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A multi-targeted liquid chromatography–mass spectrometry screening procedure for the detection in human urine of non-doping drugs commonly used by the athletes

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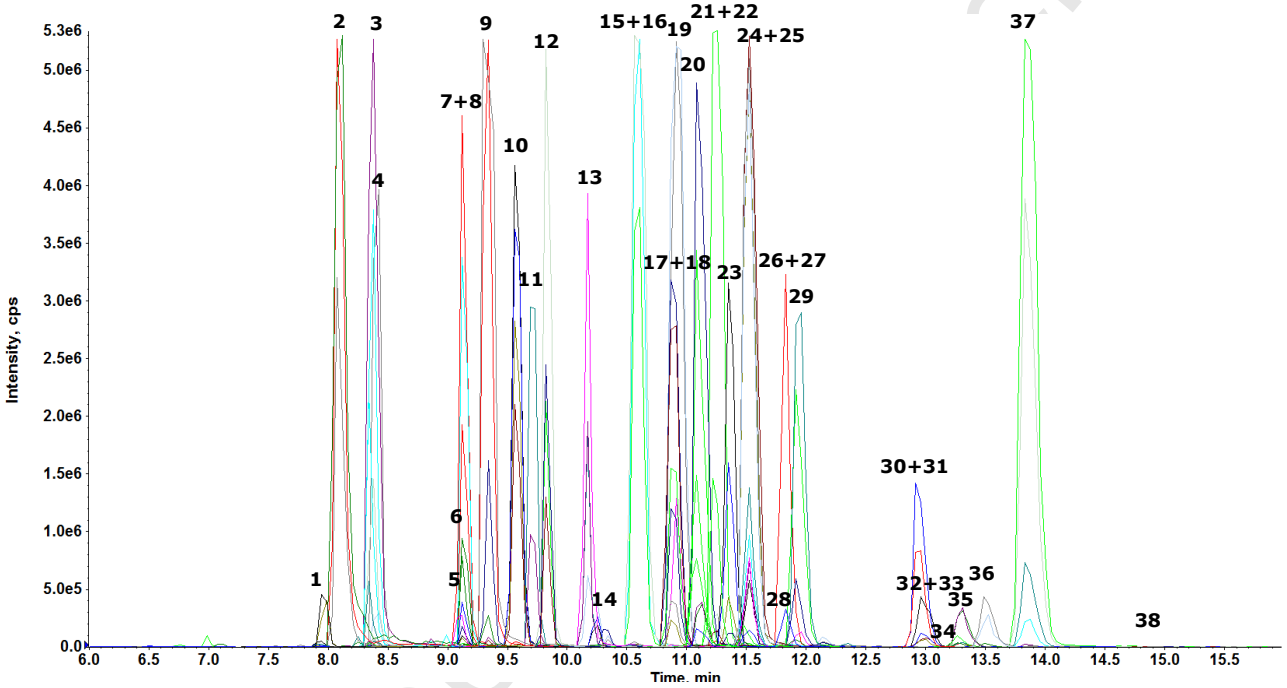

Highlights

- LC-MS/MS screening method for the simultaneous analysis in human urine of 38 drugs
- All target analytes are non-doping drugs most commonly used by the athletes.
- Method validation considered sensitivity, specificity, repeatability and robustness
- Lower limit of detection is in the range 1-50 ng mL^{-1} depending on the analyte
- Effectiveness of the method was assessed on real samples from excretion studies

A multi-targeted liquid chromatography–mass spectrometry screening procedure for the detection in human urine of drugs non prohibited in sport commonly used by the athletes

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Abstract

This work presents an analytical method for the simultaneous analysis in human urine of 38 pharmacologically active compounds (19 benzodiazepine-like substances, 7 selective serotonin reuptake inhibitors, 4 azole antifungal drugs, 5 inhibitors of the phosphodiesterases type 4 and 3 inhibitors of the phosphodiesterase type 5) by liquid-chromatography coupled with tandem mass spectrometry. The above substances classes include both the most common "non banned" drugs used by the athletes (based on the information reported on the "doping control form") and those drugs who are suspected to be performance enhancing and/or act as masking agents in particular conditions.

The chromatographic separation was performed by a reverse-phase octadecyl column using as mobile phases acetonitrile and ultra-purified water, both with 0.1% formic acid. The detection was carried out using a triple quadrupole mass spectrometric analyser, positive electro-spray as ionization source and selected reaction monitoring as acquisition mode. Sample pre-treatment consisted in an enzymatic hydrolysis followed by a liquid-liquid extraction in neutral field using *tert*-butyl methyl-ether.

The analytical procedure, once developed, was validated in terms of sensitivity (lower limits of detection in the range of 1-50 ng mL^{-1}), specificity (no interferences were detected at the retention time of all the analytes under investigation), recovery ($\geq$ 60% with a satisfactory repeatability, CV % lower than 10), matrix effect (lower than 30%) and reproducibility of retention times (CV% lower than 0.1) and of relative abundances (CV% lower than 15). The performance and the applicability of the method was evaluated by analyzing real samples containing benzodiazepines (alprazolam, diazepam, zolpidem or zoplicone) or inhibitors of the phosphodiesterases type 5 (sildenafil or vardenafil) and samples obtained incubating two of the phosphodiesterases type 4 studied (cilomilast or roflumilast) with pooled human liver microsomes. All the parent compounds,

51 together with their main phase I metabolites, were clearly detected using the analytical procedures
52 here developed.
53
54 ***Keywords**: Liquid chromatography-mass spectrometry; Drugs in sport; Azole antifungals;*
55 *Benzodiazepines; Phosphodiesterase inhibitors; Selective serotonin reuptake inhibitors.*
56

1. Introduction

The first "anti-doping list", that is the list of prohibited substances and methods in sport was published in the mid-1960s, and contained only substances active if taken immediately before or during competition (mainly stimulants and narcotics) [1-2]. Since then, the list has progressively expanded over the last 50 years, having been periodically updated first by the International Olympic Committee (IOC) and, since 2002, by the World Anti-Doping Agency (WADA). At present, "The Prohibited List International Standard" is one of the five International Standards of the World Anti-Doping Code and it is published and updated every year by the WADA. The current Prohibited List includes nine classes of compounds (*S1 Anabolic Agents, S2 Peptide hormones, growth factors, related substances and mimetics, S3 b2-agonists, S4 Hormone and Metabolic Modulators, S5 Diuretics and Masking Agents, S6 Stimulants, S7 Narcotics, S8 Cannabinoids and S9 Glucocorticoids*) three classes of methods (*M1 Manipulation of blood and blood components, M2 Chemical and Physical Manipulation and M3 Gene doping*) and two groups of compounds prohibited only in particular sports (*P1 Alcohol and P2 Beta-blockers*) [4].

The WADA list is an "open" list, this meaning that, although representative examples are reported for each class of substances, for most classes also other compounds with similar chemical structure or pharmacological activity are prohibited. In addition, to avoid the abuse by athletes of new pharmacological substances with no current approval for human therapeutic use (i.e. agents under pre-clinical or clinical development, designer drugs or compounds approved only for veterinary use), including, but not limited to, those illegally produced by clandestine laboratories and/or marketed via the Internet, a new section of the list itself, i.e. section "S0 - Non-Approved Substances" was also added in 2011 [4-5].

When a new substance/class of substances is included in the Prohibited List, the primary activity of the WADA-accredited anti-doping laboratories is to promptly develop and validate effective analytical procedures to detect the illicit intake of the newly banned compound. Methods have to be

designed to be applied worldwide, following ISO 17025 accreditation, by all WADA accredited laboratories. Together with the analytical aspects, a deep knowledge of both the metabolism/degradation and the rate and route of elimination of the new candidate drug is also necessary to select the most appropriate biological fluid, time of testing with respect to competition (i.e. either "in" and/or "out of competition"), and diagnostic markers for its intake [6].

In the last years different agents, not yet included in the Prohibited List, were given specific consideration in sport doping for the following reasons: i) according to the information available on the doping control forms their use in sports is increased; ii) scientific evidences of their direct or indirect effects on sport performances were described in literature [7-15]; and/or iii) several investigators demonstrated their capability of interfering with the analytical strategies currently adopted by the anti-doping laboratories to detect drug abuse (mainly, but not only, by modulating the activity of the enzymes involved in the phase I or II metabolism of prohibited agents) [16-23]. These classes include the azole antifungals, selective serotonin reuptake inhibitors, benzodiazepine-like substances and inhibitors of the phosphodiesterases (PDEs) type 4 and 5.

Although different methodologies to analyze benzodiazepine-like substances [24-29], antidepressants [30-34], azole antifungals [35-36] or inhibitors of the PDEs type 4 [15] and 5 [10, 37-41] in different biological fluids, pharmaceuticals, clandestine products and food supplements have already been developed, to the best of our knowledge no analytical methods for their combined detection in urine matrix, that would be compliant with the current multi-analyte procedures used in anti-doping laboratories, have been described so far.

