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## **Screening for Tamoxifen, Clomiphene and Cyclofenil in Doping Analysis**

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### **Abstract**

Purpose of this study was the identification and enclosure of the main metabolites of the anti-estrogenic drugs tamoxifen, clomiphene and cyclofenil into established, running screening procedures in doping analysis.

Excretion studies were carried out by oral application of one single therapeutical dose each of the above mentioned substances.

The analysis was performed by gas chromatography/mass spectrometry (GC/MS).

Hydroxy-methoxy-tamoxifen, hydroxy-clomiphene and hydroxy-bis-desacetyl-cyclofenil were identified as main metabolites.

The most suitable screening procedure for the enclosure of the anti-estrogenic drugs is the screening procedure for anabolic androgenic agents.

Full scan spectra and diagnostic ions for SIM-analysis are presented as well as useful informations about kinetics of hydrolysis, phase II metabolism and purification with n-pentane.

The most stable steroid profile parameters of a previous longitudinal study of the volunteer were compared with those after the application of the anti-estrogens.

### **Introduction**

Since January 2000 the anti-estrogenic drugs tamoxifen, clomiphene and cyclofenil are added to the IOC list of forbidden substances. The use is prohibited in males only.

Tamoxifen is generally used in the treatment of breast cancer and nonmalignant breast disorders. Clomiphene and cyclofenil have stimulating effects on the secretion of hypofisic gonadotropic hormones. They are mainly used in the treatment of infertility.

In males the anti-estrogenic substances may cause an increase of the endogenous production of androgens. Athletes may be encouraged to treat the adverse effects of an extensive abuse of anabolic androgenic steroids (e.g. suppression of androgens, gynaecomastia) by using anti-estrogenic drugs.

## Experimental

### *Sample preparation*

The urine samples were prepared and analyzed according to the standard operating procedure for anabolic steroids (1,2).

### *Derivatisation*

The dry residues were derivatized with 100  $\mu$ l of MSTFA/ $\text{NH}_4\text{I}$ /ethanethiol 1000:2:3 (v:w:v) and heated for 20 min at 60°C.

3  $\mu$ l of the solution were injected into the GC/MS.

### *GC/MS parameters*

GC/MS: HP 6890/HP 5973 (Hewlett Packard)

electron impact: 70 eV

column: HP Ultra I (OV-1), 17m, 0.2mm i.d., 0.11  $\mu$ m film thickness

carrier gas: 1ml helium at 180°C, split 1:10

temperature program: 180°C, 3°C per min. until 229°C, 40°C per min, 320°C

### *Urine samples*

Excretion studies were performed by one healthy male volunteer. One single therapeutical dose of tamoxifen (40 mg), clomiphene (100 mg) and cyclofenil (400 mg) was administered each. The first two days all urine samples were collected and thereafter only morning urine samples up to the point when the main metabolite was no longer detectable.

### *Metabolites*

The metabolites of tamoxifen in the urinary matrix were identified by their mass spectra as reported in the literature (3).

Hydroxy metabolites of clomiphene and bis-desacetyl-cyclofenil were identified by their mass spectra.

## Results and Discussion

### *Main metabolites*

Hydroxy-methoxy-tamoxifen was identified as main metabolite of tamoxifen (3). The major fragments of the mono-TMS-derivative are the molecular ion  $m/z$  489, the base peak  $m/z$  58 and the fragment  $m/z$  72 resulting from  $\alpha$ - and  $\beta$ -cleavage of the amino group (fig. 1).

For clomiphene the hydroxy-clomiphene was identified as main metabolite. Molecular ions of the mono-TMS-derivative are  $m/z$  493 and  $m/z$  495 for the chlorine isotopes, respectively  $\alpha$ -fission of the amino group leads to the base fragment  $m/z$  86,  $\beta$ -fission to  $m/z$  100 (fig.3).

Cyclofenil shows hydroxy-bis-desacetyl-cyclofenil as major metabolite. The molecular ion of the tris-TMS-derivative is  $m/z$  512. Base peak is  $m/z$  422 ( $M^+ - 90$ ). The third prominent ion is  $m/z$  343. Its formation and chemical structure is still under investigation (fig. 5).

