Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (6)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1999

K. CHROSTOWSKI, D. KWIATKOWSKA, A. POKRYWKA, K. BAL:
Aminoglutethimide Detection in Urine Samples
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (6). Sport und Buch Strauß, Köln, (1999) 443-454

K. Chrostowski, D. Kwiatkowska, A. Pokrywka, K. Bal

Aminoglutethimide detection in urine samples.

Department of Antidoping Research, Institute of Sport, Warsaw, Poland.

1. Abstract

Body builders started to use of aromatase inhibitors (e.g. aminoglutethimide - AG.) together with anabolic steroids, insulin and hGH in order to increased their anabolic effects. It seems likely that the new method of „improvement" of steroid doping will soon be accepted also by other sportsmen because of application of aromatase inhibitors have not been forbidden in sports yet. Until now during of doping control tests performed in body builders we found out one urine sample of the female 30 years old which contained aminoglutethimide. The aim of our study was to investigate simple method of detection of aminoglutethimide in urine samples. A capillary gas chromatographic method with nitrogen specific detection (NPD) is presented for screening measurement aminoglutethimide in urine sample. As the confirmation method we used GC/MS with and without derivatisation of MSTFA/TMCS/TMSImi. Influence of the single douse (250 mg) and long term application of AG on steroid profiling has been determined too.

2. Introduction

2.1 Essential information of the aminoglutethimide (AG) [4]

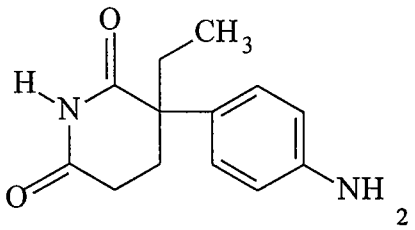


Fig 1. Chemical structure of Aminoglutethimide (AG)
3-(4-Aminophenyl)-3-ethyl-2,6-piperidinedione or 2-(p-aminophenyl)-2-ethylglutarimide) $C_{13}H_{16}N_2O_2$; mol wt 232.28.

Names of Drug:

Aminoglutethimid - ANPHARM PL, Orimeten, Cytadren - CIBA GEIGY AG,

Rodazol - GERMED. Tab. a' 250 mg.

Therapeutic dosage: 2 x 250 mg/ day up to 6 x 250 mg for months; together with hydrocortisone preparations.

Action: Adrenocortical, oestrogens and androgens suppressant that also inhibits conversion androgens to oestrogens by the aromatase enzyme system. Enzymes inhibited by aminoglutethamide: Cyp 11A1, 11B1, 11B2, (named also P450scc; P45011β; P450ald;) CYP17(P45017), CYP19 (P450arom), CYP21 (P45021) [3].

Therapeutic application: Palliative treatment of breast and prostatic cancer, Cushing's Syndrome, Hyperaldosteronismus, Anticonvulsant.

Adverse Effects: Drowsiness, ataxia, fever, skin rashes, gastro-intestinal disturbances. Most of the side effects diminish after 6 weeks administration due to enhanced metabolism of the drug. Bone-marrow depression with leucopenia, thrombocitopenia and agranulocytosis together with adrenal insufficiency may be serious side effects. Overdose may lead to central nervous system depression.

Pharmacokinetics

Aminoglutethimide is rapidly and completely absorbed following oral administration and peak of plasma concentration reach after 1 - 2 h. It is metabolise in the liver primary by acetylation to form N-acetylaminoglutethimide. The half-life at beginning of therapy as about 13 h is reported after administration of a single dose and decrease to about 7 h after 2 weeks of continuos treatment. About half of the dosage is excretion as unchanged parent compound in urine and the remainder as metabolites.

2.2. Principles of the action of Aminoglutethimide on steroidogenesis.

Aminoglutethimide exhibits inhibition on the biosynthesis pathways for steroid hormones in gonads and the adrenal cortex producers a state of „medical" adrenalectomy; by inhibitory action on peripheral aromatase blocks the conversion androgen to oestrogens.

Aminoglutethimide effects on regulation of the acute production of steroids initiated by tropic hormones (LH, FSH) in steroidgenic cells by inhibition (the first step of stereogenesis) of the enzyme complex $P450_{scc}$ - side chain cleavage of cholesterol (termed also CYP 11A1 of cytochrome P450 genes), which is localised in the mitochondrial inner membrane. Aminoglutethimide inhibits activity of this complex and results the accumulation of the cholesterol in the inner mitochondrial membrane of steroidogenic cells. But aminoglutethimide inhibit another enzymes of seroidegenesis too e.g. P45017α which carriers out two separate reactions: 17α-hydroxylation and C17-20 cleavage[7].

