Monica Mazzarino, Ilaria Fiacco, Amelia Palermo, Xavier de la Torre, Francesco Botrè

The use of cytochrome P450 inhibitors in sport. A new generation of doping masking agents?

FMSI, Antidoping Laboratory, Rome, Italy

Abstract

The activity of the CYP450 isoenzymes responsible for the phase I metabolism of the compounds included in the World Anti-Doping Agency list of prohibited substances and methods could be strongly altered by the combined administration of other drugs. Little information is available regarding the relevance of drug-drug interaction in doping control field.
This researchwas designed to investigate whether the co-administration of banned compounds with other, non-banned active principles commonly used by athletes may lead to a substantial alteration of drug metabolism and urinary excretion kinetics. The *in vitro* metabolic profile of five representative banned agents (toremifene, stanozolol, methandienone, ciclesonide, deflazacort) was assessed individually and in the presence of non banned drugs (fluoconazole, ketoconazole, itraconazole, miconazole; cimetidine, ranitidine, fluoxetine, paroxetine, nefazodone) using either human liver microsomes or CYP450 recombinant isoforms. The chromatographic separation was performed with an octadecyl reverse-phase column after liquid/liquid extraction. The detection and identification of the compounds were performed using both time-of flight and triple quadrupole mass spectrometers.
The results demonstrated that the *in vitro* metabolic profiles of the selected substrates were extensively altered in presence of ketoconazole, itraconazole, miconazole, and nefazodone; moderate variations were registered in presence of paroxetine and not significant modifications were measured in presence of ranitidine, cimetidine, fluoconazole and fluoxetine. Hence, the use of antifungals or antidepressants by athletes could lead to an incorrect interpretation of the analytical results, producing a masking effect based on the alteration of the phase I metabolism.

Introduction

The reaction of the living organism to the presence of external, biologically active compounds (“xenobiotics”) is to activate a series of processes to facilitate their elimination. Most xenobiotics are therefore biotransformed through processes involving phase I as well as phase II drug-metabolizing enzymes. According to current knowledge, cytochrome P450 system is essential for the phase I metabolism of most low molecular weight xenobiotics.
In doping control field, detection of a single urinary metabolite is sufficient to report an adverse analytical finding for most doping agents. Therefore, metabolic studies are carried out to identify the metabolites that are detectable for longer period of time after administration. These metabolites are considered the most adequate markers for the detection of drug misuse [1-5]. Changes on the catalytic activity of CYP450 isoenzymes by co-administered drugs may result in altered therapeutic and/or toxic properties of a specific substance as well as in altered excretion kinetics of the selected marker(s) of drug abuse due to drug-drug interactions.
This research was designed to investigate whether the co-administration of banned compounds with active ingredients (i.e. antifungals, antidepressants and antacids) commonly used by athletes may lead to an alteration of the drug phase I metabolic pathways. The *in vitro* metabolism protocol was optimized with five representative banned compounds (toremifene, stanozolol, methandienone, ciclesonide and deflazacort) [6] in order to obtain a good correlation with the metabolism reported in humans and subsequently utilized in presence of non banned medicaments.

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Experimental

Chemicals and reagents

3'Hydroxy-stanozolol, 4α/β-hydroxy-stanozolol, 16α/β-hydroxy-stanozolol, 6β-hydroxy-methandienone were from NMI (National Measurement Institute, Pymble, Australia). Deflazacort (Deflan®) was purchased from Laboratori Guidotti (Pisa, Italy). 21-Desacetyl-deflazacort and desisobutyryl-ciclesonide were supplied by Toronto Research Chemicals Inc. (North York, Canada). The 17-alpha-methyltestosterone (internal standard), methandienone, stanozolol and toremifene were supplied by Sigma-Aldrich (Milano, Italy). All reagents were provided by Carlo Erba (Milano, Italy). The ultrapure water used was of Milli-Q-grade (Millipore, Milano, Italy). All the reagents for the *in vitro* study and the enzymatic protein (CYP450 recombinant isoforms and human liver microsomes) were purchased from BD Biosciences (Milano, Italy).

In vitro protocols

Reaction mixtures (250 µL) were prepared in 100 mM phosphate buffer (pH 7.4), 3.3 mM $MgCl_2$ and NADPH regenerating system consisting of 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase. The substrate (or substrate + inhibitor) was added and the phase-I reaction was initiated by adding the enzymatic protein for a specific time at 37°C. One sample containing all reaction components but not the enzymatic protein was also incubated to monitor the potential non-enzymatic reactions within the incubation period. The reaction was terminated adding 250 µL of acetonitrile.

