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Metabolism of Anabolic Steroids by Recombinant Human Cytochrome P450 Enzymes

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Introduction

The cytochrome P450 enzymes (CYP enzymes) are involved in oxidative, peroxidative and reductive metabolic reactions of drugs, environmental chemicals and endogenous compounds. More than 500 CYP enzymes classified in CYP superfamily of enzymes were identified, characterised and classified into 74 families, fourteen of which are present in humans containing 33 CYP enzymes. A number of members of the CYP superfamily have been successfully expressed in bacterial, yeast, insect and mammalian cells providing an unlimited source of CYP enzymes for studying their structure, function and catalytic activity (1). Of the identified human CYP enzymes, the enzymes belonging to subfamilies CYP1A, CYP2A, CYP2B, CYP2C, CYP2D and CYP3A have been reported to catalyse hydroxylation of both endogenous and exogenous steroid hormones at different positions (2).

In the present work different formats of human recombinant CYP3A4, CYP2B6 and CYP2C9 have been investigated for *in vitro* study of metabolism of anabolic steroids. Enzyme formats used were microsomes prepared from insect cells expressing human CYP enzymes and purified recombinant human CYP enzymes. The steroids studied were testosterone, 17α-methyltestosterone, metandienone, boldenone and 4-chloro-1,2-dehydro-17α-methyltestosterone.

Experimental

Materials

Testosterone, 17α-methyltestosterone, methandienone, boldenone and NADPH were purchased from Sigma (Deisenhofen, Germany), and 4-chloro-1,2-dehydro-17α-

methyltestosterone was a gift from Jenafarm (Jena, Germany). 6β-Hydroxyl metabolites of testosterone, 17α-methyltestosterone, methandienone, 4-chloro-1,2-dehydro-17α-methyltestosterone (3) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were synthesised according to the methods described previously (4). All other reagents and solvents were of analytical grade and water was twice glass distilled before use.

Formats of human recombinant CYP enzymes

Insect cell microsomes (Baculosomes™) containing cDNA-expressed human CYP3A4 and CYP2C9, and purified recombinant *E. coli* expressed (Reco™ System) human CYP 3A4 and CYP2C9 in a reconstituted system were a gift from PanVera (Madison, USA). Human lymphoblast cell microsomes expressing human CYP2B6 were purchased from Gentest Co. (Woburn, USA). The microsomal preparations were received frozen under dry ice and were stored at -80°C until usage.

Incubation

Enzymatic reactions were carried out in glass tubes in a total volume of 500 µl at pH 7.4. The buffer used was either phosphate buffer pH 7.4 (Baculosomes™) or the Buffer Mix supplied with the purified recombinant system (Reco™ System). Stock solutions of the anabolic steroids and of the authentic 6β-hydroxyl metabolites were prepared in methanol at concentration of 1 mg/ml and were stored at –20°C. 50 µl Aliquots of the compounds investigated were added into glass tubes for incubation (final concentration 200 µM). After solvent evaporation under vacuum, 50 µl of 1 M phosphate buffer pH 7.4 and 10 µl of microsomes (0.5 mg protein/ml) were added into glass tubes. The mixture was diluted with 434 µl distilled water and pre-incubated for 3 min at 37°C. The reaction was started by addition of 6 µl of 100 mM solution of NADPH. After an incubation period of 30 min at 37°C under air, an addition of 25 µl acetonitrile stopped the reaction. The steroids and the metabolites were extracted with 5 ml t-butylmethylether (distilled over calcium hydride) in the presence of 50 mg of a solid buffer sodium bicarbonate/potassium carbonate mixture (2:1, w/w). The ether layer was transferred into glass tubes, centrifuged and evaporated to dryness under vacuum.

Incubation using purified recombinant enzyme system (Reco™ System) was performed by the same procedure as with Baculosomes™ with following modifications: the reaction mixture

contained 100 µl of the recombinant enzyme system (Reco™ System), 100 µl of the Buffer Mix supplied with the system and 290 µl of distilled water. The reaction was started by addition of 10 µl of 100 mM solution of NADPH.

Derivatisation

To obtain TMS-ethers for gas chromatography (GC)/mass spectrometry (MS) analysis, dry residue was derivatized with 60 µl of MSTFA/Imidazole (100:2, v/w) and heated for 15 min at 60°C.

GC/MS parameters

GC/MS: HP5890/HP5970

Electron impact (EI): 70 eV

Column: HP-Ultra I (OV1), 17 m, 0.20 mm diameter, film thickness of 0.11 µm

Carrier gas: helium (1 ml/min, split 10:1)

Temperature programme: 180°C, 10°C/min, 270°C, 40°C/min, 320°C, and at 320°C.

Injector temperature: 300°C.

Results and Discusion

Formation of a single metabolite was identified after *in vitro* incubation of testosterone, 17α-methyltestosterone, methandienone, 4-chloro-1, 2-dehydro-17α-methyltestosterone and boldenone with insect microsomes expressing human CYP3A4 (Baculosomes™, figures 1A, 3A, 5A, 7A and 9A), and with purified reconstituted system containing the *E. coli*_expressed CYP3A4 (Reco™ System, figures 1B, 3B, 5B and 7B). GC/MS ion chromatograms were analysed by extracting molecular or the most intense ions.

GC/MS ion chromatograms revealed single peaks at the same retention time following incubation of the parent compounds with both enzyme formats used. When experiments were performed using control microsomes the peaks corresponding to the proposed metabolites could not be detected (figures 1C, 3C, 5C, 7C, and 9 C).

The molecular ions and the fragmentation patterns of the compounds identified as metabolites indicate hydroxylation of the parent compounds. Comparison of retention times (figures 1, 3, 5 and 7) and fragmentation patterns (figures 2, 4, 6 and 8) of the metabolites and the synthesised

authentic compounds confirmed 6β-hydroxylation of testosterone, 17α-methyltestosterone, methandienone and 4-chloro-1, 2-dehydro-17α-methyltestosterone. The structure of the boldenone metabolite was not unequivocally determined, as the authentic compound was not available. The presence of the M^+ ion at m/z 446 in the mass spectrum of the metabolite of boldenone (figure 10A) confirmed hydroxylation of the parent compound, as M^+ ion in the mass spectrum of boldenone appeared at m/e 358 (figure 10B). Based on the fragmentation patterns of the metabolites of boldenone and methandienone we suggest that the boldenone metabolite identified in the incubation mixtures containing both formats of CYP3A4, is 6β-hydroxyl boldenone as presented in figure 10.

In the extracts of the incubation mixtures containing expressed CYP2C9 formats and the anabolic steroids no metabolites where detected. Of the steroids investigated, only testosterone and 17α-methyltestosterone were hydroxylated in 6β-position following incubation with human lymphoblast cell microsomes expressing CYP2B6.

These results, together with those obtained *in vivo* and reported by Schänzer *et al.* (3), suggest that 6β-hydroxylation is the preferential metabolic pathway for steroids possessing 3-one-, 4-ene-structure in the molecule. We also suggest that the electronic effects of the 3-one, 4-ene structural moiety of the steroids participate in the selectivity for the enzymatic 6β-hydroxylation of the substrates (2). This suggestion is supported by the results reported previously (3) showing that trimethylsilyl 3,5-dienol ethers of the steroids, dissolved in ethanol, are easily auto-oxidised by direct sunlight to 6-hydroxyl derivatives again with preferential formation of 6β-hydroxylated products.

Acknowledgements

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We thank PanVera, USA and Mr. A.Vodenlich for kind donation of the recombinant CYP3A4 and CYP2C9 enzymes used in this study.