### *Suitable screening procedure*

The collected urine samples after application of one single therapeutical dose of tamoxifen, clomiphene and cyclofenil were prepared and analyzed according to the screening procedure of anabolic androgenic agents (scr.4 / single ion monitoring method) and the screening procedure of conjugated heavy volatile substances (scr.2 / full scan method).

The detection of the anti-estrogenic agents was possible up to different time periods (tab.1). In general the detection in screening 4 is more sensitive than in screening 2.

Therefore the screening procedure of anabolic androgenic agents seems to be the most suitable method for enclosing the anti-estrogenic drugs.

substance	administered single dose (mg)	detection scr.4 (days)	detection scr.2 (days)
tamoxifen	40	> 35	15
clomiphene	100	9	4,5
cyclofenil	400	9	9

Tab.1: Detection of anti-estrogenic agents

The screening procedure of anabolic androgenic agents (Scr.4) was extended by enclosing three diagnostic ions for each metabolite into the appropriate ion group. Retention times and diagnostic ions for selected ion monitoring analysis are shown in tab.2, whereas GC/MS parameters are presented in the experimental part. (Under the same terms testosterone is eluted at 13.2 min, androsterone at 10.5 min and the internal standard methyltestosterone at 15.00 min.)

	metabolite of tamoxifen	metabolite of clomiphene	metabolite of cyclofenil
m/z	58, 72, 489	86, 100, 493	512, 422, 343
retention time (min)	18	18.5	17.3

Tab.2: Retention times and diagnostic ions for the analysis of the main metabolites of the anti-estrogenic agents tamoxifen, clomiphene and cyclofenil

Screening print-outs of the main metabolites of tamoxifen, clomiphene and cyclofenil are shown in fig.2, 4 and 6. Ion chromatograms for the tamoxifen- and cyclofenil-metabolite show distinct peaks in all three ion traces over the time of detection. Due to its poor fragmentation pattern the clomiphene metabolite shows only one single prominent ion (m/z 86). The other two selected diagnostic ion traces (m/z 100 and 493) have very low abundances and show only distinct signals in urine samples collected soon after application.

### *Kinetics of hydrolysis*

Sample preparation was performed according to the standard operating procedure for anabolic steroids. The hydrolysis was stopped after 0 (without incubating), 15, 30, 45, 60 minutes; 3 and 24 hours. Additionally one sample was prepared without adding the enzyme to test for the presence of unconjugated metabolites.

The conjugate of the tamoxifen metabolite was not hydrolysed without incubation. After 1 hour about 40% of the metabolite is hydrolysed. After 24 hours the hydrolysis seems to be finished. Due to the fact, that the detection of the tamoxifen metabolite after a single therapeutical application is possible over a time peroid of more than 35 days, the hydrolysis of 40% after 1 hour should be sufficient.

The hydrolysis of the conjugate of the clomiphene metabolite starts without incubation, nevertheless no unconjugated metabolite was detected. After one hour 60% of the hydrolysis is completed and after 3 hours the hydrolysis is finished. The hydrolysis time of one hour is also sufficient. Possible detection problems depend more on the poor fragmentation pattern than on incompleteness of hydrolysis.

The most rapid hydrolysis occurred with the cyclofenil metabolite conjugate. Hydrolysis starts without incubation only by adding enzyme. A small part (0,1 %) of the metabolite is excreted unconjugated. The hydrolysis is complete after 15 minutes.

### *Phase II metabolism*

The metabolites of tamoxifen and clomiphene are excreted as glucuronides. Cyclofenil metabolite is excreted in very low amounts unconjugated (0,1%), in low amounts as sulphate (5%) and the main part is excreted as glucuronide (95%).

### *Purification with n-pentane*

Extraction with n-pentane instead of tert.-butylmethylether is often used for confirmation methods of anabolic steroids (e.g. T/epiT, 19-norandrosterone) to exclude polar disturbing coeluting substances.