Analytical procedure

The purification consisted of a liquid/liquid extraction with *tert*-butylmethyl ether at pH 7.4. The organic layer was separated and evaporated to dryness. The residue was reconstituted in 50 µL of mobile phase and 10 µL were injected. All LC experiments were performed using an Agilent 1200 Rapid Resolution Series HPLC pump with binary gradient system and automatic injector (Agilent Technologies S.p.A, Milano, Italy). Chromatography separation was performed using a Discovery C18 column from Supelco (2.1 x 150 mm, 5 µm). The solvents used were: 0.1% formic acid (A) and acetonitrile (B). The gradient program started at 10% B and increased to 30% B in 10 min, after 4 min to 40% B, after 3 min to 60% B in 5 min, and finally after 4 min to 100% B for 2 min. The flow rate was set at 300 µL/min.

All LC-ESI-MS/MS experiments were performed using an API4000 triple-quadrupole instrument (Applera Italia, Monza, Italy) with positive electrospray ionization. The ion source was operated at 450 °C, the applied capillary voltage and the declustering potential were set at 4500 V and 60 V, respectively. Selected reaction monitoring and product ion scan experiments were performed employing collision-induced dissociation using nitrogen as collision gas (the collision energy values and the ion transitions were selected considering the data reported in bibliography [1-6]).

High-resolution/high-accuracy measurements were performed on an Agilent Technologies 6210 orthogonal acceleration time-of-flight mass spectrometer, equipped with an electrospray ionization source operated in positive ion mode. Nitrogen was used as the drying and nebulising gas. The drying gas flow rate and temperature were 10 L/min and 350°C, respectively. The applied capillary and fragmentor voltages were set at 4000 V and at 150 V, respectively. Mass spectra data were collected from m/z 100 to 1100. Purine (*m/z* 121.0509) and an Agilent proprietary compound (HP0921, *m/z* 922.0098) were used as internal calibrants.

Results and Discussion

In vitro phase I metabolism

The *in vitro* metabolism protocol was optimized and validated with five representative banned compounds (toremifene, stanozolol, methandienone, ciclesonide and deflazacort) starting from the procedures reported in literature [7] in order to obtain a good correlation with previously reported metabolism in humans [1-5]. Different concentrations of the selected substrates (0.5, 1.0, 5.0, 10, 50 and 100 µg/mL) and of the enzymatic protein (0.1, 0.5 and 1.0 mg/mL) and different incubation times (1, 2, 4, 8, 12 and 24 h) were tested. The best correlation with metabolism in humans was obtained using a substrate concentration of 10 µg/mL, an enzymatic protein concentration of 1 mg/mL and an incubation time of 4 h.

To characterize the enzymes involved in the phase I metabolism pathways of the compounds under investigation, the substrates were incubated with different recombinant human CYP450 isoforms (CYP3A4/5, CYP2C8/9, CYP2C19, CYP2D6, CYP2B6 and CYP2A1) using the developed *in vitro* protocol. The CYP450 isoforms involved in the metabolism of toremifene, methandienone and ciclesonide are the CYP3A4 (involved in the formation of all metabolites), the CYP2D6 (involved in the formation of the toremifene metabolites M5, M6, M8, M9 and M10 and of the methandienone metabolites M3), the CYP2C9

(involved in the formation of the toremifene metabolites M8, M9 and M10, of the methandienone metabolites M3 and M8, and of the ciclesonide metabolite M5) and the CYP2C19 (involved in the formation of toremifene metabolites M8, M9 and M10, of methandienone metabolites M3 and M8, and of the ciclesonide metabolite M5), whereas for stanozolol and deflazacort only the CYP3A4 isoformis the responsible for the phase I metabolism.

In vitro phase I metabolism inhibition

Knowledge on the potential influence of the administration of CYP450 inhibitors on the bioavailability, metabolism and excretion kinetics of a banned compound is necessary for the correct interpretation of atypical or unexpected analysis results obtained in anti-doping tests, especially in those cases where the parent drugs are extensively metabolized or are classified as threshold compounds. To preliminarily investigate whether the co-administration of CYP450 inhibitors with doping agents could produce effects analogous to those of a “traditional” masking strategy, the *in vitro* phase I metabolism of the five substrates selected was performed in presence of medicaments (antifungals, antacids and antidepressants) commonly used by athletes and that are reported to be inhibitors of the CYP3A4, CYP2D6, CYP2C9 and CYP2C19.
Figures 1-6 show the phase I metabolic profiles of toremifene (Figures 1-3), and methandienone (Figures 4-6) obtained using the human liver microsomes in absence and in presence of the antifungals (Figures 1-4), the antacids (Figures 2-5) and the antidepressants (Figure 3-6). As can be noticed for all the substrates significant alterations of the phase I metabolic profile were registered in presence of ketoconazole, miconazole, itraconazole and nefazodone. Moderate variations were, instead, registered in presence of paroxetine and not significant modifications were measured in presence of fluoconazole, fluoxetine ranitidine and cimetidine. The same results were obtained for the other compounds under investigation (data not shown).

