

Reprint from

RECENT ADVANCES  
IN DOPING ANALYSIS  
(4)

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

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D. THIEME, J. GROSSE, R.K. MUELLER:  
Observations on new Metabolites of Clostebol by High-Resolution-MS  
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## Observations on new metabolites of clostebol by High-Resolution-MS

### 1. Introduction

Aiming to the elucidation of metabolism of anabolic steroids in blood and urine, clostebol was chosen as a model compound, because

- the mass defect of the 4-chlorine atom promises an effective applicability of High Resolution Mass Spectrometry (HRMS) and
- the metabolism of clostebol is assumed to be typical for anabolic steroids and according to previous knowledge not uncommonly complex.

The main metabolism in man is characterized by excretion of 4-chloroandrosterone, 4-chloroetiocholanolone and the 3- $\alpha$ -Hydroxy-17-Keto-metabolite in urine [1]. Another recent paper [2] reported the additional occurrence of 17-epimeres, diols, dions and dihydroxylated metabolites in cattle urine. Principally all major metabolic pathways of steroids

Epimerisation and Oxydation in 17-position

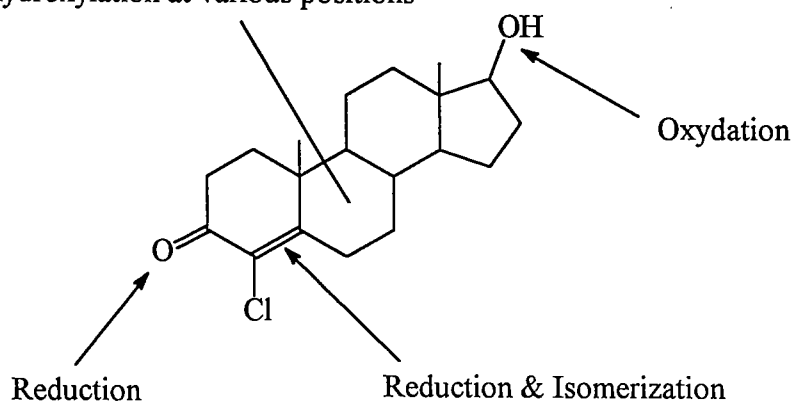
Reduction of 3-keto group

A-ring reduction with possible isomerization in positions 4 and 5

Multiple hydroxylations at various sites

are possible and should be taken into consideration.

Hydroxylation at various positions



## 2. Experimental

The high resolution mass spectrometer applied was a VG AutoSpec Ultima Q, equipped with an HP 5890 gas chromatograph.

Mode	Resolution <sup>1)</sup>	Limitation
Selected Ion Recording	5000-8000	mass range < 0.5 decade
Voltage Scan	5000	mass range < 0.5 decade
Magnet Scan	3000	scan rate > 1s
Tandem MS Multiple Reaction Monitoring	Magnet 3000 Quad 2-3amu	appropriate fragmentation

<sup>1)</sup>Resolution is defined as quotient of the mass  $m$  over the difference between two adjacent resolved masses ( $\Delta m$ ).

$$R \equiv m/\Delta m$$

### GC conditions

Chromatograph	HP 5890
Injection	split 1/10
GC column	HP Ultra 1
Oven Temperature	180°C (0 min) 6 grd/min → 310°C (4min)

Sample Preparation (according to total fraction of anabolic steroids screening)

Solid phase extraction (XAD)

Liquid-liquid extraction LLE (diethylether)

Enol-TMS-derivatisation (MSTFA/NH<sub>4</sub>I/Dithioerithrytol)

## 3. Detection of metabolites in urine

### 3.1. HRMS Survey for clostebol metabolites

The most important clostebol metabolites which are detected in routine screening procedures are 4-chloroandrostene-3-ol-17-one (B) and 4-chloro-androsterone (C). Especially the latter species is an appropriate marker because of its high concentration and long term detectability. After sublingual intake of 500µg/kg clostebolacetate, all these metabolites may be detected unambiguously for a period of 6 days, whereas 4-chloro-etiocholanolone (D) and the parent compound (A) disappear and other metabolites with corresponding elemental composition are not relevant (fig. 1).

According to probable metabolic pathways, a number of possible elemental compositions had been included into GC/HR-MS screening experiments to search for other hydroxylated, reduced and/or isomerised metabolites. The trace of 556.2991 (corresponding to hydroxylation and reduction of clostebol) shows at least two major signals of presumptive metabolites (E+F) which are not present in the blank (fig. 2).

Similarly, the species H-L (fig.3 of dihydroxymetabolites) and M-O (fig. 4 of trihydroxymetabolites) were detected at the following stages of hydroxylations.

Neither higher hydroxylated metabolites nor hydroxylations with (4-chloro-androst-) 4-ene-structures were observed.

A reliable identification and structural confirmation for each species will be very difficult because of the high number of respective substances. In addition to the confirmation of some metabolites with known mass spectra (A,B,D) the appropriate isotope ratio, correct accurate masses, useful fragmentation and a suitable time course in the observation time range are criteria for the acceptance of the presumptive metabolites.

The typical chlorine cluster is a helpful identification criterion, but all mass spectra of the hydroxylated metabolites are barely fragmented, and only an intense M-15 signal appears regularly (fig. 5).

### 3.2. Accurate Mass Measuring

High resolution mass spectrometry comprises the possibility to get information about the exact mass of fragments occurring in the spectrum. Depending on several technical details, an accuracy of better than 10ppm <sup>2.)</sup> can be obtained for GC-MS experiments with biological specimens.

In a voltage scan experiment (fig. 6) the mass spectrum of a monohydroxylated clostebol-metabolite is measured in a narrow mass range. The accurate masses of all concerning fragments and isotopes are measured, and proposals of possible elemental compositions are listed. The comparison of measured and proposed mass spectra demonstrates a good correspondence.

$$2.) \quad \text{ppm} = \Delta m/m$$

### 3.3. Tandem MS

The typical M-CH<sub>3</sub> fragmentation of the molecule may be employed in tandem MS experiments to detect all corresponding isomers with appropriate fragmentation and isotope ratios at a very low detection limit. The magnet of the first stage is set such that molecular ions and M+2 isotope ions are allowed to enter the second stage of the instrument. After induction of fragmentation in a gas cell, the occurrence of the M-15 fragment at the quadrupole is

observed. By application of an increased resolution ( $R=3000$ ) at the first stage, the specificity of HR-MS and of the tandem MS approach can be combined.

