

## RESEARCH ARTICLE

# Effects of the administration of miconazole by different routes on the biomarkers of the “steroidal module” of the Athlete Biological Passport

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## Funding information

World Anti-Doping Agency, Grant/Award Numbers: 17D07MM, 13D14MM

## Abstract

This article reports the results obtained from the investigation of the influence of miconazole administration on the physiological fluctuation of the markers of the steroid profile included in the “steroidal module” of the Athlete Biological Passport. Urines collected from male Caucasian subjects before, during, and after either systemic (i.e., oral and buccal) or topical (i.e., dermal) treatment with miconazole were analyzed according to validated procedures based on gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) (to determine the markers of the steroid profile) or liquid chromatography coupled to MS/MS (LC–MS/MS) (to determine miconazole urinary levels). The results indicate that only after systemic administration, the markers of the steroid profile were significantly altered. After oral and buccal administration, we have registered (i) a significant increase of the 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol/5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol ratio and (ii) a significant decrease of the concentration of androsterone, etiocholanolone, 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, and 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol and of the androsterone/etiocholanolone, androsterone/testosterone, and 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol/epi-testosterone ratios. Limited effects were instead measured after dermal intake. Indeed, the levels of miconazole after systemic administration were in the range of 0.1–12.5  $\mu$ g/ml, whereas after dermal administration were below the limit of quantification (50 ng/ml). Significant alteration started to be registered at concentrations of miconazole higher than 0.5  $\mu$ g/ml. These findings were primarily explained by the ability of miconazole in altering the kinetic/efficacy of deglucuronidation of the endogenous steroids by the enzyme  $\beta$ -glucuronidase during the sample preparation process. The increase of both incubation time and amount of  $\beta$ -glucuronidase was demonstrated to be effective countermeasures in the presence of miconazole to reduce the risk of uncorrected interpretation of the results.

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## KEYWORDS

antidoping analysis, athletes biological passport, confounding factors, miconazole, steroidal module

## 1 | INTRODUCTION

Azole antifungals are synthetic compounds, classified as imidazoles (e.g., ketoconazole and miconazole) and triazoles (e.g., itraconazole and fluconazole) depending on the number of nitrogen atoms in the azole ring.<sup>1,2</sup> These agents are the most used and studied antifungals; their primary mechanism of action consists in preventing the synthesis of ergosterol, a major component of fungal cell membranes, by inhibiting in a competitive manner the lanosterol 14 $\alpha$ -demethylase, a P450-dependent enzyme involved in the conversion of lanosterol to ergosterol.<sup>1-5</sup> Unfortunately, these agents are not specific for the fungal enzymes. Indeed, they can alter the biotransformation and conjugation metabolic reactions also in humans, with effects on the dynamics of the biosynthetic pathways of numerous biologically active substances, including steroids.<sup>6-18</sup> Different studies reported (i) the ability of ketoconazole to interact with both the 17 $\alpha$ -hydroxylase and 17,20-lyase activity of cytochrome P450c17, the enzyme that converts pregnenolone to dehydroepiandrosterone and progesterone to androstenedione in the human testes, ovaries, and adrenal glands<sup>6-8</sup>; (ii) the inhibitory effect of several triazole antifungals on the CYP19, responsible for the conversion of testosterone to estradiol, and androstenedione to estrone, due to their structural homology with several aromatase inhibitors, such as letrozole and anastrozole<sup>9,10</sup>; and (iii) the strong inhibitory effect of antifungals on several hepatic CYP450 isoforms (e.g., CYP3A4) and uridine 5'-diphosphoglucuronosyltransferases (UGTs) involved in the biotransformation pathways of steroids.<sup>11-18</sup>

In the doping control field, the alteration of the natural fluctuation of several endogenous steroids (currently: testosterone [T], epitestosterone [E], androsterone [A], etiocholanolone [Etio], 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol [5 $\alpha$ Adiol], 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol [5 $\beta$ Adiol], T/E, A/T, A/Etio, 5 $\alpha$ Adiol/5 $\beta$ Adiol, and 5 $\alpha$ Adiol/E) is considered, since the 1980s, a screening index of the intake of testosterone-related compounds<sup>19</sup>; consequently, any natural or synthetic substance capable of affecting the physiological levels of the target endogenous steroids might lead to a wrong interpretation of the results. For the above reason in 2016, antifungals were included by the World Anti-Doping Agency (WADA) in the Technical Document TDEAAS "Endogenous Anabolic Androgenic Steroids—Measurement and Reporting,"<sup>20,21</sup> together with other exogenous compounds (e.g., 5 $\alpha$ -reductase inhibitors, alcohol, and bacterial contamination) or physiopathologic confounding factors (e.g., pregnancy, hormonal dysfunction, and genetic polymorphism).<sup>22-29</sup> The WADA TDEAAS aims at harmonizing the approaches to the measurement and reporting of endogenous anabolic androgenic steroids (EAAS) in urine samples. In detail, the technical document reports the markers of the steroid profile, that is, T, E, A, Etio, 5 $\alpha$ Adiol, 5 $\beta$ Adiol, T/E, A/T,

## HIGHLIGHTS

- The effects of miconazole on the key parameters of the steroid profile were investigated.
- Oral and buccal administration of miconazole affect the levels of endogenous steroids.
- No effects were observed after topical (dermal) administration.
- Miconazole alters the rate of the deglucuronidation step, performed with  $\beta$ -glucuronidase.
- The effect starts to be significant at a miconazole urinary concentration greater than 500 ng/ml.

A/Etio, 5 $\alpha$ Adiol/5 $\beta$ Adiol, and 5 $\alpha$ Adiol/E, the respective population normality ranges, and how the measurements must be carried out.<sup>21</sup> The urinary concentrations of the target endogenous steroids are obtained by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS), and the data registered are then integrated into a Bayesian adaptive model, with the aim of defining the specific profile of each athlete (steroidal module of the Athletes Biological Passport [ABP]) and of revealing values out of the range of normality.<sup>30-35</sup> Data out of the range of an individual athlete's profile constitute an atypical result (Atypical Passport Finding), imposing the activation of confirmation analyses based on both GC-MS/MS and isotopic ratio mass spectrometry (GC-IRMS).<sup>21,36</sup> In this scenario, it became evident the necessity for a deeper knowledge of the exogenous and/or endogenous factors capable of generating atypical results.

We have already evaluated the effects of ketoconazole and miconazole administration on the urinary levels of the parameters of the steroid profile,<sup>37</sup> confirming the results reported in the literature for ketoconazole and showing that also miconazole is able to alter significantly the physiological fluctuations of some endogenous steroids.<sup>37</sup> This research aimed to estimate more accurately the extent of the above effect, to assess the actual impact of miconazole on the analytical strategies currently followed to detect doping by testosterone-related steroids. Urine samples collected from four male *Caucasian* volunteers before, during, and after either systemic (i.e., oral and buccal) or topical (i.e., dermal) treatment with therapeutic doses of miconazole were analyzed to characterize the baseline individual circadian and longitudinal variability profiles of the markers of the steroid profile in the presence and in the absence of the drug. This set of experiments was aimed to verify whether (i) the administration of miconazole could affect the urinary steroid profile and (ii) these changes were dependent on the dosage and/or route of administration. In parallel, the excretion profile of miconazole was also registered

after a single therapeutic dose, to establish the range of concentration responsible for the measured effects.

## 2 | EXPERIMENTAL

### 2.1 | Chemicals and reagents

T, E, and A were from Sigma-Aldrich (Milano, Italy). Etio, 5 $\alpha$ Adiol, and 5 $\beta$ Adiol were purchased from Steraloids (Newport, RI, USA); deuterated standards (testosterone-d3 [T-d3], epitestosterone-d3 [E-d3], 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol-d3 [5 $\alpha$ Adiol-d3], 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol-d5 [5 $\beta$ Adiol-d5], androsterone glucuronide-d4 [AG-d4], etiocholanolone-d5 [Etio-d5]) were obtained from the National Measurement Institute (NMI, Pymble, Australia). Miconazole nitrate and deuterated miconazole nitrate (miconazole-d5) were purchased from Toronto Research Chemicals (TRC, North York, Canada).