The experiments carried out incubating the substrates with the recombinant human CYP450 isoenzymes CYP3A4, CYP2D6, CYP2C9 and CYP2C19 in absence and in presence of the inhibitors show that the antifungals ketoconazole, miconazole and itraconazole and the antidepressant nefazodone inhibited significantly the CYP3A4 and moderately the others CYP450 isoforms involved, whereas the antidepressants paroxetine and fluoxetine inhibited significantly only the CYP2D6 isoform. Finally the antacid cimetidine inhibited moderately only the CYP3A4 isoenzyme, whereas in presence of ranitidine no inhibition was registered .

Concerning the alteration on the catalytic activity of the CYP450 isoenzymes involved in the phase I metabolism of the five banned compounds considered, the selected substrates were incubated in presence of different concentrations (0.5, 1.0, 5.0, 10, 50 and 100 µg/mL) of the CYP450 inhibitors specifically considered in this study. The results show that ketoconazole, miconazole and itraconazole, at a concentration 1/10 of the substrates, caused a decrease of more than 50% of the catalytic activity of the CYP450 isoforms involved in the formation of the representative toremifene, stanozolol, methandienone, ciclesonide and deflazacort metabolites, whereas in presence of fluoconazole a less pronounced alteration was observed. The antacid agents cimetidine and ranitidine did not produce any significant alteration of the enzymatic activities even at concentration 10 times higher than the substrates. Finally, concerning the antidepressant agents, in presence of nefazodone at a concentration 1/5 of the substrates, the catalytic activity of the CYP450 isoforms involved decreased more than 50%; paroxetine produced the same effect only at a concentration at least 5 times higher than the substrates; fluoxetine did not produce any significant alteration at concentrations up to 10 times higher than the substrates.

Conclusions

On the basis of the results obtained, the co-administration of banned compounds with the antifungals or the antidepressants could lead to an incorrect interpretation of the analytical results, producing effects analogous to those of a “traditional” masking strategy, based on the alteration of the phase I metabolic pathways. To avoid the risk of false negative results, the number of the markers of drug abuse selected during the screening analyses/routine analyses should be expanded as much as possible, ideally including also the parent compounds and all possible additional metabolites produced by alternative metabolic routes. Moreover, a monitoring study on the real occurrence of CYP inhibitors in the urine samples analyzed by the WADA-accredited laboratories would markedly enhance the statistical and epidemiological relevance of our *in vitro* observations.

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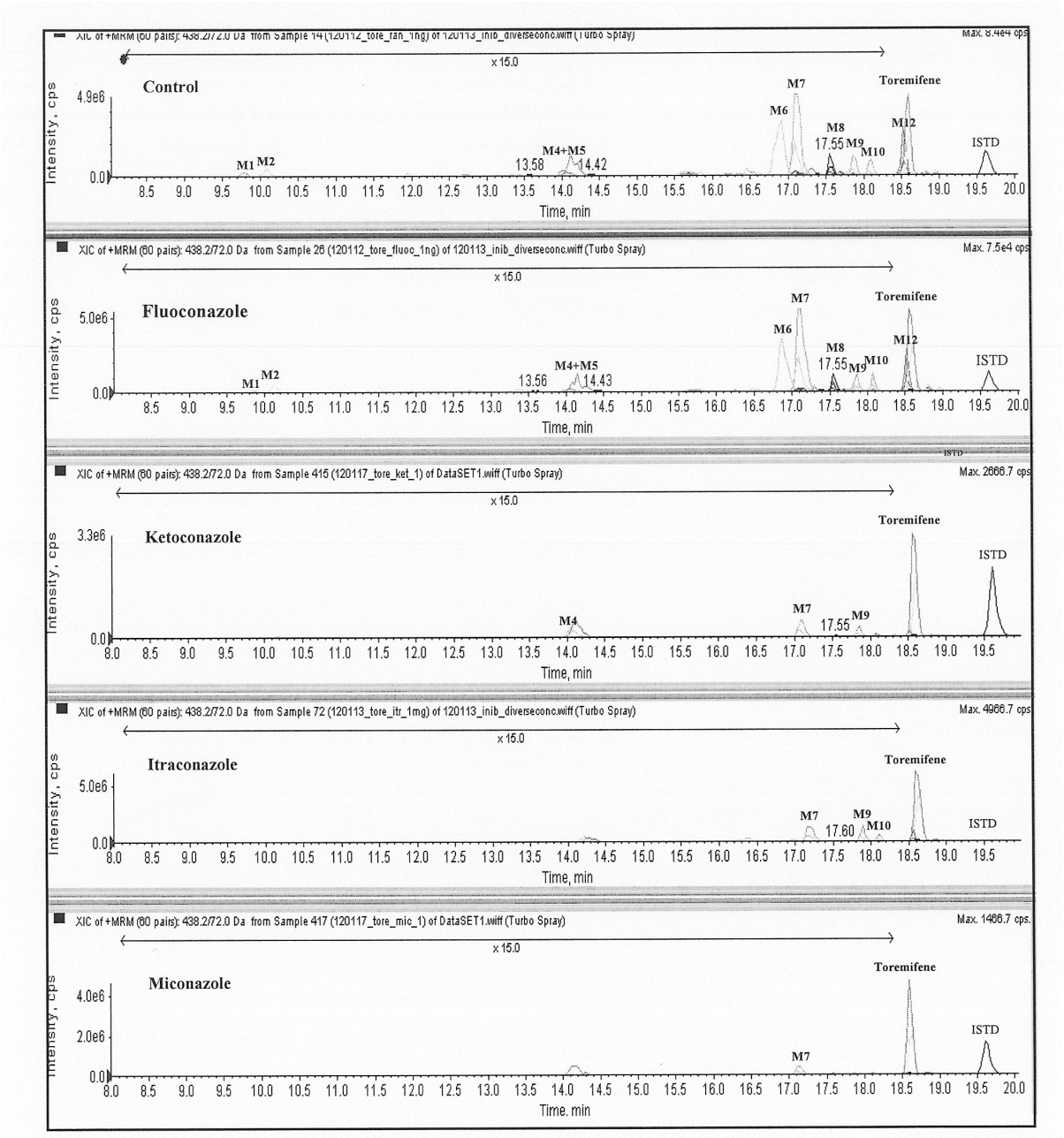