References

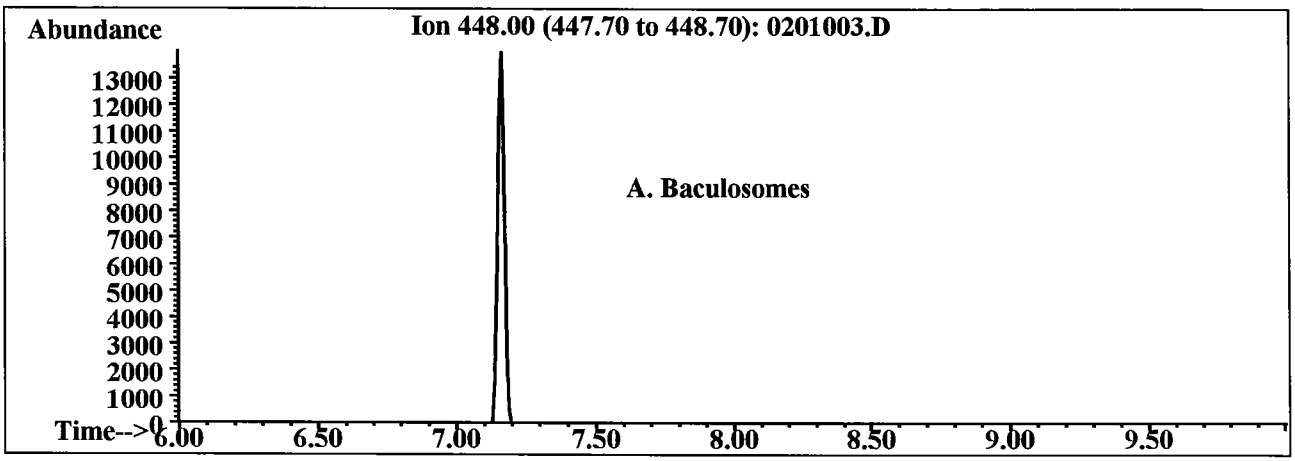
(1) Rendic S. and Di Carlo, F.: Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors: *Drug Metabolism Reviews,* 29: 413-580, 1997.

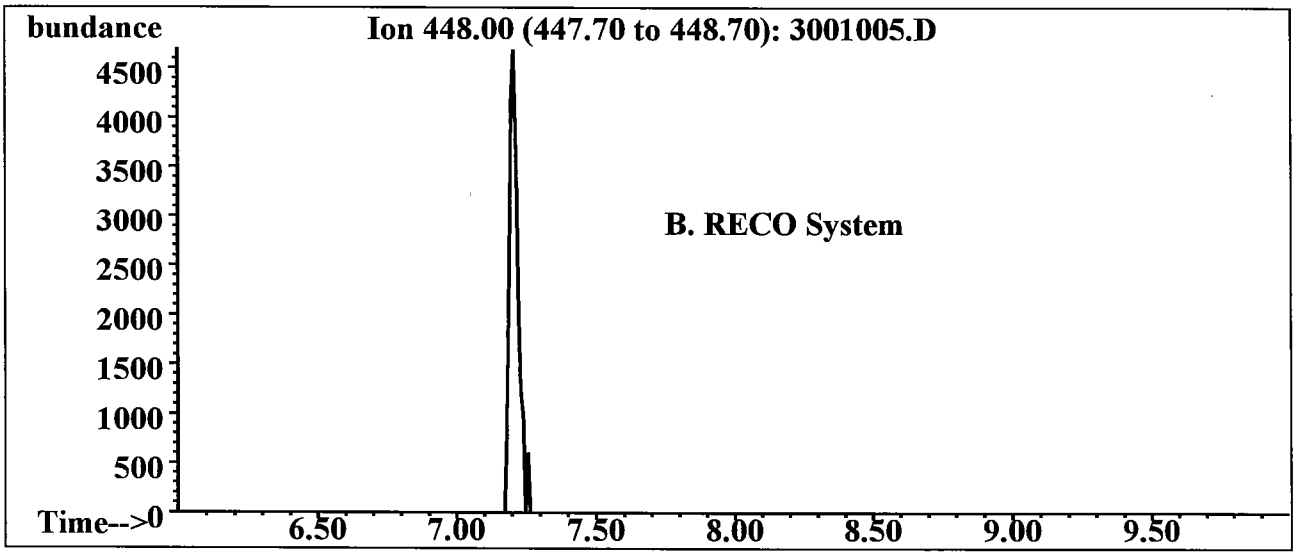
(2) Rendic, S., Nolteernsting E. and Schänzer W.: 6β-Hydroxylation of steroid hormones by recombinant human cytochrome P450 (CYP) enzymes: Xenobiotica, (submitted for publication).

(3) Schänzer, W., Horning, S., and Donike, M.: Metabolism of anabolic steroids in humans: Synthesis of 6β-hydroxy metabolites of 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone, and methandienone: *Steroids,* 60: 353-377, 1995.

(4) Donike M.: N-methyl-N-trimethylsilyl-trifluoroacetamid, ein neues silylirungs mittel aus der reihe der silylirten amide: *Journal of Chromatography,* 42: 103-104, 1969.

Fig. 1. GC/MS ion chromatograms of the bis-TMS derivative of the metabolite formed by incubation of testosterone with two different formats of human CYP3A4 enzyme (A and B) and with control microsomes (C).