Here we propose a LC-ESI-MS/MS method for the simultaneous detection of 7 selective serotonin reuptake inhibitors, 4 azole antifungal drugs, 19 benzodiazepine-like substances, 5 inhibitors of the PDE type 4 and 3 inhibitors of PDE type 5 in the urinary matrix that is in conformity with the multi-target screening procedure currently adopted by our laboratory to detect different classes of doping agents (i.e. diuretics, glucocorticoids, anti-oestrogenic agents, selective androgenic receptor modulators, designer steroids, metabolic modulators, stimulants, narcotics and

synthetic cannabinoids) [42-44]. The newly developed analytical procedure, once optimized, was validated according to ISO 17025 [45] and WADA requirements for the accredited laboratories (as detailed in the WADA International Standard for Laboratories and related technical documents [46]). The overall performance and the applicability of the proposed method was assessed by analyzing urine samples collected from patients in treatment with benzodiazepine-like substances (alprazolam, diazepam, zolpidem or zoplicone) or PDE5 inhibitors (sildenafil or vardenafil) and on samples obtained incubating the PDE4 inhibitors (cilomilast or roflumilast) with pooled human liver microsomes.

2. Experimental

2.1 Chemicals and Reagents

Alprazolam, alprazolam d5 (used as internal standard), bromazepam, brotizolam, chlordiazepoxide, citalopram, clobazam, clonazepam, diazepam, delorazepam, dapoxetine, etizolam, fluconazole, fluoxetine, flurazepam, fluvoxamine, ketoconazole, itraconazole, lorazepam, lormetazepam, 17α-methyltestosterone (used as internal standard), miconazole, nordiazepam, oxazepam, paroxetine, piclamilast, pinazepam, rolipram, triazolam, sildenafil, tadalafil, ibudilast, vardenafil, zaleplon, zimelidine, zolpidem and zoplicone were supplied by Sigma-Aldrich (Milano, Italy). Cilomilast and roflumilast were supplied by Selleck Chemicals LLC (distributed by D.B.A., Milano, Italy). Levitra® is from Bayer Pharma AG (Berlin Germany); Sildenafil EG®.

All chemicals (formic acid, acetonitrile, methanol, dimethylsufoxide, sodium phosphate, sodium hydrogen phosphate, *tert*-butyl methyl-ether) were from Carlo Erba (Milano, Italy) and Sigma-Aldrich (Milano, Italy). The enzyme β-glucuronidase (from *E. coli*) used for the enzymatic hydrolysis of conjugates, was purchased from Roche (Monza, Italy). The ultra-purified water used was of Milli-Q-grade (Millipore Italia, Vimodrone, Milano, Italy).

The reagents (sodium phosphate and tris-HCl buffers and the NADPH regenerating system consisting of magnesium chloride hexahydrate, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) and the enzymatic protein (pooled human liver microsomes) for the *in vitro* assays were purchased from BD Biosciences (Milano, Italy).

Stock solutions of the various substances were made up in methanol at a concentration of 1 mg/mL and stored in screwed cap vials at -20 °C.

The real samples utilized in this study were from previous studies carried out in our laboratory [10].

2.2 *In vitro* protocol

For *in vitro* assays stock solutions (1 mM) of the parent compounds (cilomilast or roflumilast) were prepared in dimethylsulfoxide. The incubation mixture, in a final volume of 250 µL, contains 0.1 M sodium phosphate buffer (pH 7.4), the substrates at final concentration of 10 µM, a NADPH regenerating system consisting of 3.3 mM magnesium chloride, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate and 0.4 U mL^{-1} glucose-6-phosphate dehydrogenase. After mixture pre-warming at 37 °C for 5 minutes, the reaction was started by adding 0.5 mg/mL of pooled human liver microsomes. The sample was then incubated for 2 h at 37 °C. One sample (negative control) containing all reaction components but not the enzymatic protein was also added to the batch to monitor the potential non-enzymatic reactions within the incubation period. The overall reaction was terminated by the addition of 250 µL of ice-cold acetonitrile and transferred to ice. The sample was then centrifuged at 12.000 g for 5 min before the sample purification consisting in a liquid/ liquid extraction in neutral field and evaporation to dryness. The residue was, then, reconstituted in 50 µL of mobile phase and an aliquot of 10 µL was injected on the liquid chromatography-mass spectrometry system.

2.3 Urine sample preparation

The sample preparation was based on the analytical procedure currently adopted by our laboratory to perform the multi-target screening analysis in LC-MS/MS [42-44]. Briefly, to 2 mL of urine 1.5 mL of phosphate buffer (1 M, pH 7.4), 50 µL of β-glucuronidase from *E. coli* and 10 µL of the internal standard mixture (ISTD: solution of 17α-methyltestosterone and alprazolam d5 final concentration of 50 and 5 ng mL^{-1} respectively) were added and the sample was incubated for 1 hour at 55 ºC. After hydrolysis 7 mL of *tert*-butylmethyl ether were added and the liquid/liquid extraction was carried out for 6 minutes on a mechanical shaker. After centrifugation the organic layer was evaporated to dryness at room temperature. The residue was reconstituted in 50 µL of

mobile phase and an aliquot of 10 µL was injected on the liquid chromatography-mass spectrometry system.

2.4 Instrumental Conditions

2.4.1 Liquid chromatographic conditions

The 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, Cernusco sul Naviglio, Milano, Italy). Reversed-phase liquid chromatography was performed using the conditions currently adopted by or laboratory to perform the multi-target screening analysis in LC-MS/MS [42-44]. More specifically, the chromatographic separation was performed using a Supelco Ascentis C18 column (2.1×150 mm, 5 µm) and ultra-purified water (eluent A) and acetonitrile (eluent B) containing both 0.1% formic acid as mobile phases. The flow rate was set at 250 µL min^{-1}. The gradient program started at 10% B and increased to 60% B in 7 min and then, after 6 min, to 100% B in 1 min. The column was flushed for 1 min at 100% B and finally re-equilibrated at 10% B for four minutes.

2.4.2 Mass spectrometric conditions

All experiments were performed using an Applied Biosystems (Applera Italia, Monza, Italy) API4000 instrument with positive and negative electrospray ionization. The experiments were performed using selected reaction monitoring (SRM) as acquisition mode (the ion transitions selected are reported in the Table 1), employing collision-induced dissociation (CID) using nitrogen as collision gas at 5.8 mPa, obtained from a dedicated nitrogen generator system Parker-Balston model 75-A74, gas purity 99.5% (CPS analitica Milano, Italy). The mass spectrometric parameters (declustering and needle voltages, gasses pressure, source temperature, collision cell exit potential and collision energy) were optimized by infusion of the standard solutions of the compounds under investigation at a concentration of 10 µg mL^{-1} (see the Table 1). For this purpose, a 1 mL syringe, operated by a syringe pump set at a flow-rate of 10 µL min^{-1}, was utilized. All aspects of instrument

194 control, method setup parameters, sample injection and sequence operation were controlled by the
195 Applied Biosystems Analyst software version 1.5.1.

196

197 **2.5 Validation Parameters**

198 Experiments were performed using 20 blank urines from laboratory staff to determine all
199 parameters (lower limit of detection, specificity, ion suppression/enhancement, recovery, relative
200 abundances of the characteristic ion transitions selected and retention time's repeatability and
201 robustness) required for the validation of a qualitative screening procedure.

202 For the lower limits of detection (LLOD) determination, 20 different blank urines spiked with the
203 compounds under investigation at 50 ng mL^{-1} were used. Serial dilutions were made and the LLOD
204 was reported as the lowest concentration at which a compound could be identified in all twenty
205 urines tested, with the least abundant diagnostic ion transition with a signal-to-noise (S/N) ratio
206 greater than 3 and with the ion transitions ratios fulfilled the identification criteria reported in the
207 WADA technical document TD2010IDCR [46].

208 The specificity was studied by analyzing at least 20 urine samples from laboratory staff to
209 demonstrate that no interferences are present at the retention time of the analytes under
210 investigation.

211 The effect of the urine matrix on ion suppression and ion enhancement was assessed according to
212 established protocols. Specifically the 20 different blank urines and solvent only were analyzed with
213 continuous co-infusion of the target analytes (10 μg mL^{-1} at a flow rate of 7 μL min^{-1}) via T-
214 connector.

215 For the recovery, the twenty blank urines were fortified with the compounds under investigation
216 at concentration of five times the LLOD values and extracted according to the optimized protocol
217 together with the same twenty blank urines not fortified. The twenty urines not fortified at the
218 beginning were spiked with the substances under investigation at a concentration of five times the

LLOD values into the organic layer before the evaporation. To both sets of samples, 10 μL of the ISTD working solution was added into the organic layer before the evaporation. Recovery was calculated by comparison of mean peak area ratios of the analyte and the ISTD of samples fortified prior to and after pretreatment.

The relative abundances of the characteristic ion transitions selected and the retention time repeatability was evaluated for both intermediate and intra-day assays analyzing negative urine spiked with the compounds under investigation at a concentration ten times the LLOD concentrations and at a concentration corresponding to the LLOD (see Table 2).

The robustness of the method was demonstrated by using the twenty spiked urines described above once a week for three weeks, randomly changing the instrument and the operator involved in the instrumental analysis and in the preparation of the urine samples.

3. Results and Discussion

3.1 Method development

3.1.1 Mass spectrometric conditions

Instrumental parameters in MS and MS/MS were optimized by infusing the standard solutions of the agents under investigation dissolved in the mobile phase at a concentration of 10 µg mL^{-1} using full scan and product ion scan as acquisition modes.