The derivatizing agent was a mixture of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA)/mercaptoethanol/ammonium iodide (NH<sub>4</sub>I) (1000:6:4 V/V/w) stored in screw-cap vials at 4°C for a maximum of 2 weeks. MSTFA was supplied by Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany); NH<sub>4</sub>I and mercaptoethanol were from Sigma-Aldrich (Milano, Italy). All chemicals (potassium carbonate, sodium phosphate, sodium hydrogen phosphate, sodium hydrogen carbonate, *tert*-butylmethylether, formic acid, ammonium formate, methanol, and acetonitrile) were purchased from Sigma-Aldrich (Milano, Italy). The ultra-purified water used was of Milli-Q-grade (Millipore Italia, Vimodrone, Milano, Italy).

The enzyme  $\beta$ -glucuronidase (type *Escherichia coli* K12, specific activity [37°C, 4-NP-glucuronide]:  $\geq 140$ -U/mg protein) used for the enzymatic hydrolysis of glucurono-conjugates was purchased from Roche Diagnostic (Mannheim, Germany).

Mixed-mode solid-phase extraction cartridges (Oasis<sup>®</sup> MCX, 30 mg, 30- $\mu$ m particles, 1 ml) were purchased from Waters (Milano, Italy).

Stock solutions of all the compounds under investigation and of the internal standards were prepared in methanol at a concentration of 1 mg/ml and 1  $\mu$ g/ml and stored in screw-cap vials at -20°C.

### 2.2 | Urine samples

The administration studies were approved by the local ethical committee (Comitato Etico Lazio 1, Approval Code: Prot. 1055/2014). Each subject was previously informed about the aim of the project, undersigning a written informed consent allowing the use of urines for research purposes. Volunteers were medically examined to ensure the absence of diseases. They were asked about the use of drugs, alcohol, and diet habits, including in the study only those subjects that were not taking any other drugs that could alter the physiological fluctuation of EAAS. In all treatments, recommended therapeutic dosages were used.

Urine samples were obtained from four male *Caucasian* subjects (Subjects 1, 2, 3, and 4; age 35, 25, 24, and 45 years old; and body weight 70, 80, 72, and 81 kg, respectively) in treatment with miconazole by different routes.

Multiple urine samples were collected at different times of the day: (i) for at least 5 days before the administration of miconazole, to study the physiological circadian fluctuations of each subject selected and to establish basal ranges for each marker of the steroid profile considered in the WADA technical document TDEAAS<sup>21</sup>; (ii) for at least 5 days during the treatment with miconazole, to register the alterations, if any, of the physiological circadian fluctuations of the markers of the steroid profile; and (iii) for at least 2 days after the end of the treatment, to evaluate the time necessary to re-stabilize the physiological circadian fluctuations of the markers of the steroid profile considered.

Urine samples were collected in sterile conditions every 3 h, starting with the morning urine (usually around 8:00 a.m.) and finishing with the urine in the evening (usually around 8:00 p.m.). Samples were then anonymized and stored at -20°C until analysis.

To evaluate the effects of miconazole administration on the physiological fluctuation of the markers of the steroid profile, the four subjects selected received oral, buccal, and dermatological treatments of miconazole, with a washout period of 30 days between treatments.

*Oral administration (tablets)*: 500 mg (Nizacol, 500-mg tablets, New Research S.r.l.) once a day (at 8.00 a.m.) for at least 1 week.

*Buccal administration (gel)*: 50 mg (Daktarin, 20-mg/g oral gel), five times a day (at 8.00 a.m., 11.00 a.m., 2.00 p.m., 5.00 p.m., and 8.00 p.m.) for at least 1 week.

*Dermal administration (cream)*: 10 mg (Daktarin dermatologico, 2% cream), three times a day (at 8.00 a.m., 2.00 p.m., and 8.00 p.m.) for at least 1 week.

To define the urinary excretion profile of miconazole after a single therapeutic administration, the four subjects selected received oral (500 mg), buccal (50 mg), and dermatological (10 mg) treatments with a single therapeutic dose of miconazole administered at 11.00 a.m., with a washout period of 30 days between treatments. Urines were collected every 3 hours.

### 2.3 | Sample preparation

#### 2.3.1 | Steroid profile

Sample preparation was performed following the validated procedure used at the WADA-accredited laboratory of Rome, for the detection of the EAAS in human urine.<sup>28,38</sup> Briefly, 0.75 ml of phosphate buffer (0.8 M, pH 7.4), 50  $\mu$ l of internal standards mixture (constituted by a pool of deuterated steroids: 100 ng/ml of T-d3, 25 ng/ml of E-d3, 50 ng/ml of 5 $\alpha$ Adiol-d3 and 5 $\beta$ Adiol-d5, and 2000 ng/ml of AG-d4 and Etio-d5), and 30  $\mu$ l of  $\beta$ -glucuronidase from *E. coli* were added to 2 ml of urine. The sample was then incubated for 1 h at 55°C; then, 0.5 ml of carbonate/bicarbonate buffer (1 M, pH 9) was added, and liquid/liquid extraction was carried out with 5 ml of *tert*-butylmethyl

ether for 5 min on a mechanical shaker. Samples were then centrifuged, and the organic layer was transferred to a 10-ml tube and evaporated to dryness under nitrogen stream at 75°C. The residue was reconstituted in 50 µl of the derivatizing mixture (MSTFA/mercaptoethanol/NH<sub>4</sub>I [1000:6:4 V/V/w]), and the samples were maintained at 75°C for 30 min. An aliquot of 2 µl was finally injected into GC-MS/MS system.

### 2.3.2 | Miconazole excretion profile

Sample preparation was performed following the validated procedure used at the WADA-accredited antidoping laboratory of Rome, for the detection of the different classes of prohibited compounds in human urine.<sup>28</sup> Briefly, an aliquot of 1 ml of urine was fortified with 25 µl of internal standard (ISTD, miconazole deuterated final concentration 500 ng/ml) and centrifuged (4000 g for 2 min) to separate any precipitate from the liquid phase. The sample was then incubated for 1 h at 50°C using 30 µl of β-glucuronidase and 200 µl of phosphate buffer (0.8 M, pH 7.4). Urine samples were then acidified (pH lower than 5) and purified by using the Oasis<sup>®</sup> MCX cartridges, previously conditioned with 1 ml of methanol and 1 ml of ultra-purified water. The cartridges were then washed with 1 ml of water/methanol (80/20). The compounds of interest were finally eluted using 1 ml of methanol/formic acid (95/5) containing 150 mM of ammonium formate. The organic solvent was evaporated at 40°C, and the residue resolved in 100 µl of mobile phase (initial composition). An aliquot of 5 µl was then injected into the liquid chromatography-mass spectrometry (LC-MS) systems.

## 2.4 | Instrumental conditions

### 2.4.1 | GC-MS/MS conditions

According to the validated procedure carried out in the WADA-accredited antidoping laboratory of Rome for the detection of endogenous androgenic anabolic steroids,<sup>28,38</sup> the measurements were performed on an Agilent 7890A/7000 GC-MS/MS system (Agilent Technologies, Milano, Italy), in electron impact ionization (70 eV), using a HP-1 17-m fused silica capillary column cross-linked methyl silicone, ID 0.20 mm, film thickness 0.11 µm (HP-1, Agilent Technologies, Milano, Italy). The chromatographic conditions were as follows: the carrier gas, helium at the flow rate of 1 ml/min; split ratio 1/20; the temperature program, 180°C (4.5-min hold), 3°C/min to 230°C, 20°C/min to 290°C, and 30°C/min to 320°C; the transfer line and the injection temperature were set at 280°C.

The acquisition was carried out in multiple-reaction monitoring (MRM). Calibration samples were injected every 15 samples during the sequence. The hydrolysis completeness was checked considering the area ratio A-d4/Etio-d5 (acceptance criteria fixed at 0.9), whereas the derivatization was evaluated considering the ratio androsterone mono-TMS/androsterone bis-TMS.

