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M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

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U.Mareck-Engelke, H.Geyer, U.Schindler, U.Flenker, R.Iffland* and M.Donike

Influence of Ethanol on Steroid Profile Parameters

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany
* Institut für Rechtsmedizin der Universität Köln, Germany

Abstract

Two grams of ethanol per kg body weight was orally applied to five male and six female healthy volunteers. Urine samples were taken during the application period, 24 hours before and 36 hours after.
Ethanol levels in blood and urine were determined by Headspace/GC.
The urine samples were prepared according to the screening procedure of conjugated anabolic steroids and analyzed by GC/MS.
The following steroid glucuronides were measured and quantified: androsterone (A), etiocholanolone (E), testosterone (T), epitestosterone (epiT), 11ß-OH-androsterone (OHA), 11ß-OH-etiocholanolone (OHE), 5α-androstan-3α,17ß-diol (Adiol) , 5ß-androstan-3α,17ß-diol (Bdiol), pregnandiol (Pregnd) and tetrahydrocortisol (THF).
The stability of ratios and excretion rates of endogenous steroids was investigated, the most important for judging steroid profiles in doping analysis being the ratios A/T and T/epiT.
For the female subjects the influence of ethanol, expecially for the ratios A/T and T/epiT is more obvious than for the male subjects.
The determination of blood and urine samples collected during the examination showed high levels of ethanol.
The most important fact of this investigation is that changes in steroid profile parameters, depending on alkohol consumption, are always connected with the presence of ethanol in the same urine sample.

Introduction

Effects of ethanol on the ratio between testosterone and epitestosterone have been already published by Falk, Palonek and Björkhem (2). The result of this ethanol study shows, that ethanol leads to a significant increase of the T/epiT-ratio. The values of the T/epiT-ratio were much lower than 6.
This publication raised further questions. Only 4 volunteers took part in this experiment. Only one urine sample prior to the experiment was taken. For statistical evaluation it should be better, to analyse urine samples taken over 24 hours before ethanol application to calculate individual reference ranges of the T/epiT- ratio for each volunteer.
Also missing were informations about kind of applicated alcohol, nutrition and daytime of the application.
To answer those questions the following study was performed .

Experimental

Sample preparation (1)
2 ml of urine and 20 µl of an internal standard mixture (17α-methyltestosterone 50ppm, [2,2,4,4,-$^2H_4$]-etiocholanolone 50ppm, [16,16,17,-$^2H_3$]-testosterone 2ppm, [2,2,4,4,$^2H_4$]-11ß-hydroxyandrosterone 14ppm) are added to a Amberlite XAD-2 column. The column (pasteur pipette, closed with glass pearl, bed height 2 cm) is washed with 2 ml of bidestilled water and the absorbed fraction is eluted with 2 ml of methanol. The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0.2 M sodium phosphate buffer pH 7.
To the buffer solution, 50 µl of beta-glucuronidase from E.coli is added and hydrolysis is performed for 1 h at 50°C. The buffered solution is alkalized with 250µl of 7% potassium carbonate solution to pH 9-10 and the steroids are extracted with 5 ml of tert.-butylmethylether on a mechanical shaker for 5 minutes. After centrifugation the etheral layer is transferred and evaporated to dryness under vacuo.

Derivatisation
The dry residue is derivatised with 100 µl of MSTFA/NH_4I/ethanethiol 1000:2:3 (v:w:v) and heated for 15 min at 60°C.
3 µl of the solution are injected into the GC/MS.

GC/MS parameters

GC/MS: HP 5890/HP 5971A (Hewlett Packard)
column: HP Ultra I (OV-1), 17m, 0.2mm i.d., 0.11 µm film thickness
carrier gas: 1ml helium at 180°C, split 1:10
temperature programm: 180°C, 3°C per min, 229°C, 40°C per min, 320°C

Determination of Ethanol (3)

50 µl serum or urine were filled together with 500 µl 0.04 g/l aqueous solution of t-butanol as internal standard into 20 ml headspace vials by a dilutor Eppendorf 5213. The vials were closed with butyl rubber seals and warmed up to 60°C before automatic headspace injection.