Figure 1: In vitro metabolic profile of toremifene (10 µg/mL) in absence and in presence of the antifungal agents (10 µg/mL) considered in this study. Peak identification: M1. N-demethyl-dihydro carboxy-tamoxifene, M2. dihydro-carboxy-tamoxifene, M4. dihydroxy-dihydro-toremifene, M5. carboxy-tamoxifene, M6. N-demethyl-α-hydroxy-toremifene, M7. α-hydroxy-toremifene, M8. dihydroxy-toremifene, M9. 4-hydroxy-toremifene, M10. 4'-hydroxy-toremifene, M12. N-demethyl-toremifene.

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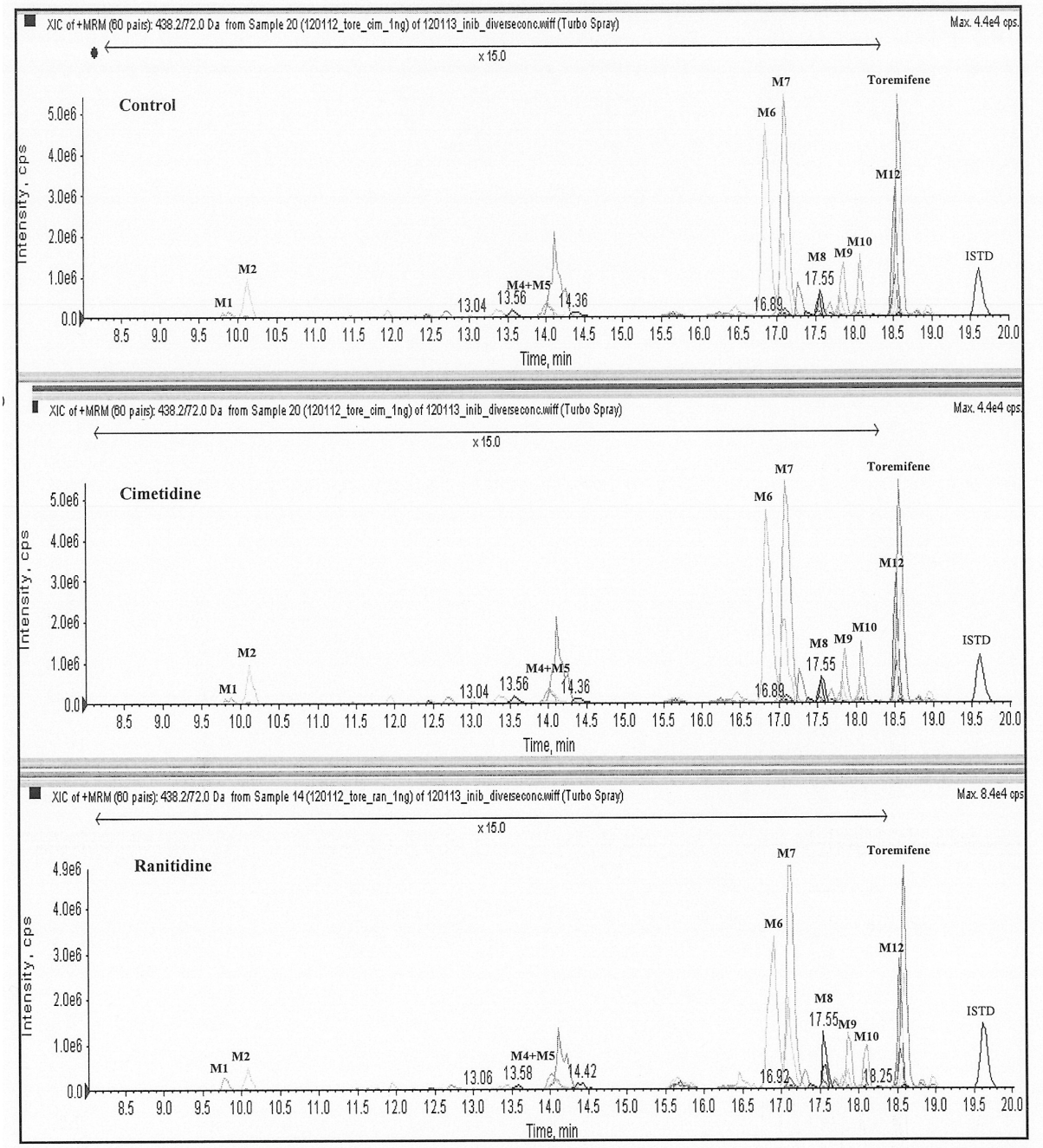

