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Modulation of phase II metabolism: A case study on 19-norandrosterone

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Abstract

We have considered the inhibitory effect of four antifungals (fluconazole, itraconazole, ketoconazole and miconazole), benzodiazepines (alprazolam, bromazepam, diazepam and triazolam) and non-steroidal anti-inflammatory drugs (diclofenac, ibuprofen, ketoprofen and nimesulide) on the enzymatic activities of the uridine diphospho glucuronosyltransferases involved in the conjugation of 19-norandrosterone.
19-Norandrosterone was incubated with pooled human liver microsomes (or recombinant uridine diphospho glucuronosyltransferases) in the absence and in the presence of the selected inhibitors. The amount of 19-norandrosterone in both free and glucuronate fraction was determined by gas chromatography coupled with low resolution mass spectrometry after sample-pretreatment consisting in an enzymatic hydrolysis (performed only for the glucuronate fraction), liquid/liquid extraction with *tert*-butyl methyl ether and derivatization to trimethylsilyl derivatives.
The *in vitro* protocol developed and optimized in this study was able to mimic the *in vivo* phase II metabolism of 19-nortestosterone. The glucuronidation of 19-norandrosterone is principally carried out by UGT2B7 (39%) and UGT2B17 (31%) isoenzymes. Among the compounds tested, ibuprofen, ketoconazole and miconazole were the most potent inhibitors of the 19-norandrosterone glucuronide formation by either pooled human liver microsomes or UGT2B7 and UGT2B17 isoenzymes. Itraconazole, diclofenac and triazolam were less effective and no effects were registered in the presence of the others agents considered.

Introduction

19-Norandrosterone (19NA) is the main phase I metabolite of 19-nortestosterone and its analogues/precursors 19-norandrostenedione and 19-norandrostenediol. Once formed, 19NA undergoes conjugation reactions catalyzed mainly by the uridine diphospho glucuronosyltransferases (UDPGTs) to form the corresponding glucuro-conjugate [1-2]. The presence of 19NA in urine is not unambiguously linked to the intentional administration of 19-nortestosterone or its precursors [3-5]. For indeed, WADA has released a specific technical document to exclude non-doping causes of its presence in urine [6].
Different factors (physiological and environmental) can alter the pharmacokinetics of a drug and therefore its metabolic profile in urine. Among them, a key role is represented by drug-drug interactions. In sports, according to the information available on the doping control forms, many athletes declare to have been using more than two different medicaments not included in the WADA prohibited list. Some of these agents, and primarily among them antifungals [8], benzodiazepines [9-10] and non-steroidal anti-inflammatory drugs (NSAIDs) [10] are reported to modulate the activity of the UDPGTs. Although it is well known that drug-drug interactions may modify the activity of metabolizing isoenzymes, to the best of our knowledge a direct investigation on the potential consequences of these interactions on the analytical strategies followed by the anti-doping laboratories to reveal the intake of doping agents had never been carried out yet. In this study we have considered the modulation of the activity of the isoenzymes involved in the glucuronidation of 19-norandrosterone by antifungals, benzodiazepines and NSAIDs.

Experimental

Chemicals and reagents
19NA and d4-19NA were from National Measurement Institute, Pymble, Australia. The inhibitors and all the chemicals were