Purification tests were conducted and extraction yields with n-pentane determined.

For the tamoxifen metabolite and the clomiphene metabolite the extraction yields with n-pentane are about 40%. For confirmation the extraction with n-pentane may lead probably to better analytical results.

The cyclofenil metabolite is not extractable with n-pentane. For confirmation it is probably possible to use purification with n-pentane as a cleaning step (pre extraction).

### *Steroid profiling*

The volunteer proceeding the excretion studies with tamoxifen, clomiphene and cyclofenil also participated in several studies concerning stability of steroid profiles (4, 5, 6).

For these studies morning urine samples were collected during one month every day (30d) and during one year one time a month (12 per year). Over a time period of 24 hours urine samples were collected every two hours even during nighttime (12 samples).

For the excretion studies with anti-estrogenic agents one single therapeutical dose of tamoxifen (40 mg), clomiphene (100 mg) and cyclofenil (400 mg) was administered each. Urine samples were collected during the first two days and thereafter only morning urine samples up to the point when the main metabolite was no longer detectable.

One of the described side effects of anti-estrogenic agents is the increase of the production of endogenous steroids.

The most stable steroid ratios androsterone/etiocholanolone (A/E), testosterone/epitestosterone (T/epiT) and 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol/5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol (Adiol/Bdiol) and the excretion rates of androsterone (A), etiocholanolone (E), testosterone (T), epitestosterone (epiT), 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol (Adiol) and 5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol (Bdiol) from excretion studies with anti-estrogenic agents and previous longitudinal studies were compared (tab.3,4).

The steroid ratios show generally low variations. An exception is the ratio Adiol/Bdiol after cyclofenil. The high decrease of this ratio results from a strong increase in Bdiol excretion. The excretion studies with the anti-estrogenic substances were performed with a single therapeutical dose, in order to get more information about the effects from anti-estrogens on steroid profile parameters other excretion studies (with more volunteers and multiple application) have to be performed.

ratio	tamoxifen (34 samples)	clomiphene (35 samples)	cyclofenil (37 samples)	30d (30 samples)	24h (12 samples)	12p/y (12 samples)
A/E	0.95 (0.14)	0.97 (0.12)	0.91 (0.14)	1.04 (0.07)	1.12 (0.09)	1.02 (0.05)
T/epiT	1.08 (0.24)	1.20 (0.27)	1.21 (0.34)	0.91 (0.10)	0.94 (0.23)	1.01 (0.14)
Adiol/Bdiol	0.20 (0.02)	0.22 (0.03)	0.09 (0.02)	0.22 (0.02)	0.24 (0.02)	0.25 (0.02)

Tab.3 : Stability of steroid profile: mean values of ratios (*StDev.*)

steroid	tamoxifen [ $\mu$ g/h]	clomiphene [ $\mu$ g/h]	cyclofenil [ $\mu$ g/h]	30d [ $\mu$ g/h]	24h [ $\mu$ g/h]	12p/y [ $\mu$ g/h]
A	89 (44)	96 (39)	86 (34)	84 (12)	97 (34)	91 (55)
E	91 (35)	99 (34)	94 (31)	82 (12)	87 (34)	91 (57)
EpiT	1.5 (0.5)	1.9 (0.9)	1.5 (0.7)	1.5 (0.2)	1.6 (0.71)	1.8 (1.1)
T	1.6 (0.5)	2.2 (1.0)	1.7 (0.5)	1.4 (0.24)	1.4 (0.54)	1.7 (1.0)
Adiol	2.5 (0.7)	3.1 (1.2)	2.6 (0.5)	2.0 (0.3)	2.4 (0.9)	2.5 (1.4)
Bdiol	13 (3.7)	15 (5.5)	29 (7.9)	9 (1.0)	10 (3.0)	10 (6.0)

Tab.4: Stability of steroid profile: mean values of excretion rates [ $\mu$ g/h] (*StDev.*)

## Conclusion

Three diagnostic ions of the most important metabolites of tamoxifen, clomiphene and cyclofenil were enclosed into the screening procedure for androgenic anabolic agents. The detection of an abuse even of a single therapeutical application of these anti-estrogenics is possible over a long time period (tamoxifen is detectable longer than 6 weeks, clomiphene and cyclofenil up to 9 days).