The comparison between positive control sample and blank (fig. 7) shows that this approach is sensitive and specific and the number of possible metabolites postulated in figures 3-4 is probably too low.

#### 4. Clostebol-metabolites in blood

The sample preparation is similar to urine treatment - 1ml of serum was cleaned by solid phase extraction and (after hydrolysis of the conjugates) by LLE with n-pentane. Formation of the enol-TMS derivatives was achieved by reaction with the derivatisation mixture described above.

The occurrence of metabolites in blood is surprisingly similar to metabolism in urine. All main urinary metabolites are detectable in blood as well (fig. 8).

Although serum concentrations are at least 20 fold lower and the available amount of blood is smaller, blood can be regarded as a useful sample material for clostebol identification because the level of interfering substances is quite low.

The differentiation between conjugated and free metabolites, structural confirmation or quantitation was not possible due to the lack of serum.

#### 5. Pharmacokinetic aspects of clostebol metabolism in blood and urine

Regarding the time course, urinary metabolites can be divided into two groups. The first group with a maximum concentration after about 12 hours comprises metabolites A,B,D and E, whereas all other hydroxymetabolites and C exhibit a maximum concentration at about 50 hours after application. The retardation of clostebol excretion indicates the probability that hydrolysis of clostebolacetate mainly determines the excretion rate. Clostebolacetate proved to be instable in (hemolytic) serum, preventing a reliable detection and quantitation. This has to be subject of further investigation.

The concentration time course in blood was comparable to observations in urine. Although the number of reliable quantified metabolites is restricted and the time range of observation was too short, it is suggested, that the same groups of metabolites occur in a very similar time course.

The common assumption, that urine is a retrospective specimen whereas serum typically reflects a momentary status is not promoted by these experiments.

## 6. Minimum requirements of identification criteria in GC/HR-MS

High resolution mass spectrometric experiments provide a higher amount of information because of their designation to small mass intervals and the resulting low rate of interference. If lower concentrations are to be detected, the increasing number of appropriate species causes the necessity to define minimum identification criteria, similar to those given for conventional GC-MS experiments. By now, only few regulations are known. The European Community [3] has accepted "criteria for identification of an analyte by GC-HRMS" which cover the case of steroid analysis in the framework of veterinary problems. It is defined, that

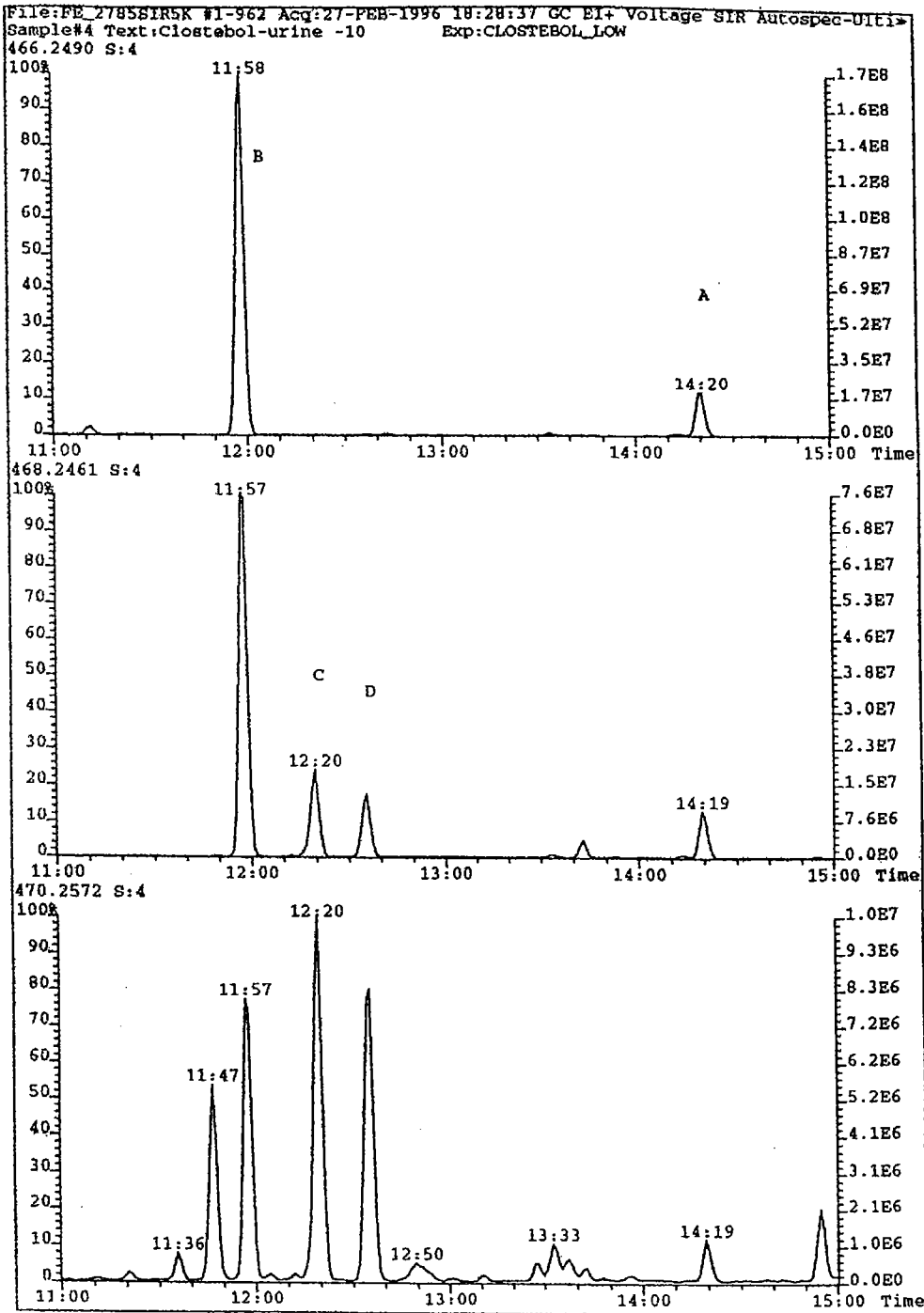
- any experiment must be carried out at a resolution better than 9500 to be classified as 'High Resolution',
- intensity ratios must comply with the standard material derivative, within a defined margin and
- accurate masses of fragments or molecular ions should be equal to the theoretical value of the corresponding derivative of the reference material, within a substance specific limit.

