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Identification of the Aromatase Inhibitor Aminoglutethimide in Doping Analysis

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Abstract

Purpose of this study was the identification and enclosure of the parent compound or the main metabolite of the aromatase inhibitor aminoglutethimide into established, running screening procedures in doping analysis.
An excretion study was carried out by oral application of one single therapeutical dose (500 mg) Orimeten® .
The analysis was performed by gas chromatography/mass spectrometry (GC/MS) according to the standard operating procedures for volatile nitrogen containing drugs (screening 1), heavy volatile nitrogen containing drugs (screening 2) and anabolic steroids (screening 4).
Aminoglutethimide is excreted almost totally as unconjugated parent compound.
In Screening 1 also small amounts of the metabolites nitroglutethimide and acetaminoglutethimide were identified, the underivatised fraction of screening 2 shows additionally chlorinated aminoglutethimide formed during the hydrolysis with hydrochloric acid in varying amounts. In screening 4 the tris-TMS and tetrakis-TMS derivatives of aminoglutethimide as well as small amounts of the metabolite acetylaminoglutethimide,tris-TMS were detected. Another substance was identified as derivatisation artefact: during silylation with MSTFA/NH_4I/ethanethiol incorporation of an ethylmercaptogroup occurred thus increasing the molecular weight by 60 amu.
The most suitable screening procedure for the enclosure of aminoglutethimide is the underivatised fraction of screening 2. Due to the fact, that in screening 2 only competition samples are analysed the additional inclosure of aminoglutethimide in the screening procedure for anabolic androgenic agents is recommended.
Full scan spectra and diagnostic ions for the analysis of aminoglutethimide in screening 2 and screening 4 are presented as well as full scan spectra of the additional identified metabolites.

Introduction

As notified by the IOC and WADA on June 1, 2001 the use of aromatase inhibitors is prohibited for males beginning on September 1, 2001.
Aromatase inhibitors are listed in the class of peptide hormones, mimetics and analogues.
The aromatase transforms testosterone and its precursor androstenedione into estrogens.
Aromatase inhibitors are therapeutically used in treatment of metastatic breast cancer in post-menopausal women because the inhibition of the biosynthesis of estrogens may lead to the degeneration of tumor tissue.
Aromatase inhibitors have been divided into three classes:

1. Aminoglutethimide
2. Non steroidal aromatase inhibitors (Anastrozol, Letrozol, Vorozol)
3. Steroidal aromatase inhibitors (Exemestane, Formestane)

For males no medical indication for the application of aromatase inhibitors exists. However, male athletes may be encouraged to treat the adverse effects of an extensive abuse of anabolic androgenic steroids (gynaecomastia) by using aromatase inhibitors.
The postulated increase of the testosterone concentration and stimulation of the testosterone biosynthesis are not scientifically proved.

Experimental

Sample preparation
The urine samples were prepared and analyzed according to the standard operating procedures for volatile nitrogen containing drugs [1], heavy volatile nitrogen containing drugs [1] and anabolic steroids [1,2]. A modification of the standard operating procedure for screening 2 (heavy volatile nitrogen containing drugs) is performed: The methanolic layer is divided and one part injected into GC/MS without derivatisation. Substances are analysed as free substances to detect mesocarb and/or its metabolite and some low concentrated stimulants. The other part of the methanolic layer is evaporated again and derivatised with MSTFA.
GC/MS parameters
GC/MS: HP 6890/HP 5973 (Hewlett Packard)
electron impact: 70 eV
Screening 1:
column: HP 5MS, 24m, 0.25mm i.d., 0.25 µm film thickness
carrier gas: helium 12 psi, split 1:8
temperature program: 0 min100°C, + 22°C / min, 2 min 330°C
injection volume: 5 µl

Screening 2:

column: HP 5MS, 24m, 0.25mm i.d., 0.25 µm film thickness
carrier gas: helium 12 psi, split 1:8
temperature program: 0 min 95°C, + 22°C / min, 2 min 330°C
injection volume: 3 µl

Screening 4:

column: HP Ultra I (OV-1), 17m, 0.2mm i.d., 0.11 µm film thickness
carrier gas: helium 12 psi, split 1:10
temperature program: 0 min 180°C, + 3°C / min, 0 min 229°C, + 40°C / min, 2 min 320°C
derivatisation: the dry residue was derivatized with 100 µl of MSTFA/NH_4I/ethanethiol 1000:2:3 (v:w:v) and heated for 20 min at 60°C
injection volume: 3 µl

Urine samples

An excretion study was performed by a healthy male volunteer. One single therapeutical dose of aminoglutethimide (500 mg) was administered. For 30 hours all urine samples were collected and thereafter only morning urine samples for in total 7 days.

Metabolites

Aminoglutethimide in the urinary matrix was identified by comparison of its mass spectrum and retention time with those of a reference standard.

The metabolites nitroglutethimide and acetaminoglutethimide described in the literature [3] were identified by their mass spectra.

Results and Discussion

Screening for volatile nitrogen containing drugs (screening 1)

Aminoglutethimide is detected almost totally as parent compound. Additional small amounts of nitroglutethimide and acetaminoglutethimide are identified (Fig.1).

The 70 eV mass spectrum of aminoglutethimide shows an abundant molecular ion (*m/z* 232) and neutral loss of ethene forms *m/z* 204. The base peak *m/z* 203 is generated by the loss of an ethyl radical and subsequent losses of CO and thereafter of HNCO lead to the abundant fragments *m/z* 175 and *m/z* 132. The proposed fragmentation pattern is based on the determination of the accurate masses of *m/z* 204, 203, 175 and 132 (Fig.2) [4].

For the other metabolites no reference compound was available. Acetylaminoglutethimide and nitroglutethimide are described in literature but no fragmentation pattern is given there. Acetylaminoglutethimide shows as molecular ion *m/z* 274. Loss of an ethyl group leads to *m/z* 245. Another significant fragment is *m/z* 217 corresponding to *m/z* 175 of aminoglutethimide. All

other fragments including the base peak at *m/z* 203 comply to the fragments of aminoglutethimide itself after loss of ketene (Fig.3).
Nitroglutethimide shows as molecular ion *m/z* 262. Loss of 28 leads to *m/z* 234, but the question is whether CO or C_2H_4 is eliminated. Other significant fragments are *m/z* 205 and 177, corresponding to *m/z* 175 and 147 (Fig.4).

It was possible to detect aminoglutethimide up to 92 hours after administration. Further investigations have shown that ether extraction at pH 9.6 instead of pH 14 leads to significantly better yields. Recovery efficiency for the ether extraction of aminoglutethimide at pH 9.6 is about 84%.

Screening for heavy volatile nitrogen containing drugs (screening 2)
Fig.5 shows the underivatised fraction analysed according to screening procedure 2. The major peak is again free aminoglutethimide. The second peak is an artefact arising during the hydrolysis with hydrochloric acid, that formed chlorinated aminoglutethimide.
Molecular ion is *m/z* 266, molecular ion minus ethyl group *m/z* 237. Other significant fragments are *m/z* 209, 181, 166 and 152. The fragmentation corresponds to that of free aminoglutethimide (Fig.6) indicating that the chlorine is bond to the aromatic moiety.
The detected peaks in the derivatised fraction of screening 2 are mixtures of different substances and/or different derivatisation products, which coelute in two major peaks. A hydroxylated metabolite was found here.