The biosynthesis of oestrogens is catalysed by microsomal enzymes known collectively as aromatase cytochrome P450arom the product of the CYP19 gene. Associated with the P450, flaviprotein and NADPH-reductase they modify A ring of androgens to the phenolic ring

characteristic to oestrogens [5]. The aromatase reaction utilises 3 mol oxygen and 3 mol NADPH for every mol of C19 steroid metabolised. All three oxygen molecules are utilised in the oxidation of methyl group to formic acid. In human tissue distribution of oestrogen biosynthesis are: in gonads-oestradiol (biosynthesis from testosterone), placenta - oestriol (from 16α-hydroxydehydroisoandrosterone), the adipose tissue - oestron (from androstenedione), as well as in the brain. As the result of the application of AG there are lack of all steroids hormones: testosterone, oestradiol, glicocorticoids and mineralocorticoids. Description of the case study of congenital aromatase deficiency in 24 years old male may supported of the idea of application of the aromatase inhibitors in body building practice. Same of details of the case are: height - 204 cm, weight 135 kg with macroorchidism. Plasma testosterone concentration = 2015 ng/ml; dihydrotestosterone =125 ng/ml; androstenedione = 335 ng/ml. Plasma LH and FSH concentration were more than 3 times above mean values[2].

AG is not very potent inhibitor. For example Fadrazol and 4-Hydroxy-androstenedione caused sustained inhibition of aromatase activity nearly 86-93%. Therefore it is possible to predict the usage of others kind of aromatase inhibitors for the doping [3].

2.3. The theoretical background of application of aminoglutethimide by body builders.

- Blockade of biosynthesis steroids in adrenal cortex suppresses catabolic effects of corticoids and increases anabolic affects induced by exogenous androgens.
- Blockade of oestrogens synthesis by aminoglutethimide may increase balance on the advantage of exogenous androgens.
- Inhibition of the aromatase enzyme system activity „prevents" conversion of exogenous androgens to oestrogens and increases (intensifies) effectiveness of androgens because androgens excess may increase aromatase activity[5].
- Prevention of development of gynecomastia by leading to lower conversion ratio of testosterone to oestrogen.

3. Material and method.

3.1. Urine samples collected up to 123 h. were obtained from the male volunteer (49 years old) after administration orally the single dose of aminoglutethimide a' 250 mg.

Additionally, we studied excretion of aminoglutethimide in urine samples of two women (76 and 50 years old) who used the drug to cure the cancer of the breasts. They applied: 2 tablets a' 250 mg per day for half a year.

3.2. Reagents and reference standards for aminoglutethimide detection

The reference substance of aminoglutethimide and glutethimide metabolite were obtained through Anpharm PL As internal standard we used diphenylamine obtained from POCH Potassium carbonate analytical grade from POCH; diethyl ether (peroxide free) - LABSCAN; sodium sulphate, anhydrous - POCH. Derivatisation procedure - just the same as in steroid profiling.

3.3. Sample preparation for aminoglutethimide detection

Procedure 1
(volatile stimulants and unconjugated narcotics)
to 5 ml of urine
+ K_2CO_3 to pH about 9,6
+ 5 µl solution of diphenylamine (1 mg/ml) (IS)
+ about 3 g Na_2SO_4 (anhydrous)
+ 1,0 ml diethyl ether (peroxide free)
extraction shake 20 min mechanical shaker
centrifuge at 3000 rpm/min for 5 min
separate organic phase
inject 2 µl to GC

3.4. Screening procedure

Analytical Parameters: Chromatograph HP 5890/NPD
GC column:16 m SE-54 (0,25 mm ID, film thickness 0,25 µm)
flow parameters

- carrier gas:	helium
- flow:	1,0 ml/min
injector parameters	
- injector mode	split 1:10
-injection volume	2 µl
- injector temperature:	270^{0}C
oven temperature program	
- initial temperature:	100 ^{0}C
- initial time :	1 min
- rate 1:	15^{0}C/ min (up to 220^{0}C)
- rate 2:	20^{0}C/min (up to 280^{0}C)
- final temperature:	280^{0}C
-final time:	7 min
NPD parameters	
- detector temperature	300^{0}C

3.5. Confirmation procedure

Analytical Parameters: GC HP 5890/MS HP 5972
GC column: 12 m SE 54 (HP-5MS) 0,20 mm ID, film thickness 0,33 µm

flow parameters	
-carrier gas:	helium
-flow:	1 ml/min
injector parameter	
- injector mode:	split 1:10
- injector volume:	2 μl
- injector temperature:	280^{0}C
oven temperature program	
- initial temperature:	100^{0}C
- initial time:	0,6 min
- rate:	15^{0}C/min to 220^{0}C 20^{0}C/up to 280^{0}C for 5 min.
- final temperature:	280^{0}C
- final time:	5 min
MDS parameters	
- detector temperature	290^{0}C
- ionisation mode:	EI, 70 eV
- acquisition mode:	Scan