GC parameters

GC: F45 Perkin Elmer
column: 2 m stainless steel 1/8" i.d. with 15% Carbowax 1500 on Chromosorb W-NAW 80-100 mesh
temperatures: oven 80°C, injector and detector 150°C

Volunteers and Experimental Protocol

Five male (age: X=31±4 years; weight: X=77±14 kg)and six female (age: X=32±5 years; weight: X=68±8 kg) volunteers participated.
24 hours before the experiment started, prior urines were collected every 4 hours, starting with the morning urine and finishing at 10 p.m.
The following day the main experiment started. At 8.30 a.m. the weight of the volunteers was determined and standardized breakfirst was eaten. Alkohol intake started at 10 a.m.. During the next four hours ethanol was applicated step by step (the aim was 2 g per kilogram body weight). Application form of the ethanol was wodka. Also, the volunteers were allowed to drink non-alkoholic beverages.
Urine samples were taken every two hours until 10 p.m..
Blood samples were taken at 3 p.m. and 6 p.m..
The next day additionally to the morning urine every 4 hours urine samples were collected, including the morning urine from the day after.

Results and Discussion

Most people drink alcohol during the evening and nighttime.The experiment started at 10 o'clock in the morning. This uncommon time for the experiment was chosen because the volunteers had to deliver urine samples every 2 hours after the beginning of ethanol intake until 12 hours later. Such a high amount of urine samples are necessary to pursue possible variations in steroid profile parameter most exactly.

The participating volunteers of the experiment should applicate orally 2 g ethanol per kg body weight by drinking vodka within 4 hours. It was only possible for two volunteers to applicate 2 g ethanol per kg body weight. 7 of 11 volunteers stopped drinking alcohol before reaching the fixed amount because of vomitting and feeling sick. The real amount of applicated ethanol for most volunteers is situated in a range between 1.4 and 1.9 g per kg body weight.

Ethanol levels in blood (serum) and urine samples were determined twice by Headspace/GC (3).
Urine samples taken during the application-period showed different amounts of ethanol (tab 1), the highest being about 2.4 g/l for a male volunteer and 1.7 g/l for a female volunteer.
Blood samples were taken two times from every volunteer (3 p.m. and 6 p.m.). Results of the analysis are shown in table 2. The highest levels of blood alcohol were about 1.8 g/l for a female volunteer and 1.7 g/l for a male volunteer.
It is possible to detect ethanol in urine up to 24 hours after application of alcohol.

In this study urine samples were collected in three parts:
1. Samples collected every 4 hours, 24 hours prior to the experiment
2. samples collected during the experiment every two hours until 10 p.m.
3. samples collected every 4 hours the day after the experiment

Former investigations prove, that the ratios A/E, A/T and T/epiT belong to the most stable steroid profile parameters (4,5,6). For the urine samples taken one day before and one day after the experiment, this is true. During the application of alcohol a variation of the ratios A/T and T/epiT was obvious. The ratio T/epiT raised, the ratio A/T decreased (tab 3,4). The ratio A/E showed no variation. These changes depend on an increase of the excretion from testosterone and a decrease of the excretion from androsterone and etiocholanolone (fig 1,2). For judging steroid profiles the ratios A/T and T/epiT are very important. In urine samples taken prior and after the experiment, these ratios were situated within the population based reference ranges based on male athletes and 1742 female athletes (7). Urine samples taken during application of ethanol showed partly increased ratios of A/T under the lower limit and decreased T/epiT-ratios higher than the upper limit.