It was possible to detect aminoglutethimide in the underivatised fraction of screening 2 up to 165 hours after administration (Fig.14). For monitoring purposes the most prominent ions at *m/z* 203, 232 and 175 are suitable.
Detected concentrations for aminoglutethimide are approximately: 125 µg/ml (7h), 15 µg/ml (47h) and 0.1 µg/ml (165h).

Screening for anabolic steroids (screening 4)
After derivatisation the tris-TMS and tetrakis-TMS derivatives of aminoglutethimide, as well as lower amounts of the metabolite acetaminoglutethimide, N,N′,O-tris-TMS were detected (Fig.7).
Aminoglutethimide, N,N,N′-tris-TMS shows as molecular ion *m/z* 448 and molecular ion minus methyl group *m/z* 433. Base peak is *m/z* 291 (Fig.8).
The major peak is due to aminoglutethimide, N,N,N′,O-tetrakis-TMS with an intense molecular ion (*m/z* 520). Further intense fragment ions are generated by the loss of a methyl radical from

the TMS moiety (*m/z* 505), the loss of ethene (*m/z* 492) or the loss of an ethyl radical (*m/z* 491). The fragments *m/z* 376 and *m/z* 262 are best explained by neutral losses of TMS-NCO and subsequently TMS-OCCH from *m/z* 491. A retro-Diels-Alder rearrangement of the molecular ion leads to *m/z* 305 (Fig.9).
Detected metabolite in low amounts is N-acetyl-aminoglutethimide, N,N′,O-tris-TMS with molecular ion *m/z* 490, molecular ion minus methyl group *m/z* 475 and molecular ion minus ethyl group *m/z* 461. Other significant ions are *m/z* 275, 261 and 232 (Fig.10).
As derivatisation artefact a during silylation with MSTFA/NH_4I/ethanethiol formed substance is presented in Fig.11. The incorporation of an ethylmercaptogroup increases the molecular weight by 60 amu. The given chemical structure is a proposed formula.
To prove the formation of aminoglutethimide+60, tetrakis-TMS the pure standard aminoglutethimide was derivatised with MSTFA/NH_4I/ethanethiol and analysed several times. Fig.12 shows the formation of aminoglutethimide+60, tetrakis-TMS. Direct after derivatisation only small traces of the substance are detectable. After 5.5 hours the peak is well detectable and after 39 hours it is the major peak in this window.
In urine samples this formation appears more rapidly. Probably the biological matrix acts as promotor for the reaction. Already in the even derivatised urine sample a distinct peak appears in ion trace *m/z* 432 eluting between epitestosterone and testosterone.

In the SIM-mode (*m/z* 520, 505, 491) the detection of aminoglutethimide, tetrakis-TMS is possible over a time period of 165 hours after administration (Fig.13).

Conclusion

In principle the standard operating procedure for heavy volatile nitrogen-containing drugs (screening 2), as well as that for anabolic steroids (screening 4), are suitable for the analysis of aminoglutethimide. Screening 2 was found to be the more sensitive method. However, since screening 2 is only performed for competition samples, both screening procedures should be used together to cover all kind of urine samples.
The acquisition of screening 2 is in the full scan mode, and extraction of the most prominent ions *m/z* 203, 232 and 175 is suitable for detecting aminoglutethimide.
In screening 4 the ions *m/z* 520, 505 and 491 should be monitored.
For confirmation we recommend a modified screening 1 procedure involving extraction of the free fraction at pH 9.6 (instead of pH 14) and salting out with sodium sulphate, followed by analysis of aminoglutethimide from the etheral layer.

Acknowledgements

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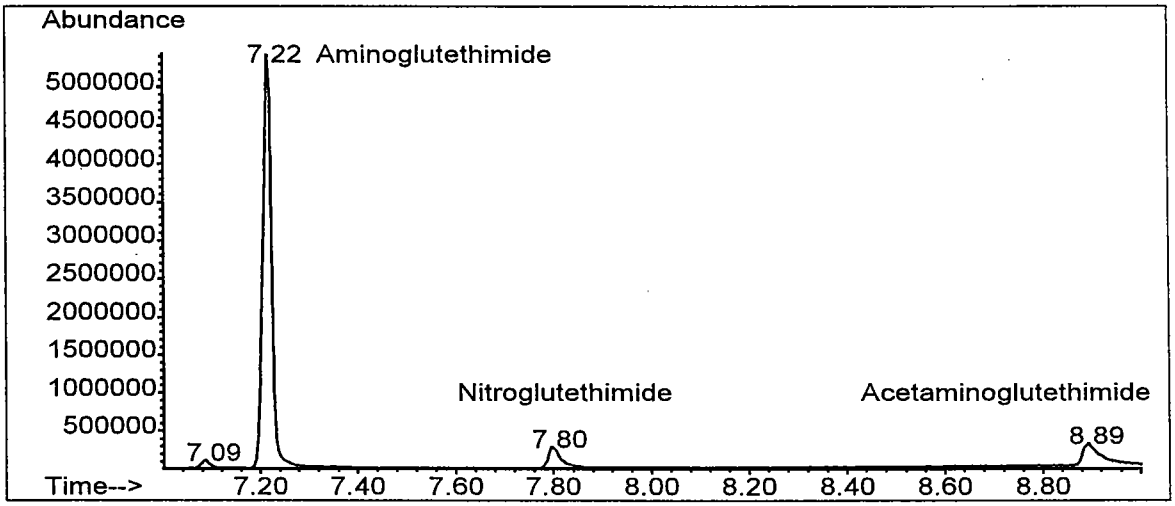

Fig 1: Screening 1 chromatogram (TIC) of a positive sample (2 hours after administration).

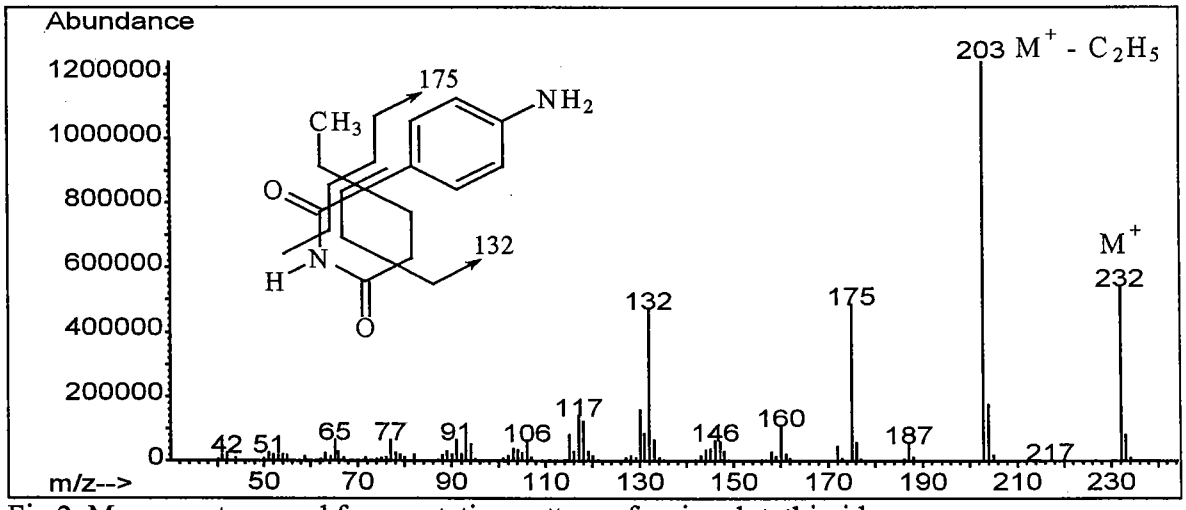

Fig 2: Mass spectrum and fragmentation pattern of aminoglutethimide.