### 2.4.2 | LC-MS/MS conditions

According to the accredited analytical method currently adopted by WADA-accredited antidoping laboratory of Rome for the detection of different classes of prohibited substances,<sup>28,39</sup> 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, Cernusco sul Naviglio, Milano, Italy). Reversed-phase LC was performed using a Supelco Discovery C18 column (2.1 × 150 mm, 5 µm). The solvents used were water containing 0.1% (v/v) formic acid (eluent A) and acetonitrile containing 0.1% (v/v) formic acid (eluent B). The gradient program starts at 10% of eluent B and increases to 60% of eluent B in 7 min and after 5 min to 100% of eluent B. The column was flushed for 3 min at 100% of eluent B and finally re-equilibrated at 10% of eluent B for 2 min. The flow rate was set at 250 µl/min. The injection volume was 10 µl. All ESI-MS/MS experiments were performed using an Applied Biosystems (Applera Italia, Monza, Italy) API4000 triple-quadrupole instrument with positive electrospray ionization. The ion source was operated at 500°C, whereas the applied capillary and declustering voltages were set at 5500 and 60 V, respectively. MS/MS experiments were performed 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 Analytica Milano, Italy). The data were acquired using selected reaction monitoring. All aspects of instrument control, method setup parameters, sample injection, and sequence operation were controlled by the Applied Biosystems Analyst software.

## 2.5 | Method validation for the determination of miconazole in urine samples

To evaluate the suitability of the LC-MS procedure for an accurate quantitation of miconazole, the analytical method currently routinely used in our laboratory was validated, according to the ISO 17025 and the WADA guidelines,<sup>40-42</sup> in terms of specificity, sensitivity (limit of detection and identification [LOD and LOI]), matrix effect, recovery, limit of quantification (LOQ), linearity, accuracy, and precision.

Calibration and quality control (QC) samples were prepared by adding the appropriate volume of stock solutions of miconazole (prepared in methanol at 10 µg/ml and 1 mg/ml) to blank urines. Calibration samples were prepared at seven concentration levels: 50, 100, 300, 500, 1000, 3000, and 5000 ng/ml; QC samples were instead prepared at three concentration levels 100, 500, and 5000 ng/ml.

The linearity was evaluated considering the coefficient of determination ( $r^2$ ). LOD, LOI, and quantitation (LOQ) were defined as the concentrations yielding a signal to noise higher than 3 and 10, respectively.

The accuracy and precision of the method were obtained by the analysis of QC samples at three different concentrations. Intraday precision was expressed as the relative standard deviation (RSD) (%) of



the estimated concentrations obtained for six replicates of the QC samples at the three different concentrations analyzed the same day. Intermediate precision is given as the RSD (%) of the estimated concentrations obtained for three replicates of the QC samples along five different days. Accuracy was instead evaluated by the relative error (%) in the estimation of the concentration for the QC samples.

The recovery was calculated by the analysis of five replicates of negative urine spiked at three different concentrations and five replicates of negative urine sample to which the same concentration of miconazole was added after extraction. The ratios of the peak areas between miconazole and the ISTD obtained from the extracted spiked samples were compared with the ratios obtained in the samples in which miconazole was added to extracted blank urine samples (representing 100% of recovery).

Matrix effect was studied by the analysis of different blank urine samples spiked at the LOQ concentration and the ISTD after the SPE extraction. Areas of miconazole and the ISTD were compared with those obtained after the analysis of a water sample spiked with miconazole after the SPE extraction.

Finally, to assess the possibility to analyze samples over the linear range, the high-level QC sample was diluted with negative urine, and the concentrations obtained after correction of the dilution were compared with those obtained for the high QC sample without dilution.

## 2.6 | Data analysis

The parameters of the steroid profile considered were T, E, A, Etio, 5 $\alpha$ Adiol, 5 $\beta$ Adiol, T/E, A/Etio, 5 $\alpha$ Adiol/5 $\beta$ Adiol, A/T, and 5 $\alpha$ Adiol/E.

The values of the urinary concentration of each target compound were calculated by the peak areas of the detected signals relative to their deuterated internal standard, using daily calibration samples obtained adding to 2 ml of the artificial urine (prepared following the protocol described by Leinonen et al.<sup>43</sup>) (i) 50 ng/ml of 5 $\alpha$ Adiol and 5 $\beta$ Adiol, 20 ng/ml of T, 10 ng/ml of E, and 2000 ng/ml of A and Etio or (ii) 200 ng/ml of 5 $\alpha$ Adiol and 5 $\beta$ Adiol, 100 ng/ml of T, 50 ng/ml of E, and 8000 ng/ml of A and Etio.

The measured concentrations of the markers of the steroid profile were reported versus the collection time to evaluate their physiological fluctuation. All data were normalized for the specific gravity applying the following formula:

$$\text{Concentration (ng/ml)} \times (1.020 - 1) / (\text{specific gravity sample} - 1).^{44}$$

All samples with a value of the specific gravity below 1.005 or above 1.035 were excluded.

To evaluate the potential alteration of miconazole on the circadian fluctuation of the markers of the steroid profile, boxplots representing the statistical distribution of the urinary levels of the endogenous steroids before, during, and after drug administration were reported. The significance of the potential alterations was calculated using the Mann–Whitney test, whereas the statistical significance was set at  $P < 0.01$ .

The values of the urinary concentration of miconazole were calculated by the peak areas of the detected signals relative to its deuterated internal standard, using daily calibration samples obtained by adding to negative urine 100, 500, or 5000 ng/ml of miconazole.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Determination of the circadian variation of the markers of the steroid profile

To study the physiological individual circadian and longitudinal fluctuations of the target compounds described in the TDEAAS and to establish basal ranges for each subject considered, urine samples were collected before the treatment with miconazole for at least 5 days. Urines were analyzed using the validated protocol currently adopted by our laboratory to determine the markers of the steroid profile.<sup>29,39</sup> The urinary concentration of each endogenous steroid selected was then plotted versus the urine collection time.

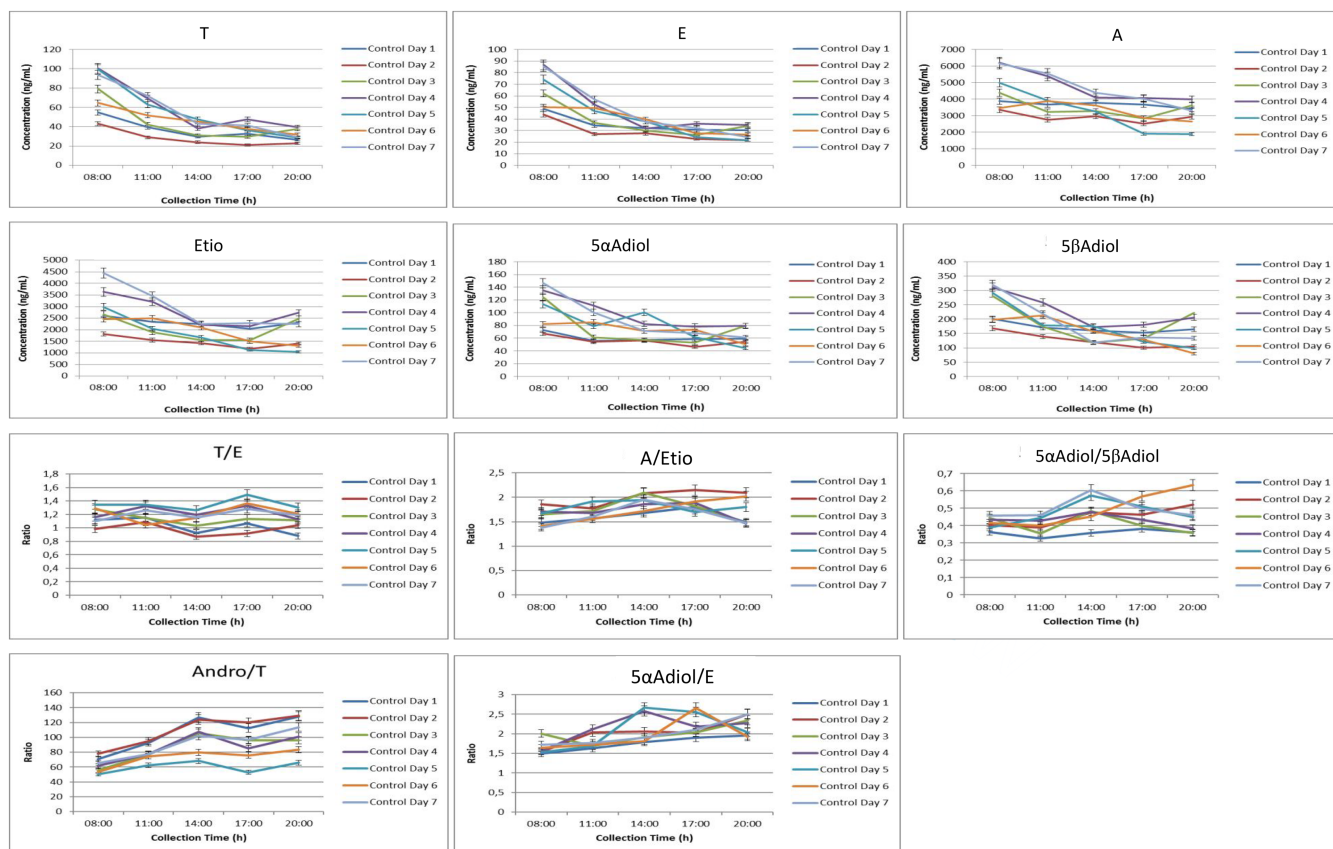
Figure 1 reports the results of the longitudinal and circadian study for Subject 1; as it can be seen, not significant variability of the urinary concentration values from urine samples collected from the same subject at the same time was registered; on the contrary, the concentration values of each steroid showed marked interindividual differences, confirming the results obtained in previous investigations.<sup>45–50</sup> Nonetheless, the circadian trend was qualitatively the same for all subjects: the excretion was maximal in the morning and decreased significantly along the day, especially for T and E. Regarding T/E, A/Etio, and 5 $\alpha$ Adiol/5 $\beta$ Adiol ratios, no significant differences were observed along the day and between days, confirming the data reported in the literature<sup>45</sup> supporting the appropriate selection of markers for the “steroidal module” of the ABP.<sup>30–35</sup> Similar results were registered for Subjects 2, 3, and 4 (data not shown).