3.6. Steroid profiling.

3.6.1. Reagents and reference steroids

Testosterone (T) and Epitestosterone (E) were purchase from Steraloids; methyltestosterone from Fluka; the mixture of deuterised internal standard solution: 17α-methylotestosterone, [2,2,4,4-^{2}H4] etiocholanolone, [16,6,17-^{2}H3]testosterone, [16,6,17-^{2}H3] epitestosterone and [2,2,4,4-^{2}H4]-11β-hydroxyandrosterone was obtained from Prof. W. Schänzer Cologne; Methanol A.R. (POCH Poland); diethylether A.R. - LABSCAN; MSTFA (Machery Nagel Germany); β-glucuronidase ex e. coli from Boeringer Mannheim (Germany); TMS-imidazole (Pierce, USA; TMS-J and dithioerithritol (Aldrich USA); XAD-2 resine (Serva Germany); RIA kits (SEROZYME) for LH determination were supplied by Serono.

3.6.2. Sample preparation for steroids determination

Urine samples were processed to routine procedure used in the laboratory for the analysis of conjugated and free fractions of steroids (Donike et al..[1]).

3.6.3. LH determination

Urinary LH was determined with SERONO assay kits (Serono U.K). Assay calibration was performed according to manufacturer's instruction; three quality control samples were included tin each run.

4. Results.

4.1. Detection of aminoglutethimide in urine samples

Our investigation revealed that amonoglutethimide appears in urine mainly as a unchanged parent compound together with same metabolites in lower concentrations.

Fig. 1 indicated one pick of aminoglutethimide in screening procedure one with GC (NPD) and calibration curve with striate line from 1.0 up to 12 μg/ml of the standard concentrations. Higher concentration were calculated by appropriate dilution of the samples. Limits of quantitation we assessed as 0.35 μg/ml, which we found out at 123 h after application of 250 mg AG. By the confirmation procedure with GC/MS (illustrated at fig. 2), we found one main peak of parent compound of aminoglutethimide (m/z 203, 232, 175) and two small peaks of acetylaminoglutethimide (m/z 203, 245, 274) and glutethimide (m/z 189, 117, 217). AG could be detected according to the free fraction for steroid procedure IV A. After the derivatisation MSTFA/TMCS/TMSImi. (100:5:2) we found out tetra -TMS Aminoglutethimide (m/z: 291, 433, 276, 448), di-TMS Aminoglutethimide (m/z 219, 361, 376) and mono-TMS Aminoglutethimide (m/z 275, 304, 247) as was illustrated on Fig 3.

4.2. Influence of aminoglutethimide on steroid profile in urine samples

Fig 4 presents aminoglutethimide excretion curve in urine after application of the single dose 250 mg by the male volunteer (49 years old). After 24 h there was decreased the concentration up to 50% maximal value and after 123 h we could detected 0,35 μg/ml. Fig 4 presented the influence of the aminoglutethimide on and T/E ratios, testosterone and epitestosterone (in ng/ml) and LH (in U/l) changes concentrations in urine. Our observation indicated that LH concentration increased after application of aminoglutethimide. It may be result rather low concentration of oestrogens after aromatase inhibition than low testosterone concentration which had been the low value during all the time of observations. We did not found any influence after application of aminoglutethimide on T/E and T/LH ratios. Fig 5 presented the effects of long term effects by 6 months of the administration of aminoglutethimide (two tables a' 0.250 daily) due to of the breast cancer in two women (50 and 76 years old) in urine samples. In two cases we found out high LH concentrations (higher in younger one) which corresponded to higher concentrations of the drug in the case.

5. Conclusion:

- Augmentation of anabolic steroids effects by application of aminoglutethimide or other aromatase inhibitors seems to be quite possible in body builders practice.
- Detection and identification of aminoglutethimide in urine samples by using screening one and confirmation procedure by GC/MS is simple and reliable method.

5. Reference:

1. Donike M., S. Rauth, A Wolansky: Reference Ranges of Urinary Endogenous Steroid Profile . 11-th Cologne Workshop on Dope Analysis. Proceeding 1991: 69-83,

2. Morishmina A., M.M. Grumbach, E.R. Simpson, C. Fisher, K. Qin: Aromatase Deficiency in Male and Female Siblings Caused by a Novel Mutation and Physiological Role of Estrogens. J. Clin. Endocrin. and Metabol. 1995. 80,12: 3689-3698,

3. Rendic S.: Drug Interaction in Biosynthesis and Metabolism of Steroid Hormones. The Role of Human Cytochrome P450s. In: Recent Advances in Doping Analysis (4) Proceedings of the Manfred Donike Workshop 14th Cologne Workshop on Doping Analysis. 1996: 17-22,