Any of these items are not perfectly suitable for doping analysis, and adaptations has to be made, to define appropriate quality standards, because uncritical extrapolations of low-resolution MS regulations to the different technical principle and lower concentration ranges are not justified.

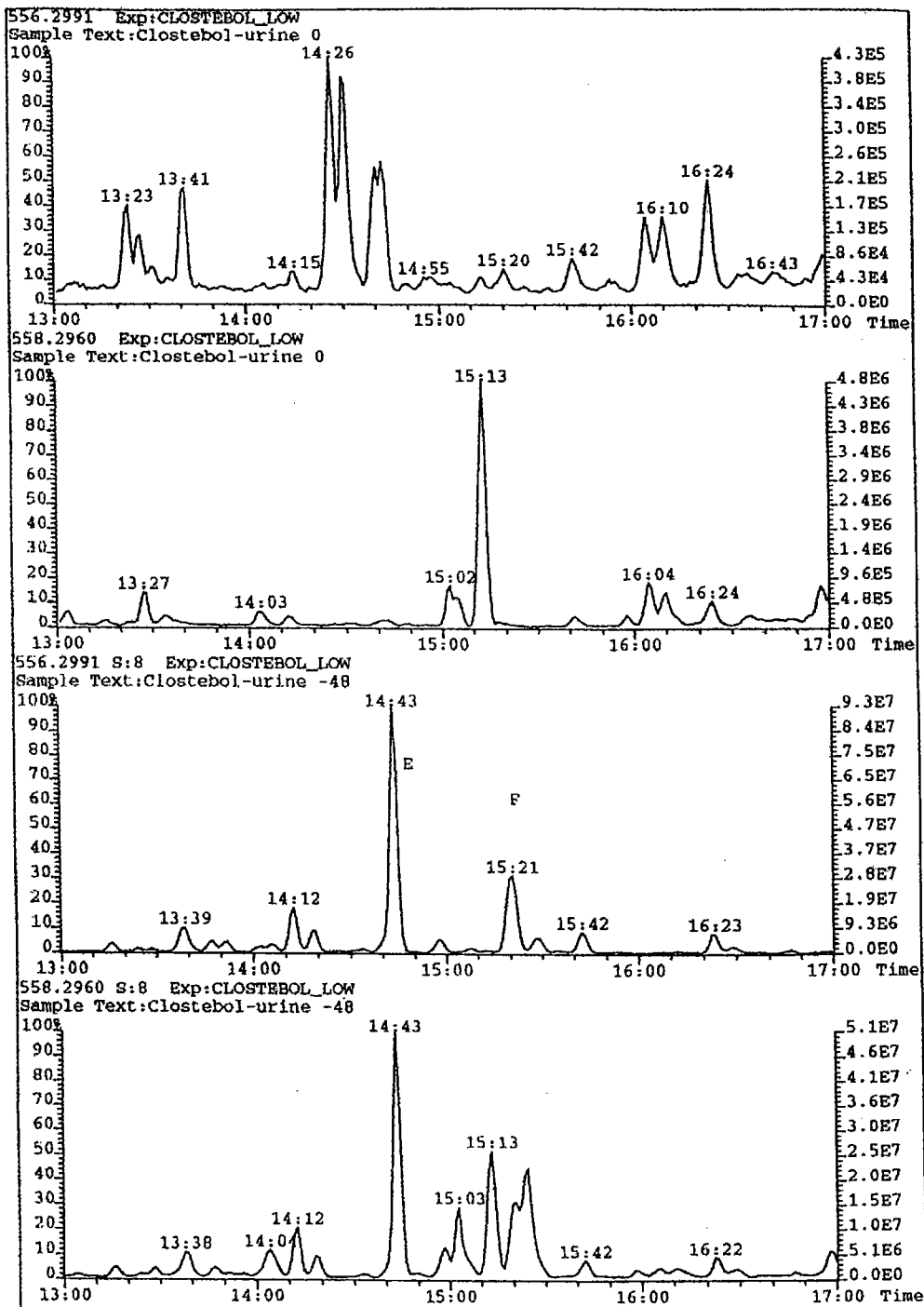
## Conclusions

- 1.) Peak to noise ratios of chromatographic signals of clostebol and its metabolites can be increased considerably by application of High Resolution Mass Spectrometry.
- 2.) Metabolism of clostebol in urine is very complex. More than 15 metabolites, including multiple hydroxylated metabolites, occurred in the screening.
- 3.) Metabolism in blood and urine is very similar-the same major metabolites appear, showing similar time courses.
- 4.) The number of relevant species (including interfering background substances) increases with decreasing detection limits. Therefore, minimum requirements for substance identification by HRMS should be defined.