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supplied by Sigma-Aldrich (Milano, Italy). The enzyme β-glucuronidase from *E. coli* was from Roche (Monza, Italy).
The reagents for the *in vitro* studies and the enzymatic proteins were from BD Biosciences (Milano, Italy).
In vitro protocol
The incubation mixture, total volume of 250 µL, contains 0.1 M sodium phosphate buffer (pH 7.4), the substrate in dimethylsulfoxide (DMSO) at final concentration of 20 µM, 8 mM magnesium chloride, 25 µg/mL alamethicin and 2mM cofactor Uridine 5'-Di-Phospho-α-D-glucuronic acid. Samples were pre-warmed at 37°C for 5 minutes and 0.5 mg/mL of Human Liver Microsomes (HLM) or c-DNA expressed UGT isoforms were added and the reaction was started. After incubation for 10 minutes at 37°C, 250 µL of ice-cold acetonitrile were added to stop reactions; and the samples were centrifuged at 12000 rpm for 5 minutes. One sample (negative control) containing all reaction mixture components except proteins was added.
Analytical procedure
Samples obtained by the *in vitro* incubation were added with 1 mL of phosphate buffer (1 M, pH 7.4) and 200 ng/mL of d4-19NA and liquid/liquid extraction was carried out with 5 mL of n-pentane for 5 minutes on a mechanical shaker. After centrifugation, the organic layer was transferred to a 10 mL tube and evaporated to dryness. The residue was derivatized at 70°C for 20 min, using 50 mL of N-methyl-N-trimethylsilyl-trifluoroacetamide/ammonium iodide/dithierythritol (1000:2:4:v/w/w), and 1 µL was injected into the GC-MS.
The aqueous layer was added with 50 mL of ß-glucuronidase from *E. coli* and 200 ng/mL of d4-19NA. The samples were incubated for 1 hour at 50 °C. After hydrolysis, to alkalize the sample 1 mL of carbonate/bicarbonate buffer (0.8 M, pH 9), was added and liquid/liquid extraction was carried out with 7 mL of n-pentane for 5 minutes on a mechanical shaker. After centrifugation, the organic layer was transferred to a 10 mL tube and evaporated to dryness. To the residue 50 mL of the derivatizing mixture (N-methyl-N-trimethylsilyl-trifluoroacetamide/ammonium iodide/dithierythritol (1000:2:4:v/w/w)) were added and the sample was incubated at 70°C for 20 min. Then 1 µL was injected into the GC-MS.
Quantitative analysis was performed on an Agilent Technologies 5890/5973A (Milano, Italy), in electron impact ionisation (70 eV), using a 17 m fused silica capillary column cross-linked methyl silicone, ID 0.20 mm, film thickness 0.11 µm. The carrier gas was helium (flow rate: 1 mL/min, split ratio 1:10), and the temperature program was as follows: 180°C (hold 4.5 min), 3°C/min to 230°C, 20°C/min to 290°C, 30°C/min to 320°C; the transfer line and injector temperature was set at 280°C. Acquisition was carried out in selected ion monitoring. The diagnostic ions at *m/z* 405 and at *m/z* 409 were monitored for 19-norandrosterone and deuterated 19-norandrosterone respectively. The values of the concentration of 19-norandrosterone was calculated by the peak areas of the detected signals relative to the deuterated internal standard.

Results and Discussion

In vitro glucuronidation of 19NA
To set up the *in vitro* protocol, different solvents (methanol, DMSO and acetonitrile), buffer types (phosphate and tris-HCl) and pHs (5.0, 7.4, 8.0), concentrations of the substrate (1, 5, 10, 20, 40, 60, 80, 100 and 200 mM) and of the enzymatic protein (0.1, 0.2, 0.5 and 1.0 mg/mL) and different incubation times (5, 10, 20, 30, 60 and 120 min) were tested. The best results were obtained using DSMO as substrate solvent, the phosphate buffer at pH 7.4, a substrate concentration of 20 mM, an enzymatic protein concentration of 0.5 mg/mL and an incubation time of 10 min.
Figure 1A reports the results obtained incubating 19NA with HLM using the *in vitro* protocol set up and optimized in this study. As can been noticed 19-norandrosterone undergoes extensive glucuronidation (90-95%) confirming that the *in vitro* model developed and optimized in this study provides a good representation of the conjugation reactions in humans [7-8].
To define the relative contribution of individual UGT isoforms to the phase II metabolic rate, 19-norandrosterone was incubated in the presence of the different UGT isoforms selected in this study (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B15 and UGT2B17) using the *in vitro* protocol here developed. The data obtained show that the isoenzymes principally involved in the glucuronidation of 19-norandrosterone are the UGT2B7 (39%) and the UGT2B17 (31%). The UGT1A3 (7%), UGT1A4 (15%), UGT2B4 (5%) and UGT2B10 (3%) isoforms are involved in a lesser degree; whereas the others isoforms tested were not involved in the formation of 19-norandrosterone glucuronide (see Figure 1B).
In vitro glucuronation inhibition studies
To investigate the capacity of medicaments commonly used by athletes in modulating the *in vitro* glucuronidation of 19-norandrosterone, inhibition studies were performed. 19-Norandrosterone was incubated with either pooled human liver microsomes or cDNA expressed UGT isoforms (UGT2B7 and UGT2B17) mainly involved in the formation of 19-norandrosterone glucuronide, in the absence and in the presence of different concentrations (0.4, 2, 4, 10, 20, 40, 100