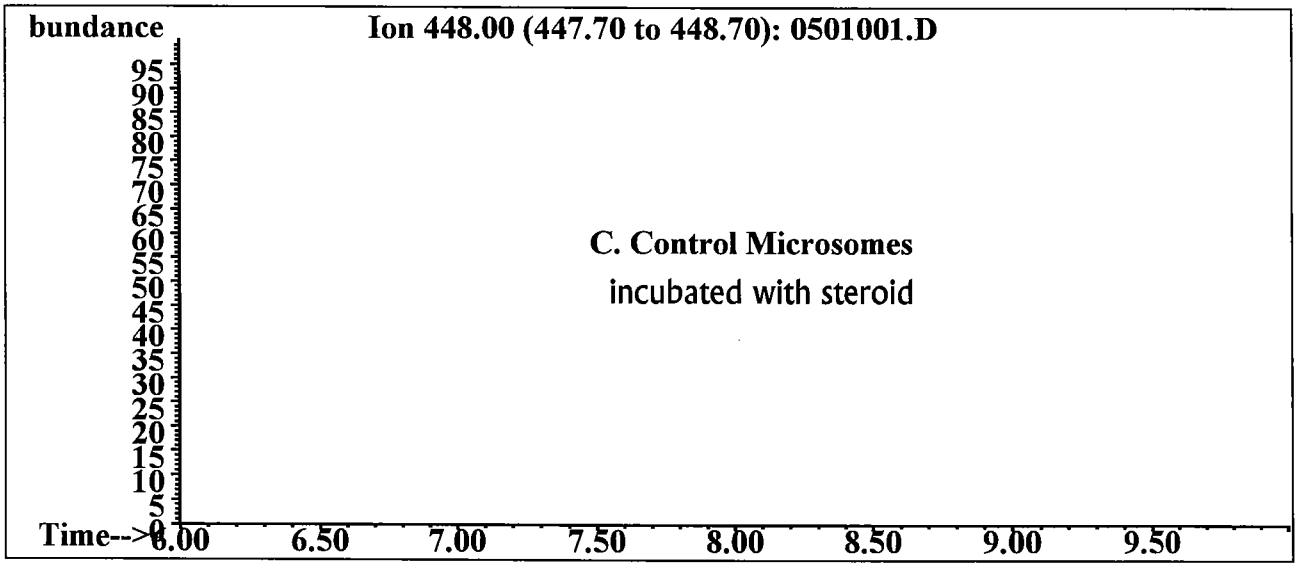
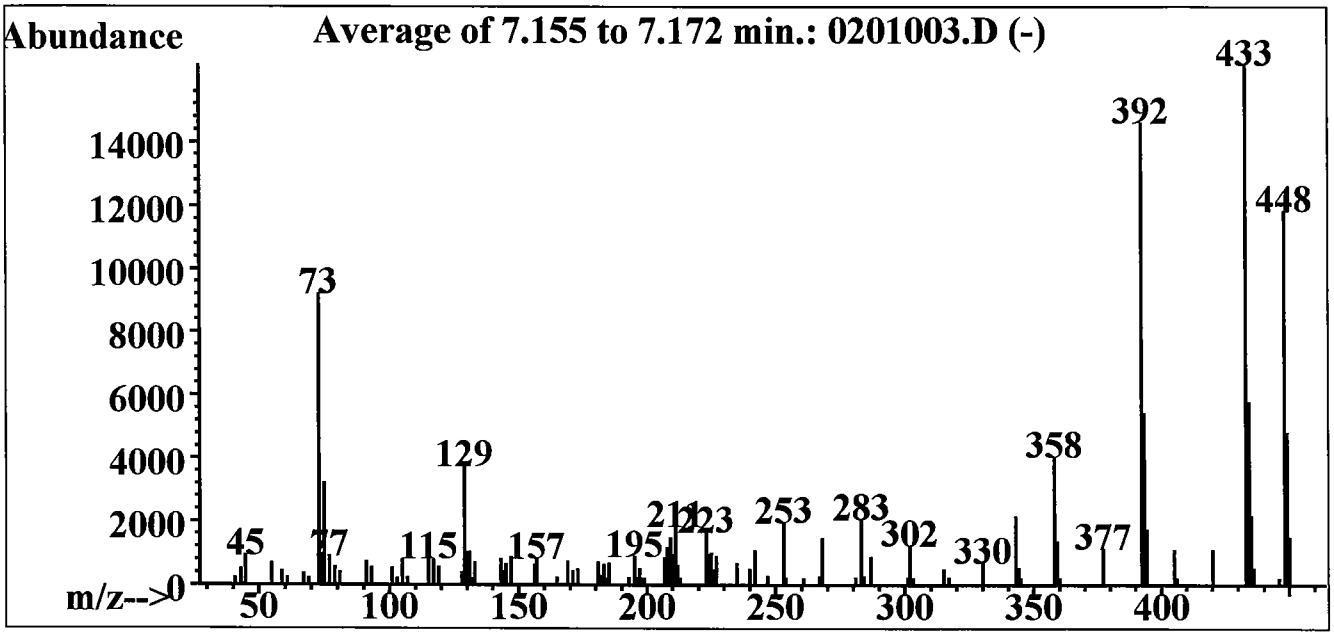
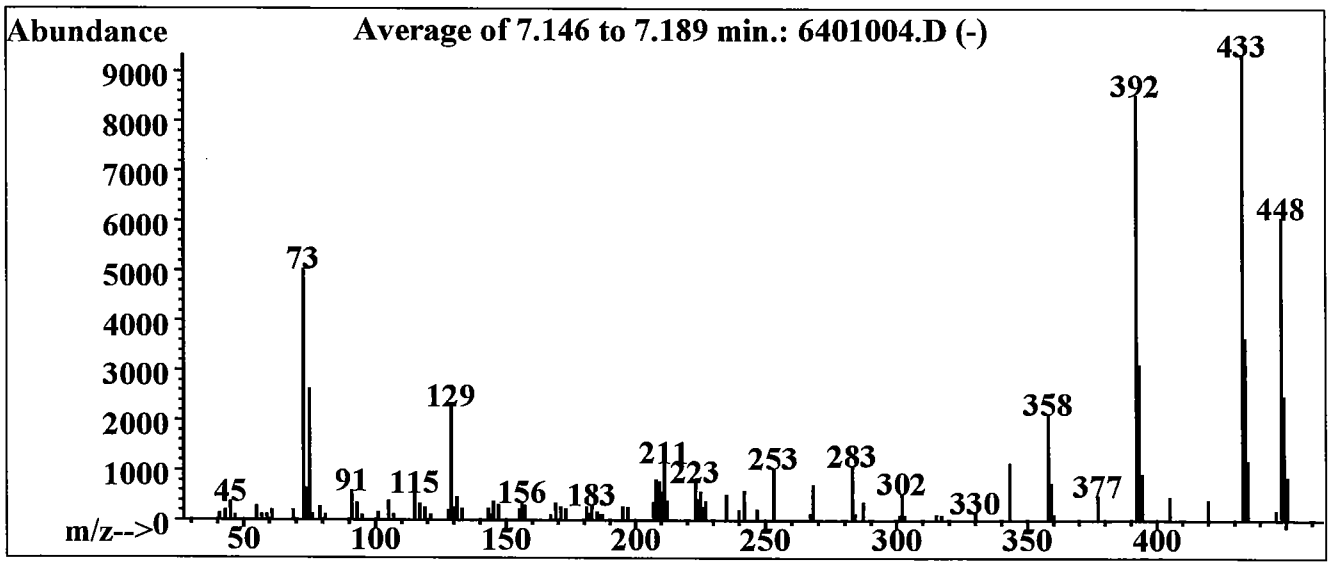


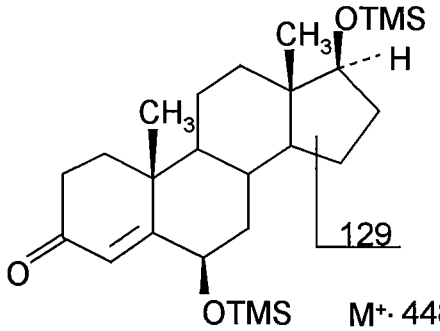
Fig. 2. EI-spectra of the bis-TMS derivative of 6ß-hydroxytestosterone formed by incubation of testosterone with hyman CYP3A4 (A) and of synthetic 6ß-hydroxytestosterone (B).

A



B





Structure of 6ß-hydroxytestosterone bis-TMS.

Fig. 3. GC/MS ion chromatograms of the bis-TMS derivative of the metabolite formed by methyltestosterone incubation with two different formats of human CYP3A4 enzyme (A and B) and with control microsomes (C).