4. Reynolds J.E.F editor.: MARTINDALE . The Extra Pharmacopoeia, Edition 31. London 1996: 541,

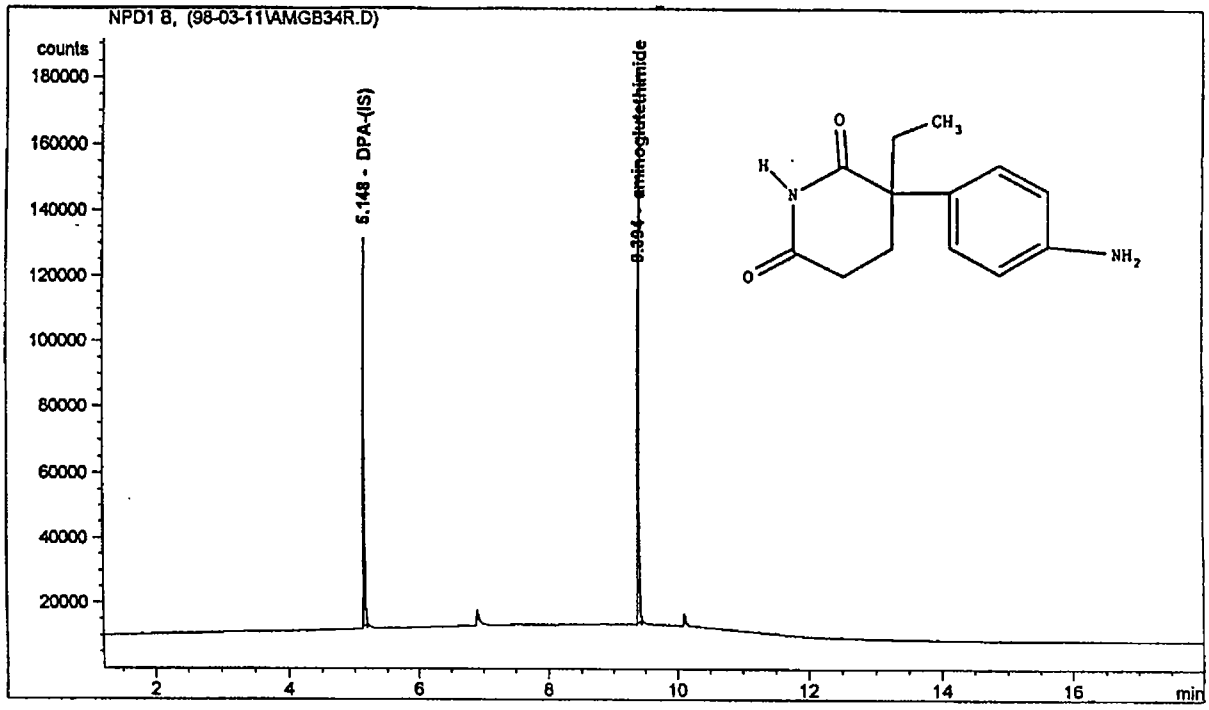
5. Simpson E.R. et al.: Aromatase Cytochrome P450, The Enzyme Responsible for Oestrogen Biosynthesis. Endocrine Reviews. 1994. 15, 3: 342-355,

6. Stillman S.C., B.A.J. Evans , I.A. Hughes: Androgen dependent stimulation of aromatase activity in genital skin fibroblasts from normal and patients with androgen insensivity. Clin Endocrin. 1991.35: 533-538,

7. Stocco D.M., Clark BJ.: Regulation of the Acute Production of Steroids in Steroidogenic Cells. Endocrine Reviews. 1996.17.3: 221-239.

Fig. 2.

aminoglutethimide, urine 34h



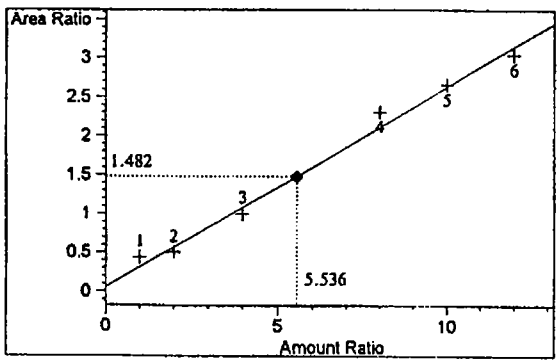
Sample ISTD Information:

ISTD #	ISTD Amount [ug/ml]	Name
1	1.00000	DPA-(IS)

RetTime [min]	Type		Area counts*s	Amt/Area ratio	Amount [ug/ml]	Grp	Name
5.148	BB	I	1.45411e5	1.00000	1.00000		DPA-(IS)
9.394	BB		2.15525e5	3.73533	5.53642		aminoglutethimide