Figures

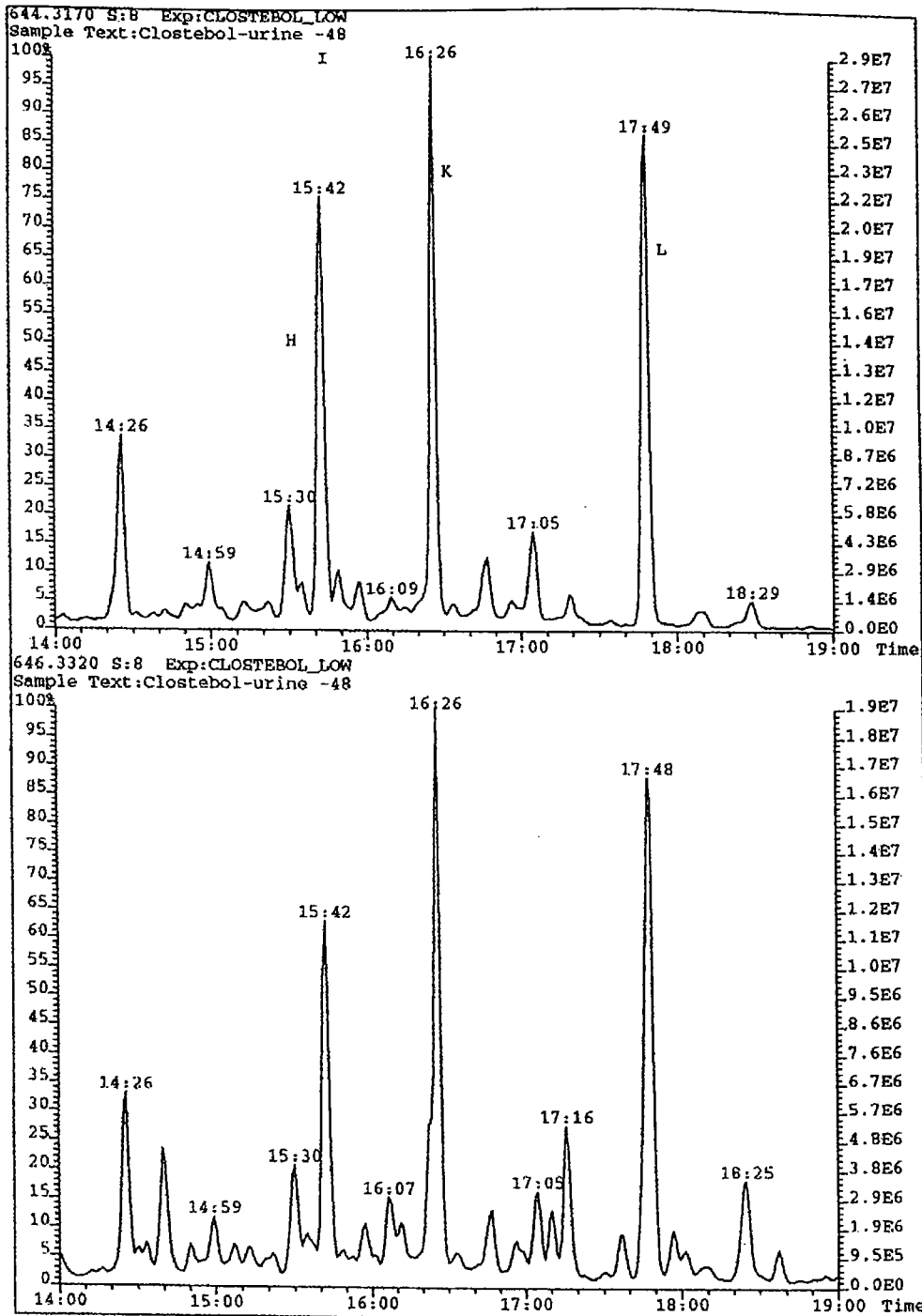


1.) Selected ion recording chromatograms of a high resolution MS screening. Clostebol (A=Parent compound and metabolites B-D are most useful for a routine screening.

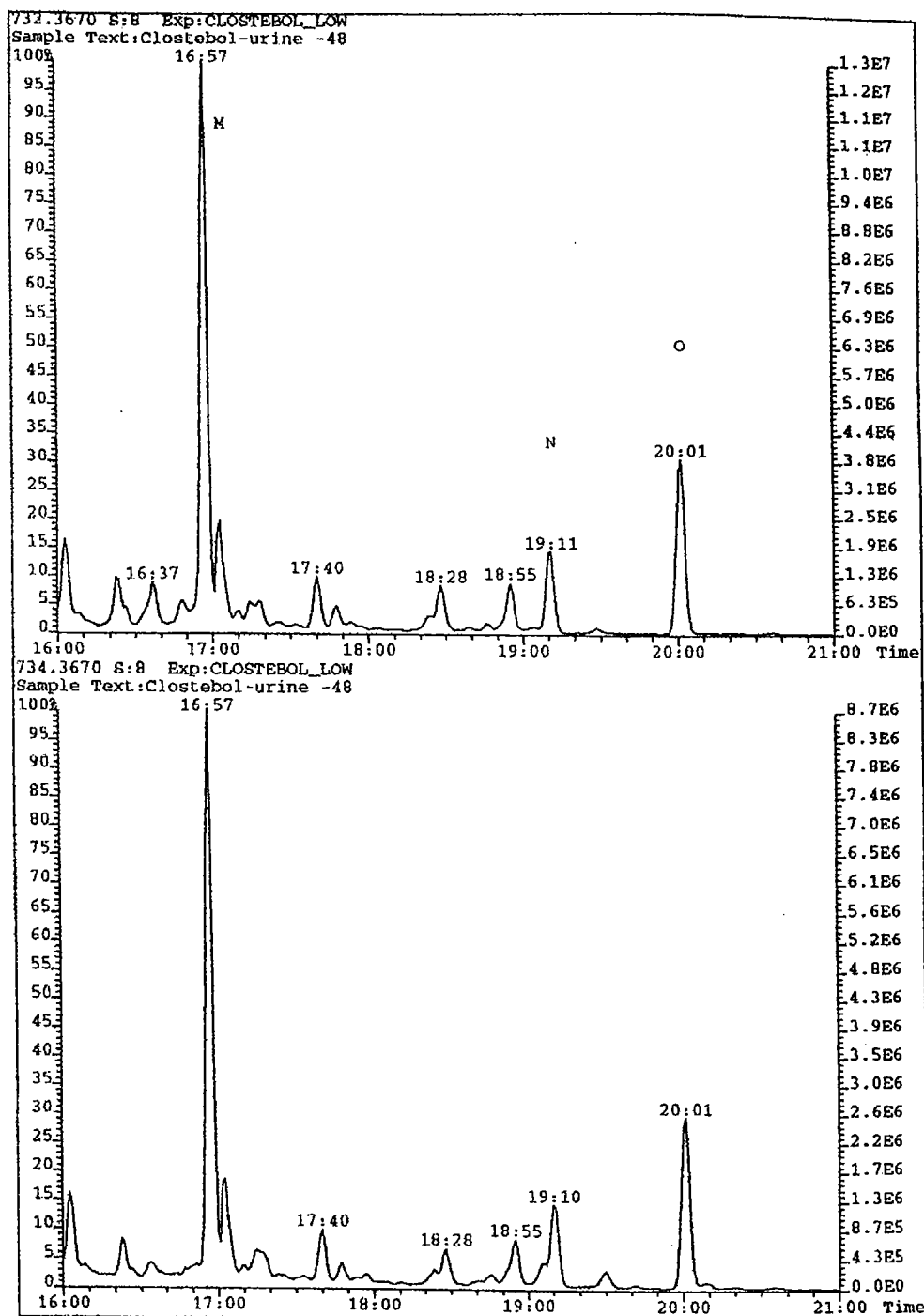


2.) Selected ion recording chromatograms to survey monohydroxylated clostebol metabolites. Molecular ions 556.299 of the possible metabolite and the corresponding isotope signal of a positive control (lower windows) are compared with a blank (upper windows).