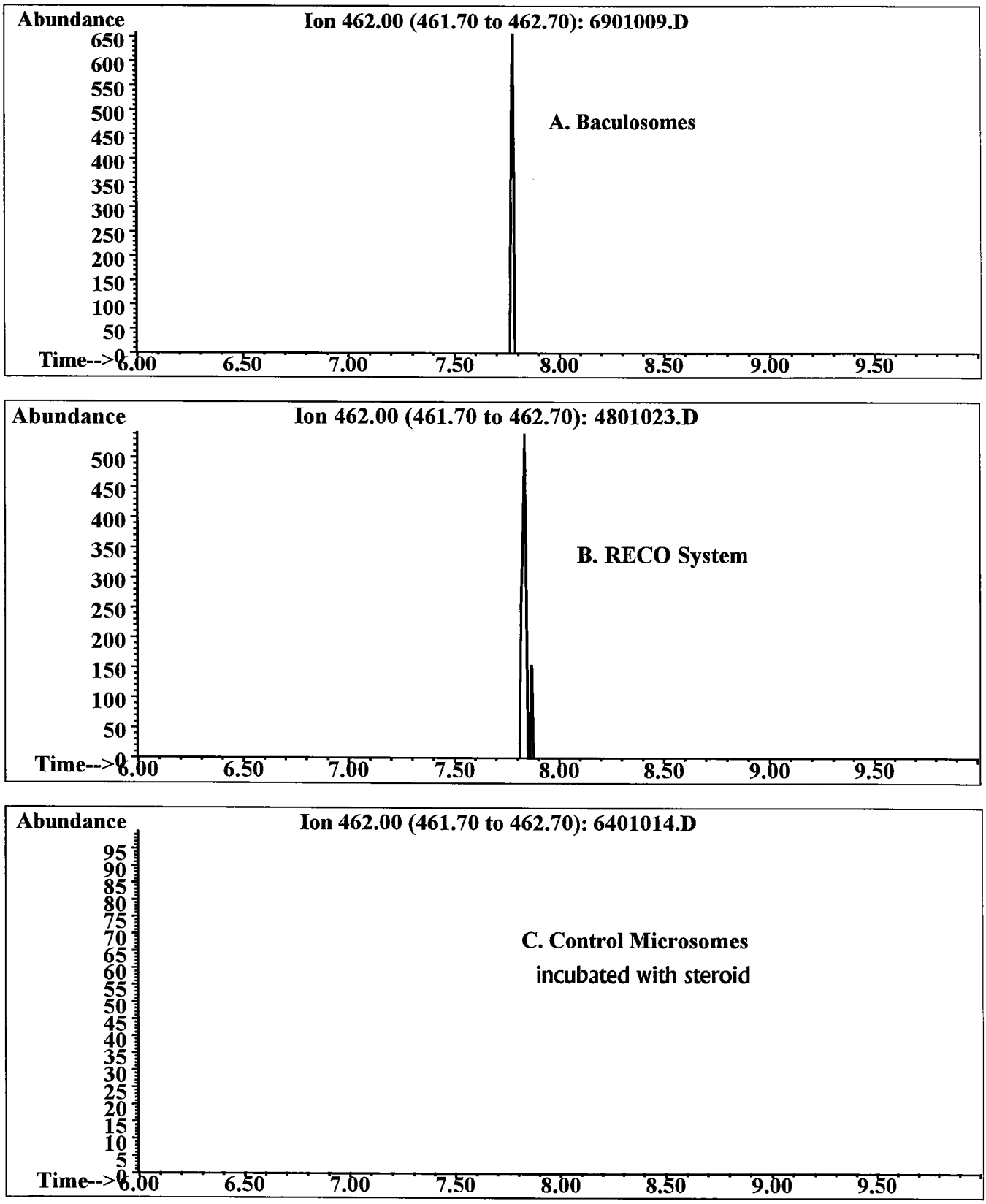
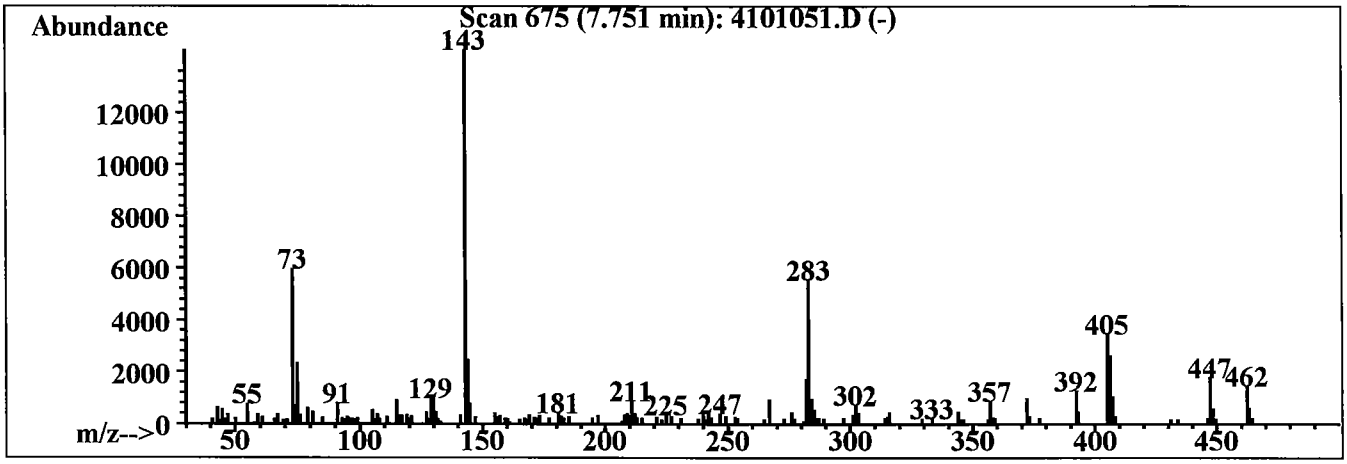
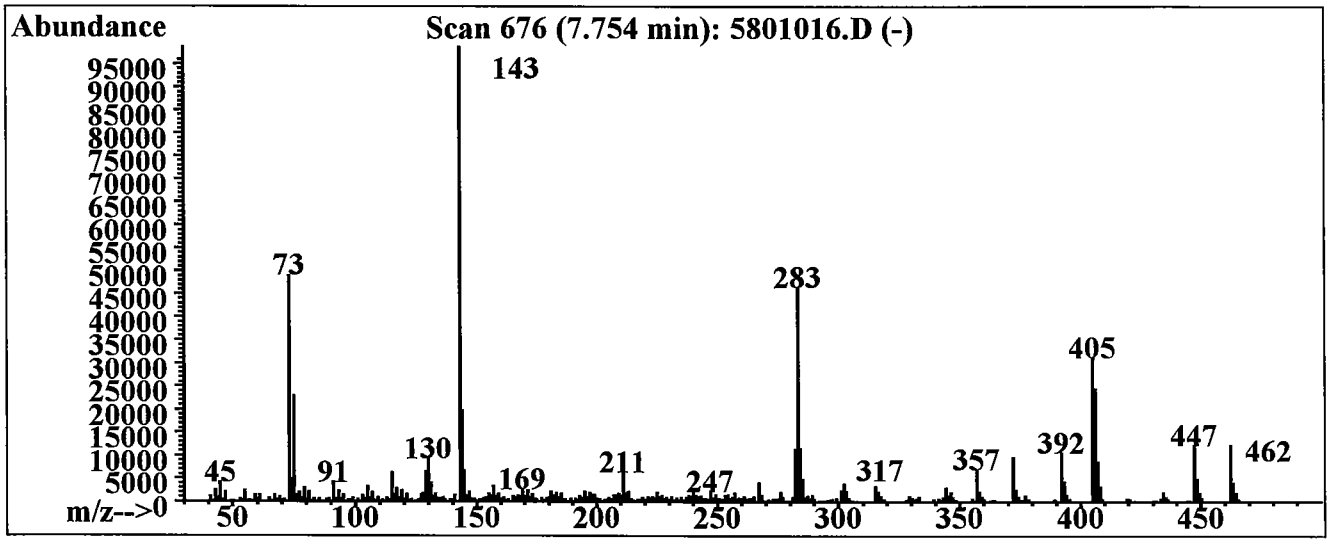


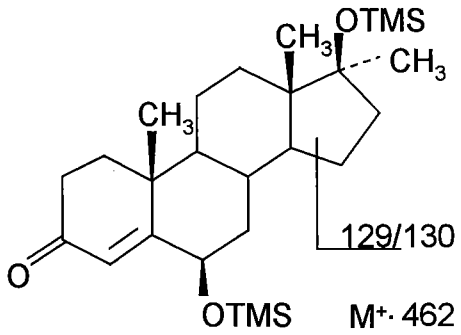
Fig. 4. EI-spectra of the bis-TMS derivative of 6ß-hydroxymethyltestosterone formed by incubation of methyltestosterone with hyman CYP3A4 (A) and of synthetic 6ß-hydroxymethyltestosterone (B).