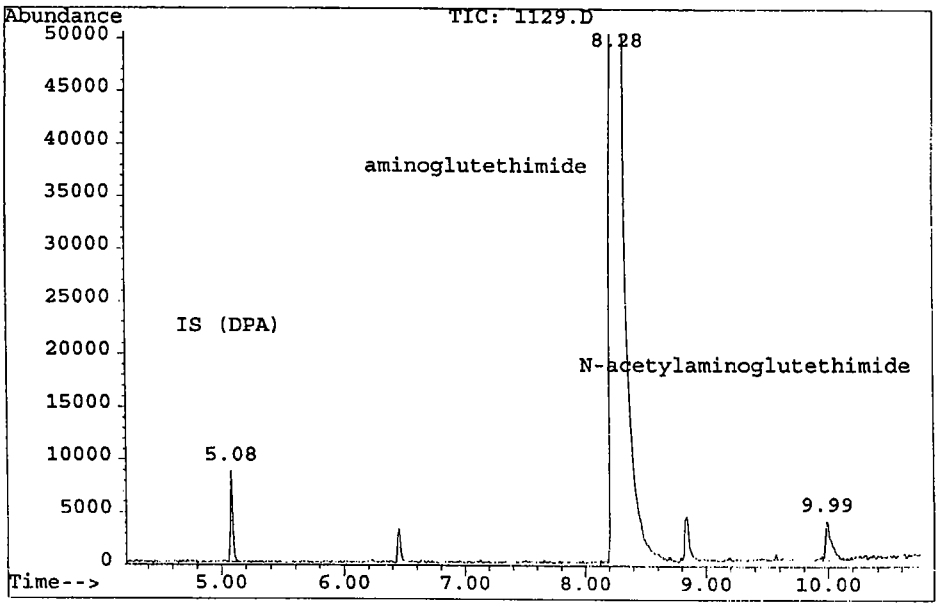
Totals without ISTD(s) : 5.53642

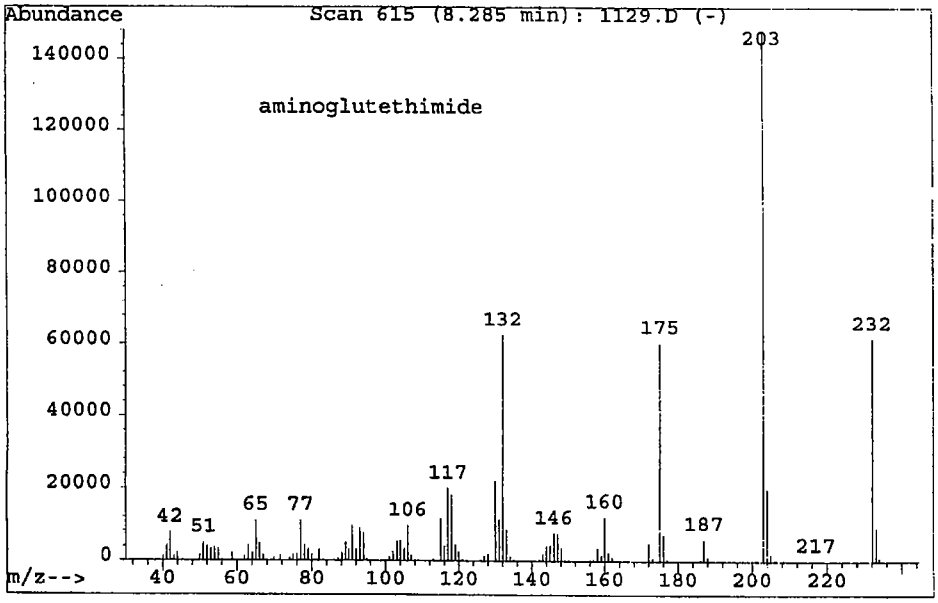
Calibration Curves



aminoglutethimide at exp. RT: 9.407
NPD1 B,
Correlation: 0.99557
Residual Std. Dev.: 0.12561
Formula: y = mx + b
m: 2.57390e-1
b: 5.71545e-2
x: Amount Ratio
y: Area Ratio

Fig. 3.