and 200 µM) of the non-prohibited medicaments (the antifungals fluconazole, itraconazole, ketoconazole and miconazole; the benzodiazepines alprazolam, bromazepam, diazepam and triazolam; the non-steroidal anti-inflammatory drugs diclofenac, ibuprofen, ketoprofen and nimesulide) considered in this study. The results obtained are reported in Figures 2A-C. Concerning the antifungals, as can be noticed from the plots reported in Figure 2A, only ketoconazole and miconazole are able to significantly inhibit (more than 20%) the conjugation rate of 19-norandrosterone at concentrations corresponding to their plasma concentration range (1-10 mM) following administration of a therapeutic dose., Itraconazole is able to inhibit significantly (more than 20%) only the UGT2B7 isoform at concentrations corresponding to its plasma concentration range (1-10 µM) following administration of a therapeutic dose. The UGT2B17 isoform was, instead, significantly inhibited only in the presence of concentration of itraconazole much higher than 10 µM and therefore no significant alteration were measured in the presence of HLM. Whereas fluconazole does not show inhibitory capabilities on both UGT2B7 and UGT2B17 isoforms also at the highest concentrations (200 µM) evaluated in this study.
Concerning the non-steroidal anti-inflammatory drugs, as can be noticed in Figure 2B only ibuprofen is capable to alter significantly (more than 20%) the 19-norandrosterone glucuronidation at concentrations corresponding to its plasma level range (40-200 µM) following administration of a therapeutic dose. Diclofenac is able to alter significantly (more than 20%) the catalytic activity of UGT2B7 and UGT2B17 only at concentrations much higher than its plasma level range (40-200 µM) following administration of a therapeutic dose. Whereas, ketoprofen and nimesulide do not show inhibitory capabilities on both UGT2B7 and UGT2B17 isoforms also at the highest concentrations (200 µM) evaluated in this study.
Finally, concerning the benzodiazepines considered in this study, only triazolam is able to moderately (less than 20%) alter the catalytic activity of UGT2B7 and UGT2B17 at concentration corresponding to its plasma concentration range (0.4-4 µM) following administration of a therapeutic dose, and therefore in the presence of HLM significant alteration was registered only at concentration much higher than 4 µM (Figure 2C). Whereas, alprazolam, diazepam and bromazepam do not show inhibitory capabilities on both UGT2B7 and UGT2B17 isoforms also at the highest concentrations (200 µM) evaluated in this study.