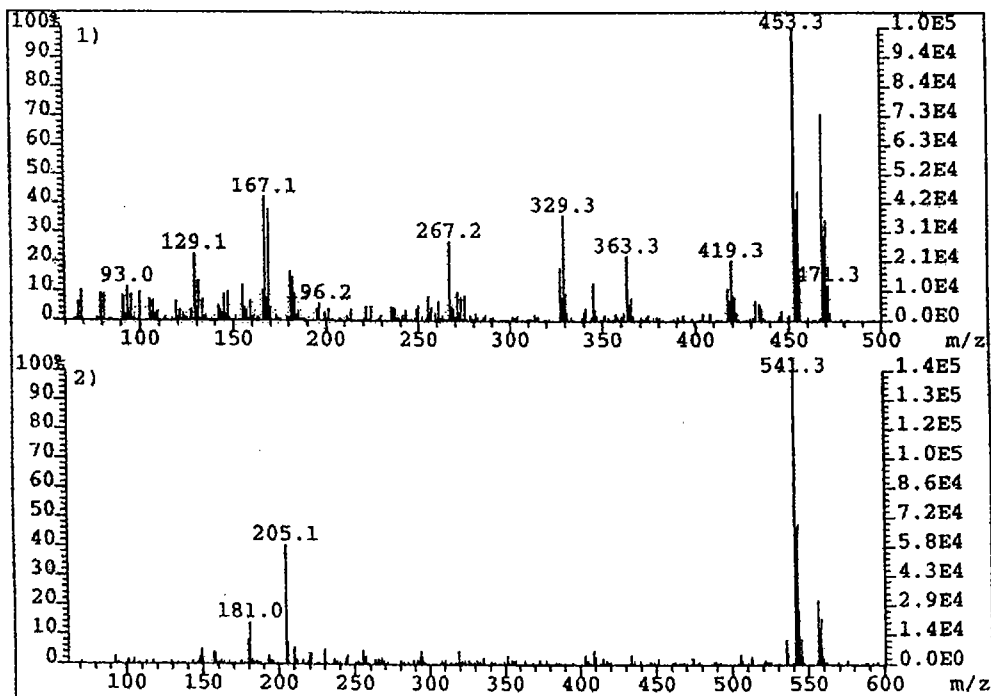
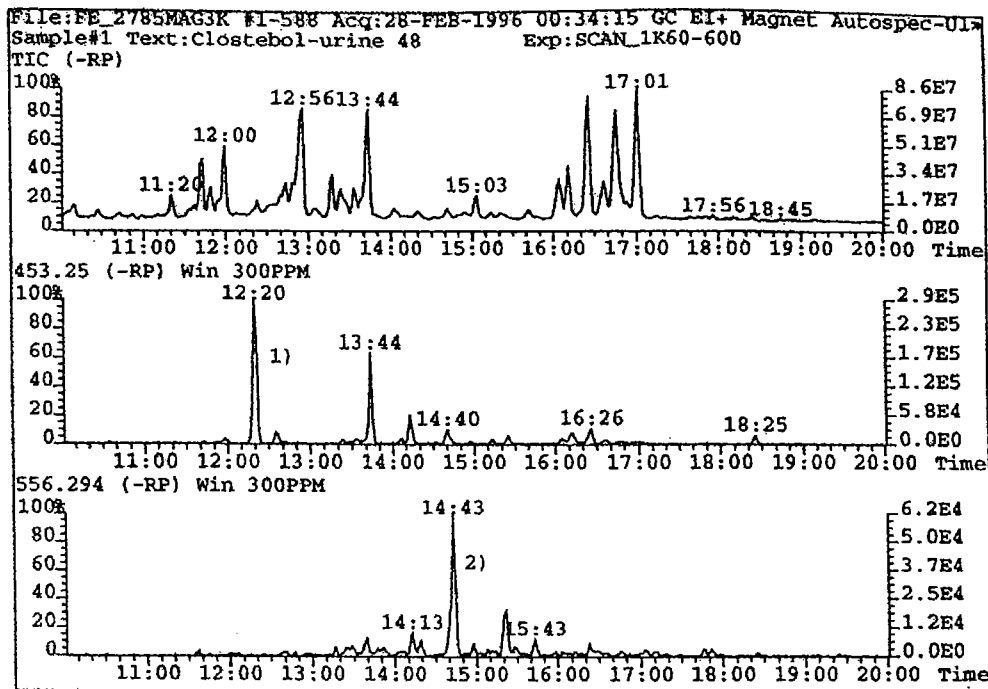




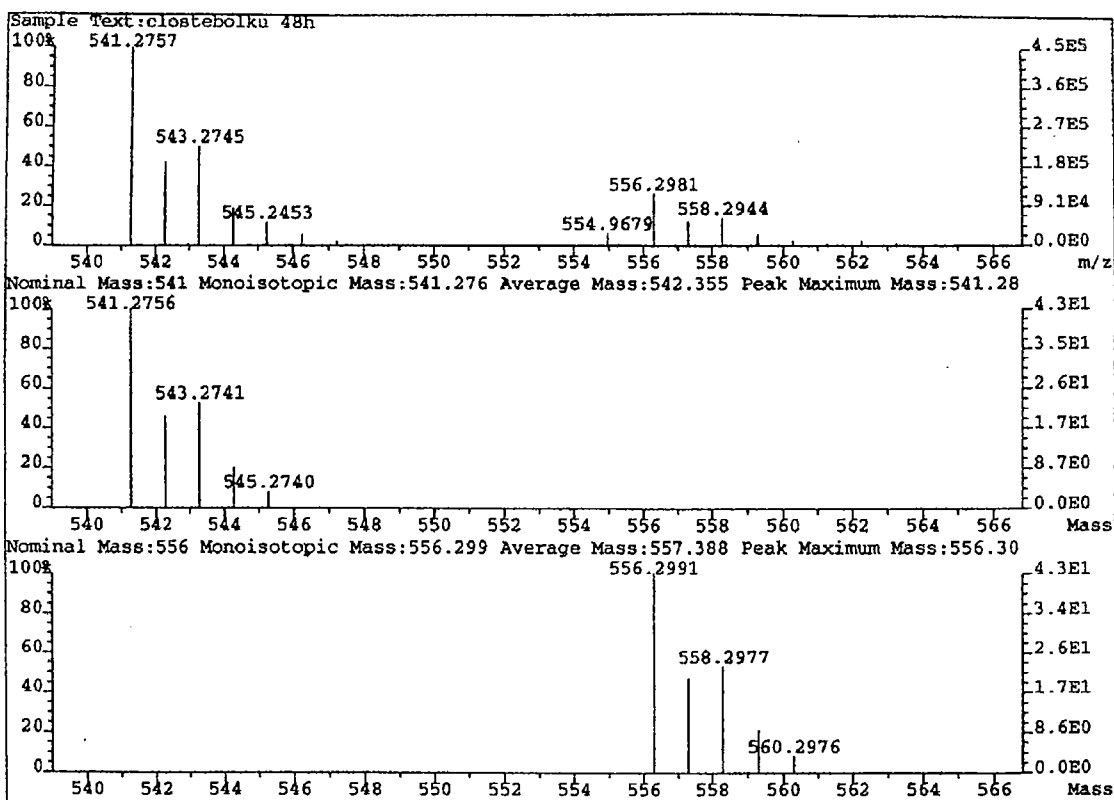
3.) Selected ion recording chromatograms to survey dihydroxylated clostebol metabolites. Molecular ion 644.317 of the possible metabolite and the corresponding isotope signal of a positive control are figured.