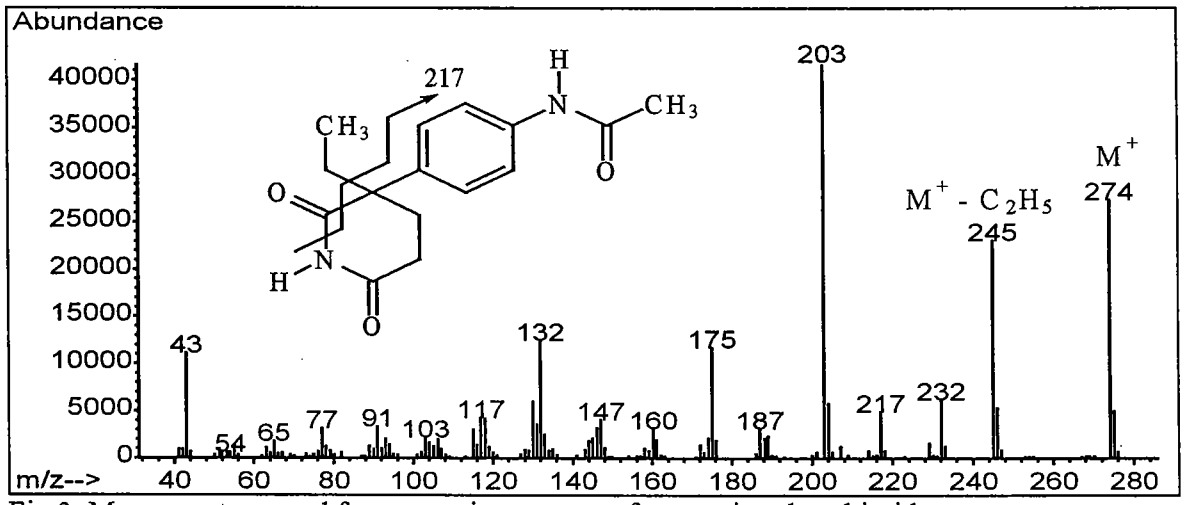

Fig 3: Mass spectrum and fragmentation pattern of acetaminoglutethimide

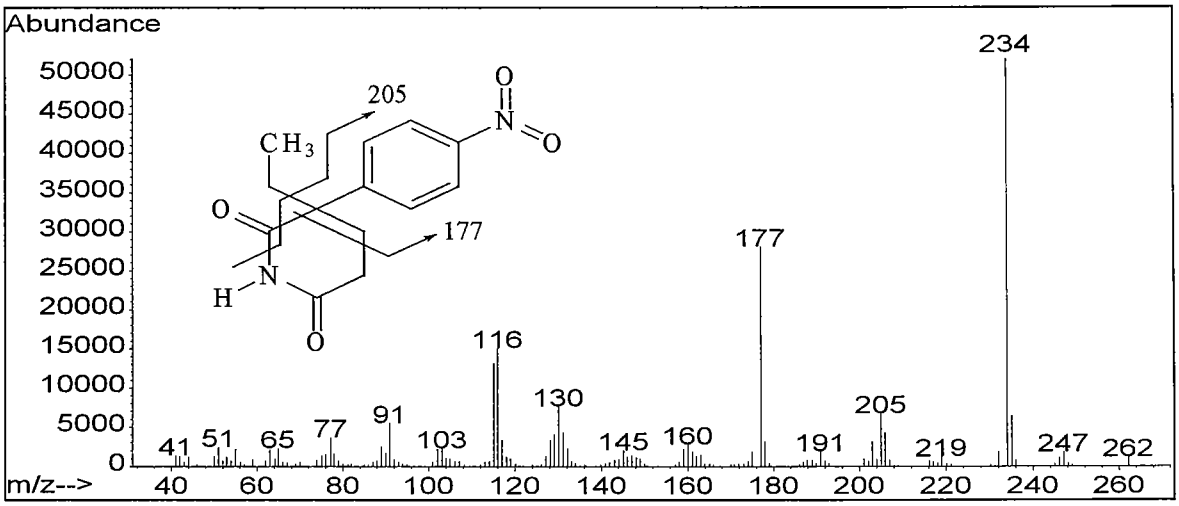

Fig 4: Mass spectrum and fragmentation pattern of nitroglutethimide

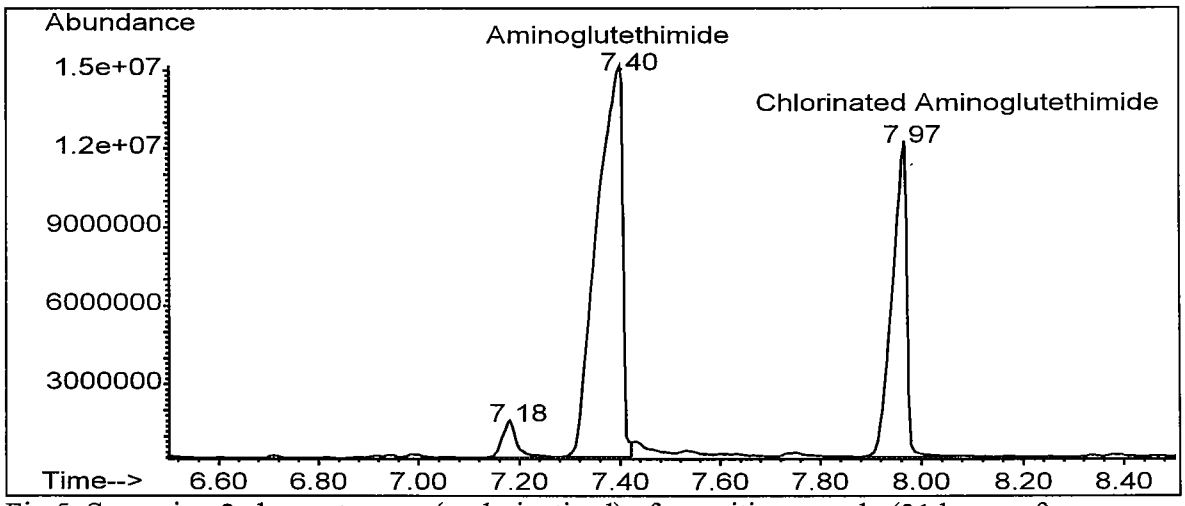

Fig 5: Screening 2 chromatogram (underivatised) of a positive sample (21 hours after administration).