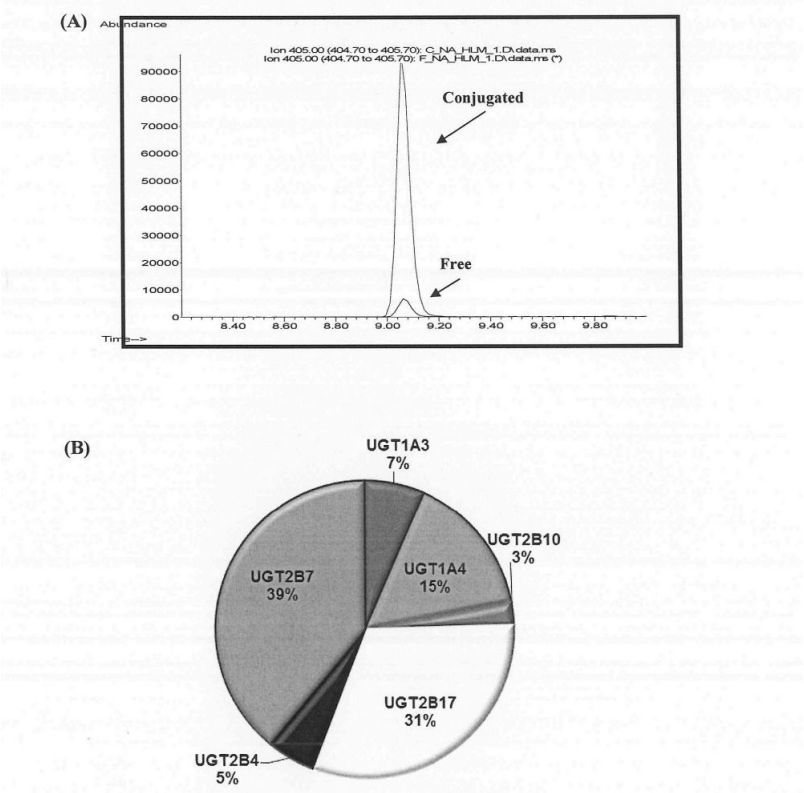

Figure 1: *In vitro* metabolic profile of 19-norandrosterone (20 µM) using the *in vitro* protocol set up in this study and reported in the experimental part **(A)**; characterization of the UGT isoforms involved in the 19-norandrosterone glucuronidation using the *in vitro* protocol set up in this study and reported in the experimental part **(B)**.