4.) Selected ion recording chromatograms to survey trihydroxylated clostebol metabolites. Molecular ion 732.367 of the possible metabolite and the corresponding isotope signal of a positive control are figured.



5.) Full magnet scan mass spectrum of the main metabolite C compared to a typical poor fragmented spectrum of a monohydroxylate metabolite E.



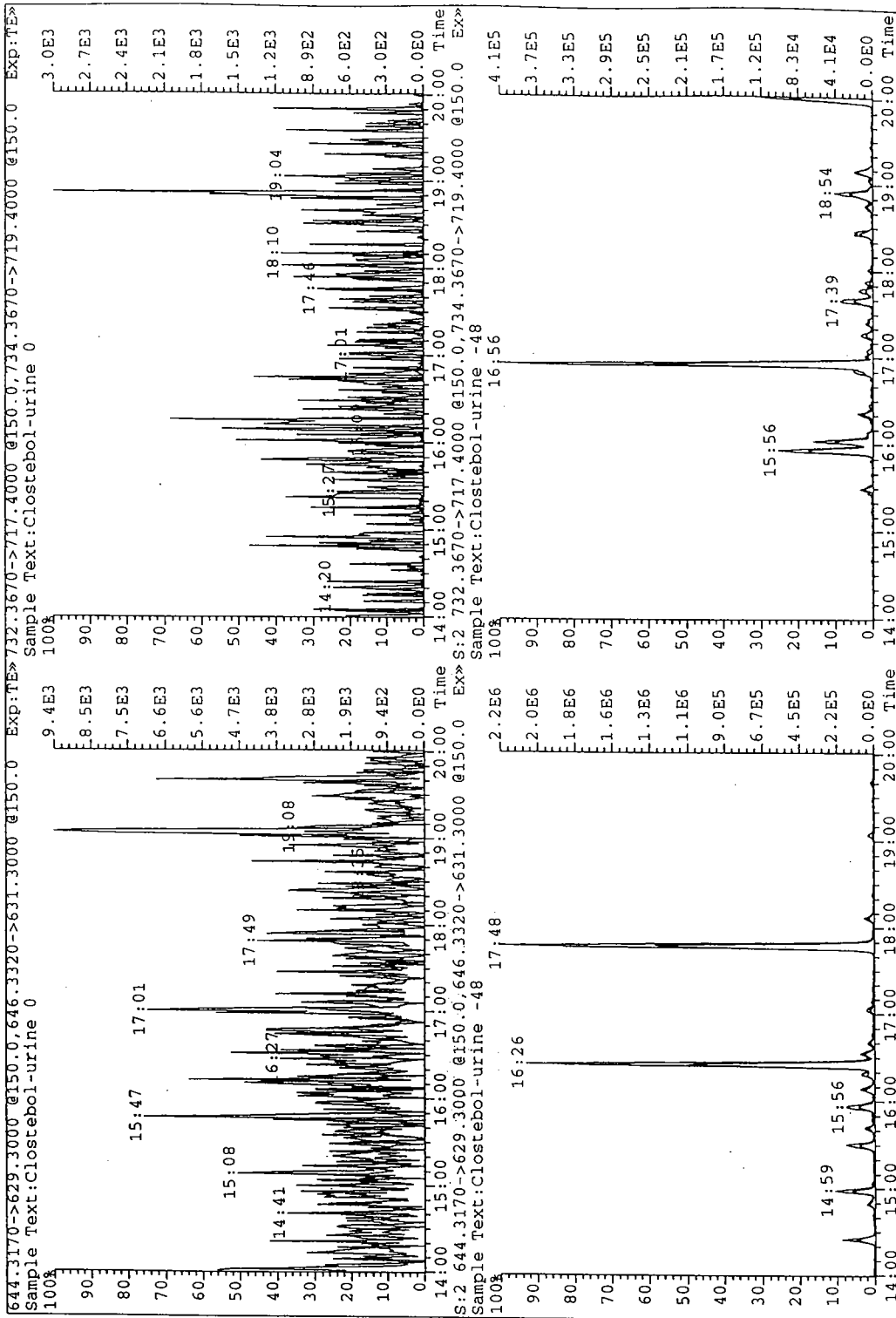
Elemental Composition

Date : 7-MAR-1996

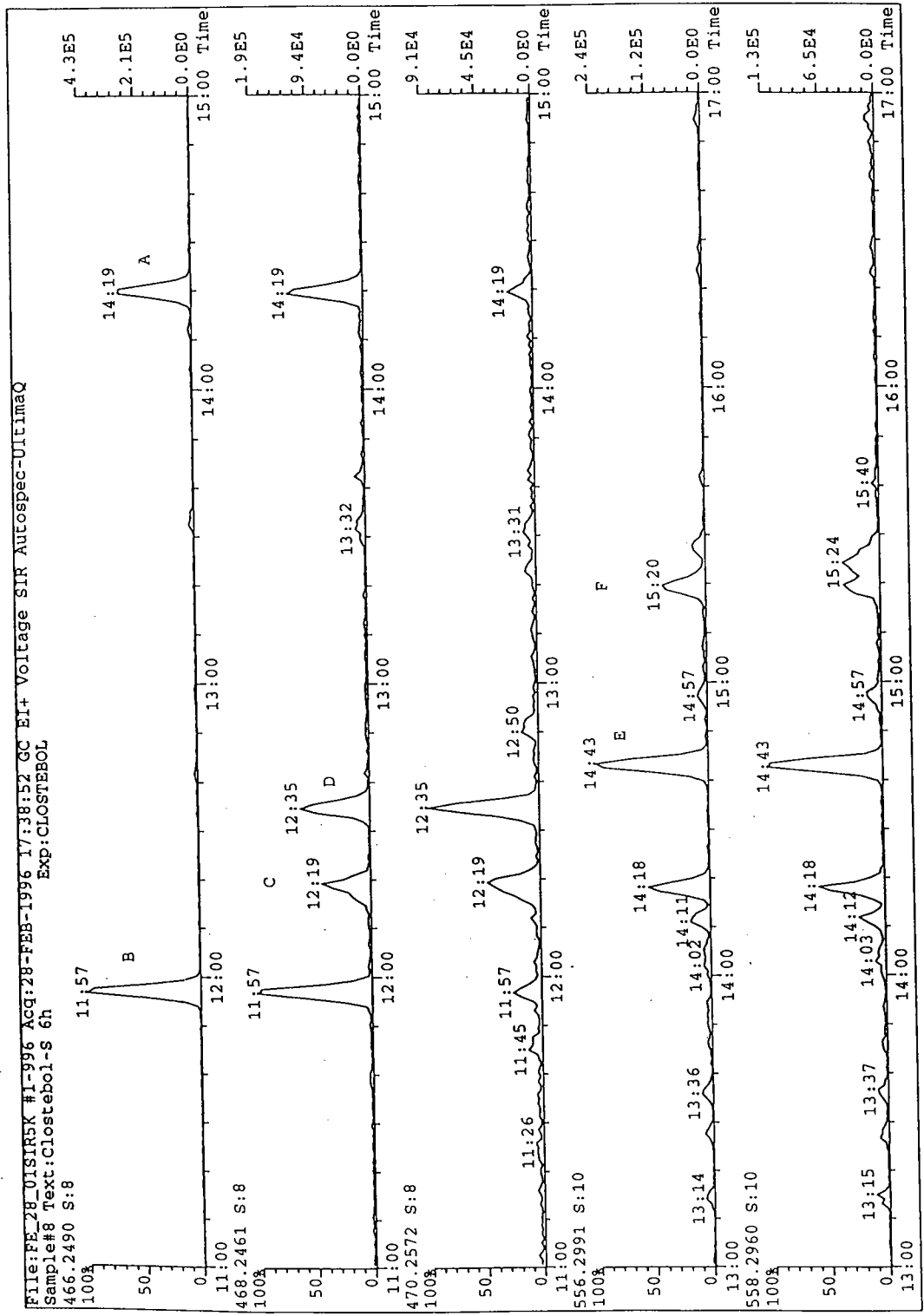
File:MR06\_92VOLK Ident:189-192 Win 300PEM Acq: 7-MAR-1996 15:30:41  
 Autospec-UltimaQ EI+ Voltage EPM:541 EPI:681460 TIC:2507962 Flags:NORM  
 Sample Text: clostebolku 48h  
 Heteroatom Max: 20 Ion: Both Even and Odd  
 Limits:

Mass	mDa	PEM	Calc. Mass	DBE	C	H	N	O	Si	Cl
556.299545	10.0			-0.5	0	0	0	0	0	1
				20.0	200	400	5	5	5	1