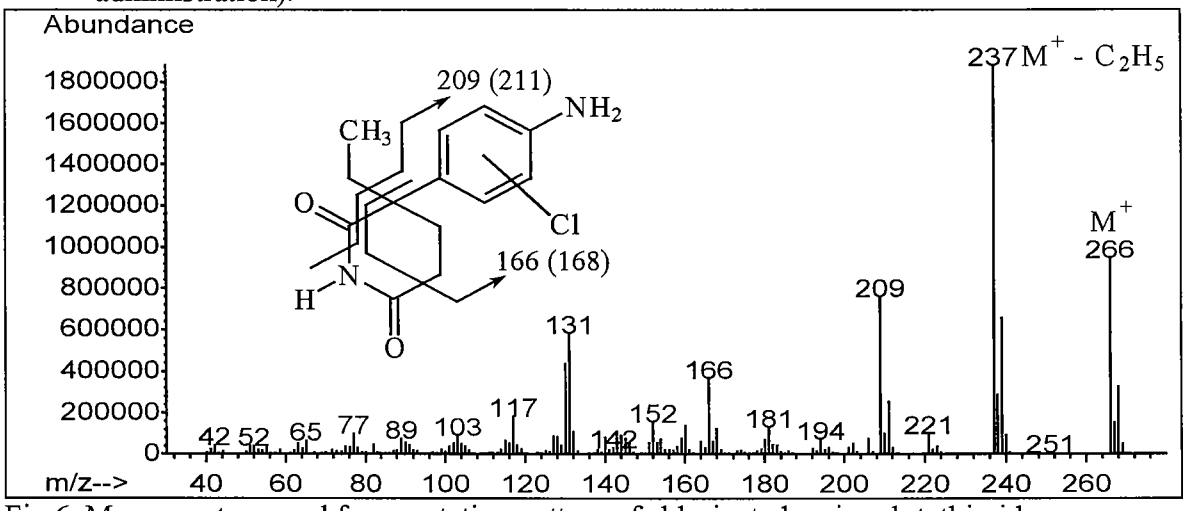

Fig 6: Mass spectrum and fragmentation pattern of chlorinated aminoglutethimide

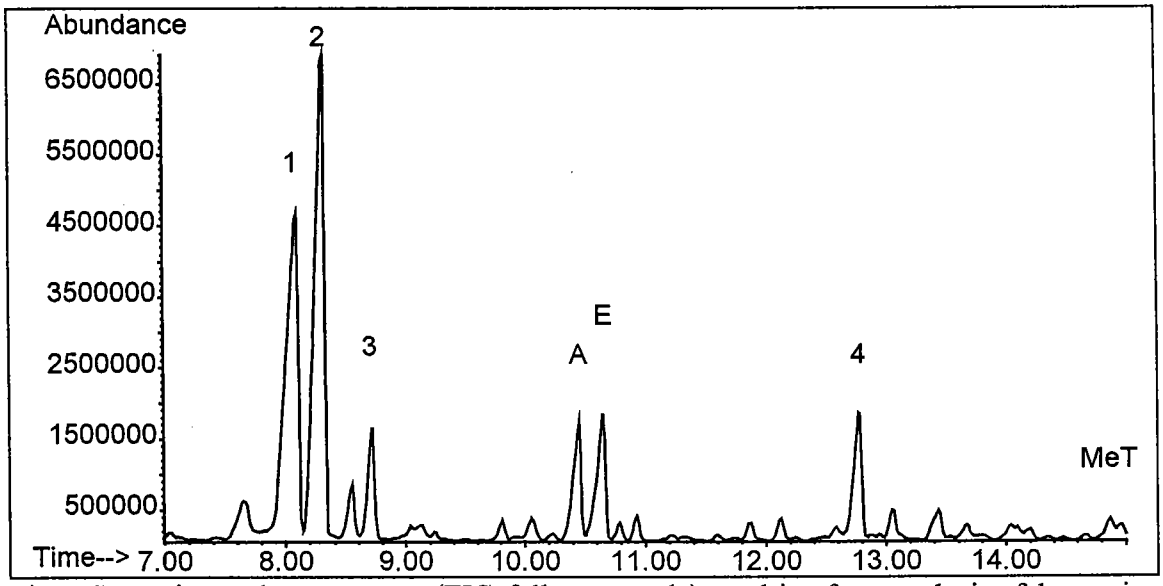

Fig 7: Screening 4 chromatogram (TIC, full scan mode) resulting from analysis of the conjugated fraction of a positive sample (46.5 hours after administration)

1) Aminoglutethimide, N,N,N'-tris-TMS (M = 448)
2) Aminoglutethimide, N,N,N',O-tetrakis-TMS (M = 520)
3) N-Acetyl-aminoglutethimide, N,N',O-tris-TMS (M = 490)
4) Aminoglutethimide + 60, N,N,N',O-tetrakis-TMS (M = 580) = Ethylmercapto-aminoglutethimide, N,N,N',O-tetrakis-TMS

A) Androsterone, O,O'-bis-TMS (M = 434)
E) Etiocholanolone, O,O'-bis-TMS (M = 434)
MeT Methyltestosterone, O,O'-bis-TMS (M = 446)

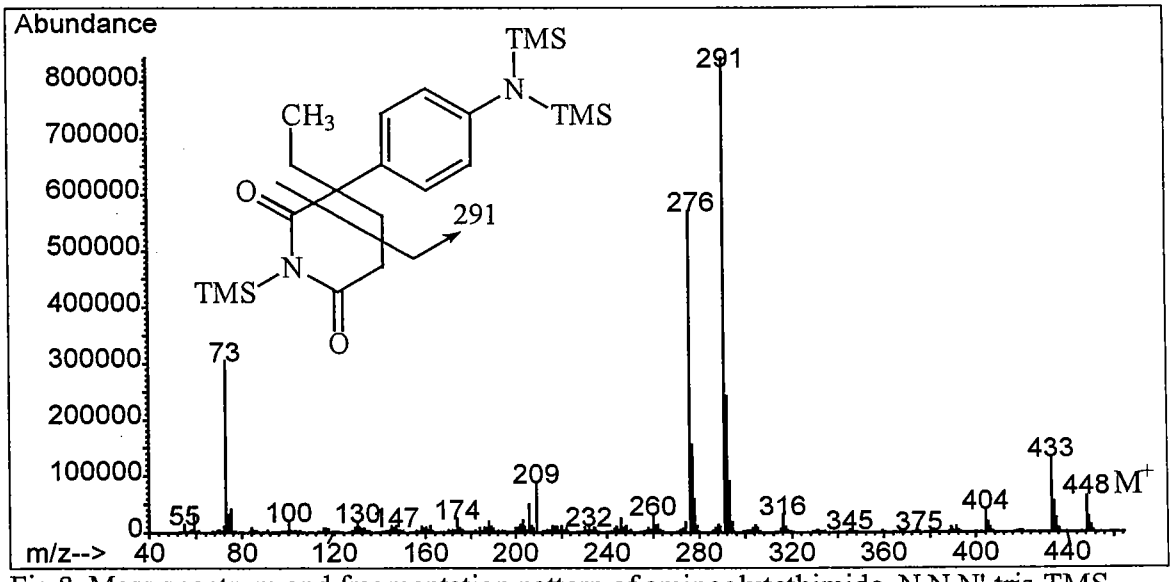

Fig 8: Mass spectrum and fragmentation pattern of aminoglutethimide, N,N,N'-tris-TMS

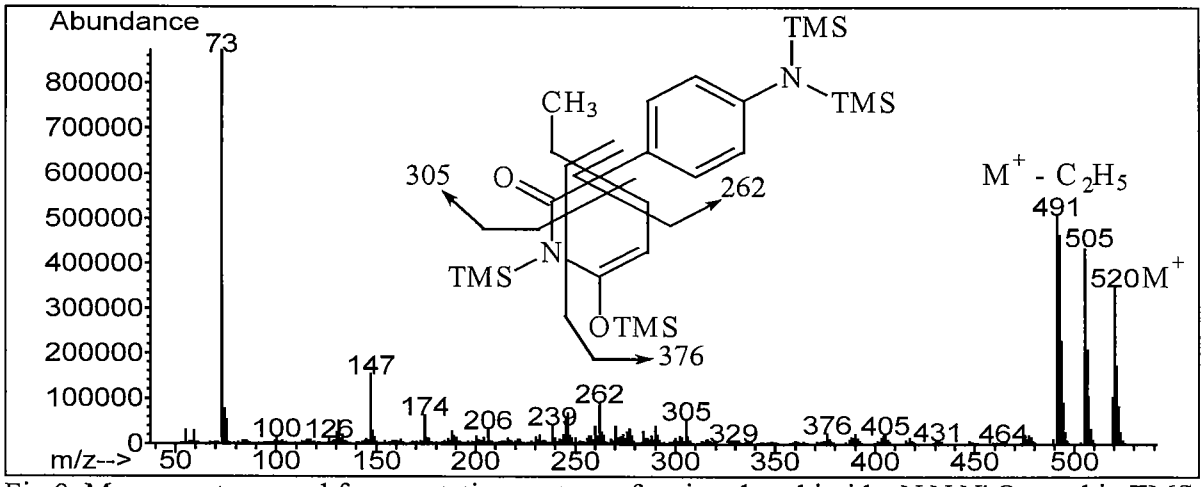

Fig 9: Mass spectrum and fragmentation pattern of aminoglutethimide, N,N,N',O-tetrakis-TMS.

