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# RECENT ADVANCES IN DOPING ANALYSIS (12)

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# Validation of the determination of tibolone in human urine and saliva using GC-MS for doping control and clinical studies

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## INTRODUCTION

Tibololone (TIB, Livial®) is a synthetic steroid structurally related to 19-norsteroids, such as noretisterone. Due to its estrogenic potency and androgenic activity, TIB could be used by athletes as doping agent. TIB is quickly metabolized into 3α/3β-hydroxy-TIB by dehydrogenase enzymes in the intestine and the liver[1-3] (Fig 1). These metabolites are weak estrogens and have a half-life of approximately 7 hours. In the endometrium, 3-OH-tibolone is metabolized by 3-OH-dehydrogenase-isomerase to the $\Delta^4$-isomer (7α-methyl-noretisterone), which is a weak to moderate progestagen and androgen; its half-life was not yet estimated[1-3]. Monitoring steroid hormones in saliva rather than in urine, blood spot, or serum specimens has several well-documented advantages for biobehavioral researchers[4-5]. Collection techniques are less invasive than vein punction, affording researchers the ability to conduct repeated sampling over the course of minutes, hours, days, etc. Also saliva is not considered a class II biohazard. Salivary levels of some steroids are believed to accurately represent the biologically active fraction in the blood circulation.

TIMMER *et al.*[1-3] described the difficulty to determine the pharmacokinetic parameters of TIB and it's $\Delta^4$-isomer metabolite in plasma due to it's pre-dose plasma concentration being lower than the limit of quantitation of the technique (0.1ng/mL). The authors reported also that the $C_{max}$ of TIB and $\Delta^4$-isomer are below 1ng/mL. Our goal is to report a simple, rapid and specific method for the extraction of TIB from human saliva and its subsequent analysis using GC-MS. In order to investigate the pharmacokinetic parameters of TIB in saliva and compare the results with those reported in the literature for plasma. Extraction of TIB and it's metabolites from urine was also evaluated for doping control monitoring.

## EXPERIMENTAL

*Sample extraction and derivatization:* 1 mL of a blank saliva was spiked with TIB and 17α-methyl-5α-androstane-3α,17β-diol (IS, 10 ng). 1mL of 0.2M sodium phosphate buffer pH 7.0 and 4mL of TBME:$CH_2Cl_2$ (7:3, v:v) were added (shaken and centrifuged). The organic phases were transferred and evaporated to dryness ($N_2$/40°C). The residues were dried in a desiccator and derivatized with 40μL of MSTFA / TMSim (100:2, v:v) and heated for 20 min at 60°C. *Apparatus and chromatographic conditions*: The analyses were performed with the same conditions and equipments that are used in our lab for steroids doping control analysis[6].

*Assay validation*: A full pre-study validation routine has been performed, including sensitivity, specificity, linearity, accuracy, repeatability and reproducibility.

*Pharmacokinetic study:* A healthy female volunteer with age: 27, body mass: 50kg, height: 1.63cm was selected. Before (0 min) and after a single oral administration of 2.5 mg of Livial® tablet (NV, Organon, Holland, The Netherlands), saliva samples were spit into test tubes immediately before and at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 min following drug administration. Saliva samples were immediately prepared after collection.

*Urine analysis*

The urine of the volunteer involved in pharmacokinetic study in saliva was collected. Urine samples were submitted to a hydrolysis step (*E. coli*) followed by TBME liquid-liquid extraction at pH 9.0[7]. The same derivatization procedure of saliva analysis was used. Rio de Janeiro Federal Hospital Ethics Committee of Clinical Investigation approved the clinical protocol (protocol number: 080/01).

## RESULTS

Representative chromatograms of SIM analysis of the saliva samples spiked with TIB are shown in Fig. 2. There are no chromatographic peaks with S/N>3 interfering with that of TIB or internal standard (IS). TIB and IS were well resolved from each other, with retention times of 6.17 and 5.89 min, respectively. The mass spectrum of bis-OTMS TIB is characterized by the ion m/z 456 (molecular ion), and by ions m/z 441 and 301 (Fig. 3). In human saliva, regression analysis yielded linear correlation ($r^2$ >0.99) over the concentration range (0.1 to 6.0 ng/mL) and equation curve y = 0.0083x + 0.0004. The goodness of fit was evaluated by means of analysis of variance ($F_{test}$, $\alpha = 0.05$). The mean recoveries of TIB were 55.0 ± 20.0% (0.3 ng/mL, n=5), 75.0 ± 4.8% (2.4 ng/mL, n=5), 70.0 ± 10.9% (4.5 ng/mL, n=5). Mean recovery of IS was 72.5 ± 4.8% (30 ng/mL, n = 15). The intra-day repeatability was between 19.4% and 2.7% over the 0.3 and 4.5 ng/mL concentration range of TIB. The present method