### 3.2 | Evaluation of the effects of the administration by different routes and doses of miconazole on the fluctuation of the markers of the steroid profile

As already outlined before, the effects of miconazole on the individual circadian fluctuation of the parameters of the “steroid profile” described in the WADA Technical Document TDEAAS were evaluated after oral, buccal, and dermal administration of therapeutic doses of drugs containing miconazole.

Results were expressed in terms of median and first and third quartiles, and extreme values are represented by boxplots. The significance of the potential alterations was calculated using the Mann–Whitney test, whereas the statistical significance was set at  $P < 0.01$ .

Figure 2a–c reports the boxplot representing the distribution of the measurements obtained analyzing the urines collected from Subject 1 before, during, and after oral, buccal, or dermal administration



**FIGURE 1** Circadian fluctuations of the parameters of the steroid profile specified in the WADA TDEAAS for Subject 1 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

of miconazole. As it can be noticed, (i) after oral administration of 500 mg of miconazole once a day for 5 days, significant alteration was registered for the  $5\alpha\text{Adiol}/5\beta\text{Adiol}$  ratio, A/Etio ratio, A, Etio, A/T ratio, and  $5\alpha\text{Adiol}/\text{E}$  ratio, whereas moderate variations were registered for  $5\alpha\text{Adiol}$  and  $5\beta\text{Adiol}$  (see Figure 2a); (ii) after buccal administration of 50 mg of miconazole five times a day for 5 days, significant alteration of the urinary levels of  $5\alpha\text{Adiol}/5\beta\text{Adiol}$  ratio, A/Etio ratio, A, Etio, A/T ratio,  $5\alpha\text{Adiol}/\text{E}$ ,  $5\alpha\text{Adiol}$ , and  $5\beta\text{Adiol}$  was measured (see Figure 2b); and finally, (iii) after dermal administration of 10 mg of miconazole three times a day for 5 days, no significant alteration was registered in the urinary levels of the parameters of the steroid profile, with the exception of  $5\beta\text{Adiol}$  and the  $5\alpha\text{Adiol}/5\beta\text{Adiol}$  ratio for which a moderate alteration was measured (see Figure 2c).

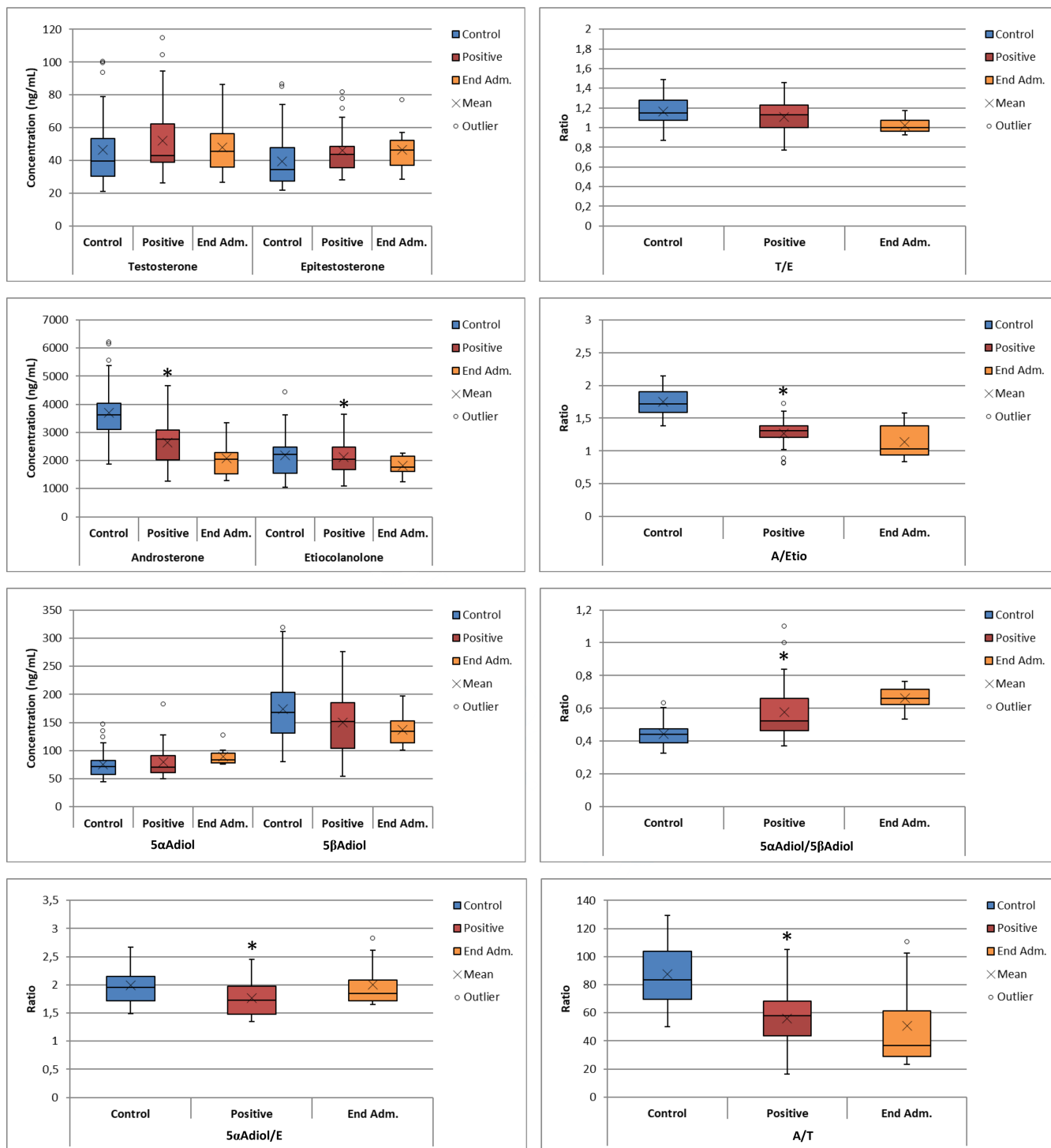
The alterations on the physiological fluctuations of the parameters of the steroid profile are visible after few hours from drug administration; furthermore, after repeated doses, several differences were noticed indicating that accumulation of miconazole occurs especially in the case of buccal administration. Similar results were also obtained for Subjects 2, 3, and 4 (data not shown).