Abundance
TIC: 1129.D
8.28
aminoglutethimide
IS (DPA)
N-acetylaminoglutethimide
5.08
9.99
Time-->
5.00
6.00
7.00
8.00
9.00
10.00


Abundance
Scan 615 (8.285 min): 1129.D (-)
aminoglutethimide
203
132
175
232
117
65
77
106
160
42
51
146
187
217
m/z-->
40
60
80
100
120
140
160
180
200
220

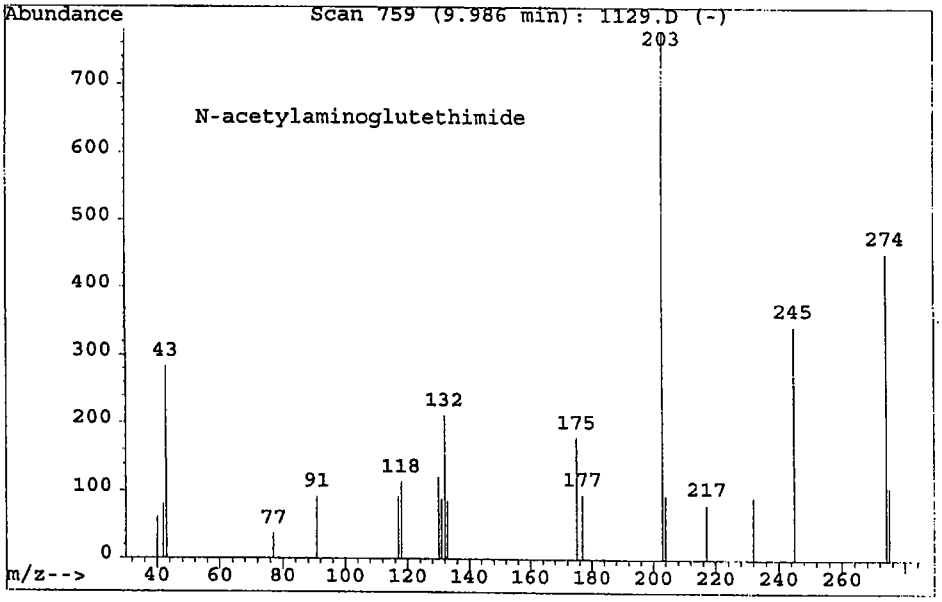

Abundance
Scan 759 (9.986 min): 1129.D (-)
203
N-acetylaminoglutethimide
274
245
43
132
175
118
91
177
217
77
m/z-->
40
60
80
100
120
140
160
180
200
220
240
260

Fig. 4.