In addition to the above mentioned main metabolites of the three anti-estrogenic substances, more metabolites were identified. Their chemical structure and fragmentation pattern will be described in further proceedings.

## Acknowledgements

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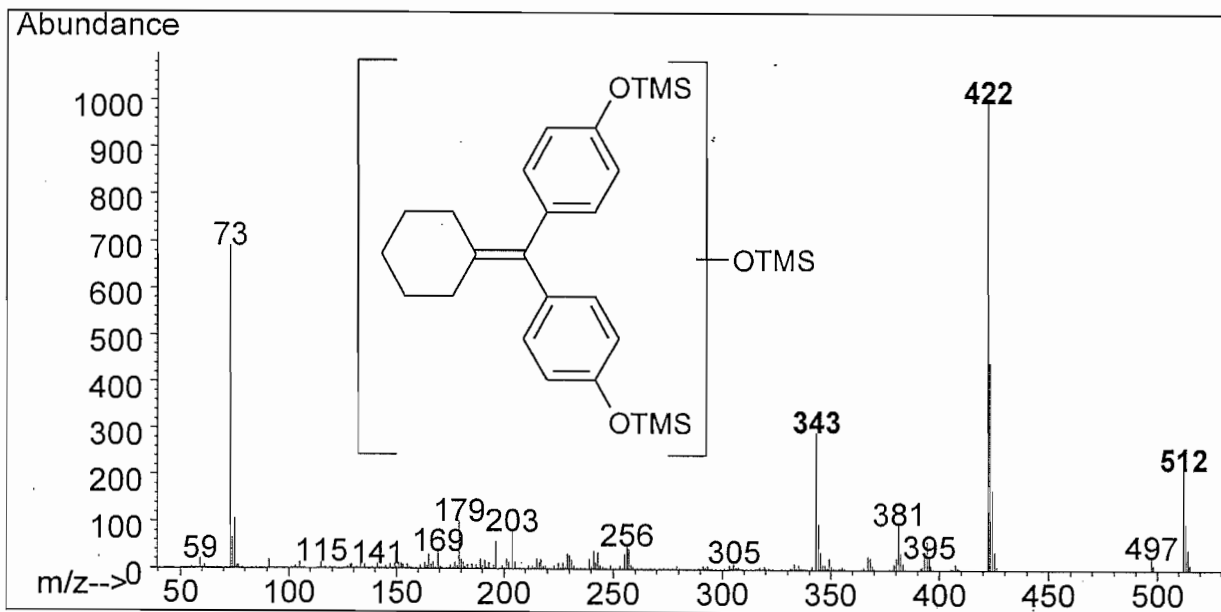


Fig. 5: EI mass spectrum and formula of hydroxy-bis-desacetyl-cyclofenil, O,O',O''-tris-TMS, M = 512