Judging the steroid profiles of the urine samples taken during ethanol intake, it is also obvious, that the ratios A/T and T/epiT are situated outside of the subject based reference range (5,6,8,9).
The ratios A/T and T/epiT taken at 3 different times (24 hours prior to consumption, 8 hours after beginning of the experiment and 24 hours after beginning of the experiment) were statistically evaluated (Friedman-Test). The statistical result is a high significant change of the steroid profil parameters A/T and T/epiT between urine samples taken during alcohol consumption and urine samples taken prior and/or after the experiment. For female volunteers this statistical significance is much stronger than for male volunteers (Tab 5,6).

Urine samples showing changes of steroid profile parameters, depending on the application of alcohol, always contain some ethanol (fig 1,2).

Conclusion

Influence of ethanol application can lead to a significant increase of the ratio T/epiT and decrease of the ratio A/T, possibly situated outside the popular based reference ranges.
Those changes depend on an increase of the excretion of testosterone and on a decrease of the excretion of androsterone.
It is also possible to detect ethanol in urine samples delivered after consumption of high amounts of alcohol.
Changes in steroid profile parameters (most important: T/epiT) depending on alkohol consumption are always connected with a presence of ethanol in the same urine sample.

It is very important for judging a high T/epiT-case in dope control to analyse additionally ethanol in this urine sample.

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Table 1: Influence of Ethanol on steroid profile parameters

Ethanol levels [g/l] in urine samples collected during alcohol intake

F1 - F6 = female volunteers M1 - M5 = male volunteers

EtOH appl. = probable amount of alcohol applicated (g/kg body weight)

		Ethanol [g/l]					
Date	Time (h)	F1	F2	F3	F4	F5	F6
EtOH	appl.	1.8	1.4	2	1.6	1.7	1.7
15.12.93	10.00	0	0	0	0	0	0
	12.00	0.56	1.39	0.55	0.86	0.45	0.75
	14.00	1.05	1.61	1.41	1.51	1.71	1.32
	16.00	0.94	1.19	1.74	1.11	1.59	1.23
	18.00	0.67	1.13	1.34	0.97	1.11	1.23
	20.00	0.43	0.62	1.37	0.73	0.63	0.15
	22.00	0.16	0.23	0.73	0.14	0.37	0.04
16.12.93	10.00	0	0	0	0	0.08	0

		Ethanol [g/l]				
Date	Time (h)	M1	M2	M3	M4	M5
EtOH	appl.	1.9	1.9	1.9	2	1.7
15.12.93	10.00	0	0	0	0	0
	12.00	0.49	0.10	1.05	1.02	0.45
	14.00	1.33	0.88	1.72	0.92	1.69
	16.00	1.10	1.04	1.08	0.84	2.41
	18.00	0.78	0.69	0.79	0.63	1.30
	20.00	0.52	0.22	0.80	0.53	0.78
	22.00	0.24	0	0.35	0.27	0.34
16.12.93	10.00	0	0	0	0.02	0.01

Table 2: Influence of Ethanol on steroid profile parameters

Ethanol levels [g/l] in blood samples (serum)

F1 - F6 = female volunteers M1 - M5 = male volunteers

EtOH appl. = probable amount of alcohol applicated (g/kg body weight)

	Ethanol [g/l]					
Time	F1	F2	F3	F4	F5	F6
EtOH appl.	1.8	1.4	2	1.6	1.7	1.7
15.00 h	1.23	1.29	1.83	1.55	1.52	1.33
18.00 h	0.78	0.79	1.25	1.15	0.99	0.80

	Ethanol [g/l]				
Time	M1	M2	M3	M4	M5
EtOH appl.	1.9	1.9	1.9	2	1.7
15.00 h	1.29	1.03	1.45	1.39	1.72
18.00 h	0.84	0.47	0.98	0.96	-