The experiments were, first, performed in full scan mode in order to examine the ionization behavior of the agents considered in this study. No signals were obtained in full scan spectrum acquired in negative mode; abundant signal was instead recorded in positive mode. Only the protonated molecular ion $[M+H]^+$ was observed in the MS spectrum obtained in positive ionization; adduct ions were not observed. The signals of the protonated molecular ions were optimized evaluating different declustering and needle voltages and different gasses (curtain, gas 1 and gas 2) pressures. Optimal results were obtained using a curtain gas pressure of 25 psi, a source temperature of 500 °C, an ion source gas 1 pressure of 35 psi, an ion source gas 2 pressure of 40 psi, a declustering voltage of 80 V, a collision cell exit potential of 10 V and a needle voltage of 5500 V.

To study the dissociation routes of the different substances and to select characteristic mass spectral fragments, the same standard solutions were infused, using product ion scan as acquisition mode and different collision energies (20, 25, 30, 35, 40, 45, 50, 55 and 60 eV). The protonated molecular ion $[M+H]^+$ undergoes significant fragmentation only at collision energy higher than 35 eV for the class of benzodiazepine-like substances, azole antifungals and inhibitors of PDE type 5, whereas lower collision energies are sufficient to fragment the inhibitors of the PDE type 4 and the selective serotonin reuptake inhibitors. Figures 1A-E show the product ion spectra of several compounds under investigation. As can be noticed, the dissociation routes for each class of substances are strictly linked to the substituents present in the basic structure. More in details:

- The benzodiazepine-like substances (as shown in Figure 1A, reporting the product ion spectra obtained at a collision energy of 35 eV) showed the following behavior: (i) bromazepam and diazepam lose carbon monoxide from the 7- membered ring, followed by the loss of the halogen in the 7-position of the fused benzene ring; (ii) clobazam and clonazepam were found to have the major product ion to be the result of loss of CH_2CO and of NO_2 respectively in the 7-position of the fused benzene ring; (iii) flurazepam loses the alkyl groups; (iv) lorazepam and oxazepam were found to have the major product ion to be the result of loss of water followed by the loss of carbon monoxide from the 7- membered ring; finally (v) the chlorine- containing triazolam loses chlorine and the diatomic nitrogen corresponding to the opening of the five-membered nitrogen-containing ring. The others benzodiapepine-like substances studied, for which the product ion spectra are not reported in Figure 1A, are characterized by similar dissociation routes, confirming the results reported in literature [24-29].

- The selective serotonin reuptake inhibitors (as shown in Figure 1B, reporting the product ion spectra obtained at collision energy of 25 eV) showed fragmentation patterns that follow from dissociation routes strictly depending on the specific molecular structure; the only common transition is referred to the loss of the end-of-chain amine residue.

- The azole antifungals (Figure 1C, reporting the product ion spectra obtained at collision energy of 45 eV) showed a fragmentation pattern in which the main dissociation route is characterized by the breakdown of the molecule in correspondence of the imidazole (ketoconazole and miconazole) or triazole (fluconazole and itraconazole) ring and of the dichlorophenyl or difluorophenyl group.

- The PDE4 inhibitors (Figure 1D, reporting the product ion spectra obtained at collision energy of 40 eV) showed common fragmentation pathways for cilomilast, rolipram and piclamilast, characterized by the loss of the cyclopentene followed by the loss of ammonia, methyl radical and carbon monoxide for rolipram, by the loss of HCN, water and carbon monoxide for

cilomilast and by the cleavage of the amide bond for piclamilast; fragmentation of roflumilast follows similar dissociation routes, characterized by the loss of the methylenecyclopropane and HCl followed by the cleavage of the amide bond, confirming the data reported by Thevis *et al.* [15].

- Finally, the product ion spectra of the PDE5 inhibitors, sildenafil and vardenafil, are characterized by common dissociation routes, showing the production of ion fragments derived from the breakdown of the molecule between the phenyl and pyrimidyl ring confirming the data reported by Strano-Rossi *et al.* [10]. Tadalafil follows different dissociation routes characterized by the breakdown of the molecule at the 1,3-benzodioxole ring [10].

The mass spectrometric conditions for the metabolites of the compounds under investigation for which no reference standards are available in our laboratory were obtained considering the data reported in literature and the fragmentation behaviors of the parent compounds because similar fragmentation are expected. The ion transitions utilized to developed the SRM method (see Table 1 for the ion transitions selected) were obtained by calculating the protonated molecular ion $[M+H]^+$ of the potential metabolite and by selecting the diagnostic ions found in the product ion spectra of the parent compounds.

3.1.2 Chromatographic and sample pre-treatment conditions

The chromatographic conditions and the sample pretreatment were optimized starting from the conditions used for the multi-target screening procedure currently adopted by our laboratory to detect diuretics, glucocorticoids, anti-estrogenic and androgenic agents, synthetic cannabinoids, adrenergic agents, designer steroids and stimulants. For this purpose, a standard mixture containing all the compounds under investigation was added to 2 mL of ultra-purified water at a concentration ten times the LLOD value. The sample was, then, analyzed using the sample pre-treatment and the chromatographic conditions currently utilized by our laboratory and reported in the experimental part and the mass spectrometric parameters described above. Figure 2 reports the results obtained, as can be seen all the compounds are clearly detected with a satisfactory chromatographic retention,

sensitivity and peak shape. In addition all the compounds evaluated were extracted with a recovery higher than 60% and a satisfactory repeatability (CV % lower than 10).

3.2 Method validation

The newly developed method was validated according to the ISO 17025 and WADA-guidelines [45-46]. For this purpose, repeatability of relative retention time (according to the WADA technical document TD2010IDCR [46] in case of a non-isotopic internal standard the relative retention time shall not differ by more than 1% from that of the same substance in the spiked urine sample) and of relative ion abundance, specificity, carry over, ion suppression/enhancement and lower limit of detection were measured.

The analyses performed on the 20 negative samples confirmed that the methods did not show significant interferences and therefore it has an adequate selectivity. Carry-over was tested by analyzing the negative urine samples after positive samples obtained adding to the negative urines the compounds under investigation at a concentration ten times higher than their LLOD value. The procedure was carried out twice and showed that no carry-over was occurring by analyzing a negative sample right after a positive sample. In addition, the configuration of HPLC auto-sampler, using continuous flushing of the needle, offered minimal or even zero carry-over to all analyses.

The test for ion suppression/enhancement effects by post column split-infusion of analytes yielded no significant matrix effect (lower than 35%) at the retention times of the analytes under investigation and internal standard while 20 different urine samples were injected (see Table 1).

The lower limits of detection were in the range of 10-50 ng mL^{-1} for the class of azole antifungals, 1-5 ng mL^{-1} for the benzodiazepine-like substances, the selective serotonin reuptake inhibitors and for the inhibitors of PDE type 4, and 2-5 ng mL^{-1} for the inhibitors of PDE 5 (see Table 2), these values being in agreement with the data reported by previous investigators.

Finally, for all the compounds under investigation, good repeatability of the relative retention times (CV% lower than 0.1) and of relative abundances of selected ion transitions (CV% lower than 15) were measured for both intermediate and intra-day assays

3.3 Analysis of real samples and sample obtained after incubation with human liver microsomes

The performance and the applicability of the newly developed method in detecting the agents considered in this study in real cases was evaluated by analysing real samples containing benzodiazepines (alprazolam, diazepam, zolpidem or zoplicone) or inhibitors of PDE type 5 (sildenafil or vardenafil) and samples obtained after incubation of cilomilast or roflumilast with pooled human liver microsomes. In Figures 3-5 are reported the results obtained analysing the real samples containing alprazolam (Figure 3A), diazepam (Figure 3B), zolpidem (Figure 4B), zoplicone (Figure 4C), sildenafil (Figure 5B) or vardenafil (Figure 5C). The results obtained are in conformity with those reported by previous investigators [10, 23-29], more in details, in the urine sample containing alprazolam, the alprazolam hydroxylated metabolite was detected in high concentration, whereas the intact compounds was present only in low amount (Figure 3A); the urine sample collected after diazepam administration contains the metabolite nordiazepam and the parent compound, whereas the second ones contains also the metabolic product oxazepam (Figure 3B); the urine samples collected after administration of zolpidem (Figure 4B) or zoplicone (Figure 4C) contain the parent compounds in concentration much higher than their metabolites (acid metabolite for zolpidem and demethylated metabolite for zoplicone). Finally, concerning the inhibitors of the PDE5, as can be noticed in Figures 5B-C, the urines collected after 18 hours from the administration of a single dose of sildenafil or vardenafil contain the intact compounds and their main metabolites (hydroxylated and de-alkylated metabolites) in concentration much higher than the LLOD of the analytical procedures here proposed.

356 In Figures 6A-B are instead reported the results obtained analyzing the sample obtained after
357 incubation of cilomilast and roflumilast with pooled human liver microsomes. For both compounds
358 the oxydated metabolites were detected confirming the results reported by Thevis *et al.* [15].
359

4. Conclusions

The data presented in this study show the capability and suitability of the newly developed and validated LC-ESI-MS/MS procedure to detect simultaneously the intake of drugs belonging to the classes of selective serotonin reuptake inhibitors, of azole antifungal drugs, of benzodiazepine-like substances and of the inhibitors of the phosphodiesterases type 4 and 5. These agents can be easily included in the LC-MS/MS multi-analyte screening procedure currently adopted by our laboratory to detect different classes of banned compounds (30 diuretics, 17 glucocorticoids, 6 anti-oestrogenic agents, 4 selective androgenic receptor modulators, 7 synthetic cannabinoids, 2 beta-adrenergic agents and 5 designer steroids, 3 narcotics, 2 metabolic modulators and 9 stimulants), without compromising the necessary analytical requirements. The analytical procedure has been fully validated, tested on real samples.