A



B





Structure of 6ß-hydroxymethyltestosterone bis-TMS.

Fig. 5. GC/MS-chromatograms of the bis-TMS derivative of the metabolite formed by incubation of metandienone with two different formats of human CYP3A4 enzyme (A and B) and with control microsomes (C).

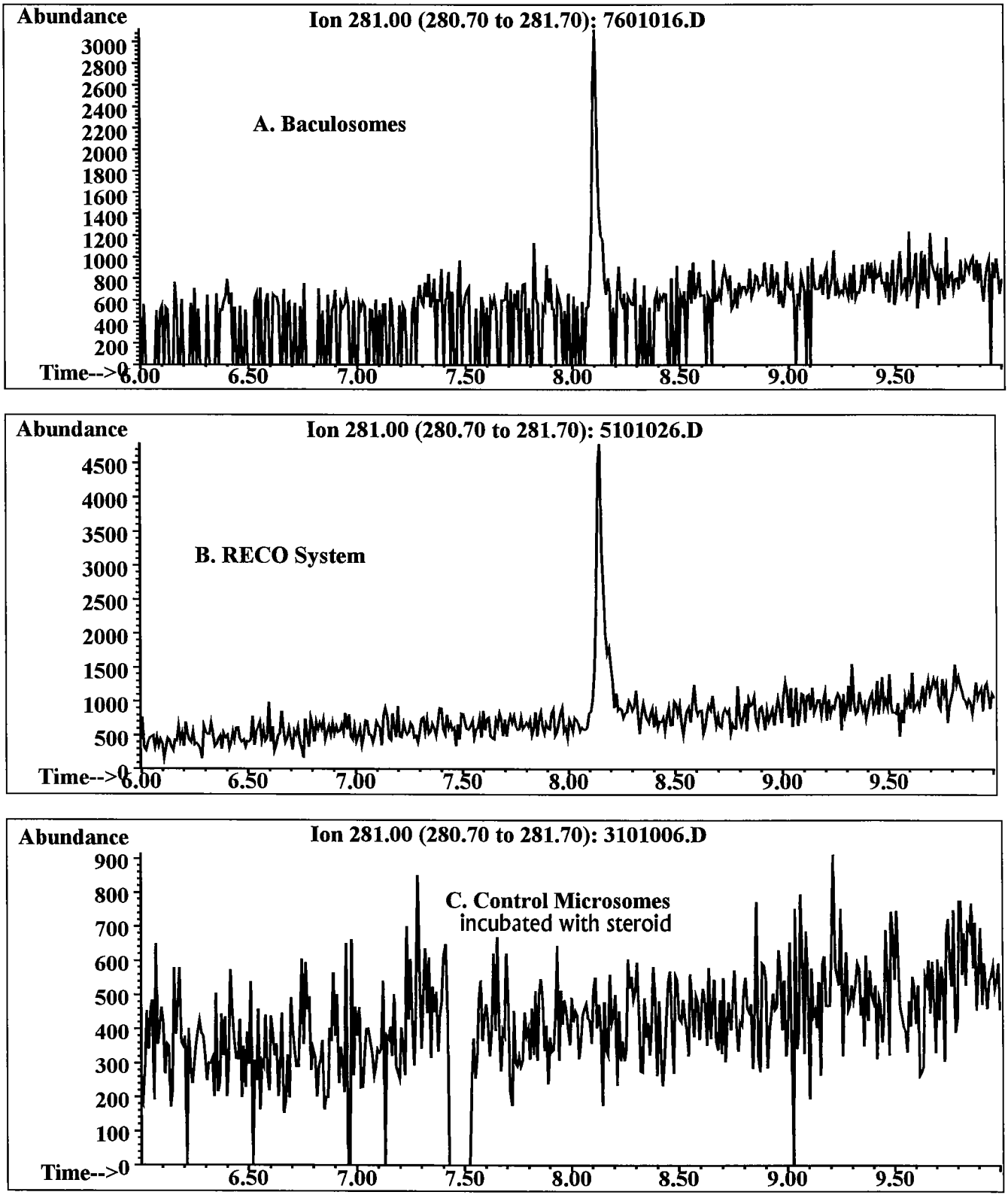
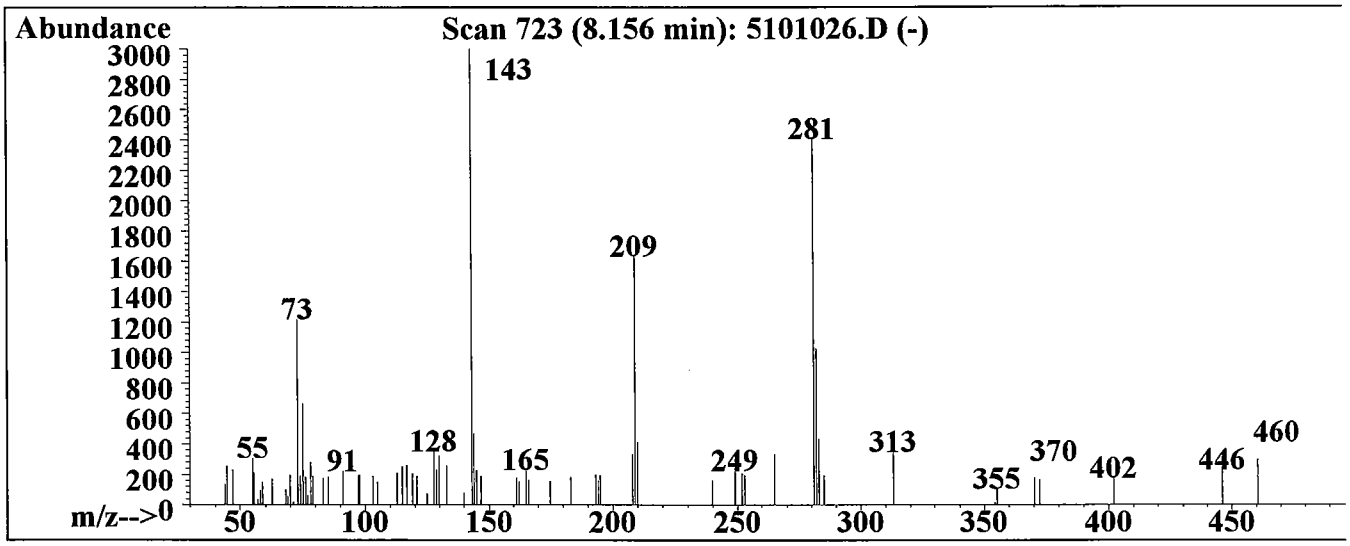
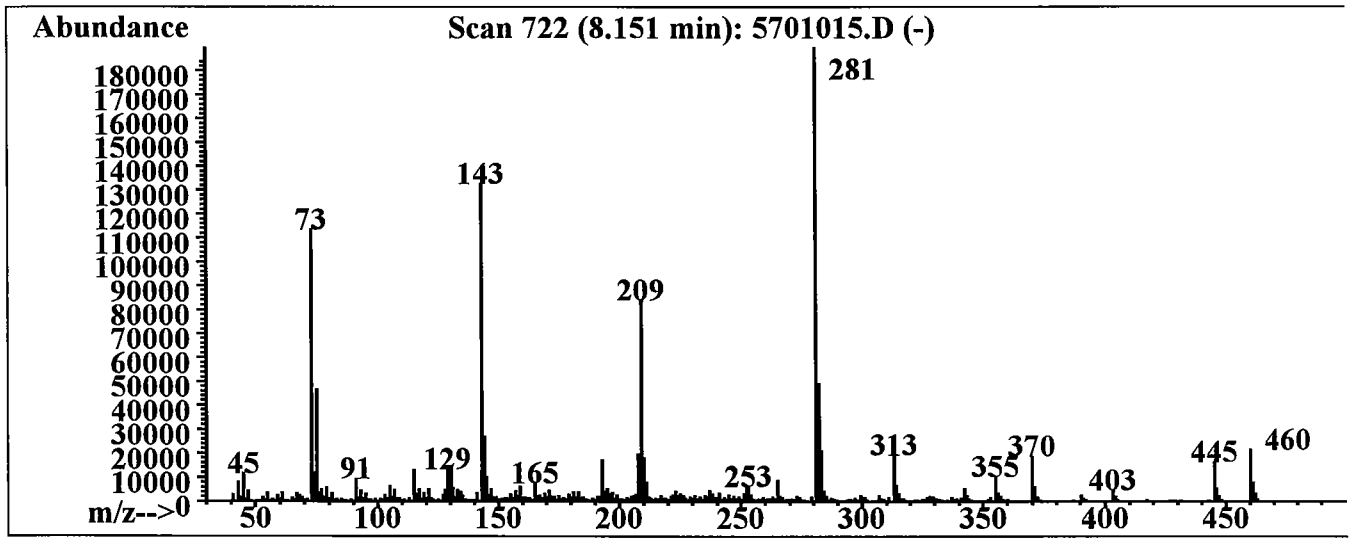