Figure 3 reports the values of A-d4/Etio-d5 ratio (used to evaluate the completeness of the enzymatic hydrolysis during the sample preparation process) expressed in percentage versus the collection time, in the urine samples collected before and during the oral or buccal administration of miconazole for Subject 1. The A-d4/Etio-d5 ratio

ranged between 30% and 70% in the urines collected after oral administration and between 20% and 70% in the urines collected after buccal administration, indicating that the enzymatic hydrolysis in the samples collected after oral and buccal treatments with miconazole was not complete. The dermal application, instead, does not impact the hydrolysis completeness; indeed, the A-d4/Etio-d5 ratio is in the range of 95% to 100%. It follows that the alteration on the markers of the steroid profile registered after oral and buccal administration of miconazole could be mainly explained by the alteration of the kinetics/efficacy of deglucuronidation of the endogenous steroids by  $\beta$ -glucuronidase by miconazole.<sup>51</sup> Similar results were registered for Subjects 2, 3, and 4 (data not shown).

To confirm the above observation and to propose the countermeasures to adopt in case of presence of miconazole in the urine samples collected in the occasion of doping control tests, urines collected before and after oral, buccal, or dermal administration were incubated using either different incubation times (0, 1, 2, 4, 8, and 24 h) or different amount of  $\beta$ -glucuronidase solution (0, 30, 60, 90, 180, 300, and 450  $\mu\text{l}$ ). Figure 4a–c reports the results obtained in one of the urines collected before drug administration (without miconazole) and in the urine collected after 9 h from the first oral administration of miconazole (Figure 4a), 24 h from the first buccal administration of miconazole (Figure 4b), or 9 h from the first dermal administration of miconazole (with miconazole) (Figure 4c). For the sample collected

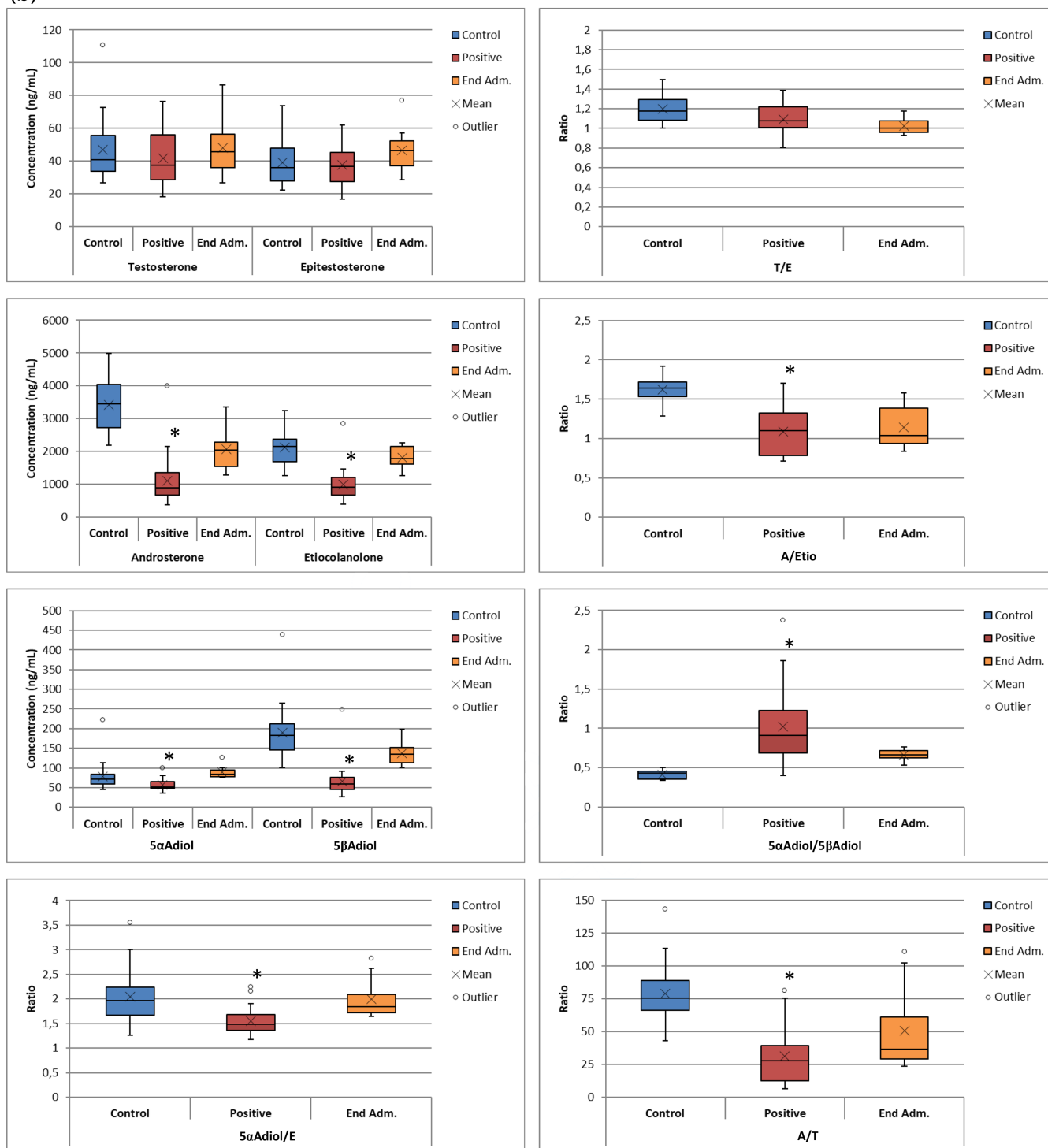
(a)



\* P&lt;0.01

**FIGURE 2** (a) Boxplot representing the statistical distribution of the markers of the steroid profile in the urines collected from Subject 1 before during and after oral administration of 500 mg of miconazole once a day. The number of measurements included in each box plot is 25. (b) Boxplot representing the statistical distribution of the markers of the steroid profile in the urines collected from Subject 1 before during and after buccal administration of 50 mg of miconazole five times a day. The number of measurements included in each box plot is 25. (c) Boxplot representing the statistical distribution of the markers of the steroid profile in the urines collected from Subject 1 before during and after dermal administration of 10 mg of miconazole three times a day. The number of measurements included in each box plot is 25 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

(b)



\* P<0.01

FIGURE 2 (Continued)

after oral or buccal administration, increasing either the incubation time or the amount of enzyme, the levels of the markers of the steroid profile and the ratio A-d4/Etio-d5 increased significantly reaching maximum values after 8 h of incubation and/or by using 180  $\mu$ l of the enzyme solution (see again Figure 4a,b). No differences were instead

registered in the case of dermal administration, being the enzymatic hydrolysis already complete with the standard procedure (see again Figure 4c). These countermeasures were then applied to all the samples collected after oral and buccal treatments with therapeutic doses of miconazole. The results obtained showed that using both 8 h of

(c)