556.299545	0.0	0.0	556.299522	4.0	27	57			5	1
	-0.4	-0.8	556.299109	5.0	28	53		3	3	1
	0.5	0.9	556.300033	11.0	30	45	4	2	1	1
	-1.3	-2.4	556.298233	15.5	36	43	1	2		1
	1.4	2.5	556.300909	0.5	22	55	3	3	4	1
	-1.8	-3.2	556.297766	5.5	26	51	3	2	3	1
	1.8	3.3	556.301376	10.5	32	47	1	3	1	1
	-2.2	-3.9	556.297353	6.5	27	47	3	5	1	1
	2.2	4.0	556.301789	9.5	31	51	1		3	1
	-2.7	-4.8	556.296890	16.0	34	41	4	1		1
	2.7	4.9	556.302252	0.0	24	57		4	4	1

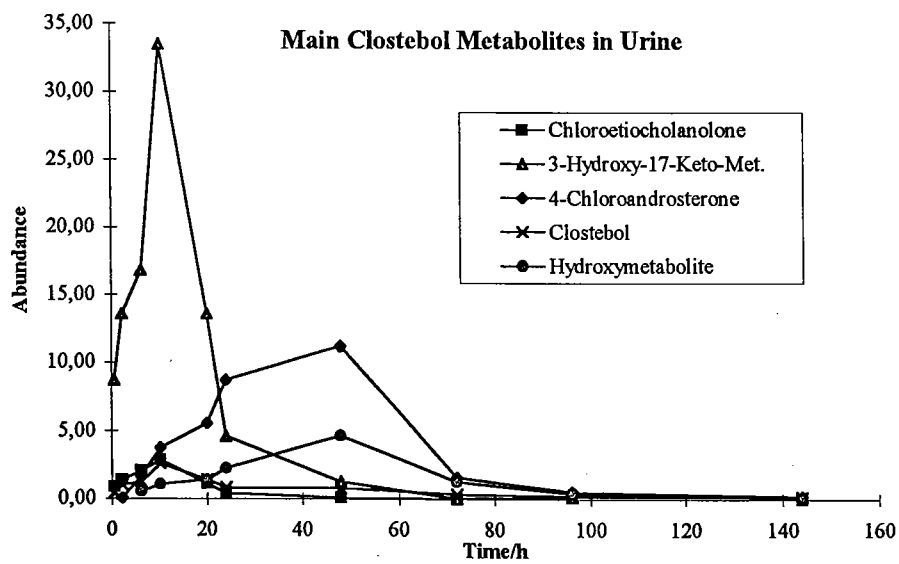
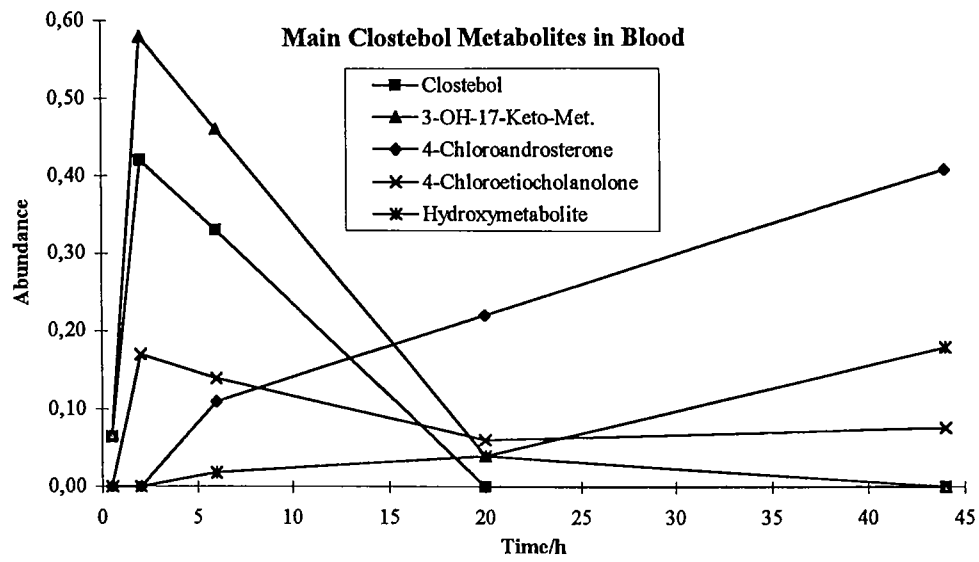
6.) Narrow range voltage scan - mass spectrum of metabolite E. An accurate mass measurement was performed and structural proposals are listed to confirm the suggested elemental composition. The measured spectrum is compared with theoretical values for accurate masses and isotope clusters.