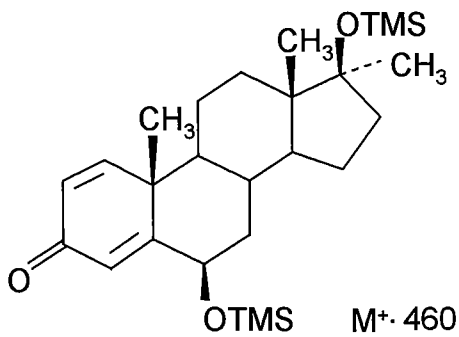
Fig. 6. EI-spectra of the bis-TMS derivative of 6ß-hydroxymetandienone formed by incubation of metandienone with hyman CYP3A4 (A) and of synthetic 6ß-hydroxymetandienone (B); molecular ion at m/z 460.

A



B





Structure of 6ß-hydroxymethandienone bis-TMS, M^{+} 460.

Fig. 7. GC/MS ion chromatograms of the bis-TMS derivative of the metabolite formed by incubation of 4-chloro-1,2-dihydro-17α-methyl-testosterone with two different formats of human CYP3A4 enzyme (A and B) and with control microsomes (C).

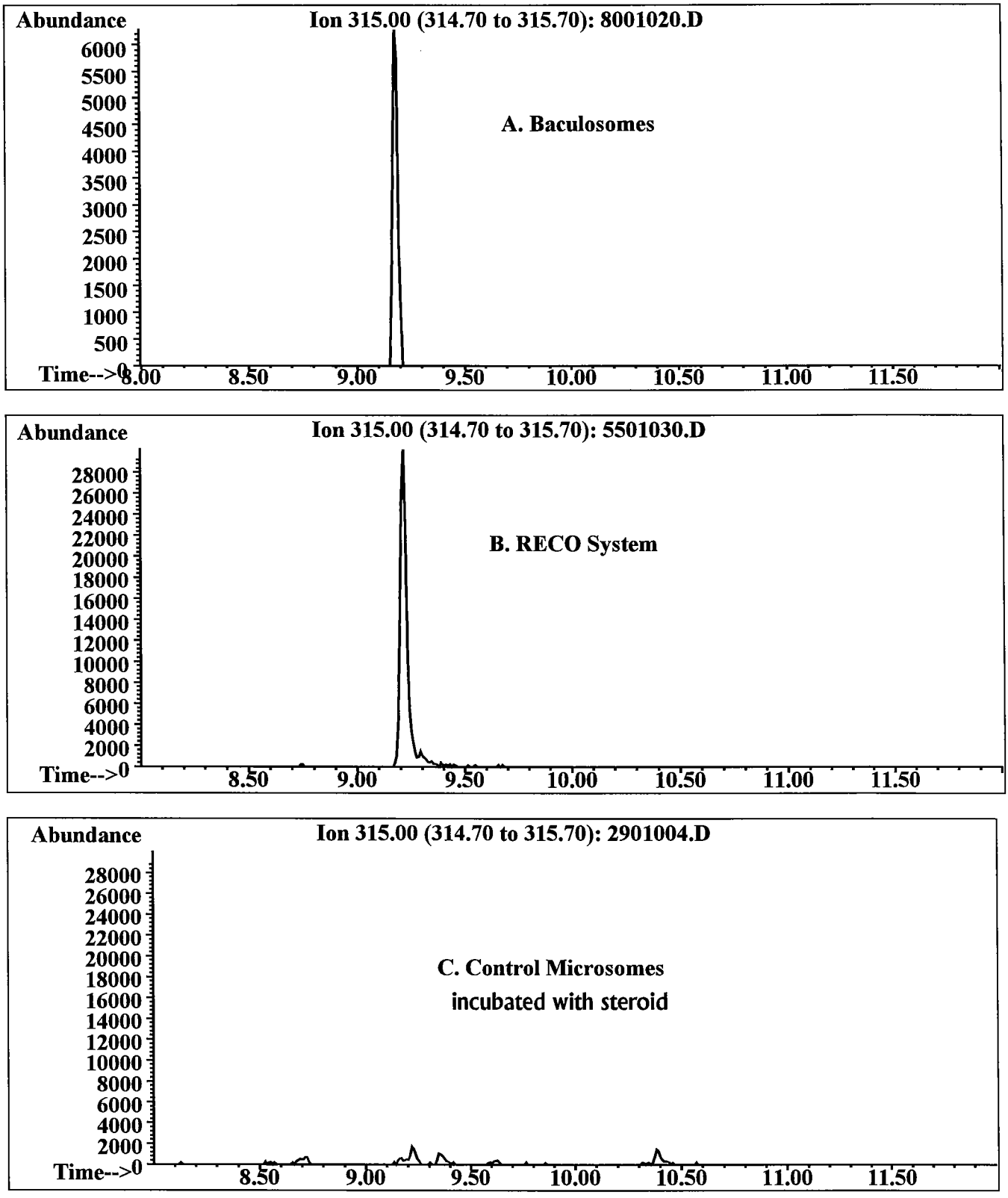
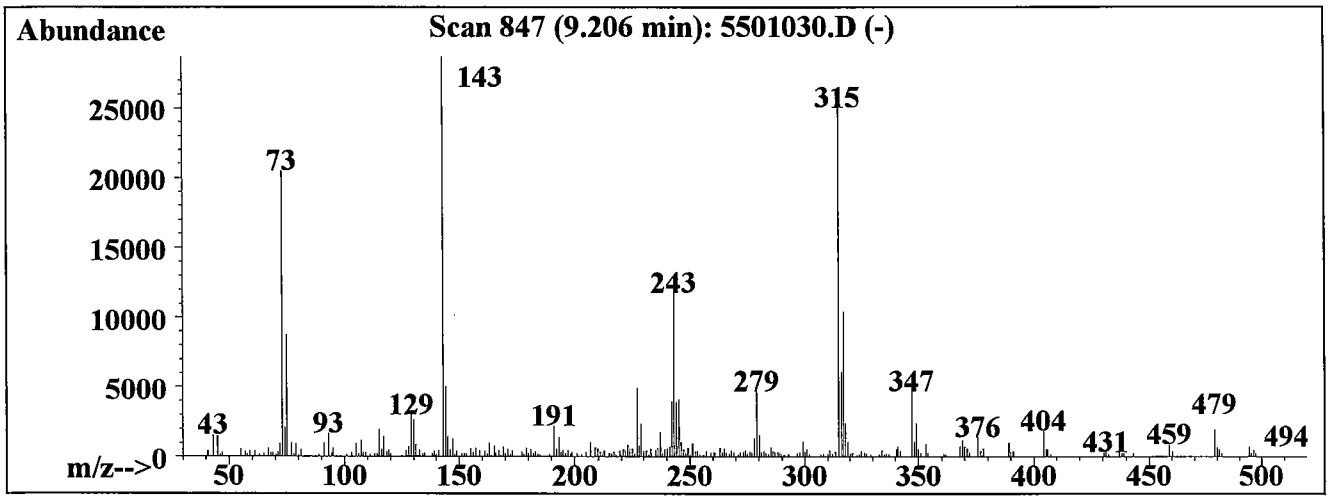
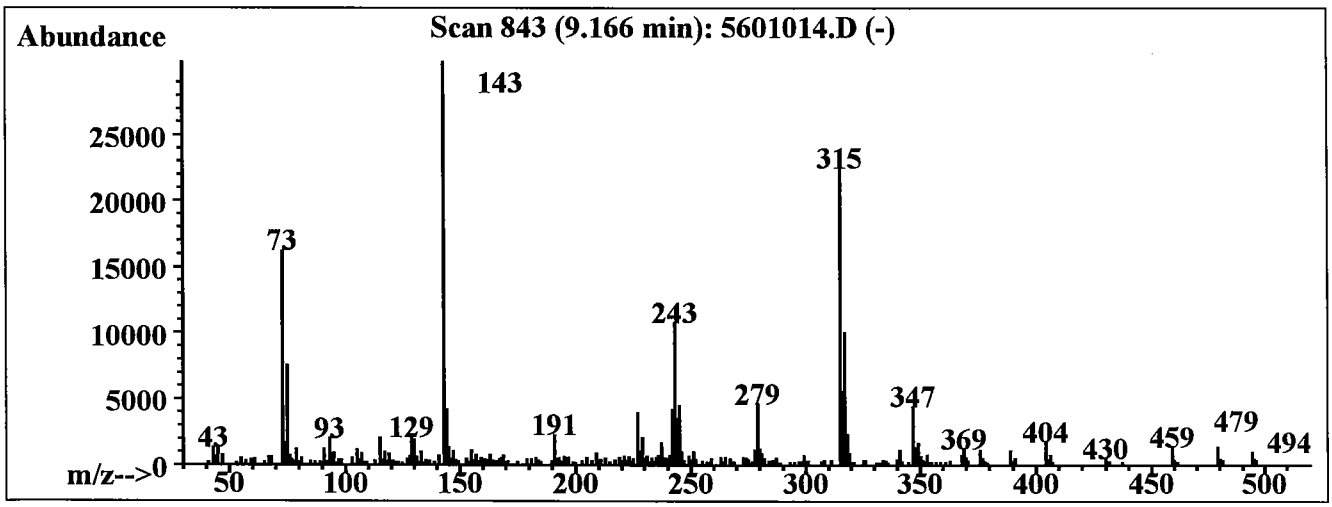