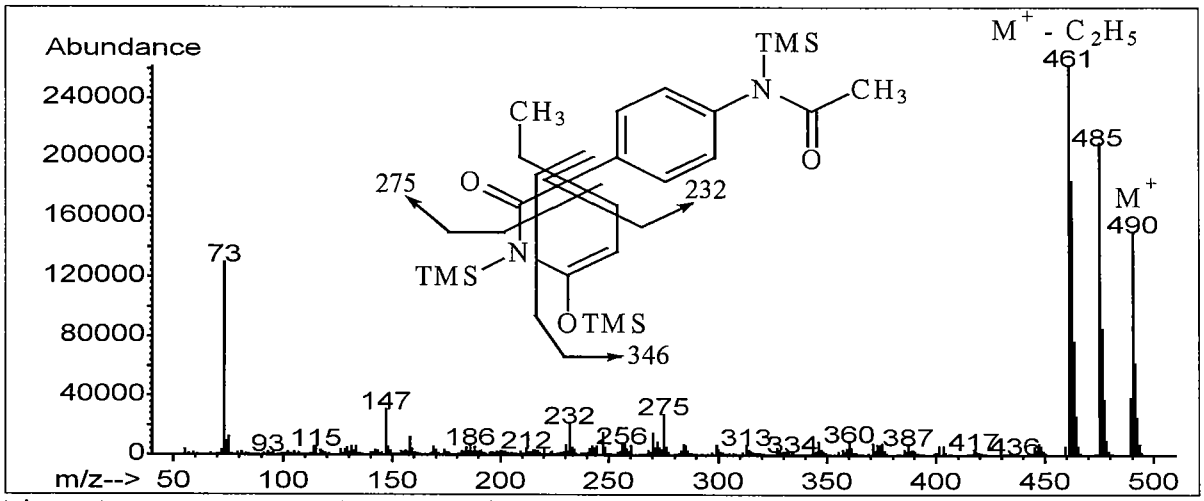

Fig 10: Mass spectrum and fragmentation pattern of N-acetyl-aminoglutethimide, N,N',O-tris-TMS.

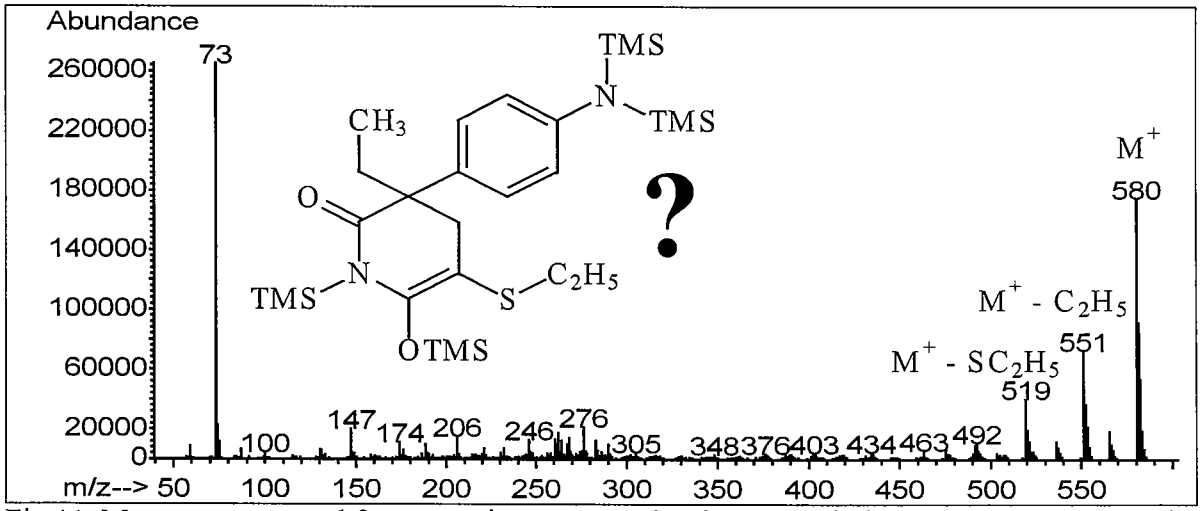

Fig 11: Mass spectrum and fragmentation pattern of aminoglutethimide+60, N,N,N',O-tetrakis-TMS (M = 580) = ethylmercapto-aminoglutethimide, N,N,N',O-tetrakis-TMS