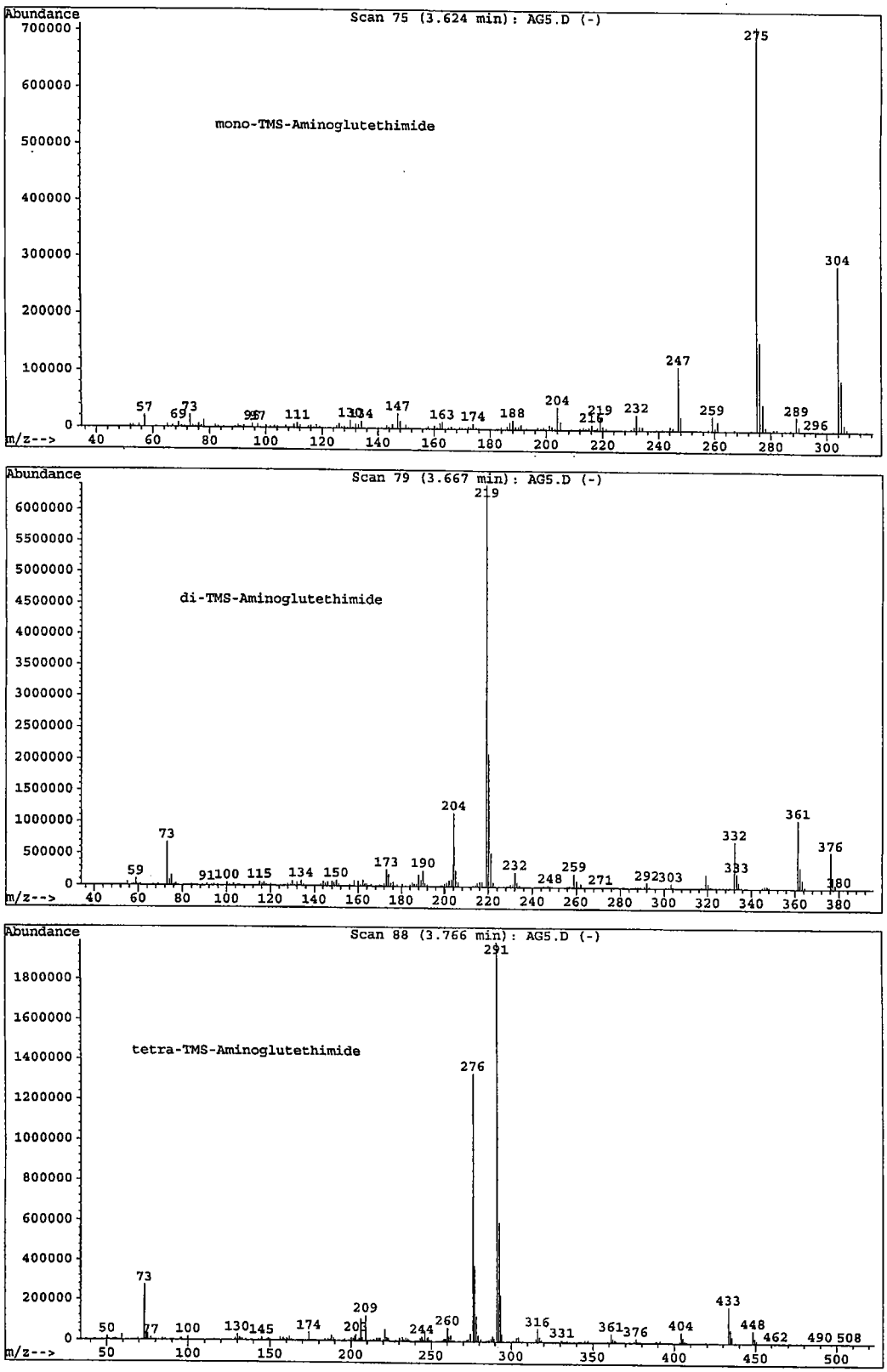

Abundance
Scan 75 (3.624 min): AG5.D (-)
mono-TMS-Aminoglutethimide
275
304
247
204
m/z-->
Abundance
Scan 79 (3.667 min): AG5.D (-)
219
di-TMS-Aminoglutethimide
204
73
361
332
376
m/z-->
Abundance
Scan 88 (3.766 min): AG5.D (-)
291
tetra-TMS-Aminoglutethimide
276
73
209
433
m/z-->

Fig. 5.

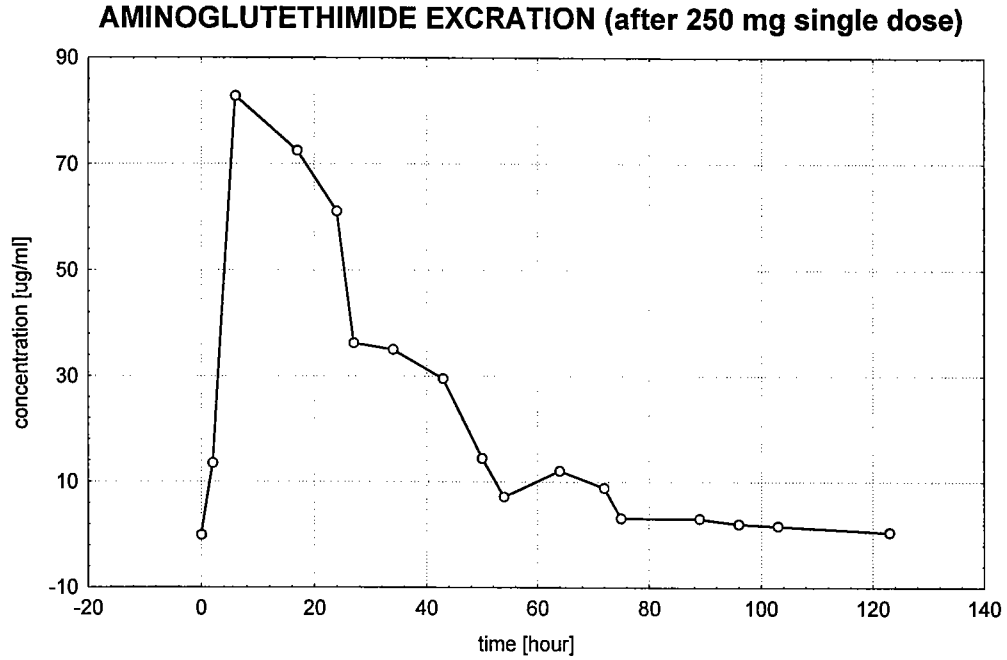

AMINOGLUTETHIMIDE EXCRATION (after 250 mg single dose)
concentration [ug/ml]
time [hour]
90
70
50
30
10
-10
-20
0
20
40
60
80
100
120
140

Fig. 6.