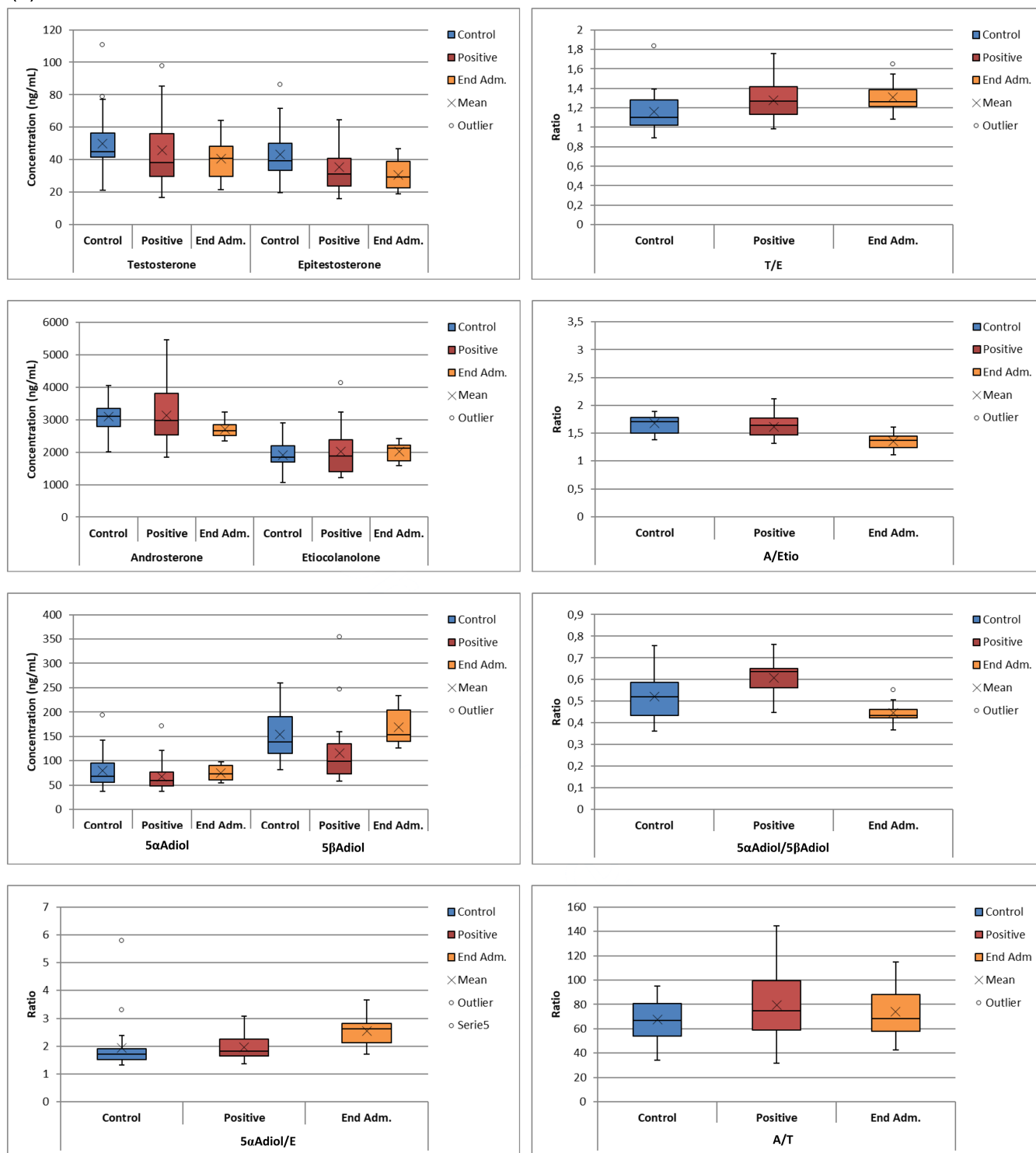
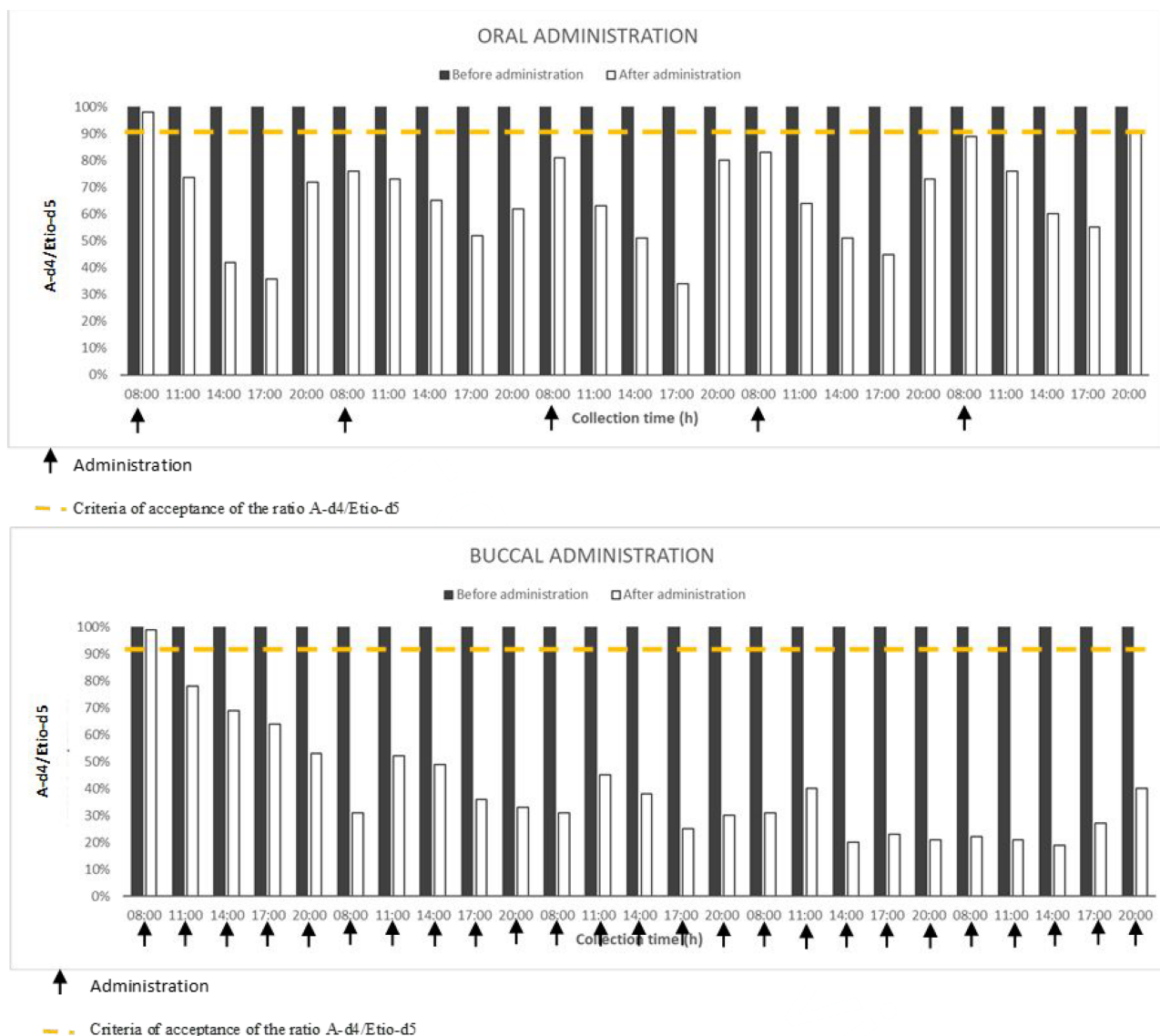


FIGURE 2 (Continued)

incubation and 180  $\mu$ l of the enzyme, it is possible to obtain an increase of the measured concentration of the markers of the steroid profile and of the ratio A-d4/Etio-d5 in all samples analyzed independently from the miconazole levels (see the results for Subject 1 after oral administration reported in Figure 5; similar results were obtained after buccal administration and for the other subjects studied).

### 3.3 | Excretion profile of miconazole

The observations reported before outline that the entity of the enzymatic hydrolysis inhibition could be linked to the urinary levels of miconazole. Indeed, the enzymatic hydrolysis was not complete in the samples collected after oral and buccal treatments with miconazole.



**FIGURE 3** Enzymatic hydrolysis completeness (A-d4/Etio-d5) before miconazole administration (without miconazole, black bar) and during oral or buccal administration of miconazole (with miconazole, white bar) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

On the contrary, no effect on the kinetics/efficacy of the enzymatic hydrolysis was observed during dermal administration, indicating that this phenomenon might be dependent on miconazole urinary concentration. To evaluate in more detail this observation, the completeness of enzymatic hydrolysis was correlated to the urinary concentration of miconazole by conducting excretion studies with a single dose of the drug.

The analytical method used to determine miconazole in urine was validated in terms of specificity, sensitivity, LOQ, linearity, recovery, matrix effect, accuracy, and precision.