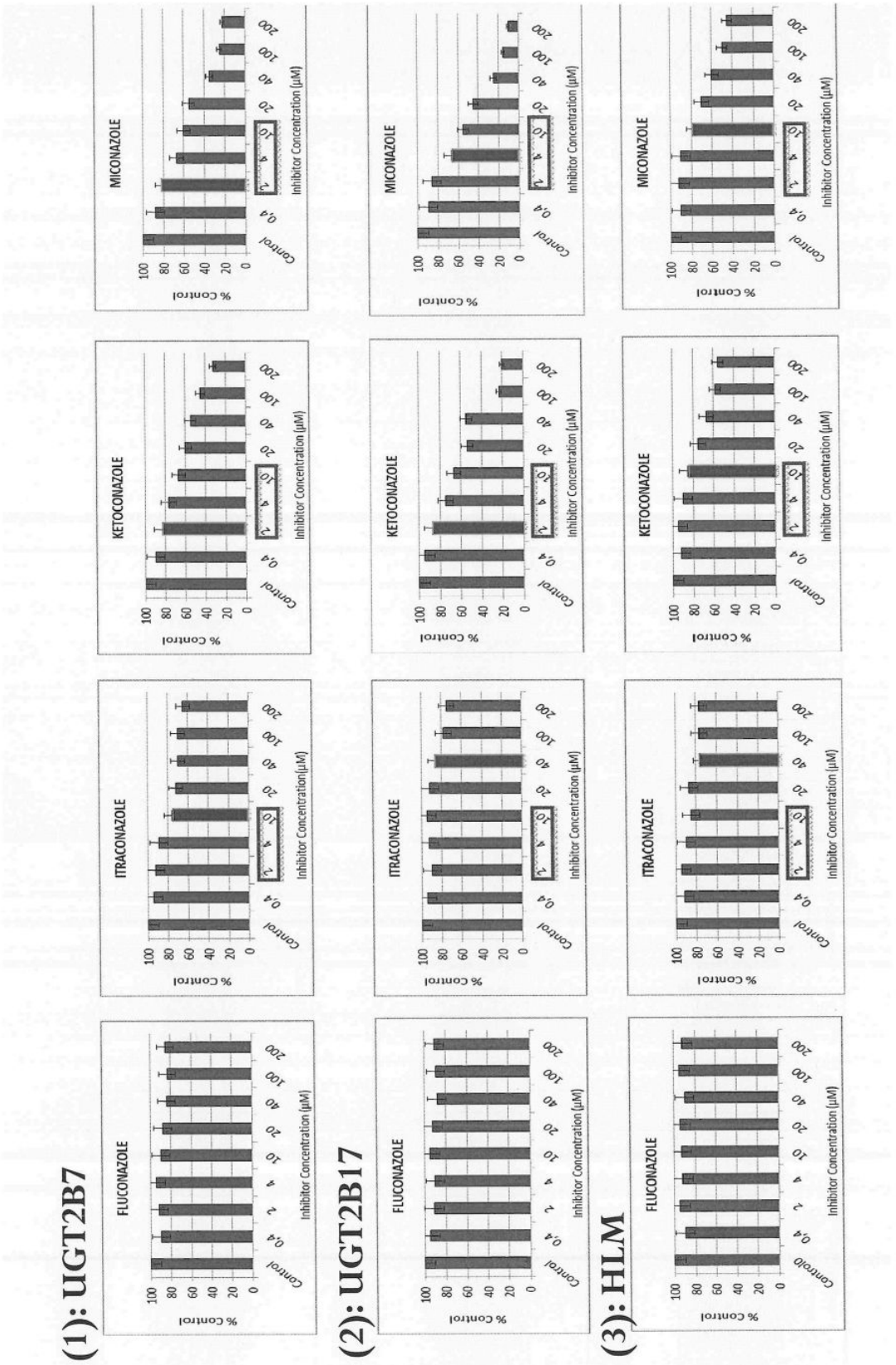

Figure 2A: Alteration of the catalytic activity of the UGT isoenzymes involved in the formation of 19-norandrosterone glucuronide in the presence of different concentration (0.4, 2, 4, 10, 20, 40, 100 and 200 µM) of the antifungal **(A)** considered in this study. Each point represent the mean value of three independent determinations± the deviation standard of the analytical method.