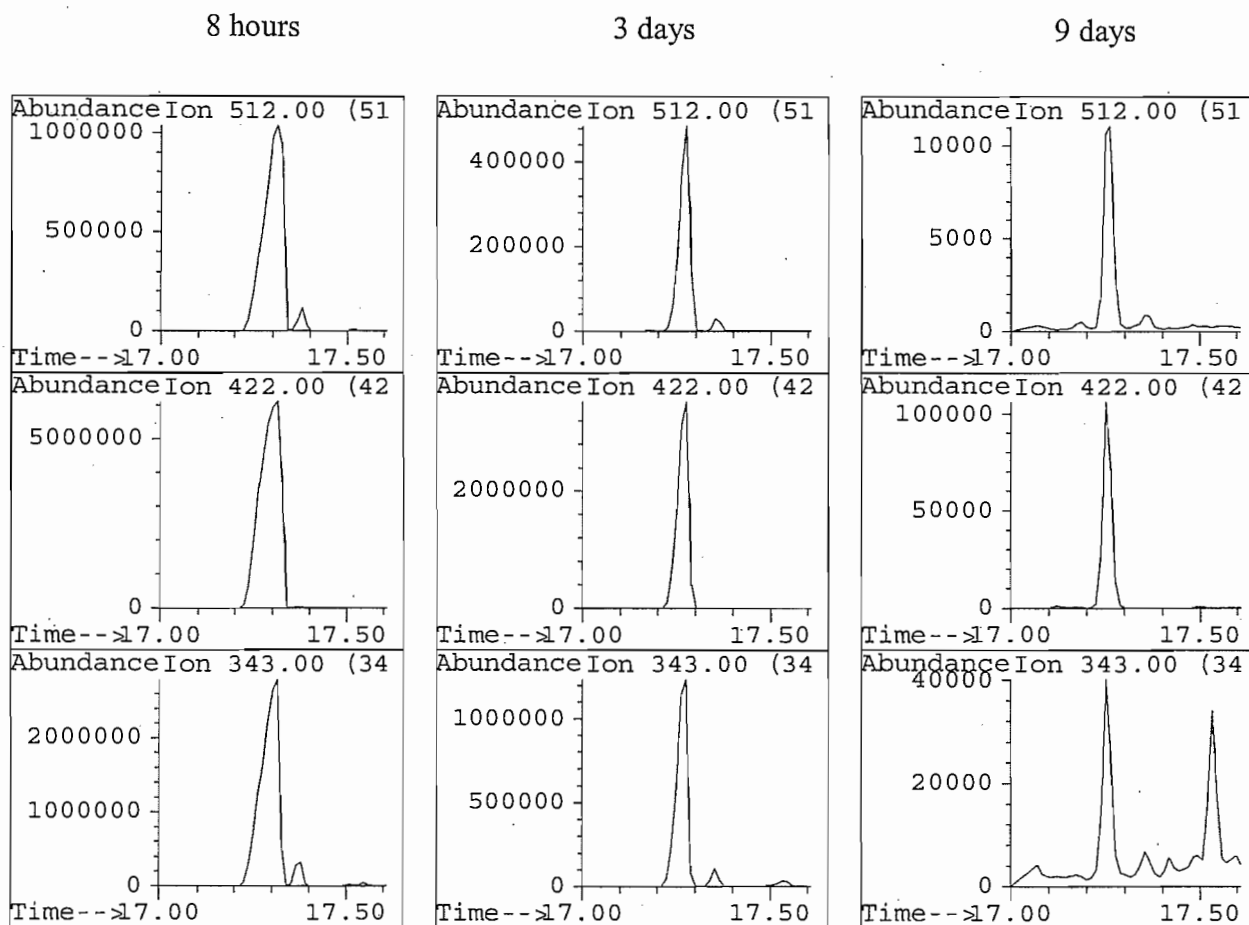


Fig. 6: Screening for the main metabolite of cyclofenil