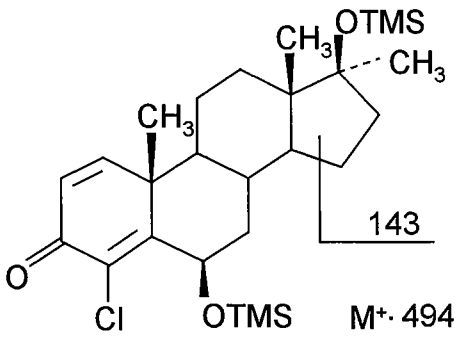
Fig. 8. EI-spectra of the bis-TMS derivative of 6ß-hydroxy-4-chloro-1,2-dihydro-17α-methyltestosterone formed by incubation of 4-chloro-1,2-dihydro-17α-methyltestosterone with hyman CYP3A4 (A) and of synthetic 6ß-hydroxy-4-chloro-1,2-dihydro-17α-methyltestosterone (B).

A



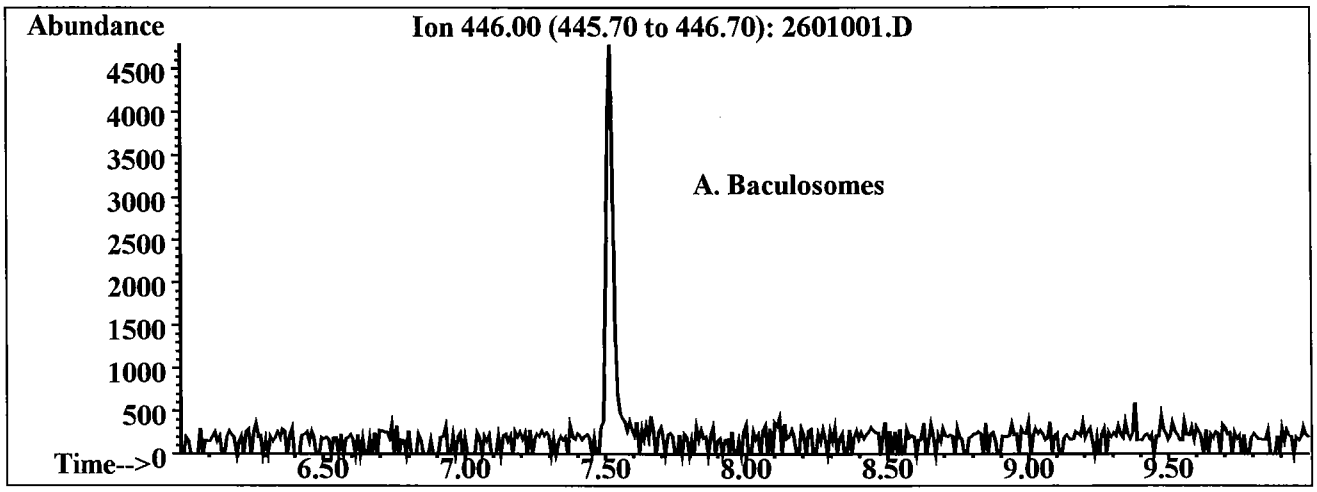
B





Structure of 6ß-hydroxy-4-chloro-1,2-dihydro-17α-methyltestosterone bis-TMS.

Fig. 9. GC/MS ion chromatogram of the bis-TMS derivative of the metabolite formed by incubation of boldenone with human CYP3A4 enzyme (A) and with control microsomes (C).