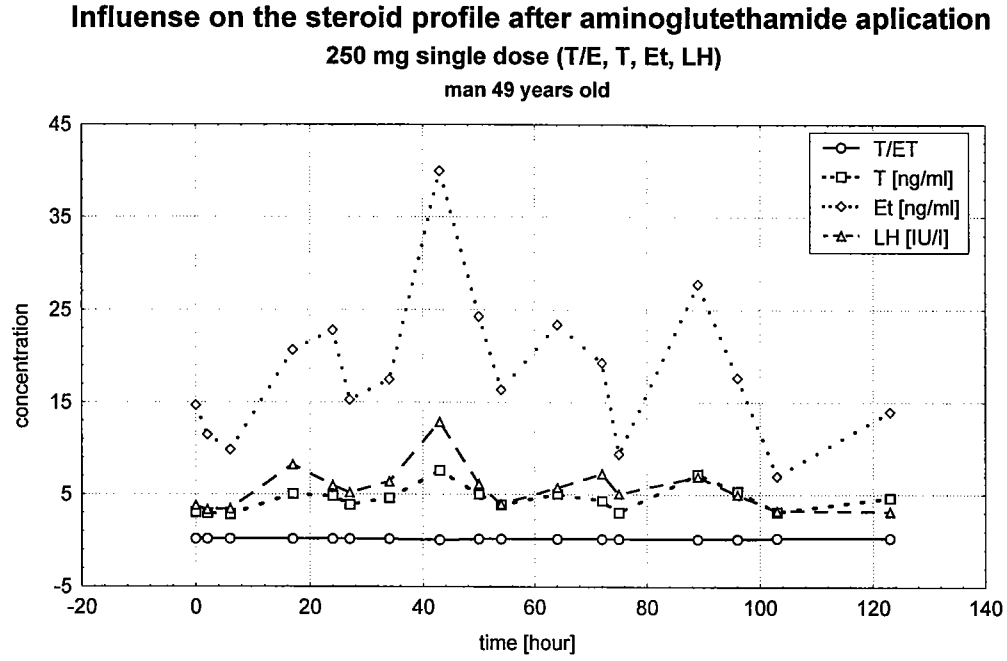
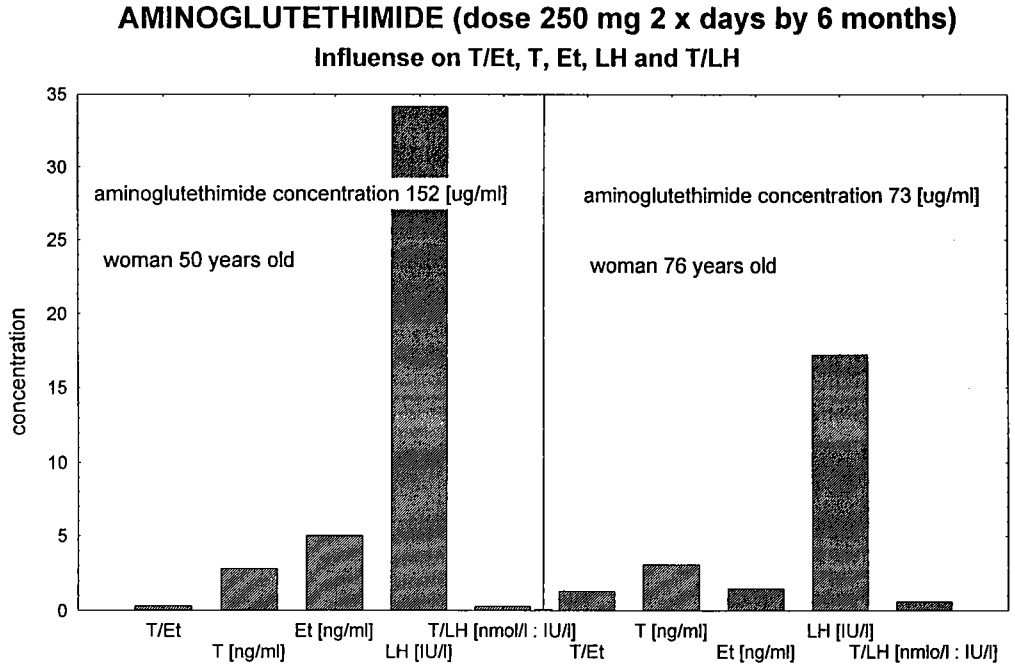

Influense on the steroid profile after aminoglutethamide aplication
250 mg single dose (T/E, T, Et, LH)
man 49 years old
concentration
time [hour]
T/ET
T [ng/ml]
Et [ng/ml]
LH [IU/l]
45
35
25
15
5
-5
-20
0
20
40
60
80
100
120
140

Fig. 7.


AMINOGLUTETHIMIDE (dose 250 mg 2 x days by 6 months)
Influense on T/Et, T, Et, LH and T/LH
concentration
35
30
25
20
15
10
5
0
aminoglutethimide concentration 152 [ug/ml]
woman 50 years old
aminoglutethimide concentration 73 [ug/ml]
woman 76 years old
T/Et
T [ng/ml]
Et [ng/ml]
LH [IU/l]
T/LH [nmol/l : IU/l]
T/Et
T [ng/ml]
Et [ng/ml]
LH [IU/l]
T/LH [nmlo/l : IU/l]