Figure 2: In vitro metabolic profile of toremifene (10 µg/mL) in absence and in presence of the antacid agents (10 µg/mL) considered in this study. Peak identification: M1. N-demethyl-dihydro carboxy-tamoxifene, M2. dihydro-carboxy-tamoxifene, M4. dihydroxy-dihydro-toremifene, M5. carboxy-tamoxifene, M6. N-demethyl-α-hydroxy-toremifene, M7. α-hydroxy-toremifene, M8. dihydroxy-toremifene, M9. 4-hydroxy-toremifene, M10. 4'-hydroxy-toremifene, M12. N-demethyl-toremifene.

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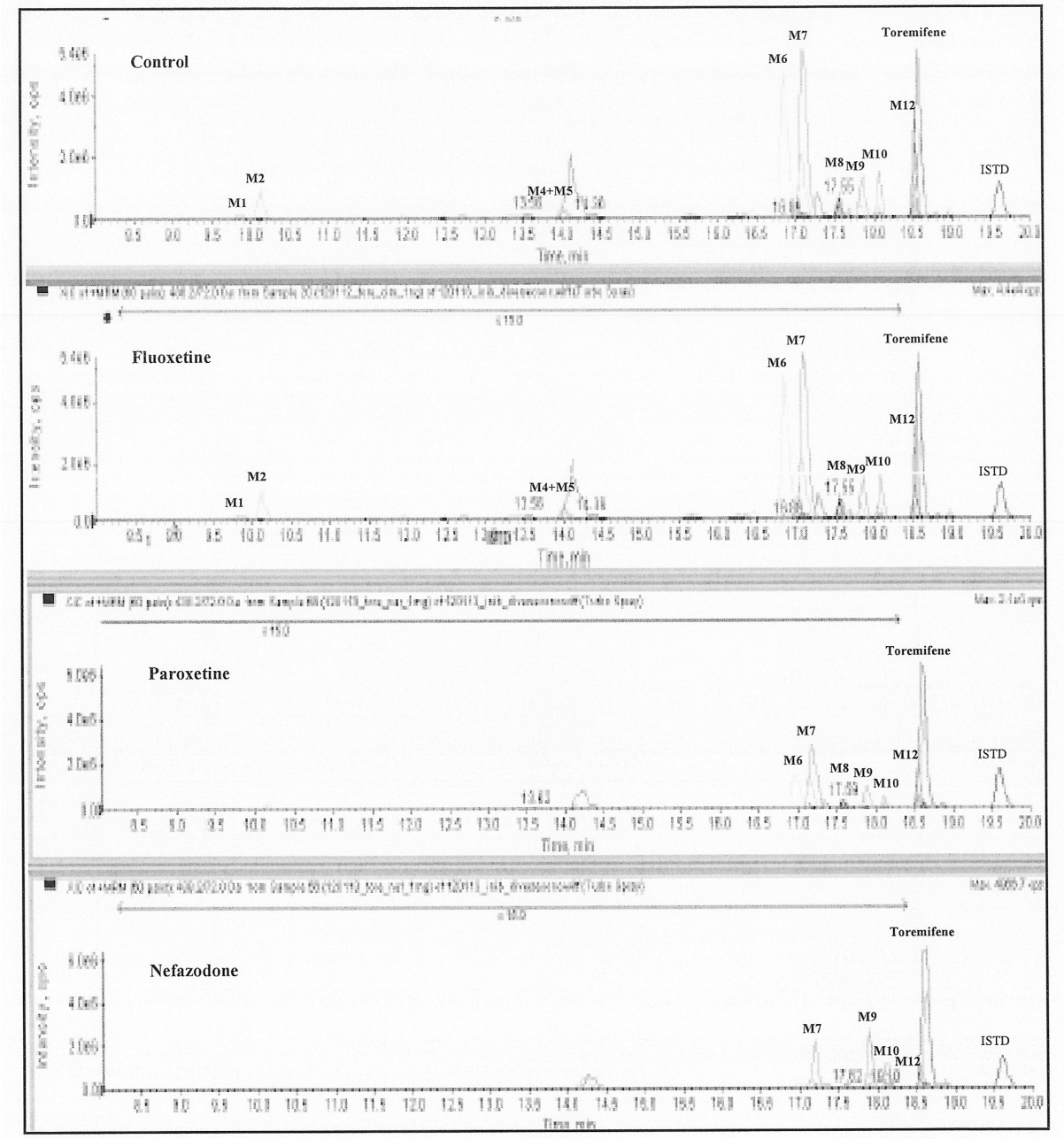