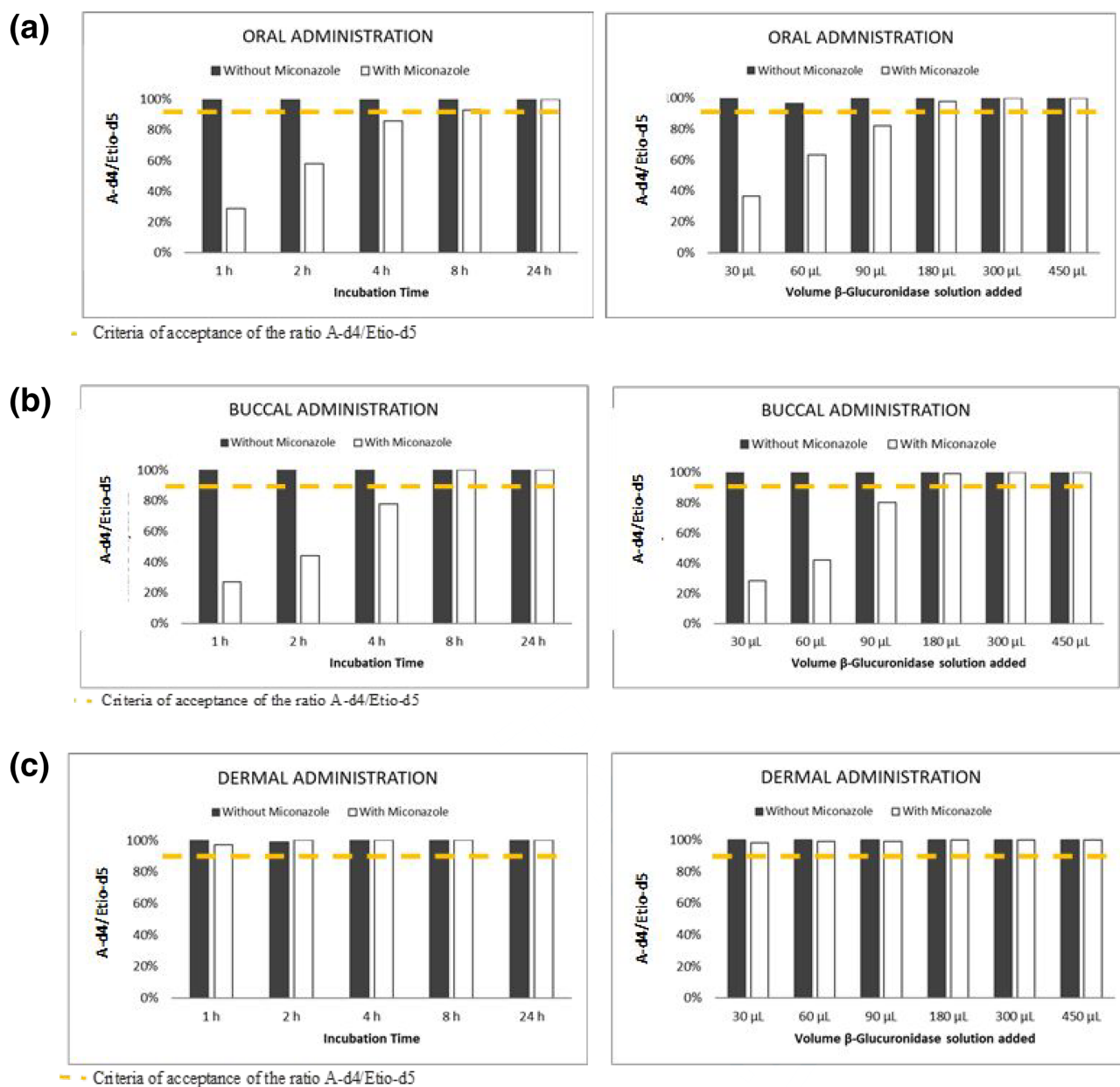
No significant interferences were observed at the retention times of miconazole and ISTD in the chromatograms of the corresponding ion transitions in negative urine samples. LOD, LOI, and LOQ were 5, 10, and 50 ng/ml, respectively. The analytical method developed

was shown to be linear in the studied range of concentrations (50–5000 ng/ml) with  $r^2$  exceeding 0.95.

Recoveries greater than 65% were measured; no correlation was observed between extraction recoveries and concentrations. The matrix effect was found to be lower than 30%.

Regarding intraday and intermediate precisions, RSDs were below 20% for the lowest QC samples and below 15% for the other two QC samples. As for accuracy, relative errors were better than 20% for the lowest QC samples and better than 15% for the other two QC samples. Finally, there was no significant difference between the concentrations of the highest QC samples and the concentrations detected in the same QC samples diluted with water and corrected for dilution, indicating that the analysis of samples over the linear range by dilution is feasible.