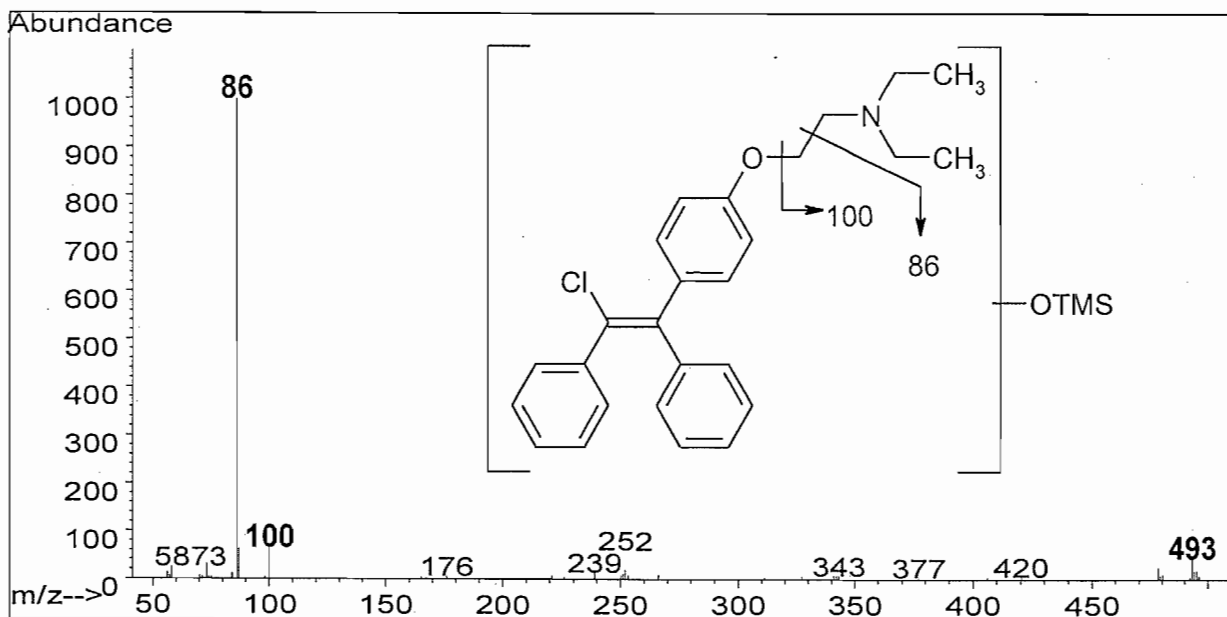


Fig. 3: EI mass spectrum and formula of hydroxy-clomiphene, O-TMS, M = 493 (495)