The analytical procedure here presented ensures the possibility to screen for, by a unique assay, all the most common “non-doping” drugs used by the athletes (based on the information reported on the “doping control form”), and other drugs who are suspected to be performance enhancing and/or masking agents in particular conditions. The application of this method would therefore significantly increase the available information on the actual (ab)use of the above classes of drugs among athletes.

Finally, the overall performance of the method suggests that it could be successfully applied not only for routine use in anti-doping laboratories, but also for various applications in the field of clinical and forensic toxicology. In future we intend to include in the newly developed procedure other components of the classes of substances here studied.

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520 **Table 1**

521 Mass spectrometric conditions: precursor ion (Q1), product ions (Q3) and collision energy (CE)

ID	Compound	Q1 (*m/z*)	Q3 (*m/z*)	CE (*eV*)
Azole antifungals				
16	*Fluconazole*	307	70, 169, 200, 220, 238	45, 40, 35, 35, 35
31	*Ketoconazole*	532	82, 447, 421, 490	60, 55, 55, 50
38	*Itraconazole*	706	256, 451, 492	65, 55, 55
33	*Miconazole*	417	69, 82, 159	55, 50, 40
Benzodiazepine like substances				
24	*Alprazolam*	309	205, 381	40, 35
-	*Hydroxy-alprazolam*	325	227, 279	40, 35
10	*Bromazepam*	316	182, 209	35, 35
-	*Hydroxy-bromazepam*	332	286, 314	40, 35
27	*Brotizolam*	395	279, 314	40, 35
4	*Chlordiazepoxide*	300	227, 241	35, 25
28	*Clobazam*	302	225, 260	40, 35
21	*Clonazepam*	316	223, 270	40, 35
-	*7-ammino-clonazepam*	286	121, 222	40, 35
29	*Diazepam*	285	222, 257	40, 35
26	*Delorazepam*	305	242, 277	40, 40
25	*Etizolam*	343	289, 314	45, 35
7	*Flurazepam*	389	289, 316	45, 35
20	*Lorazepam*	321	275, 303	35, 25
34	*Lormetazepam*	335	288, 317	35, 25
17	*Nordiazepam*	271	243, 208	40, 35
18	*Oxazepam*	287	241, 269	35, 25
35	*Pinazepam*	310	241, 292	45, 35
22	*Triazolam*	343	279, 308, 315	40, 35, 35
-	*Hydroxy-triazolam*	359	176, 331	45, 35
15	*Zaleplon*	306	236, 260	35, 25
2	*Zoplicone*	389	217, 245	40, 25
3	*Zolpidem*	308	235, 263	35, 30
-	*Zolpidem phenyl-4-carboxylic acid*	338	151, 192	40, 35
Selective serotonin reuptake inhibitors				
9	*Citalopram*	325	58, 109, 262, 307	35, 35, 30, 25
13	*Dapoxetine*	306	117, 157, 183, 261	35, 35, 30, 25
6	*Fluoxetine*	310	259, 290	30, 25
12	*Fluvoxamine*	319	62, 71, 87, 302	35, 35, 35, 25
11	*Paroxetine*	330	70, 151, 192	35, 30, 25
14	*Sertralin*	307	159, 276	30, 25
1	*Zimelidine*	318	194, 273	30, 25
Inhibitors PDE4				
32	*Cilomilast*	344	203, 230, 249, 276	35, 30, 25, 25
-	*Hydroxy-cilomilast*	360	276, 342	30, 25
30	*Piclamilast*	382	152, 220	40, 30
37	*Roflumilast*	403	167, 241, 367	40, 35, 25
-	*Roflumilast-N-oxide*	419	241, 383	30, 25
19	*Rolipram*	276	163, 191, 208	45, 40, 30
-	*Demethyl-rolipram*	262	103, 131, 177, 194, 177	45, 45, 40, 35, 30
-	*Hydroxy-rolipram*	292	274, 257, 208	45, 40, 30
36	*Ibudilast*	231	92, 119, 132, 145	45, 40, 40, 35
Inhibitors PDE5				
8	*Sildenafil*	475	58, 100, 311	50, 45, 35
-	*Sildenafil hydroxylated*	491	100	45
-	*Syldenafil demethylated*	461	100	45
23	*Tadalafil*	390	69, 135, 302	40, 35, 30
5	*Vardenafil*	489	58, 72, 151, 461	50, 50, 45, 30
-	*Vardenafil deethylated*	461	151	35

522

Table 2

Retention time (Rt), Lower limits of detection (LLOD), recovery and matrix effect of the compounds under investigation

Compound	**Rt** (min)	**LLOD** (ng mL^{-1})	**Recovery** (%)	**Matrix effect** (%)
Azole antifungals				
Fluconazole	10.7	10	62	28
Ketoconazole	12.7	50	58	22
Itraconazole	14.9	50	62	32
Miconazole	13.4	10	61	33
Benzodiazepine like substances				
Alprazolam	11.1	5	72	33
Bromazepam	9.6	5	75	32
Brotizolam	11.5	5	68	35
Chlordiazepoxide	8.4	5	77	30
Clobazam	11.9	5	69	28
Clonazepam	11.2	5	76	35
Diazepam	12.2	5	74	35
Delorazepam	11.8	5	72	33
Etizolam	11.5	5	75	35
Flurazepam	9.1	5	76	35
Lorazepam	11.1	5	76	30
Lormetazepam	12.0	5	75	31
Nordiazepam	11.1	5	68	29
Oxazepam	11.0	5	72	35
Pinazepam	13.3	5	66	25
Triazolam	11.5	5	69	28
Zaleplon	10.6	5	65	28
Zoplicone	7.6	5	66	25
Zolpidem	8.1	1	69	33
Selective serotonin reuptake inhibitors				
Citalopram	9.3	3	78	32
Dapoxetine	10.2	3	75	30
Fluoxetine	9.0	5	77	33
Fluvoxamine	9.8	1	82	33
Paroxetine	9.7	5	77	35
Sertralin	10.3	5	76	30
Zimelidine	8.0	5	74	
Inhibitors PDE4				
Cilomilast	11.5	1	65	28
Piclamilast	12.9	5	68	25
Roflumilast	13.8	1	69	26
Rolipram	10.6	2	66	27
Ibudilast	13.6	5	62	27
Inhibitors PDE5				
Sildenafil	9.1	2	59	33
Tadalafil	11.4	2	62	32
Vardenafil	8.4	5	66	31

6. FIGURE LEGENDS

Figure 1.

Molecular structure and product ion spectra of eight benzodiazepine-like substances (bromazepam, clobazam, clonazepam, diazepam, flurazepam, lorazepam, oxazepam and triazolam) **(A)**, of the selective serotonin reuptake inhibitors considered **(B)**, of the azole antifungals considered **(C)**, of four inhibitors of the PDE type 4 (cilomilast, piclamilast, roflumilast and rolipram) **(D)** and of the inhibitors of the PDE type 5 (sildenafil, vardenafil and tadafinil) **(E)**. The mass spectrometric parameters used were those reported in the results and discussions part, whereas the collision energy was set at 35 eV for the benzodiazepine-like substances, at 25 eV for the selective serotonin reuptake inhibitors, at 45 eV for the azole antifungals and at 40 eV for the inhibitors of the phosphodiesterases type 4 and 5.

Figure 2.

Extracted ion chromatograms of blank urine spiked with the compounds under investigation at a concentration of 100 ng mL^{-1}. The sample was analyzed using the analytical procedure (pre-treatement and instrumental conditions) reported in the experimental part. Peak identification: *1. zimelidine, 2. zoplicone, 3. zolpidem, 4. chlordiazepoxide, 5. vardenafil, 6. fluoxetine, 7. flurazepam, 8. sildenafil, 9. citalopram, 10. bromazepam, 11. paroxetine, 12. fluovoxamine, 13. dapoxetine, 14. sertralin, 15. zaleplon, 16. fluconazole, 17. oxazepam, 18. nordiazepam, 19. rolipram, 20. lorazepam, 21. alprazolam, 22. clonazepam, 23. tadalafil, 24. triazolam, 25. etizolam, 26. brotizolam, 27. cilomilast, 28. delorazepam, 29. clobazam, 30. lormetazepam, 31. diazepam, 32. piclamilast, 33. ketoconazole, 34. miconazole, 35. pinazepam, 36. ibudilast, 37. roflumilast, 38. itraconazole.*

Figure 3.

Extracted ion chromatograms of a blank urine and urine samples collected after administration of alprazolam **(A)** or after administration of diazepam **(B)** at different collection times (2h and 12h after administration). The samples were analyzed using the analytical procedure (pre-treatment and instrumental conditions) reported in the experimental part.

Figure 4.

Extracted ion chromatograms of a blank urine **(A)** and urine samples collected after administration of zolpidem **(B)** or zoplicone **(C)**. The samples were analyzed using the analytical procedure (pre-treatment and instrumental conditions) reported in the experimental part.

Figure 5.

Extracted ion chromatograms of urine samples collected before **(A)** and after 18 hours from the administration of a single dose of sildenafil EG® (25 mg of sildenafil) **(B)** or 18 hours from the administration of a single dose of Levitra® (5 mg of vardenafil) **(C)**. The samples were analyzed using the analytical procedure (pre-treatment and instrumental conditions) reported in the experimental part.

Figure 6.

Extracted ion chromatograms of the samples obtained after incubation of cilomilast **(A)** or roflumilast **(B)** without and in the presence of pooled human liver microsomes. The samples were analyzed using the analytical procedure (pre-treatment and instrumental conditions) reported in the experimental part.