Table 3: Changes of steroid profile parameters during intake of alcohol

F1 - F6 = female volunteer 1-6

time = time after beginning of the experiment

time	F1		F2		F3	
	T/epiT	**A/T**	**T/epiT**	**A/T**	**T/epiT**	**A/T**
24 h prior (mean)	2.41	283	0.18	1468	0.58	133
+ 2	2.44	177	0.19	999	0.73	83
+ 4	2.90	93	0.25	631	1.18	41
+ 6	4.65	21	0.21	673	2.32	16
+ 8	6.00	20	0.19	634	3.06	19
+ 10	5.20	38	0.26	405	1.88	34
+ 12	5.41	62	0.25	419	2.01	24
+ 24	1.71	311	0.15	1579	0.57	145

time	F4		F5		F6	
	T/epiT	**A/T**	**T/epiT**	**A/T**	**T/epiT**	**A/T**
24 h prior (mean)	0.47	566	3.85	157	1.22	130
+ 2	0.62	306	5.00	71	1.78	67
+ 4	0.77	190	5.00	43	2.57	39
+ 6	1.40	80	6.00	25	4.85	16
+ 8	2.12	48	17	14	5.76	13
+ 10	2.31	47	22	6	2.92	37
+ 12	1.83	60	16	17	1.16	150
+ 24	0.25	488	2.46	221	1.17	158

Table 4: Changes of steroid profile parameters during intake of alcohol

M1 - M5 = male volunteer 1-5

time = time after beginning of the experiment

time	M1 T/epiT	M1 A/T	M2 T/epiT	M2 A/T	M3 T/epiT	M3 A/T
24 h prior (mean)	1.79	37	0.64	52	1.62	41
+ 2	2.27	32	0.65	48	1.36	35
+ 4	2.08	25	0.75	35	1.66	26
+ 6	2.35	21	0.74	35	1.78	30
+ 8	2.73	18	0.95	29	2.80	33
+ 10	2.63	16	0.99	26	2.22	38
+ 12	2.90	19	0.82	50	1.91	35
+ 24	1.33	56	0.51	84	1.08	95

time	M4 T/epiT	M4 A/T	M5 T/epiT	M5 A/T
24 h prior (mean)	0.84	78	1.06	72
+ 2	1.32	35	1.35	50
+ 4	1.38	27	1.41	36
+ 6	1.82	21	1.48	34
+ 8	2.85	17	1.99	30
+ 10	3.18	22	2.18	23
+ 12	2.40	31	2.10	27
+ 24	0.86	117	0.96	105

Table 5: Influence of Ethanol on steroid profile parameters

Statistical evaluation of changes in steroid profil parameters for female

F1 - F6 = female volunteer 1-6

p = statistical significance (*** = $p \leq 0.05$ = high significant)

T/epiT	- 24 h	+ 8 h	+ 24 h
F1	1.45	6.00	1.71
F2	0.17	0.26	0.15
F3	0.62	3.06	0.57
F4	0.41	2.31	0.25
F5	2.06	22	2.46
F6	1.17	5.76	1.16
p		0.0094	***

A/T	- 24 h	+ 8 h	+ 24 h
F1	299	20	311
F2	1376	405	1579
F3	127	16	145
F4	673	48	488
F5	198	6	221
F6	158	13	150
p		0.0111	***

Table 6: Influence of Ethanol on steroid profile parameters

Statistical evaluation of changes in steroid profil parameters for male

M1 - M5 = male volunteer 1-5

p = statistical significance (*** = $p \leq 0.05$ = high significant)

T/epiT	- 24 h	+ 8 h	+ 24 h
M1	1.78	2.73	1.33
M2	0.61	0.99	0.51
M3	1.72	2.80	1.08
M4	0.82	3.18	0.86
M5	1.08	2.18	0.96
p		0.0150	***