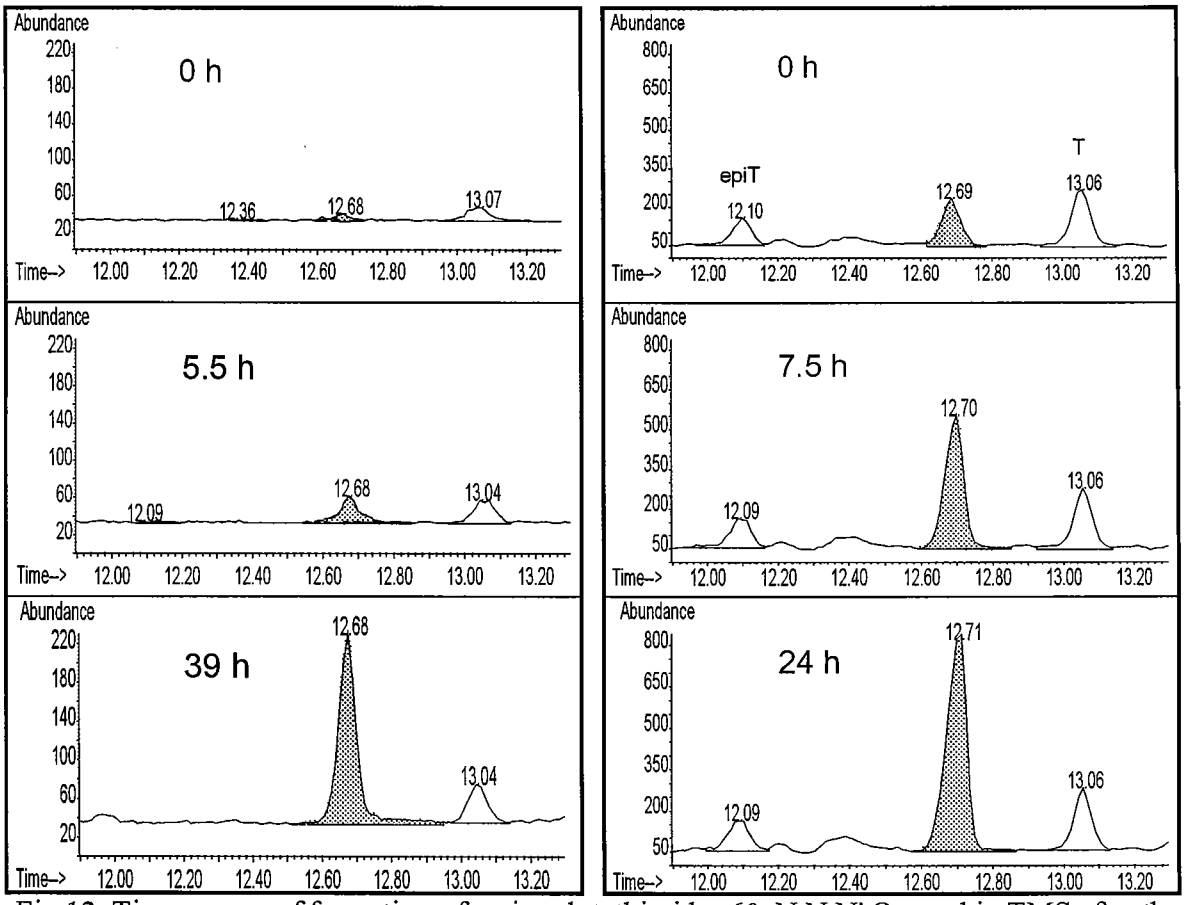

Fig 12: Time course of formation of aminoglutethimide+60, N,N,N',O-tetrakis-TMS after the derivatisation in the T/epiT window monitorung *m/z* 432. Left: pure aminoglutethimide (peak at 13.04 min from d_3-T); right: excretion study 24 hours

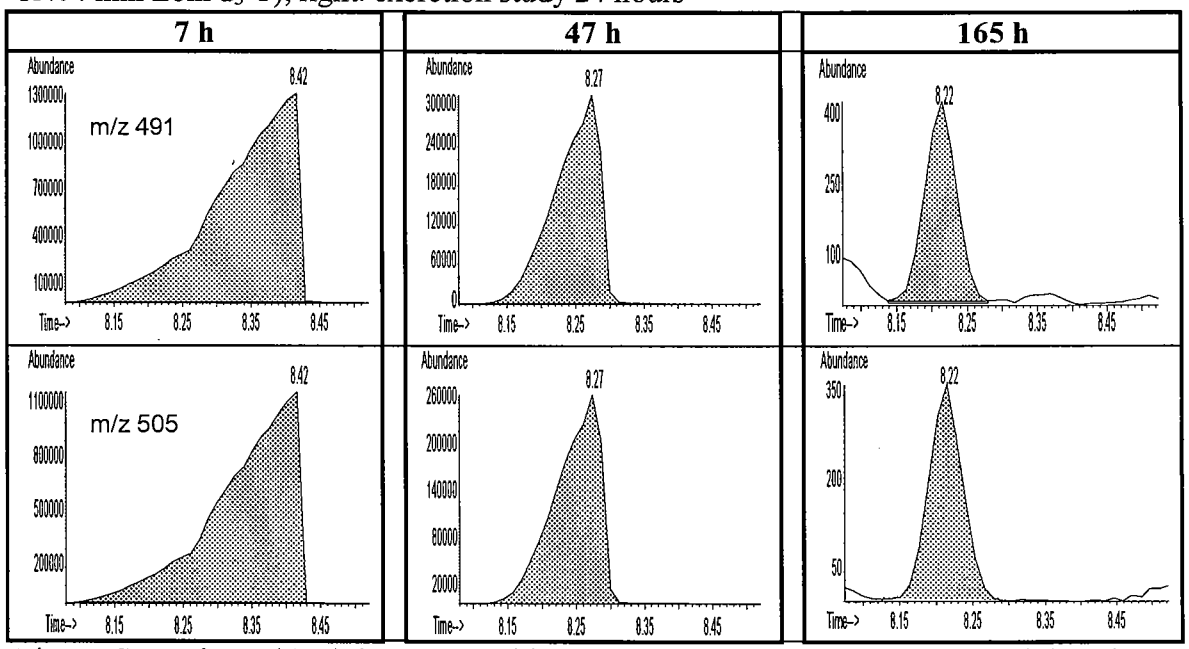

Fig 13: Screening 4 (SIM) for three positive samples at different times after administration.

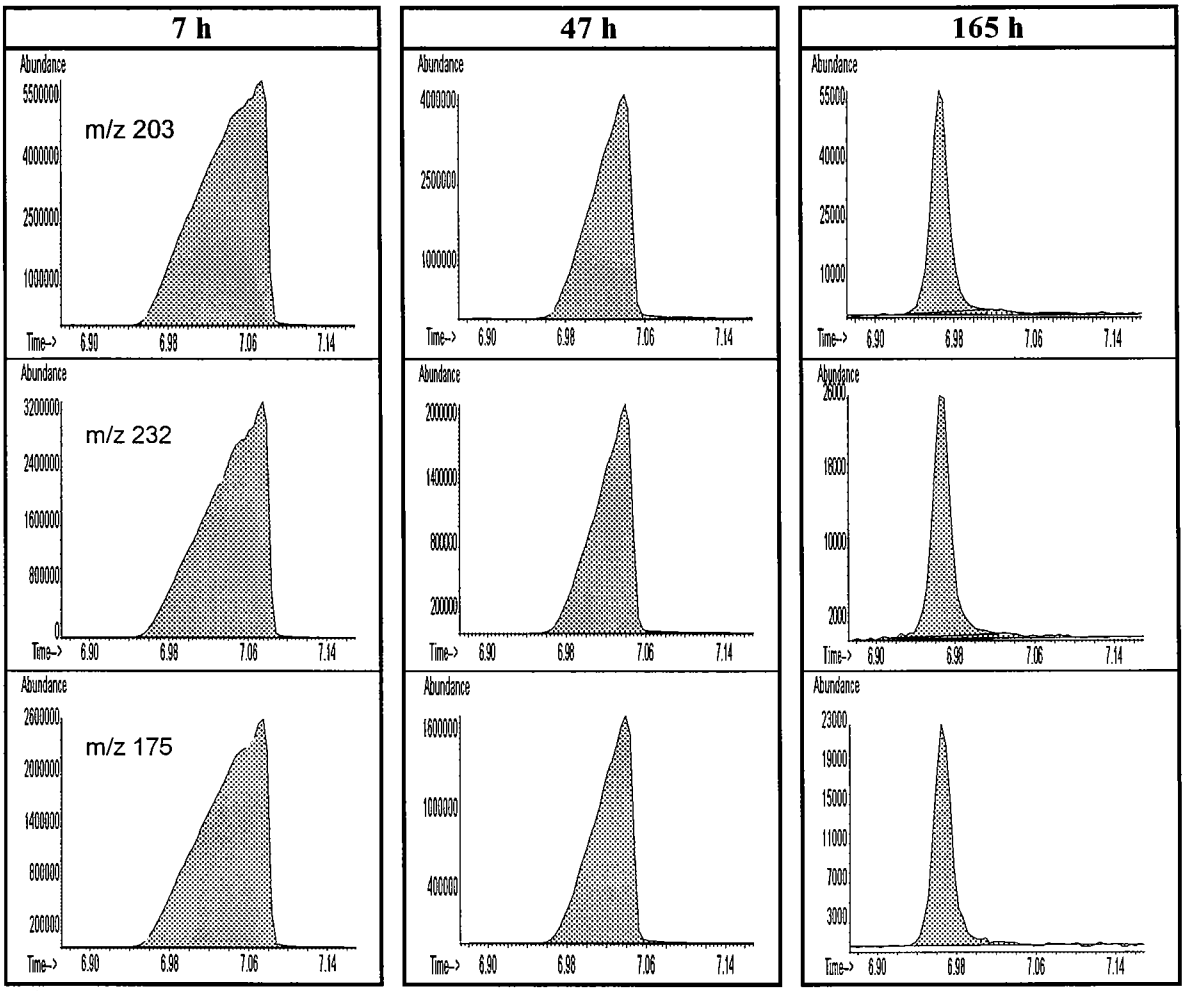

Fig 14: Results of screening 2 analysis (underivatised) for three positive samples taken at different times after administration.