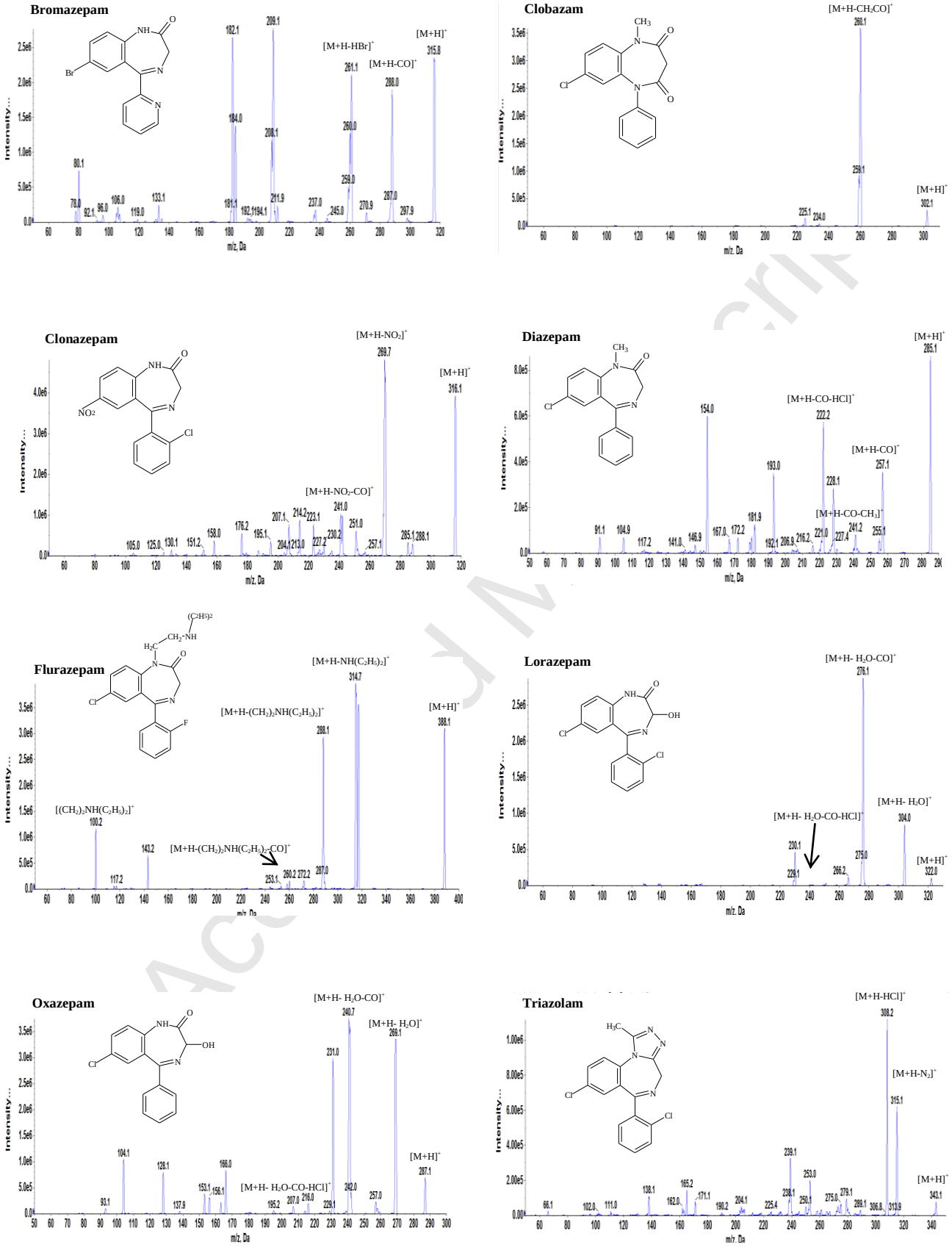


Figure 1A

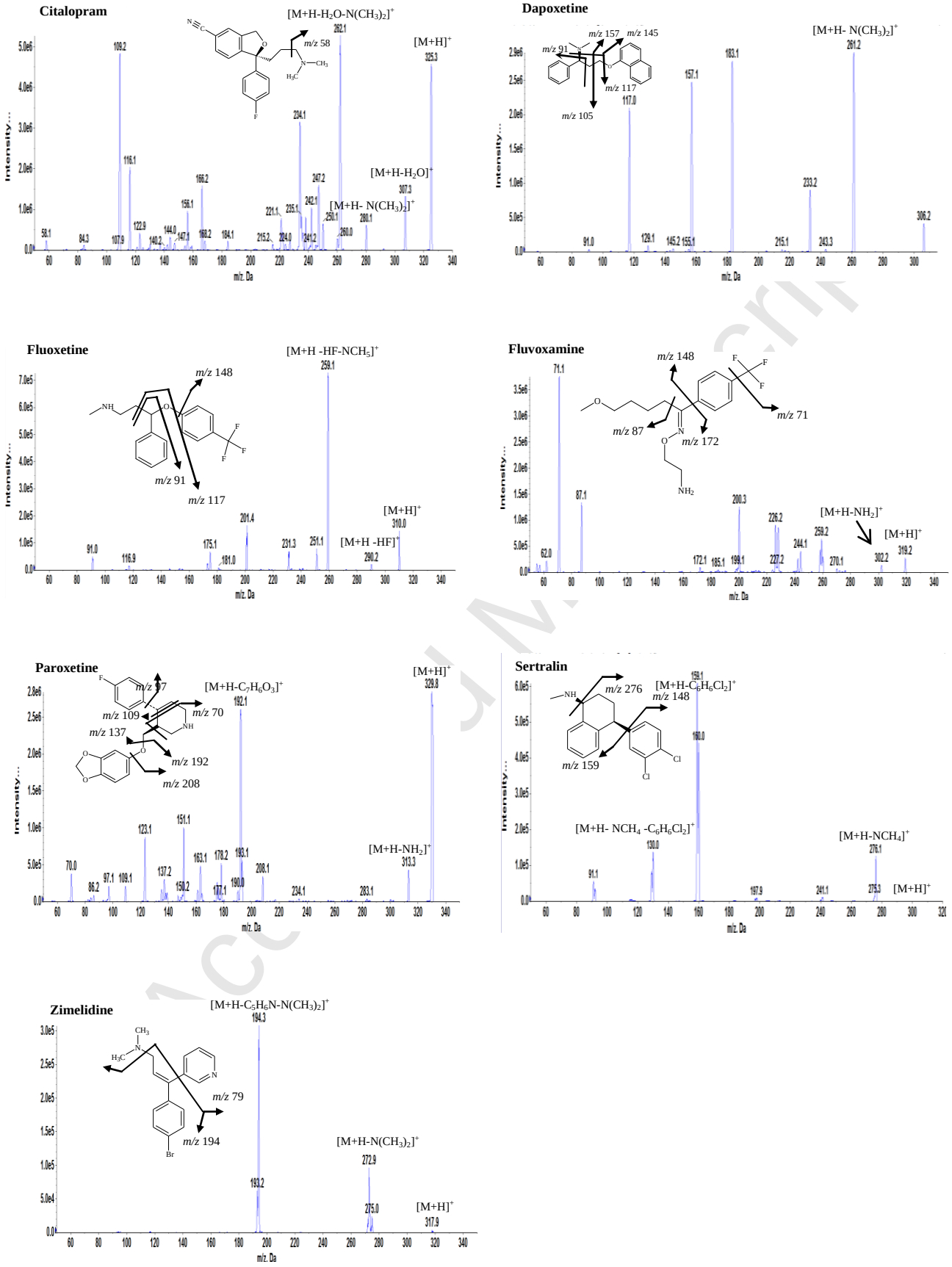


Figure 1B