Figure 3: In vitro metabolic profile of toremifene (10 µg/mL) in absence and in presence of the antidepressant agents (10 µg/mL) considered in this study. Peak identification: M1. N-demethyl-dihydro carboxy-tamoxifene, M2. dihydro-carboxy-tamoxifene, M4. dihydroxy-dihydro-toremifene, M5. carboxy-tamoxifene, M6. N-demethyl-α-hydroxy-toremifene, M7. α-hydroxy-toremifene, M8. dihydroxy-toremifene, M9. 4-hydroxy-toremifene, M10. 4'-hydroxy-toremifene, M12. N-demethyl-toremifene.

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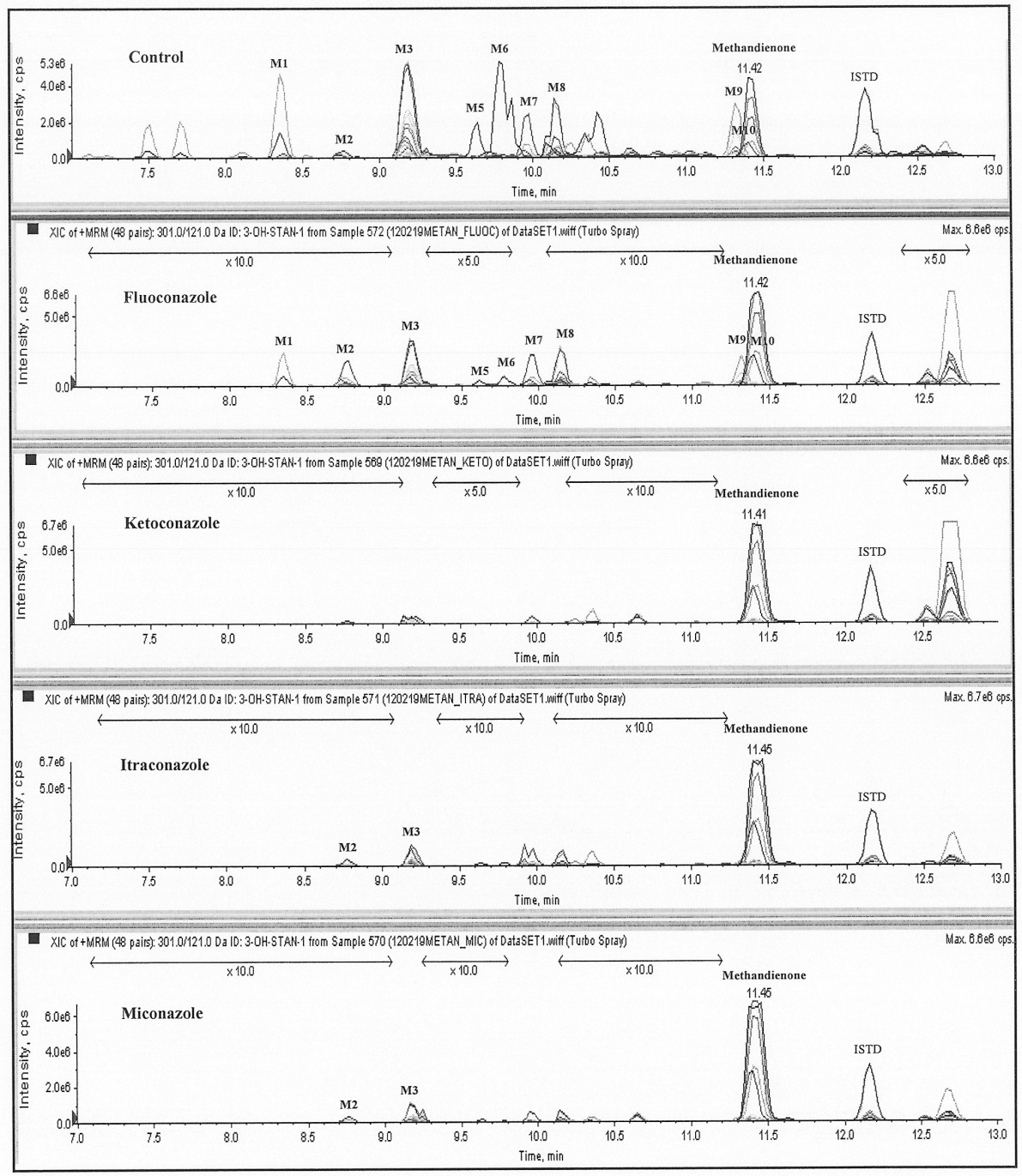