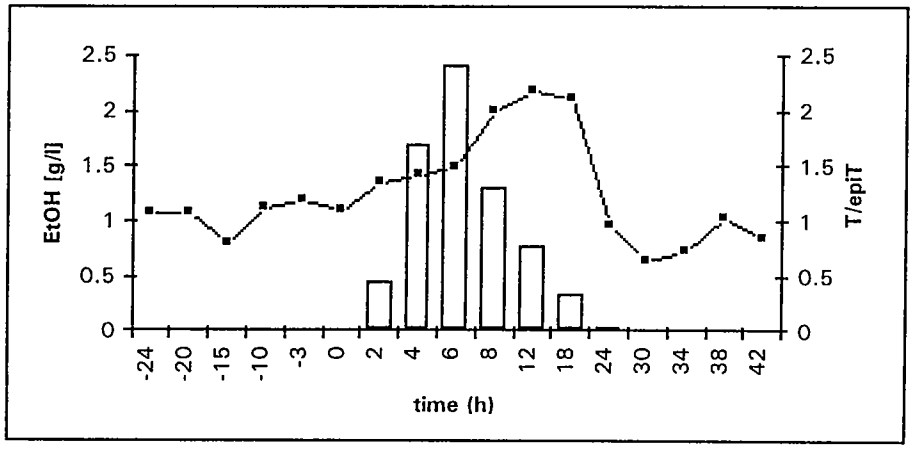
A/T	- 24 h	+ 8 h	+ 24 h
M1	38	16	56
M2	53	26	84
M3	39	26	95
M4	96	17	117
M5	74	23	105
p		0.0067	***

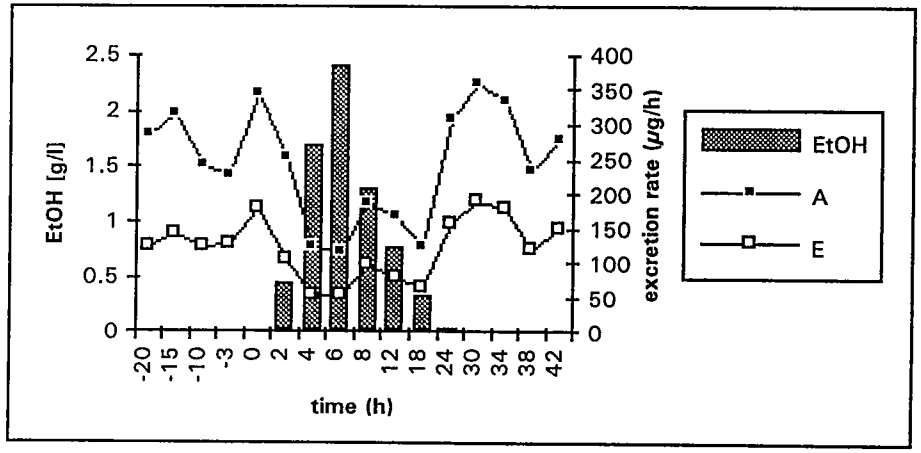
Remark: Calculated ratios from 3 different times were evaluated statistically (Friedman-Test)