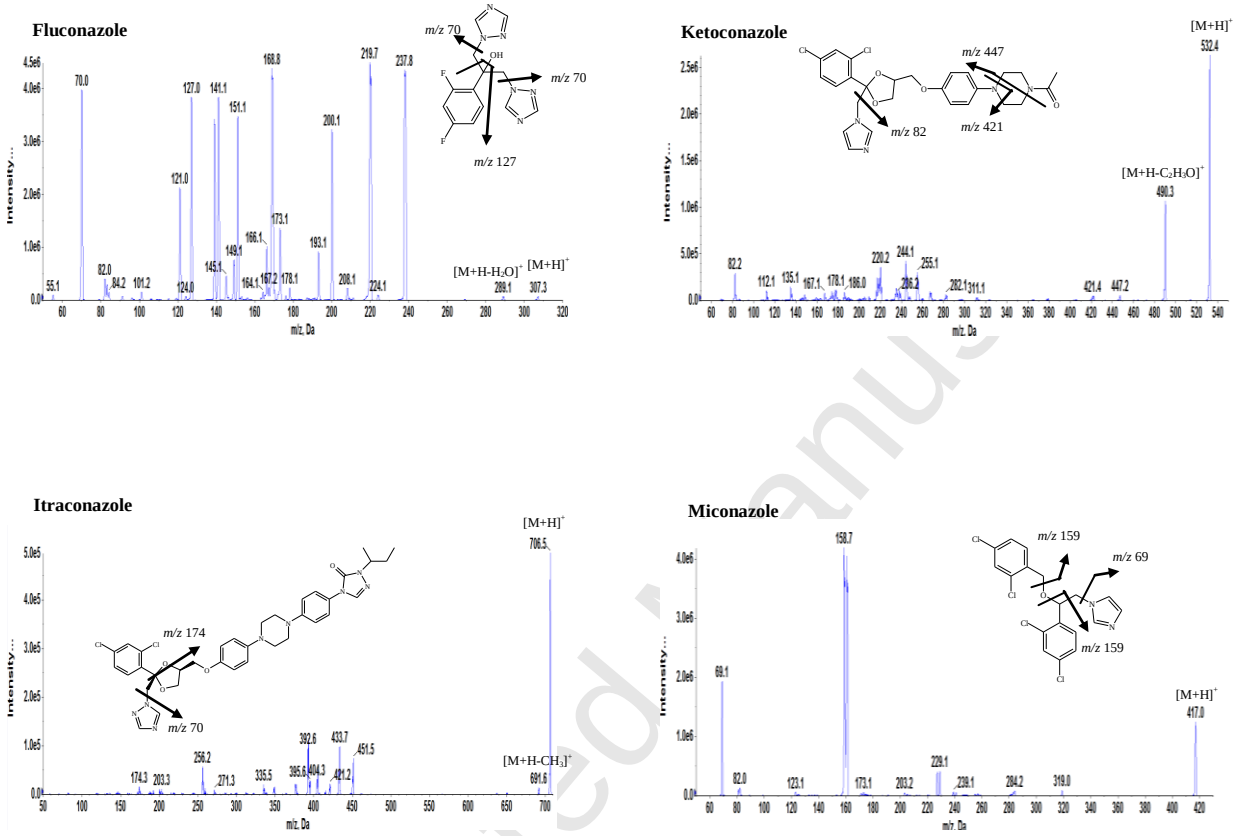


Figure 1C

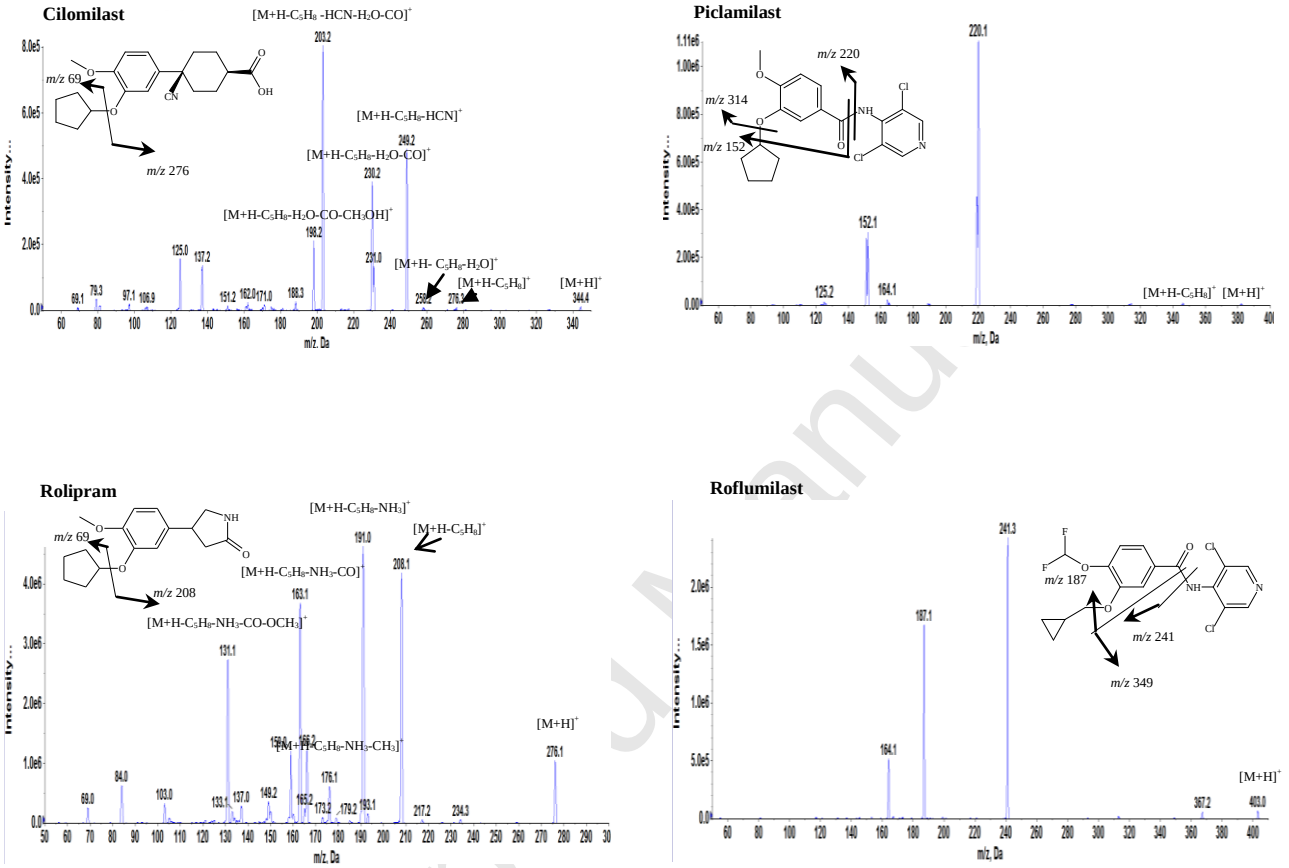


Figure 1D

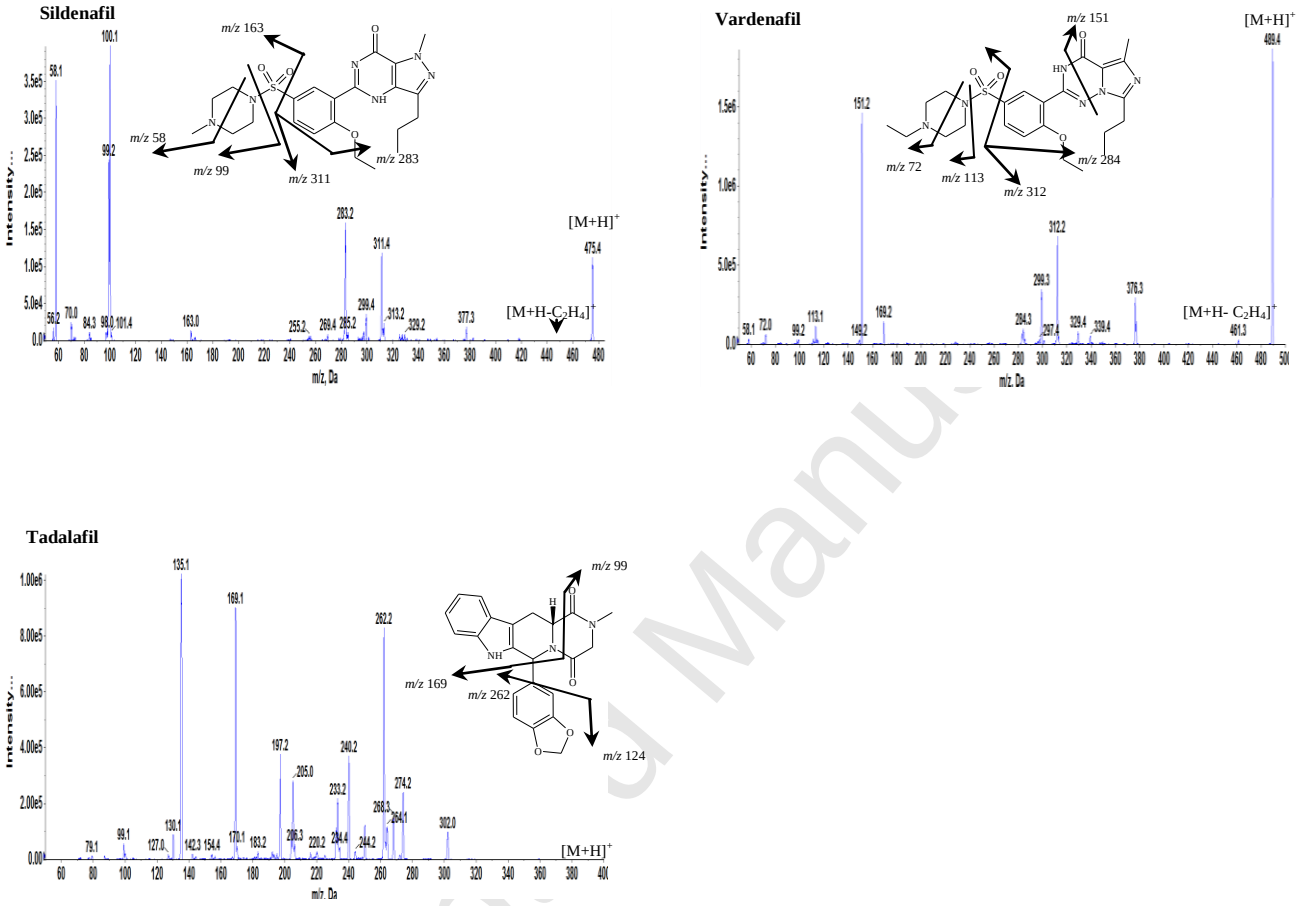