Figure 4: In vitro metabolic profile of methandienone (10 µg/mL) in absence and in presence of the antifungal agents (10 µg/mL) considered in this study. Peak identification: M1. dihydroxy-methandienone, M2. monohydroxy-methandienone, M3. 6β-hydroxy-methandienone, M5. monohydroxy-dehydro-methandienone, M6. monohydroxy-dehydro-methandienone, M7. monohydroxy-methandienone, M8. monohydroxy- methandienone, M9. 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one, M10. 17,17-dimethyl-18-norandrosta- 1,4,13-trien-3-one.

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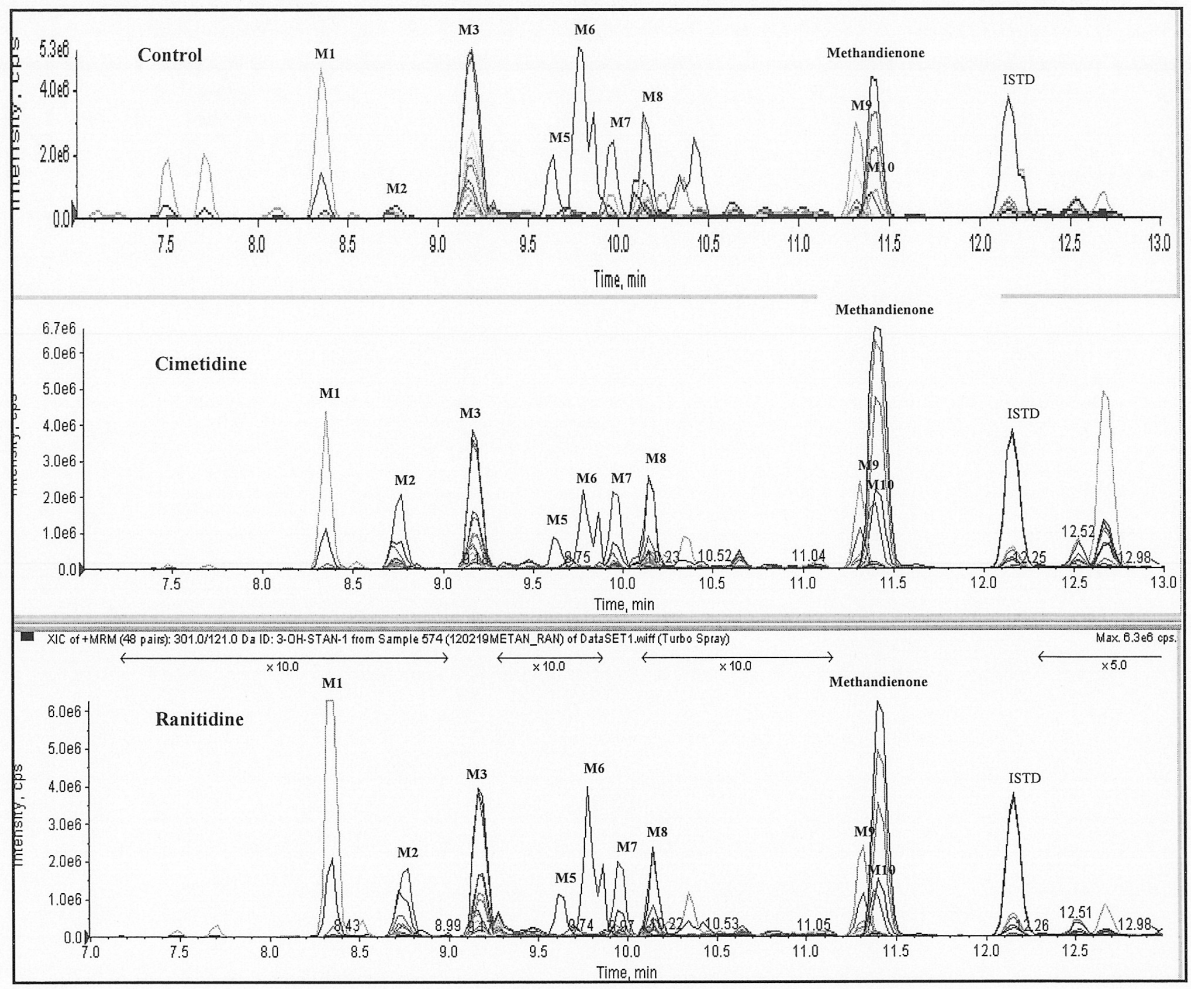

Figure 5: In vitro metabolic profile of methandienone (10 µg/mL) in absence and in presence of the antacid agents (10 µg/mL) considered in this study. Peak identification: M1. dihydroxy-methandienone, M2. monohydroxy-methandienone, M3. 6β-hydroxy-methandienone, M5. monohydroxy-dehydro-methandienone, M6. monohydroxy-dehydro-methandienone, M7. monohydroxy-methandienone, M8. monohydroxy- methandienone, M9. 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one, M10. 17,17-dimethyl-18-norandrosta- 1,4,13-trien-3-one.