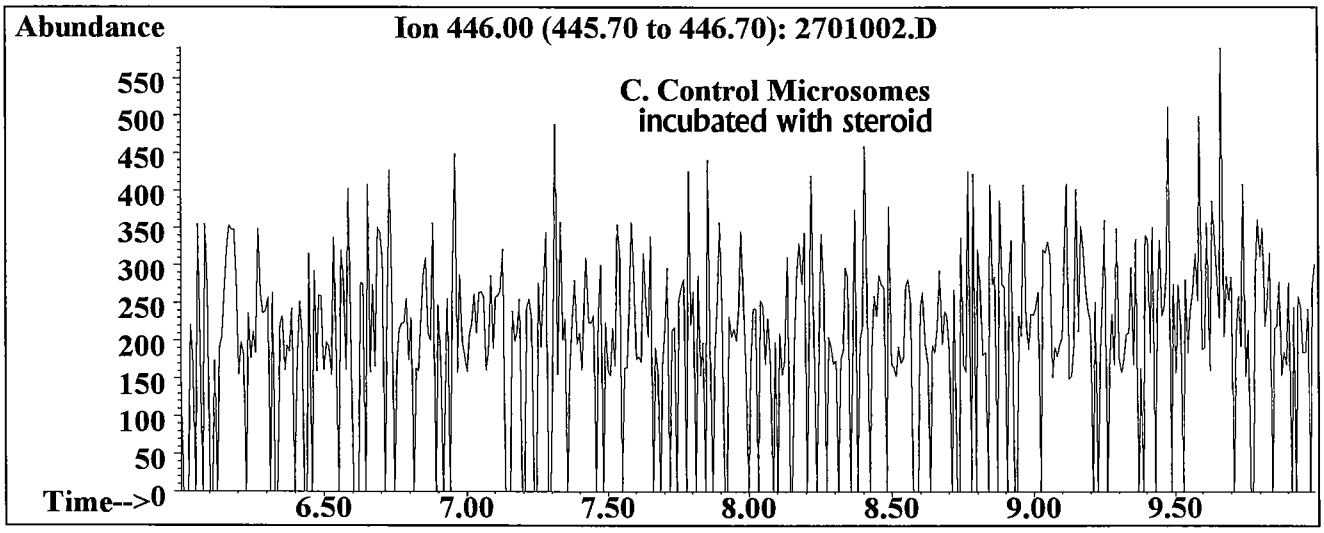
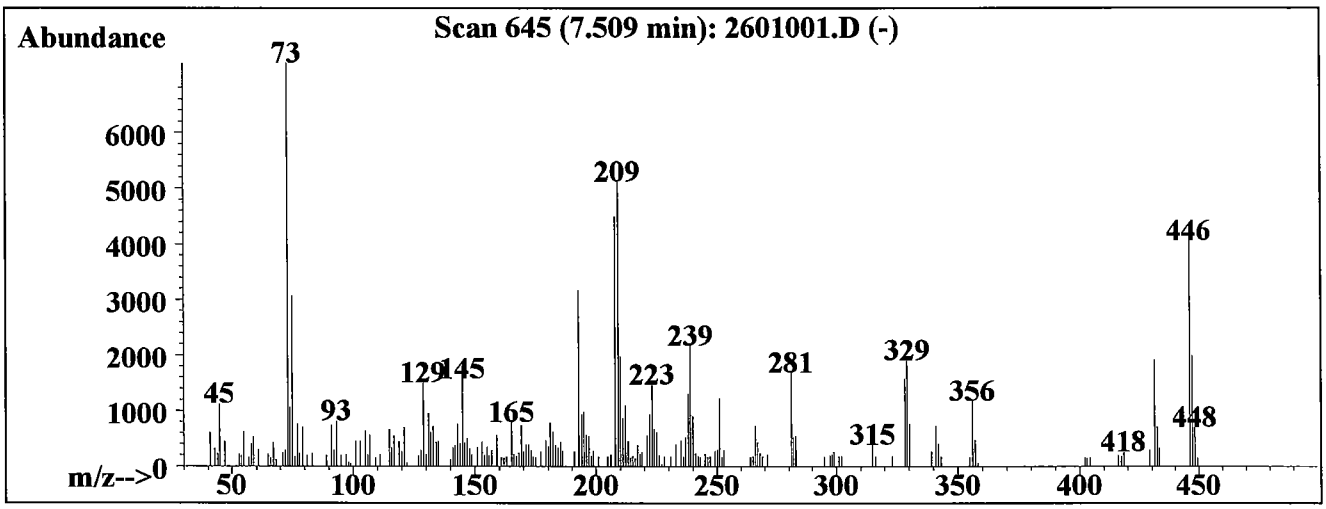


Fig. 10. EI-spectra of the bis-TMS derivative of 6ß-hydroxyboldenoneformed by incubation of boldenone with hyman CYP3A4 (A) and of parent compound boldenone (B).

A



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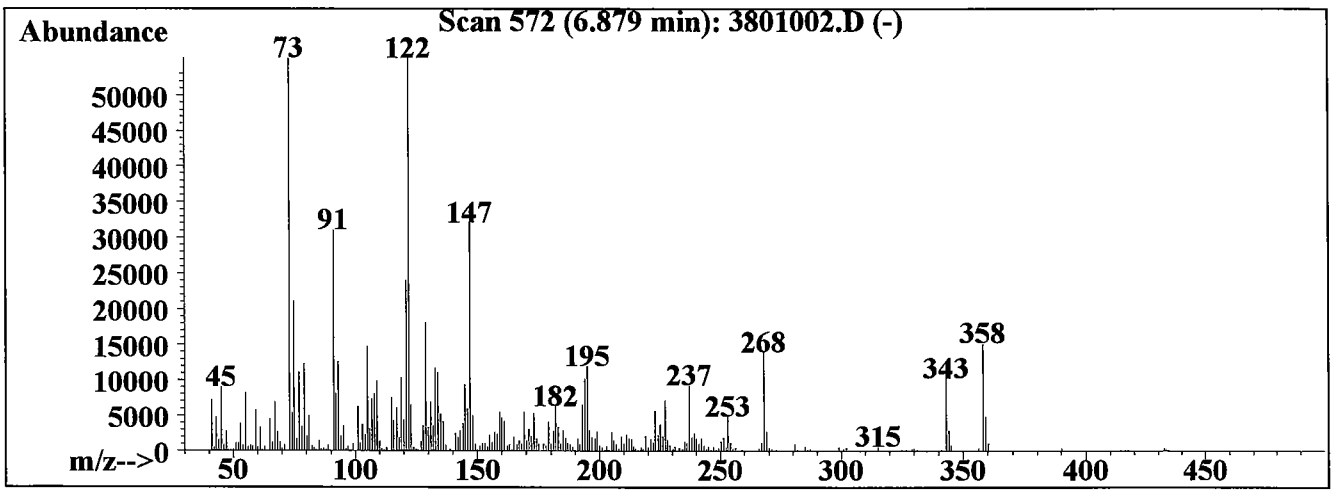
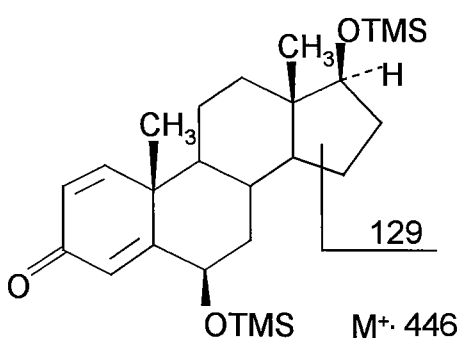
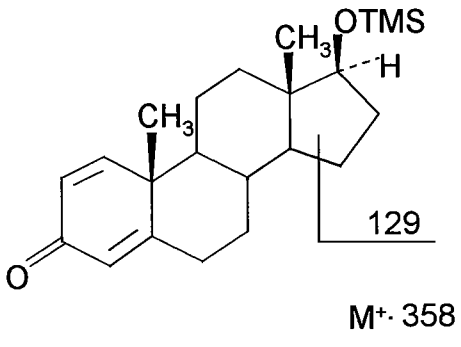




Structure of hydroxyboldenone bis-TMS.



Structure of boldenone mono-TMS.