Figure 1E

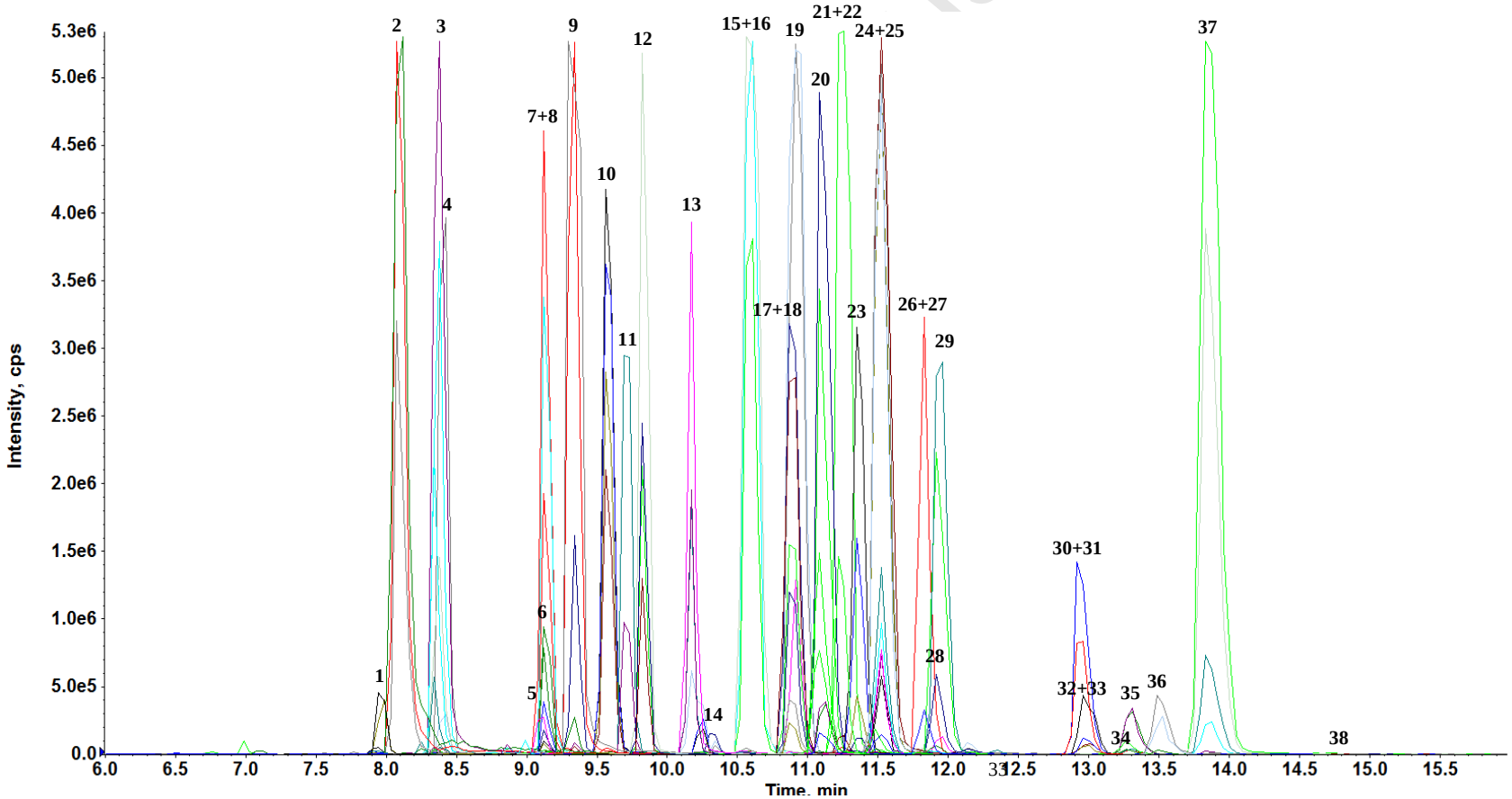
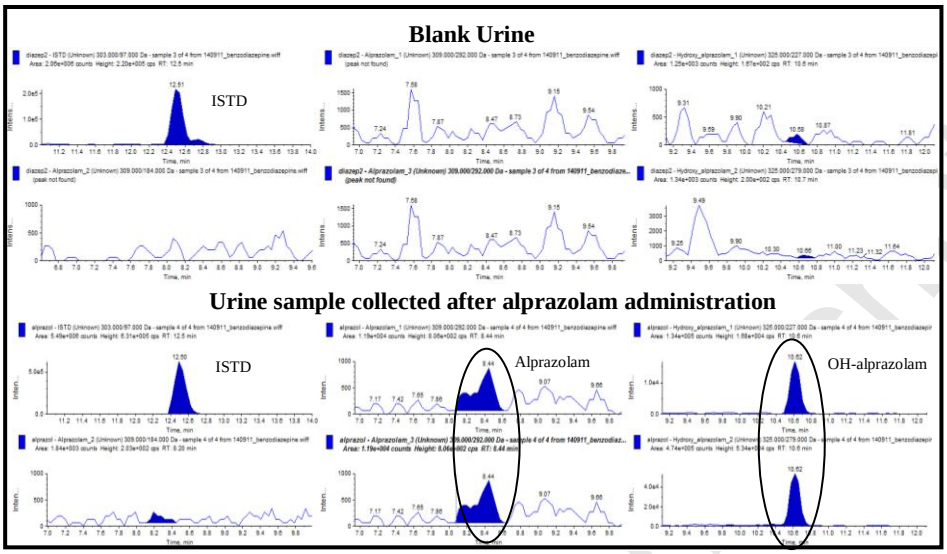


Figure 2

(A)



(B)