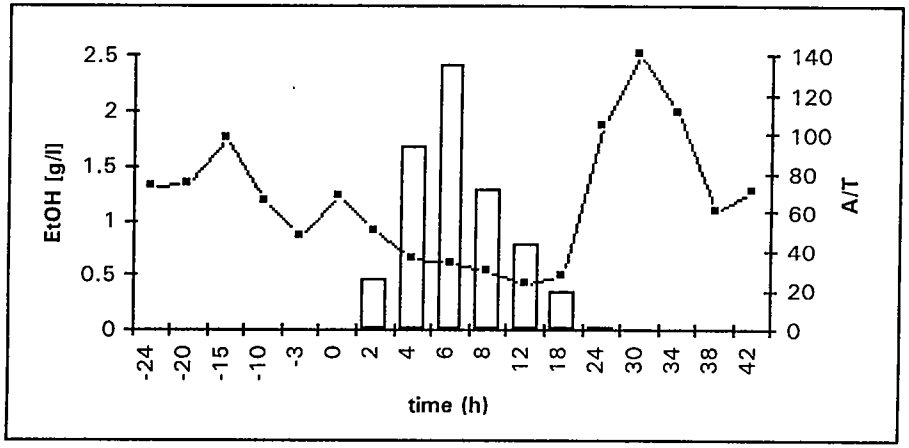
-24 h: 24 hours prior to consumption

+ 8 h: 8 hours after beginning of the experiment

+24 h: 24 hours after beginning of the experiment







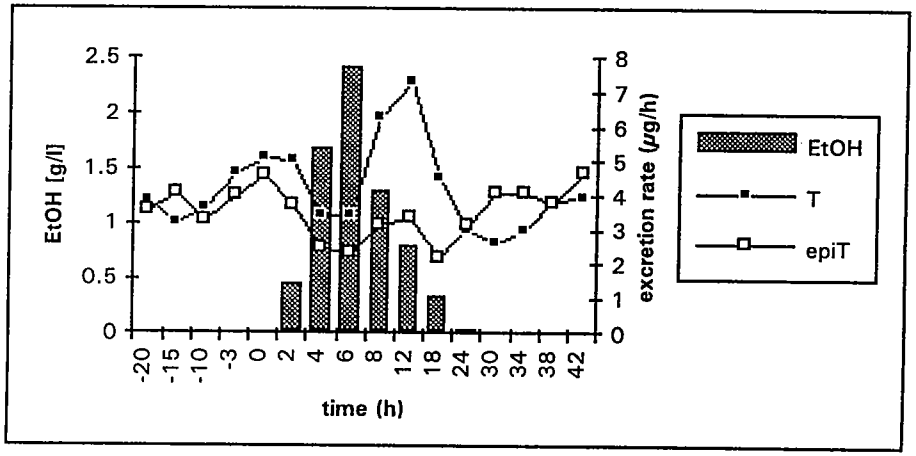
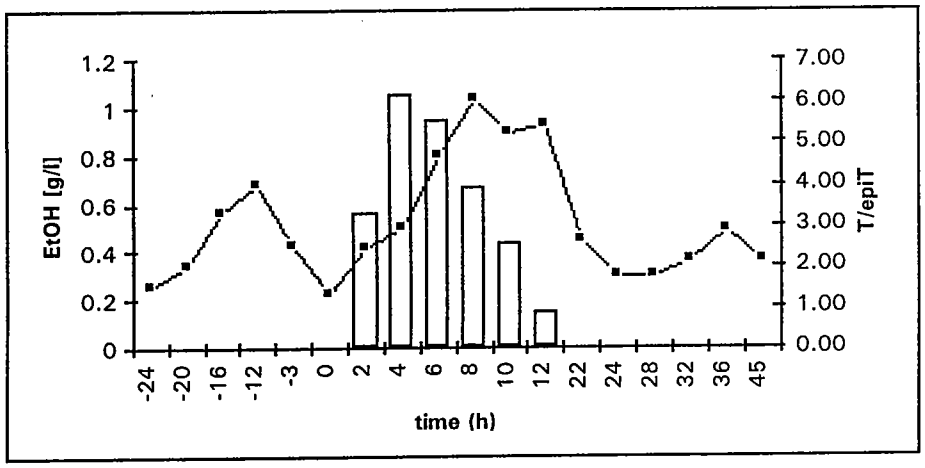
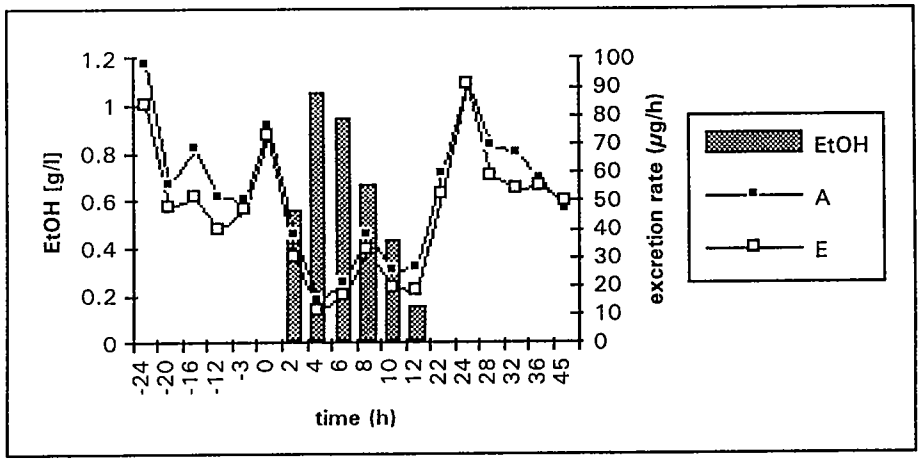