Notice to the incorporation of ethylmercapto groups during trimethylsilylation with MSTFA/NH_4I/ethanethiol

Since the early nineties the Cologne Lab uses ethanethiol instead of dithioerythreitol (Cleland's reagent) as antioxidant in the trimethyliodosilane reaction mixture [1]. This is due to the fact that from time to time routine samples contained ghost peaks interfering with the steroid profile which were assigned to polymeric oxidation products of dithioerythreitol.

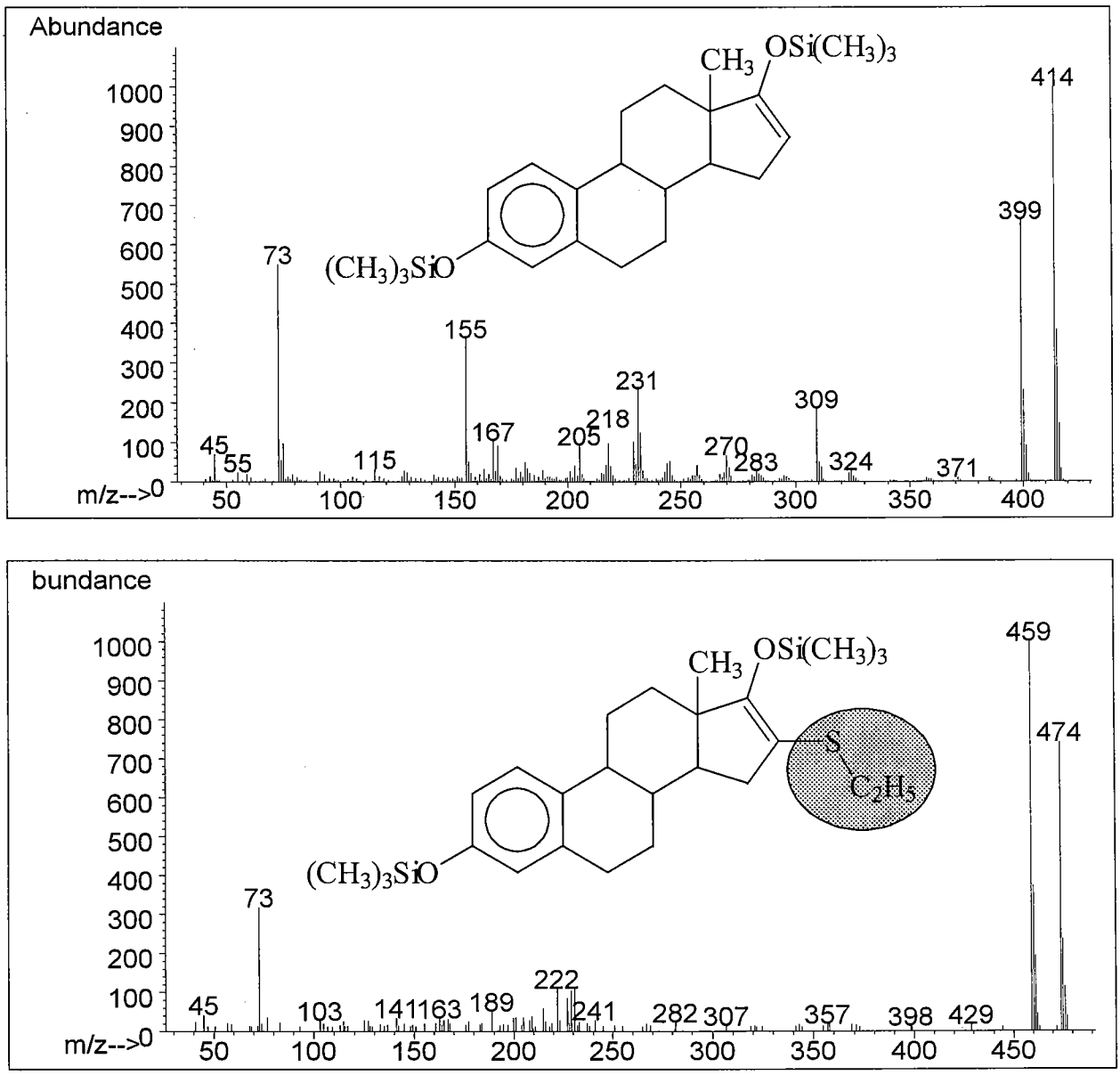

Fig.1: Mass spectra of Estrone, bis-TMS (upper) and of Estrone+60, bis-TMS (lower)

Nevertheless, sometimes chromatograms were obtained with a lack of certain steroids, and additional peaks were detected which showed molecular ions of the missing steroids increased by 60 amu or 148 amu when mercaptoethanol was used. Thus, incorporation of an ethylmercapto or trimethylsiloxyethylmercapto group was assumed as presented for estrone in Fig. 1. The structure was not entirely elucidated but assigned by the following: enol acetates react with halogenes or activated halogen to α-halo

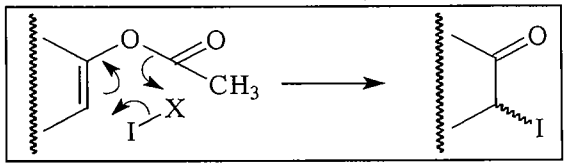

Fig. 2: Formation of α-iodo ketones [2].

ketones [2] (Fig. 2), as observed when enolised 17-keto steroids were treated with iodine in an antioxidant free TMIS reaction mixture (Fig. 3. The D-ring fragment *m/z* 155 is missing in the spectrum of ethylmercapto estrone indicating that the D-ring is affected by an incorporation. Finally, the α-substituted ketones are again enolised to stable products as there is no further hydrogen in the α-position of the ketone.