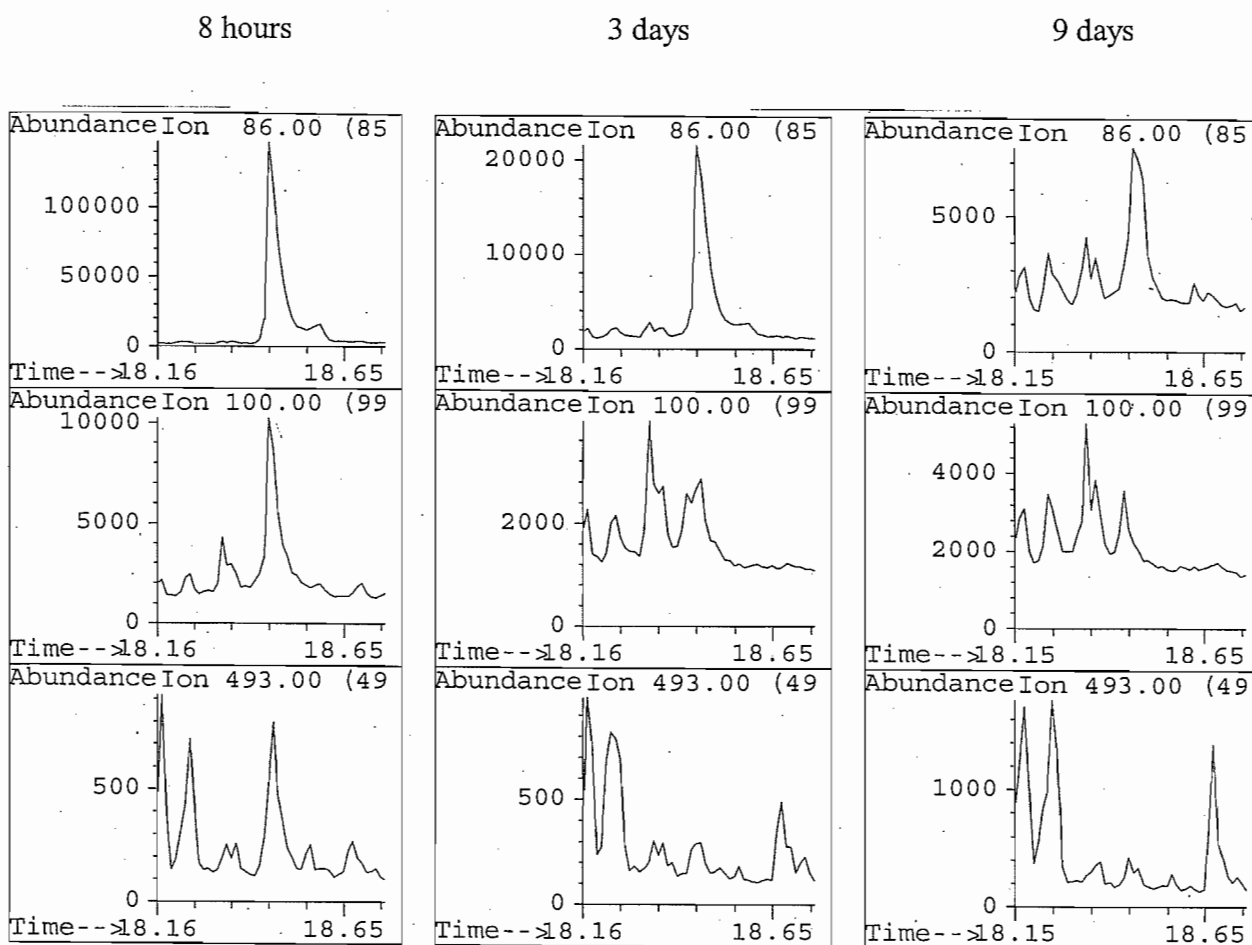


Fig. 4: Screening for the main metabolite of clomiphene

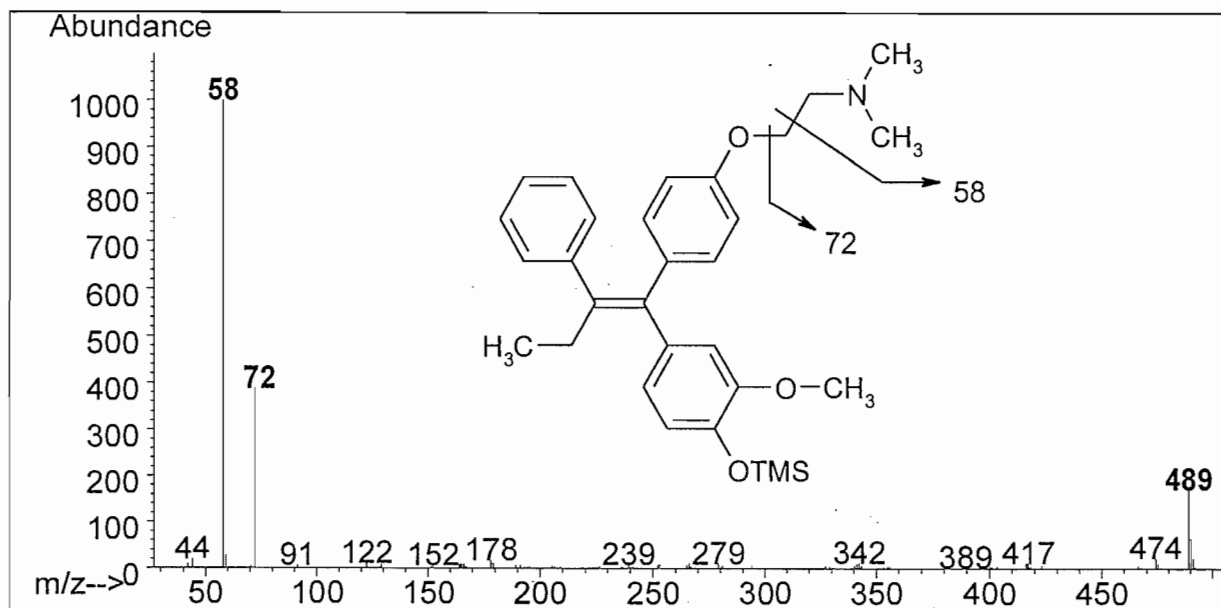


Fig. 1: EI mass spectrum and formula of hydroxy-methoxy-tamoxifen, O-TMS, M = 489

