internal reference compounds) of urine samples of volunteer P10 before, during and after the T-gel application are presented. All six T/E values during the application exceeded the WADA cut off limit of 4. The C/C/IRMS analysis only delivered 4 adverse findings, i.e. delta/delta differences above three (8), when And was chosen as target compound. The analysis of testosterone as target compound delivered adverse findings, i.e. delta/delta differences > 3, for all samples “on T-gel”.

In Table 6, T/E values and the corresponding GC/C/IRMS results of urine samples of volunteer P9 before, during and after the T-gel application are presented. Volunteer P9 has performed an intermittent application of T-gel. With the target compound And, the delta/delta

differences did not exceed the value of three, i.e. the administration of T-gel could not be detected with GC/C/IRMS. The analysis of T and Adiol as target compounds led to two and three delta/delta differences above three respectively. The delta/delta differences between Bdiol and 11OHA are much smaller than the differences between Adiol and 11OHA. These results once again indicate that T is most probably converted mainly to 5-alpha-steroids after transdermal application, due to the high 5-alpha-reductase activity of the skin.

	T/E	E-11OHA	A-11OHA	T-11OHA
P10/VV 4	0.55	1.46	0.69	0.8
P10/VV 7	0.60	1.21	0.36	1.03
P10/AP 4	**6.33**	**1.98**	**2.83**	**3.49**
P10/AP 10	**13.79**	**3.04**	**3.61**	**4.27**
P10/AP 15	**8.13**	**2.93**	**3.77**	**4.35**
P10/AP 21	**9.52**	**2.54**	**2.93**	**3.64**
P10/AP 27	**9.90**	**3.98**	**4.29**	**4.63**
P10/AP 31	**28.42**	**2.04**	**3.58**	**4.56**
P10/NB 6	1.43	1.13	1.15	2.99
P10/NB 13	1.00	0.51	-0.01	1.42

Tab. 5: T/E ratios and GC/C/IRMS data (delta/delta-differences) of urine samples of vounteer P10 before (VV), during (AP) and after (NB) a 6 week continuous application of testosterone gel (grey area: values during application).

Furthermore the results of the GC/C/IRMS analyses indicate that for the detection of the misuse of T-gel the target compounds T and Adiol may be much more suitable than And, Etio and Bdiol.

	T/E	A-11OHA	T-11OHA	Adiol-11OHA	Bdiol-11OHA
P09/VV 8	3.36	-0.60	0.10	1.10	1.30
P09/VV 19	1.99	0.40	0.80	2.00	1.10
P09/AP 4	**4.21**	**2.40**	**4.30**	**6.00**	**2.70**
P09/AP 6	**5.66**	**0.80**	**4.30**	**3.40**	**1.80**
P09/AP 8	2.60	-0.10	1.80	1.90	0.30
P09/AP 10	2.66	-1.10	0.40	1.20	-0.70
P09/AP 14	**4.64**	**1.30**	**3.20**	**4.40**	**1.40**
P09/AP 16	**3.99**	**0.30**	**2.90**	**3.10**	**0.80**
P09/AP 21	2.18	0.10	1.30	1.90	0.60
P09/AP 23	2.49	-0.20	1.70	2.10	0.50
P09/NB 1	2.45	-1.20	0.70	0.90	-0.30
P09/NB 5	2.56	-0.20	0.90	2.90	1.10

Tab. 6: T/E ratios and GC/C/IRMS data (delta/delta-differences) of urine samples of vounteer P9 before (VV), during (AP) and after (NB) a 6 week intermittent application of testosterone gel (grey area: values during application).

4 Conclusions

According to the actual results of the investigation the following can be concluded:

- The most discriminating parameters of the steroid profile for the detection of the misuse of T-gel are the ratios T/E, Adiol/E, And/E.
- The misuse of testosterone gel can be detected by the use of individual reference ranges. Limits of population based reference ranges (e.g. T/E > 4) are not sufficient.
- For the IRMS suitable target compounds are T and Adiol

5 Acknowledgements

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