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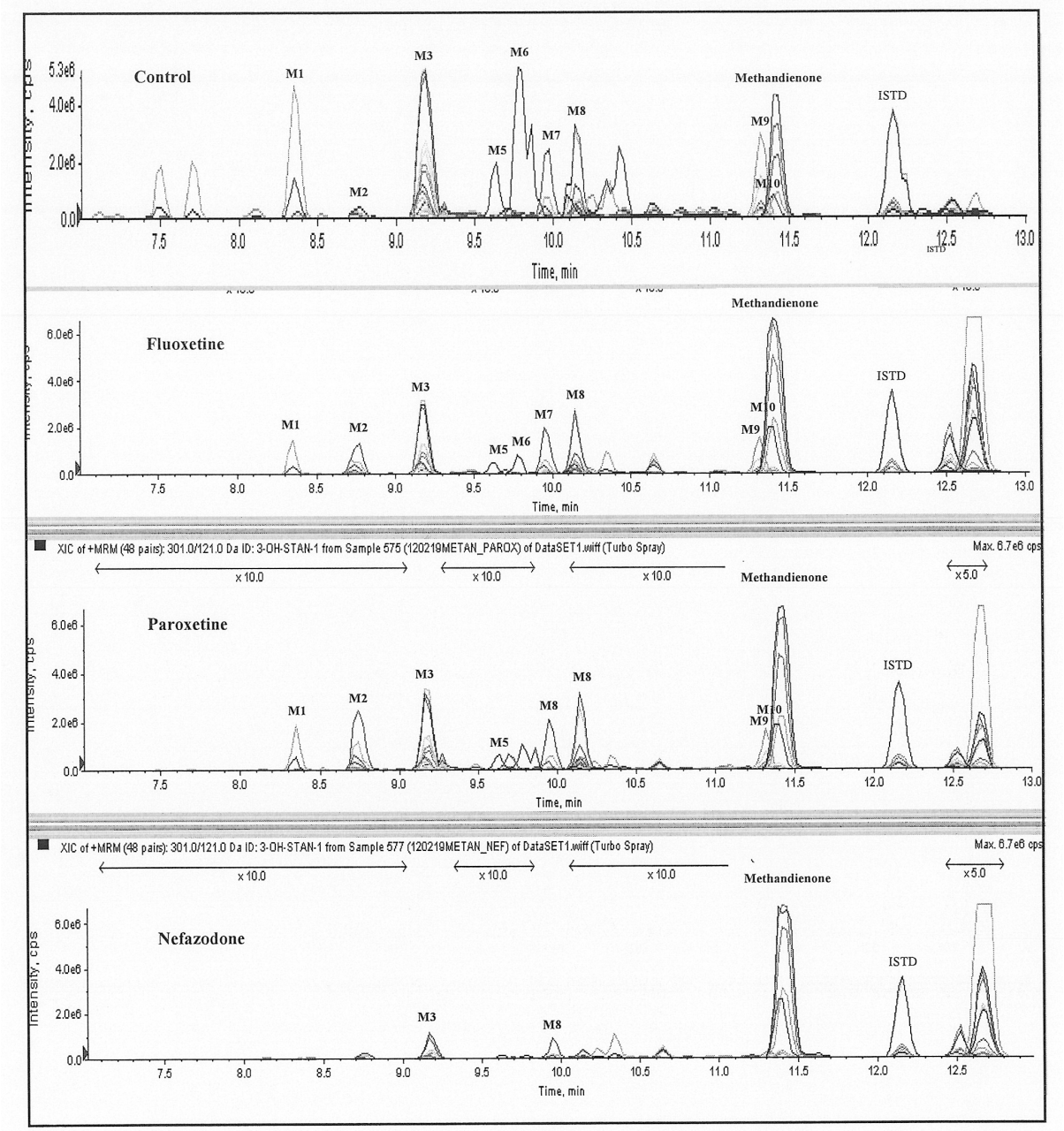

Figure 6: In vitro metabolic profile of methandienone (10 µg/mL) (right) in absence and in presence of the antidepressant agents (10 µg/mL) considered in this study. Peak identification (methandienone): M1. dihydroxy-methandienone, M2. monohydroxy-methandienone, M3. 6β-hydroxy-methandienone, M5. monohydroxy-dehydro-methandienone, M6. monohydroxy-dehydro-methandienone, M7. monohydroxy-methandienone, M8. monohydroxy-methandienone, M9. 17β-hydroxymethyl-17α-methyl-18-norandrosta- 1,4,13-trien-3-one, M10. 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one.

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