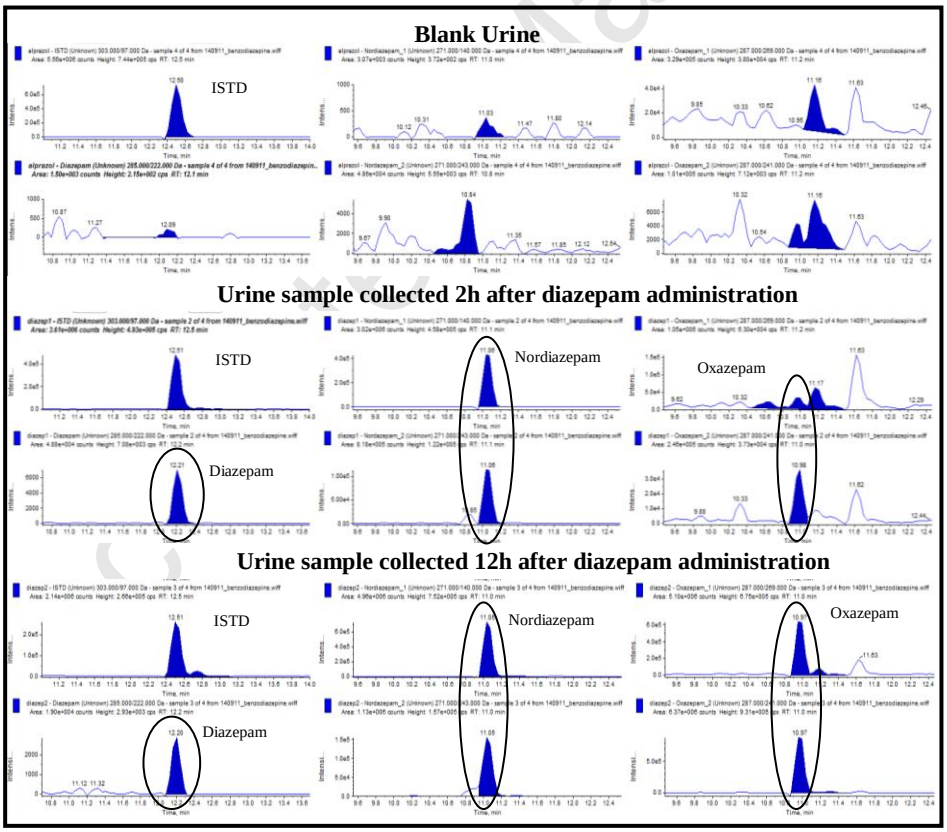


Figure 3

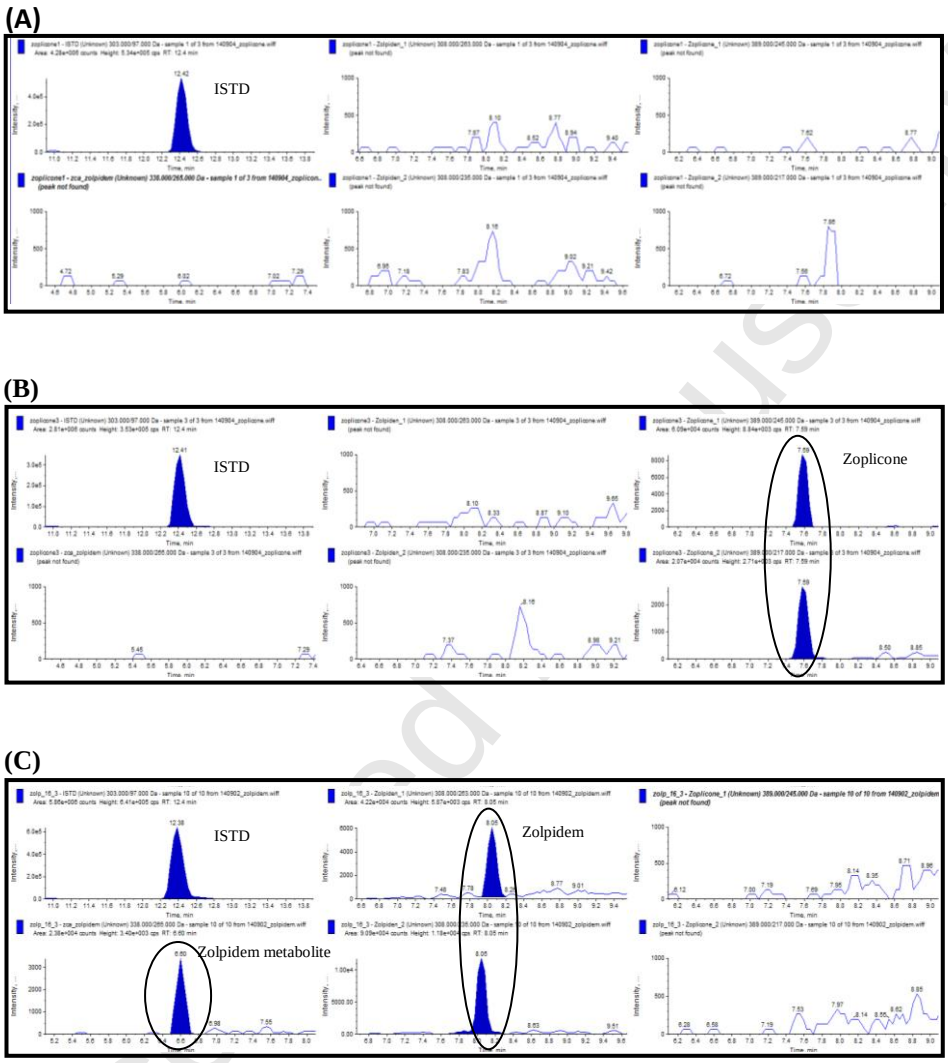


Figure 4

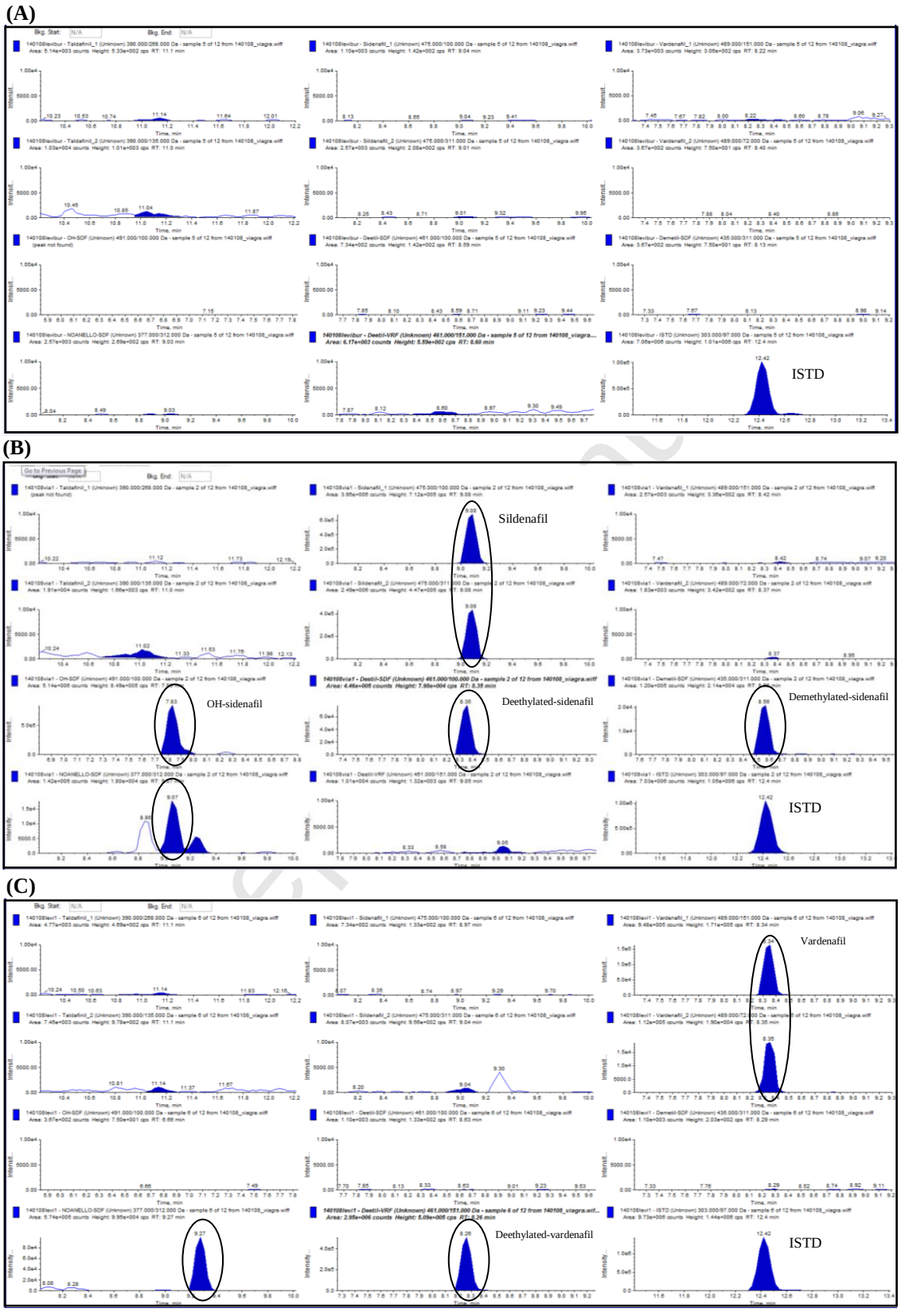


Figure 5

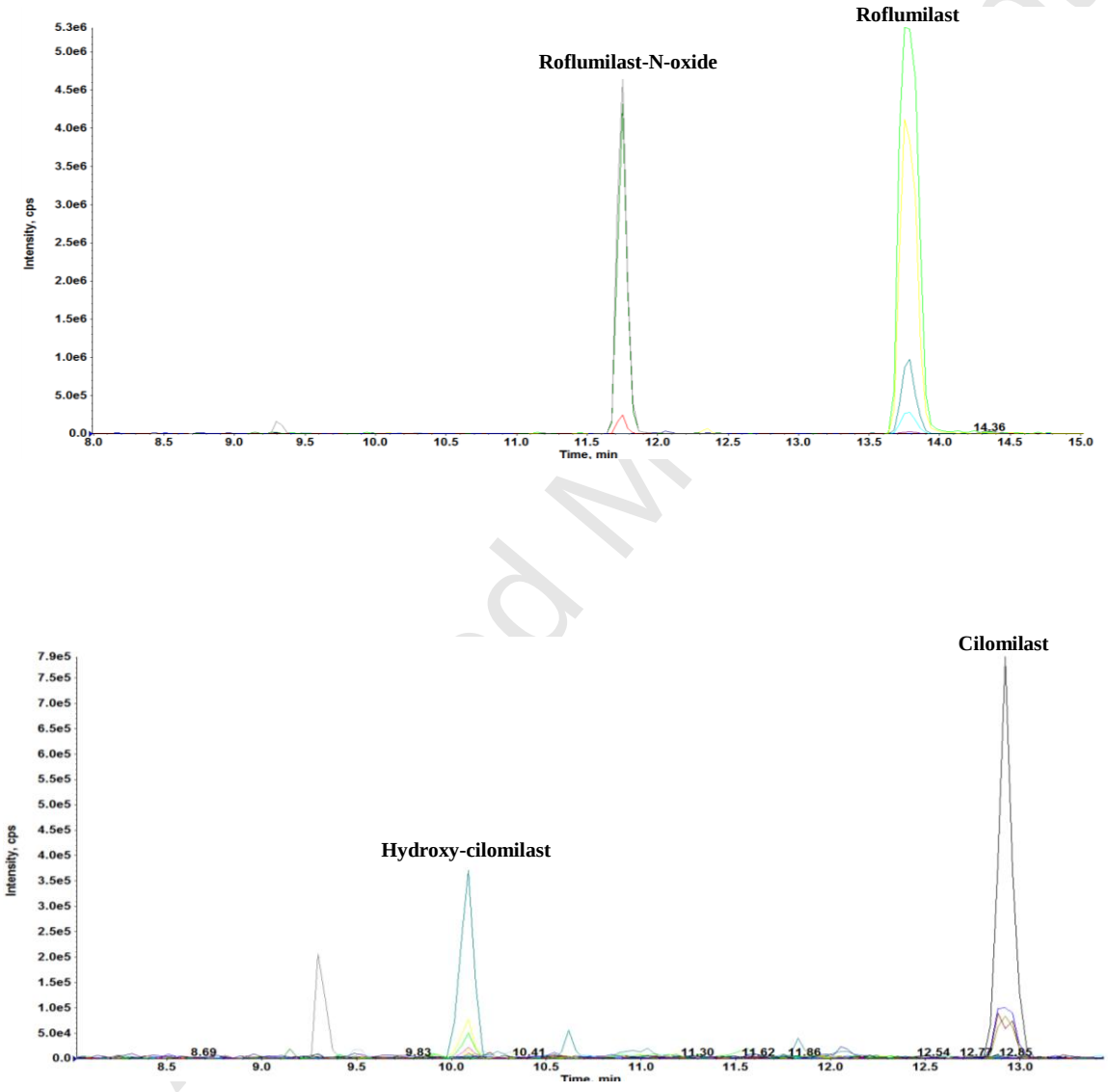


Figure 6