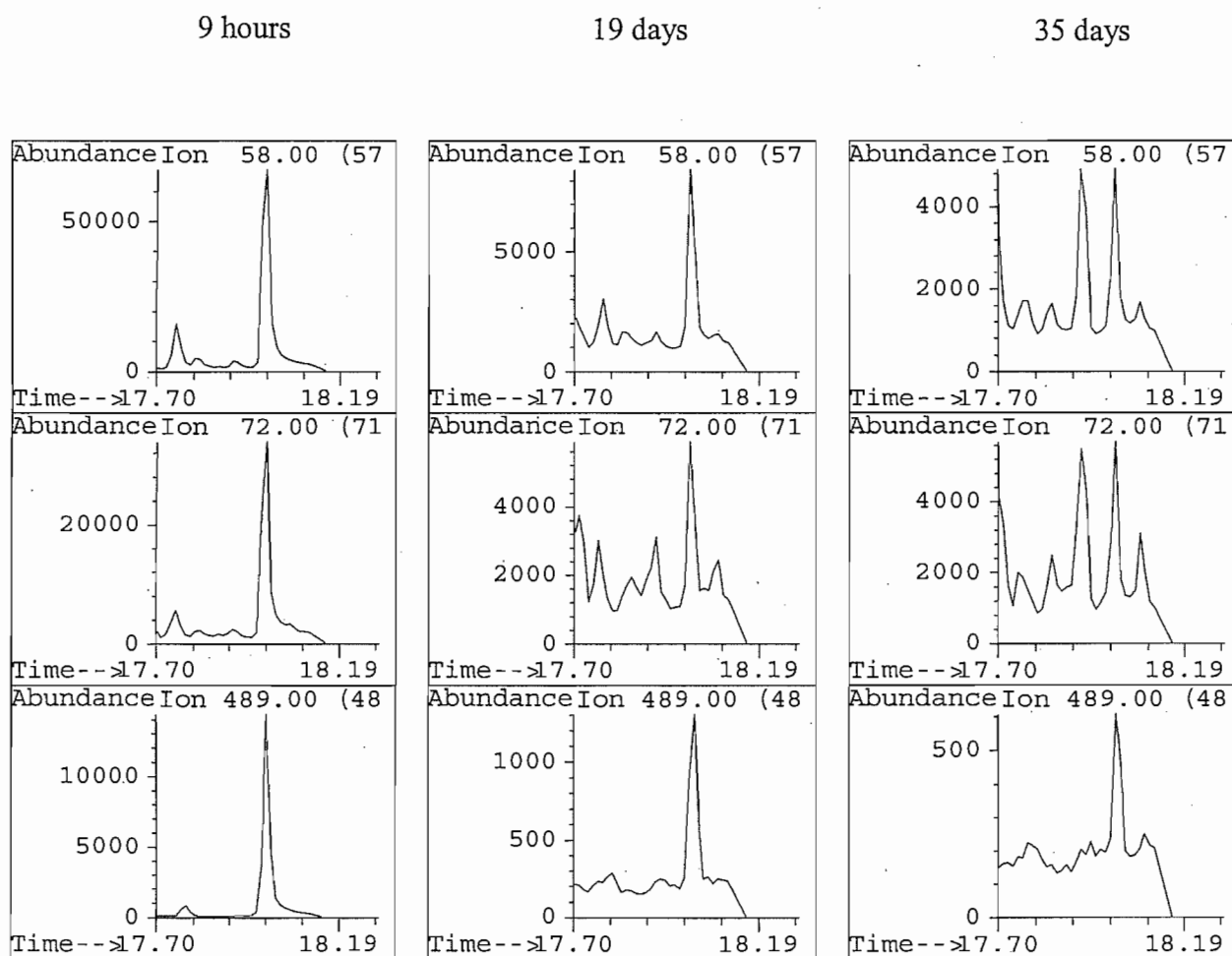


Fig. 2: Screening for the main metabolite of tamoxifen