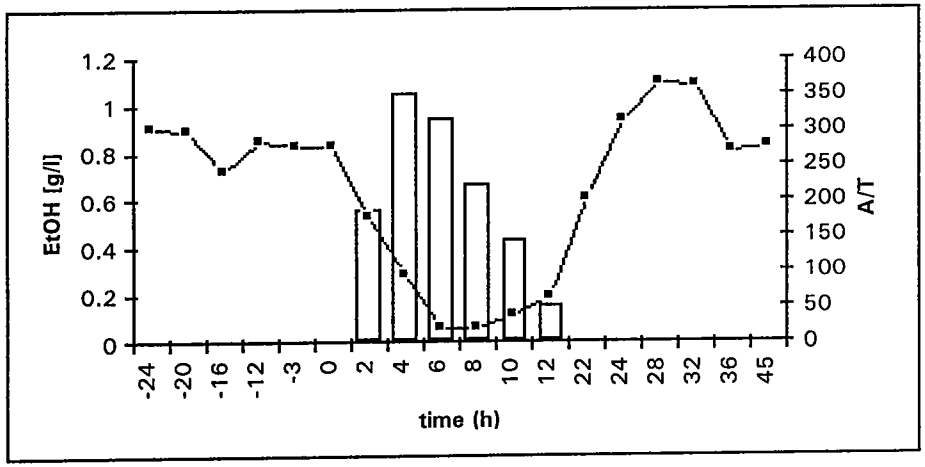
Fig 1:Influence of Ethanol on steroid profil parameters

changes of ratios and excretion rates of endogenous steroids during application of alcohol

volunteer M5







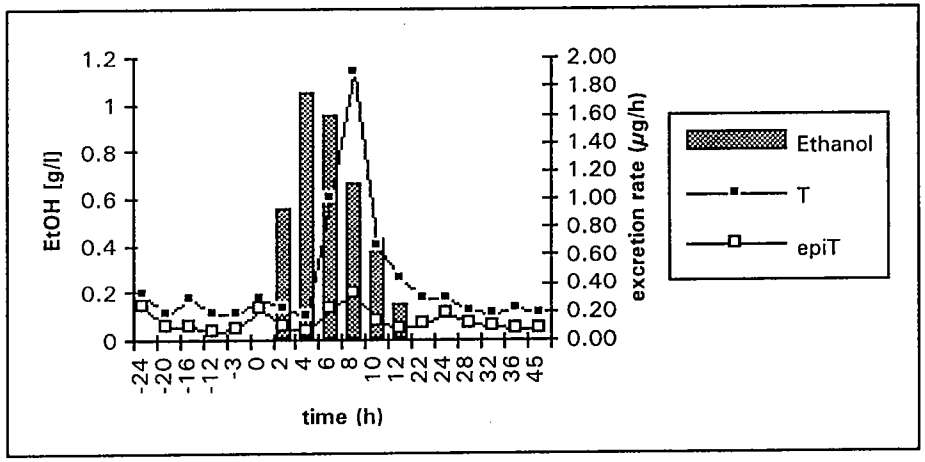


Fig 2: Influence of Ethanol on steroid profil parameters
changes of ratios and excretion rates of endogenous steroids during application of alcohol
volunteer F1