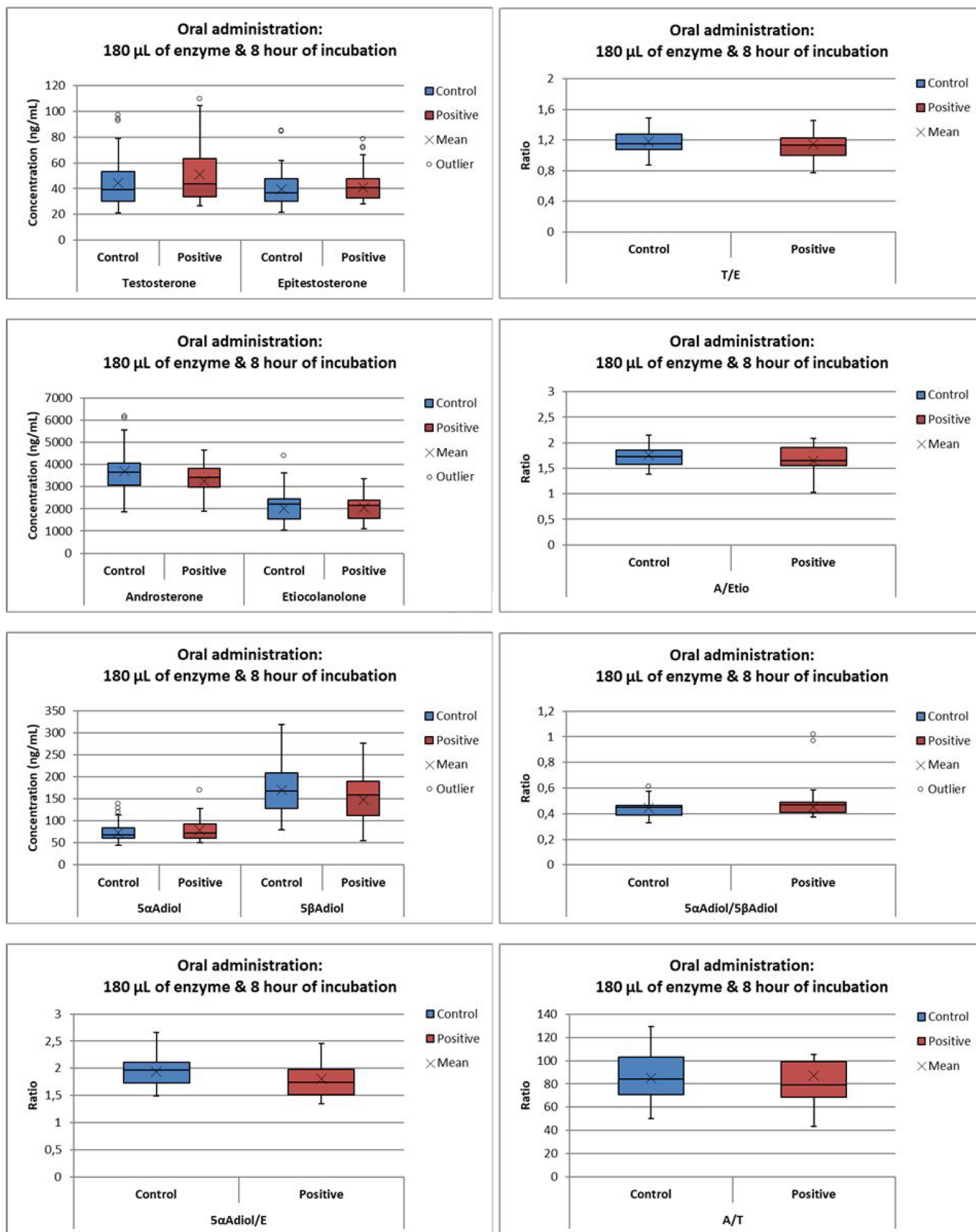




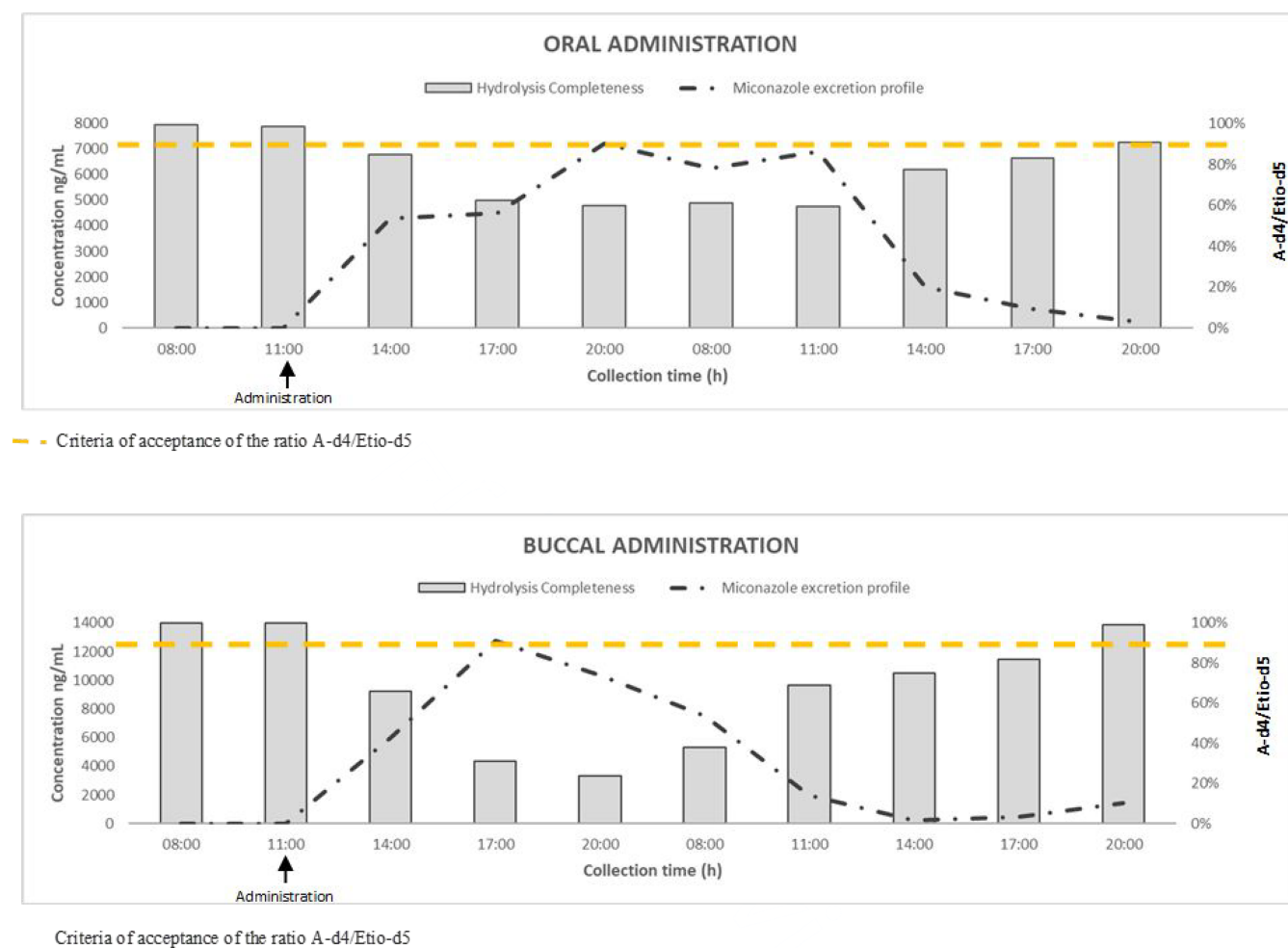
**FIGURE 4** Evaluation of the enzymatic hydrolysis completeness (A-d4/Etio-d5) using different incubation time and enzyme solution volume in one urine samples collected before miconazole administration (without miconazole, black bar, gray line) and in one urine sample collected (a) after 9 h from the first oral administration of miconazole (with miconazole, white bar, black line); (b) after 24 h from the first buccal administration of miconazole (with miconazole, white bar, black line); and (c) after 9 h from the first dermal administration of miconazole (with miconazole, white bar, black line) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

According to these results, the developed method was used for an accurate quantitation of miconazole. In detail, to define miconazole excretion profile, urine samples collected after a single administration of miconazole by different routes were analyzed by using the validated procedure. Figure 6 reports the values of A-d4/Etio-d5 ratio (expressed in percentage versus the collection time) measured after oral and buccal administration of miconazole overlapped with the excretion profile of miconazole in Subject 1. The excretion profile of miconazole varied depending on the

administration route; the excretion rate was much higher after buccal than oral administration. Miconazole reached the maximum of excretion after 6 h from buccal administration reaching levels of 12.5  $\mu\text{g/ml}$  and after 9 h from the oral administration reaching levels of 7  $\mu\text{g/ml}$ . Miconazole was found in urine for more than 48 h after both oral and buccal administration at levels ranging from 0.1 to 7 and from 0.1 to 12.5  $\mu\text{g/ml}$ , respectively. On the contrary, after dermal administration, the levels of miconazole were below the LOQ (50 ng/ml).



**FIGURE 5** Boxplot representing the statistical distribution of the markers of the steroid profile in the urines collected from Subject 1 before and after oral administration of 500 mg of miconazole once a day. Enzymatic hydrolysis was performed with 180 μL of β-glucuronidase for 8 h at 55°C. The number of measurements included in each box plot is 25 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 6** Excretion profile of miconazole after oral or buccal administration of a single therapeutic dose of miconazole received at 11.00 a.m. overlapped with enzymatic hydrolysis completeness (A-d4/Etio-d5) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Regarding the A-d4/Etio-d5 ratio, it started to decrease after 3 h from miconazole administration reaching minimum values of 60% after 9 h from oral administration and 20% after 6 h from buccal administration; its value came back to 90% to 95% after 30 h from both oral and buccal administration when miconazole levels were lower than 1 µg/ml and higher than 0.5 µg/ml.

## 4 | CONCLUSIONS

Our results showed that after oral and buccal administration of miconazole, the physiological fluctuations of the markers of the steroid profile currently monitored by the WADA-accredited antidoping laboratories to screen the misuse of testosterone-related compounds were significantly altered, whereas no effects were registered after topical administration, as a consequence of the low circulating levels of the drug. Indeed, the levels of miconazole after systemic

administration were in the range of 0.1–12.5 µg/ml, whereas after dermal administration, drug concentration was below the LOQ (50 ng/ml). Significant alteration started to be registered at concentrations of miconazole higher than 0.5 µg/ml.

The observed effects can be mainly explained by the effect of miconazole on the kinetics/efficacy of the enzymatic hydrolysis during the sample preparation process; indeed, the increase of both incubation time and amount of β-glucuronidase was demonstrated to be a useful countermeasure in the presence of miconazole to reduce the risk of uncorrected interpretation of the results.

The observations obtained in this study underline the importance to screen for miconazole during the routine analysis carried out by the WADA-accredited antidoping laboratories to decrease the risk of uncorrected interpretation of the analysis results. Indeed, the administration of miconazole might be problematic not only for the interpretation of the steroid profile but also for the determination of synthetic steroids (e.g., 19-norandrosterone)

and other prohibited compounds excreted in urine mainly as glucuronides.

## ACKNOWLEDGEMENT

This project has been supported by research grants of the World Anti-Doping Agency (project code: 13D14MM and 17D07MM).

## DATA AVAILABILITY STATEMENT

Data available on request from the authors

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**How to cite this article:** Mazzarino M, Comunità F, de la Torre X, Molaioni F, Botrè F. Effects of the administration of miconazole by different routes on the biomarkers of the “steroidal module” of the Athlete Biological Passport. *Drug Test Anal*. 2021;13(10):1712-1726. <https://doi.org/10.1002/dta.3121>