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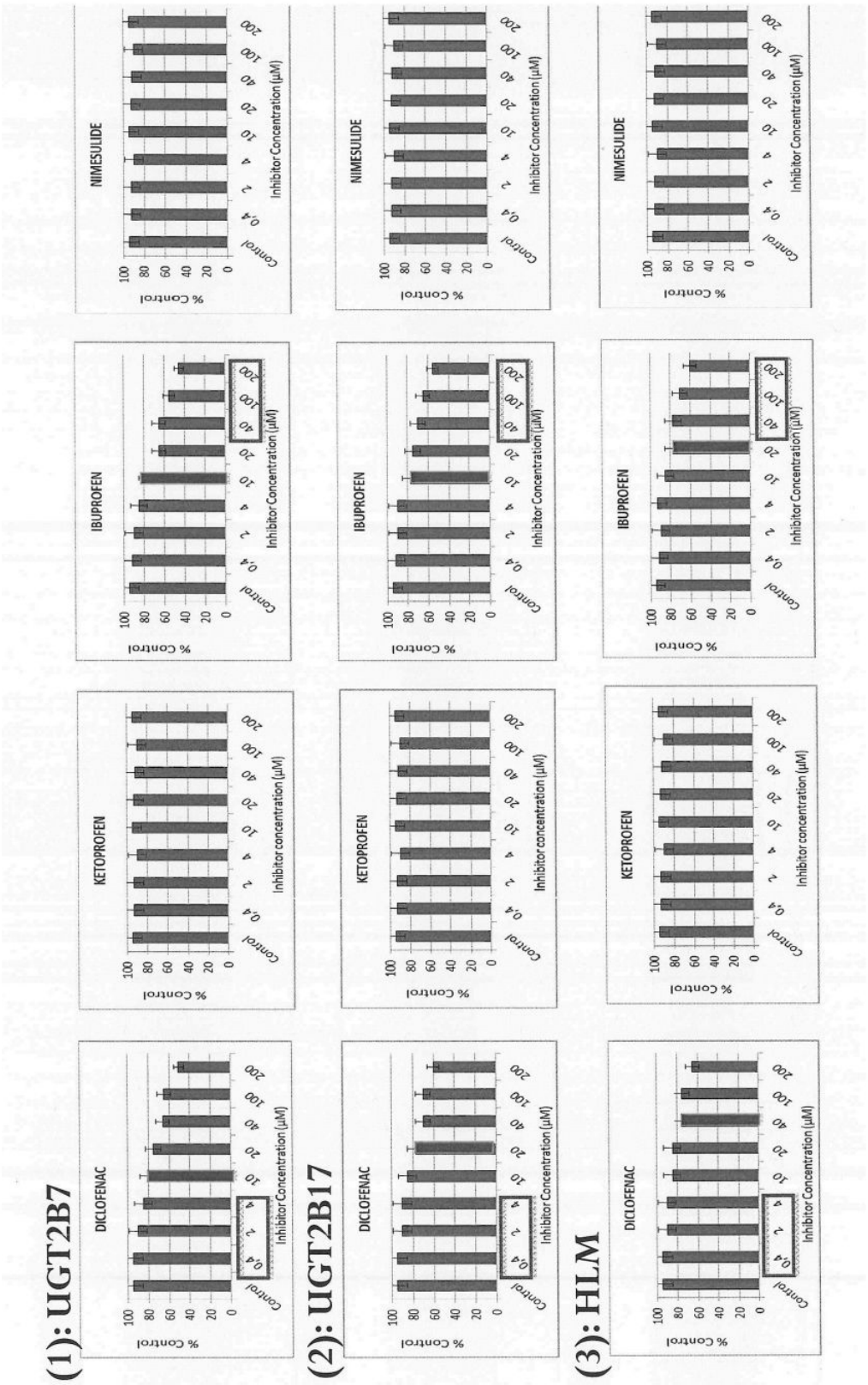

Figure 2B: Alteration of the catalytic activity of the UGT isoenzymes involved in the formation of 19-norandrosterone glucuronide in the presence of different concentration (0.4, 2, 4, 10, 20, 40, 100 and 200 µM) of the non-steroidal antinflammatory drugs **(B)** considered in this study. Each point represent the mean value of three independent determinations± the deviation standard of the analytical method.