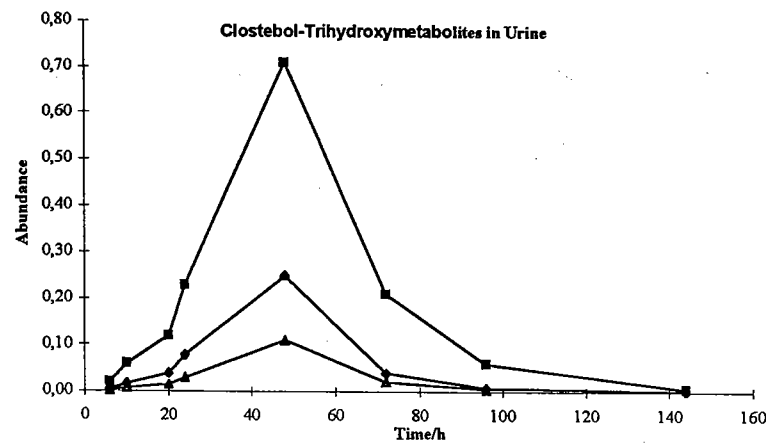
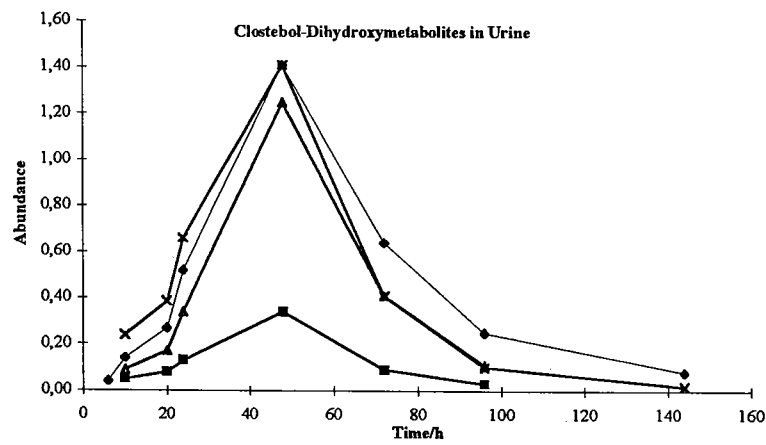
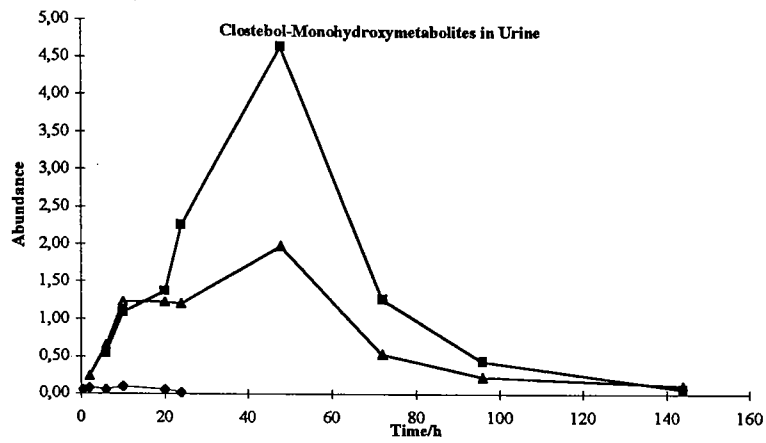
7.) Tandem MS experiment (multiple reaction monitoring=MRM-Q) to compare dihydroxylated (left column) and trihydroxylated (r.h.s.) clostebol metabolites with the corresponding blank (upper windows). The loss of a methyl group of the concerning molecular ion and isotope is detected.



8.) HR-MS examination of clostebol and its metabolites in serum. Upper three windows show the metabolites A-D in the 6h fraction, metabolites E-F at 44h after application is figured below.



9.) Time courses of clostebol main metabolites in blood and urine



10.) Time courses of various clostebol hydroxy-metabolites in urine



## References

1. Schänzer, W. and M. Donike, *Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites*. Analytica Chimica Acta, 1993. 275: p. 23-48.
2. Leysens, L., et al., *Metabolites of 4-chlorotestosterone acetate in cattle urine as diagnostic markers for its illegal use*. Journal of Chromatography B, 1994: p. 43-54.
3. Stephany, R.W., *Commission Decision of 14 July 1987 laying down the methods to be used for detecting residues of substances having a hormonal action and of substances having a thyrostatic action*. Official Journal of the European Communities, 1987. L223: p. 18-36.