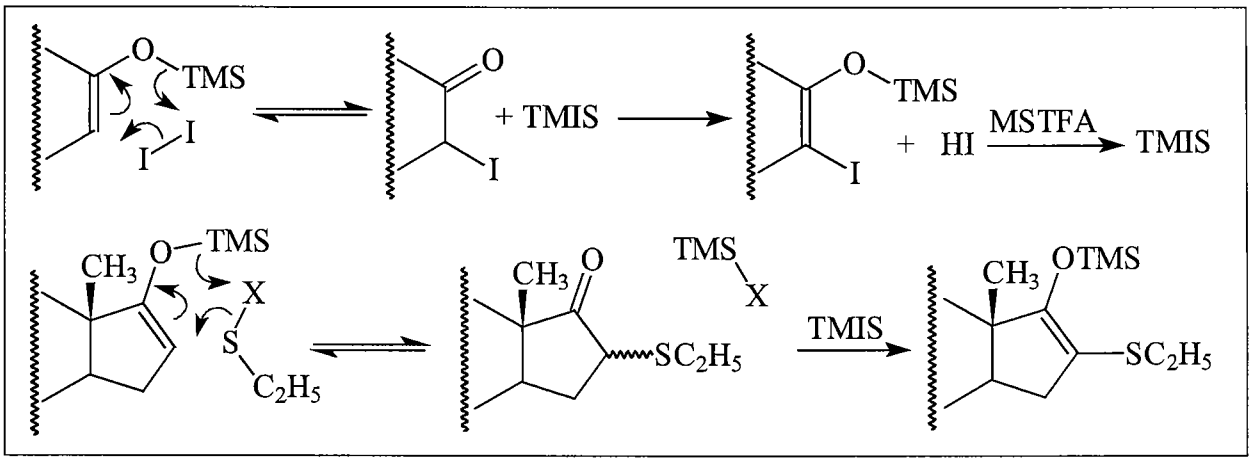

Fig. 3: Reaction schemes for the formation of α-iodo and α-ethylmercapto ketones

The formation of '+60' products is best detected by monitoring the ethylmercapto derivative of the internal standard 2,2,4,4-tetradeutero-etiocholanolone ($M^+ = 498$). Besides that, special attention must be paid to oxandrolone and 17-epi-oxandrolone (hereinafter referred to as oxandrolones) when analysed in the total fraction of screening 4: though (normally) enolised oxandrolones are not detectable, the ethylmercapto substituted and enolised oxandrolones ($M^+ =$

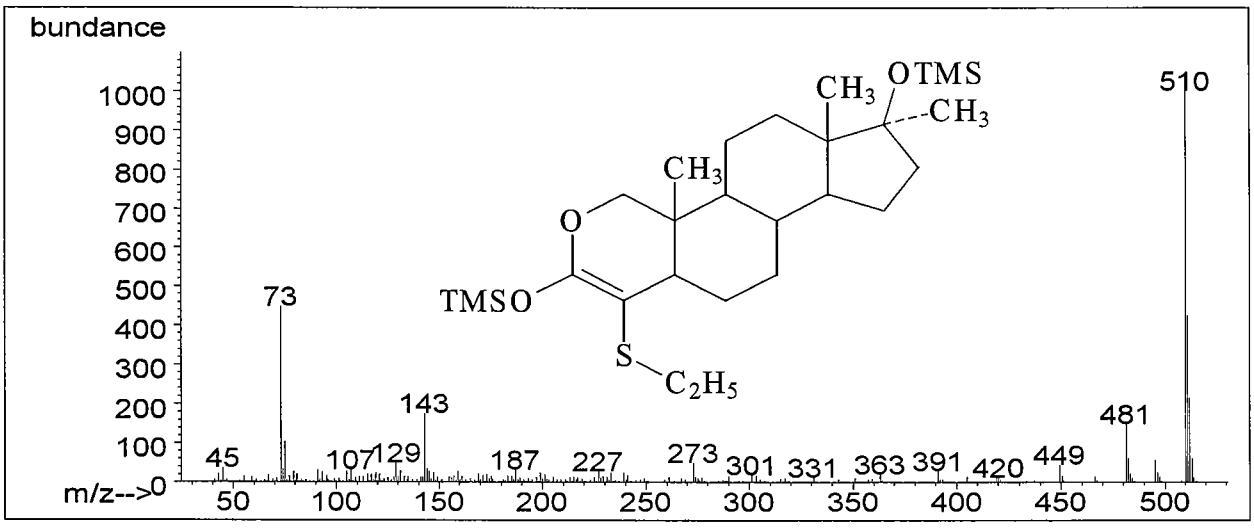

Fig. 4: Mass spectrum of ethylmercapto oxandrolone, bis-TMS.

510) are formed very rapidly. A mass spectrum is presented in Fig.4: here the loss of an ethyl radical is observed (*m/z* 481).A normal time course of the conversion of oxandrolones in MSTFA/NH_4I/ethanethiol to their enolised ethylmercapto derivatives is presented in Fig. 5.

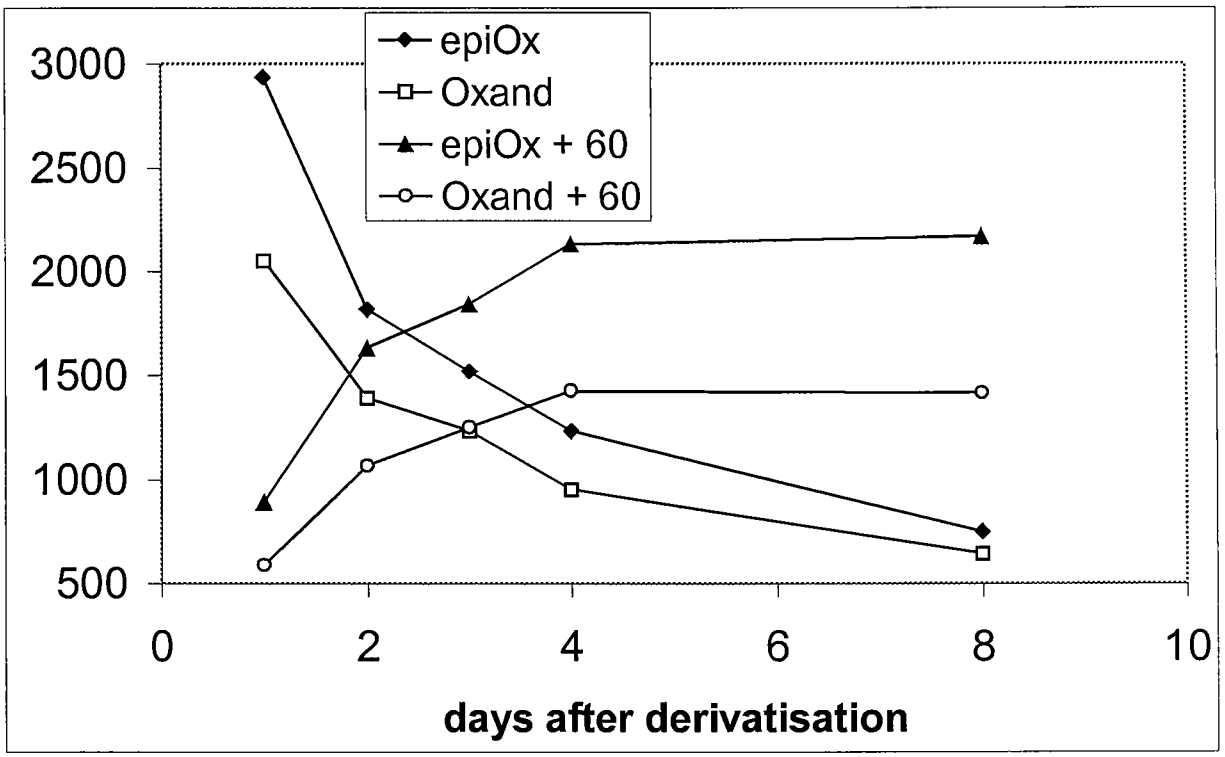

Fig. 5: To areas of methyltestosterone *m/z* 446 (ISTD) related areas of the oxandrolones *m/z* 143 and their enolised ethylmercapto derivatives *m/z* 510 versus days after derivatisation of a urinary standard.

The decomposition of the oxandrolones might occur faster as in the example above. Moreover, it becomes obvious that the decrease of oxandrolones continues while the content of the '+60' products appears constant after 4 days. The rapid reaction of aminoglutethimide and oxandrolones obviously depends on their structure: the first is a lactame, the second a lactone. Thus, their properties are similar, but different in terms of the ketone function.

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