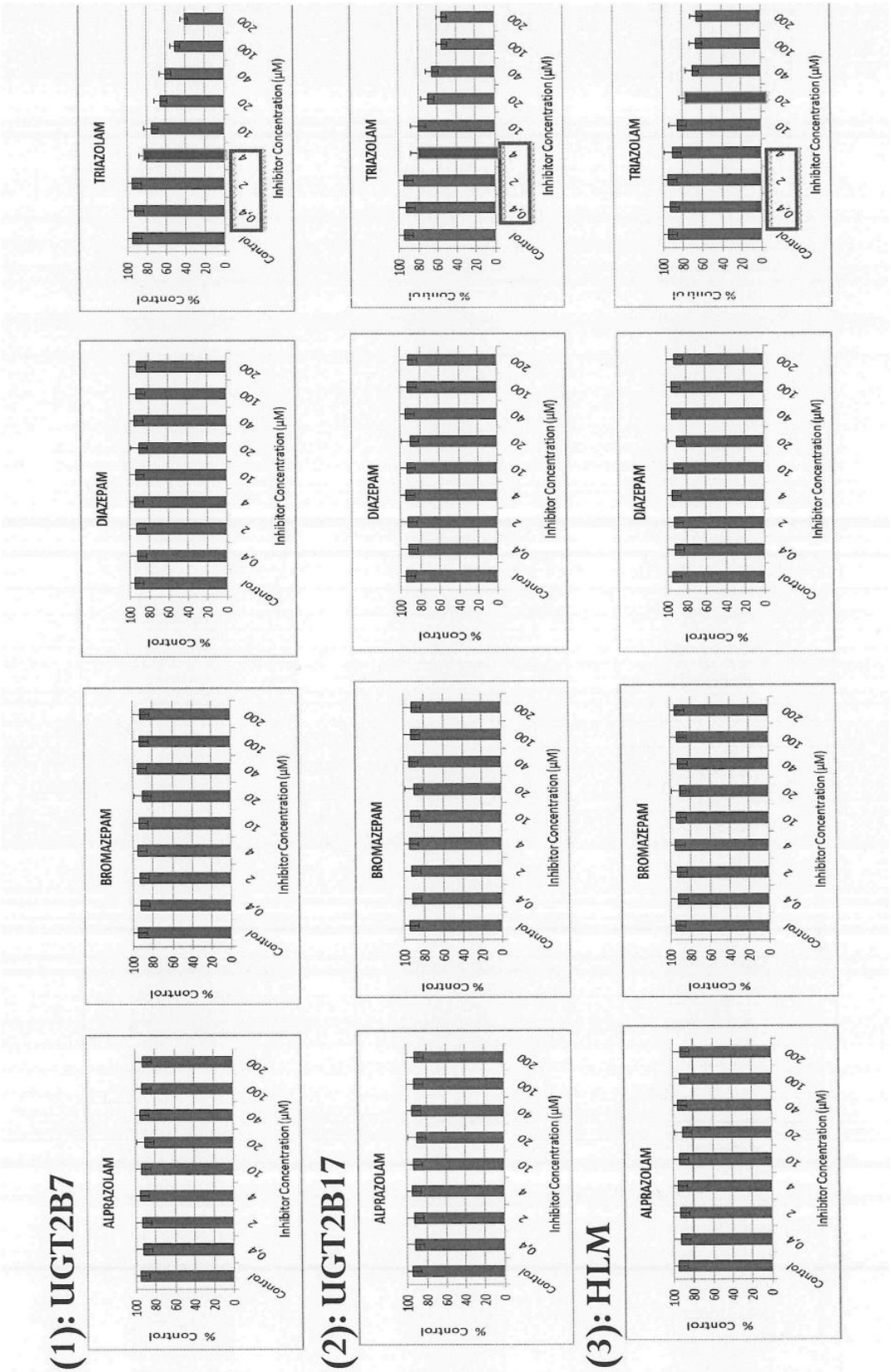

Figure 2C: Alteration of the catalytic activity of the UGT isoenzymes involved in the formation of 19-norandrosterone glucuronide in the presence of different concentration (0.4, 2, 4, 10, 20, 40, 100 and 200 µM) of the benzodiazepines **(C)** considered in this study. Each point represent the mean value of three independent determinations± the deviation standard of the analytical method.

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Conclusions

The data obtained showed that inhibitory drug-drug interactions at the level of drug phase II metabolism might occur between 19-norsteroids and permitted drugs. To prevent the risk of potential “false negative” results, we propose, in case of concentration of 19-norandrosterone close to or lower than 2 ng/mL, to investigate the presence of non-prohibited agents able to modulate the activity of the UGT2B isoenzymes. This countermeasure together with the evaluation of the genotype could be useful in the interpretation of the analytical results.
In future, we plan (i) to monitor the real occurrence of antifungals, NSAIDs and benzodiazepines in the samples collected in occasion of doping control test, and (ii) to confirm our *in vitro* observation by conducting *in vivo* studies.

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Acknowledgements

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