was applied to determine the saliva concentrations of TIB during a pharmacokinetic study in a healthy volunteer who orally received 2.5 mg of TIB. The pharmacokinetic parameters for TIB in plasma that could be reliably estimated were $T_{max}$ (1.5 ± 0.7 h) and $C_{max}$ (1.3 ± 0.7 ng/mL)[3] due to the fact that the plasma concentrations of TIB could only be measured in the circulation during the first 6h after administration (2.5 mg)[3]. The $C_{max}$ and $T_{max}$ of TIB observed in saliva were 4ng/mL and 5min, respectively (data not shown). The method was adequate for monitoring saliva concentration profiles of TIB during the 1 h sampling period. It means that saliva could be an alternative matrix to perform clinical studies. The method was also used to elucidate the excretion of TIB and it's metabolites in urine (Fig 4). From the best of our knowledge, this is the first report of TIB metabolites in urine. In this study, 3β-OH-TIB was excreted in a higher amount than the other metabolites (Fig 4).

## CONCLUSION

The developed method proved to be useful and reliable for the determination of TIB in human saliva. The results demonstrate that the pre-treatment procedure is simple, rapid, and specific, avoiding degradation of the drug. This method, validated for concentrations ranging from 0.3 to 6.0ng/mL has a good reproducibility and accuracy and is useful for clinical therapeutic drug monitoring, considering saliva as an alternative matrix to perform clinical studies. The recovery from urine with TBME at pH 9.0 shows that the extraction screening 4B is suitable for the clean-up of TIB and its metabolites from urine, but as the derivatization procedure adopted in screening 4B was not able to derivatize these compounds, they cannot be screened in doping routine work by 4B.

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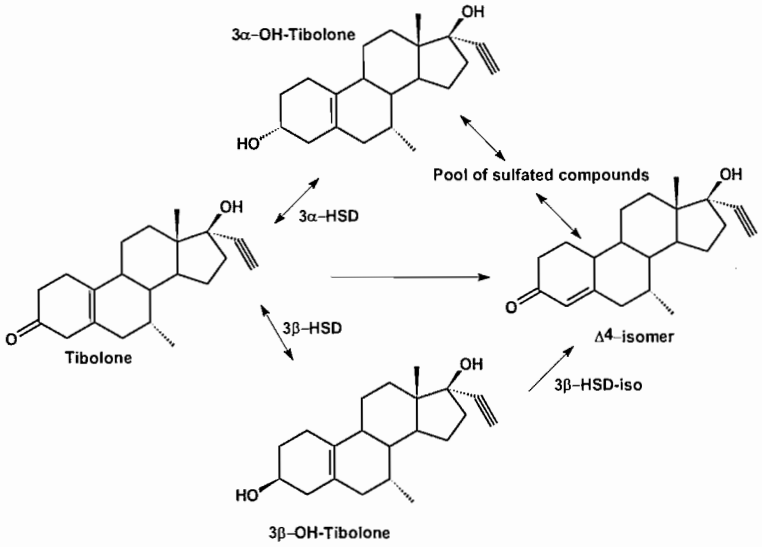


**Fig. 1.** Major pathways of tibolone metabolism in humans. 3α/3β-hydroxysteroid dehydrogenase (HSD), 3βHSD-iso and 3β-hydroxysteroid dehydrogenase/isomerase[3].

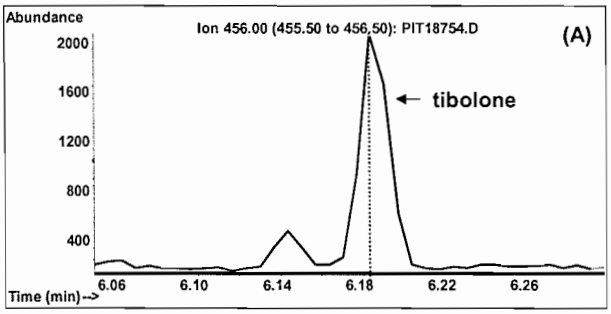


**Fig. 2**. GC/MS ion chromatograms of SIM analysis of bis-O-TMS tibolone (m/z 456) (A) saliva spiked with tibolone (1 ng/mL) and (B) blank saliva sample.

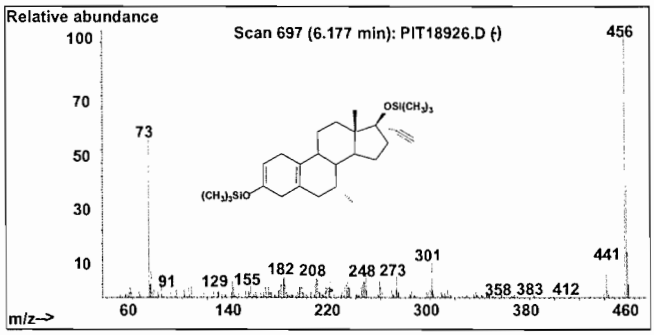


**Fig. 3.** Mass spectrum (GC-MS) of bis-O-TMS tibolone.

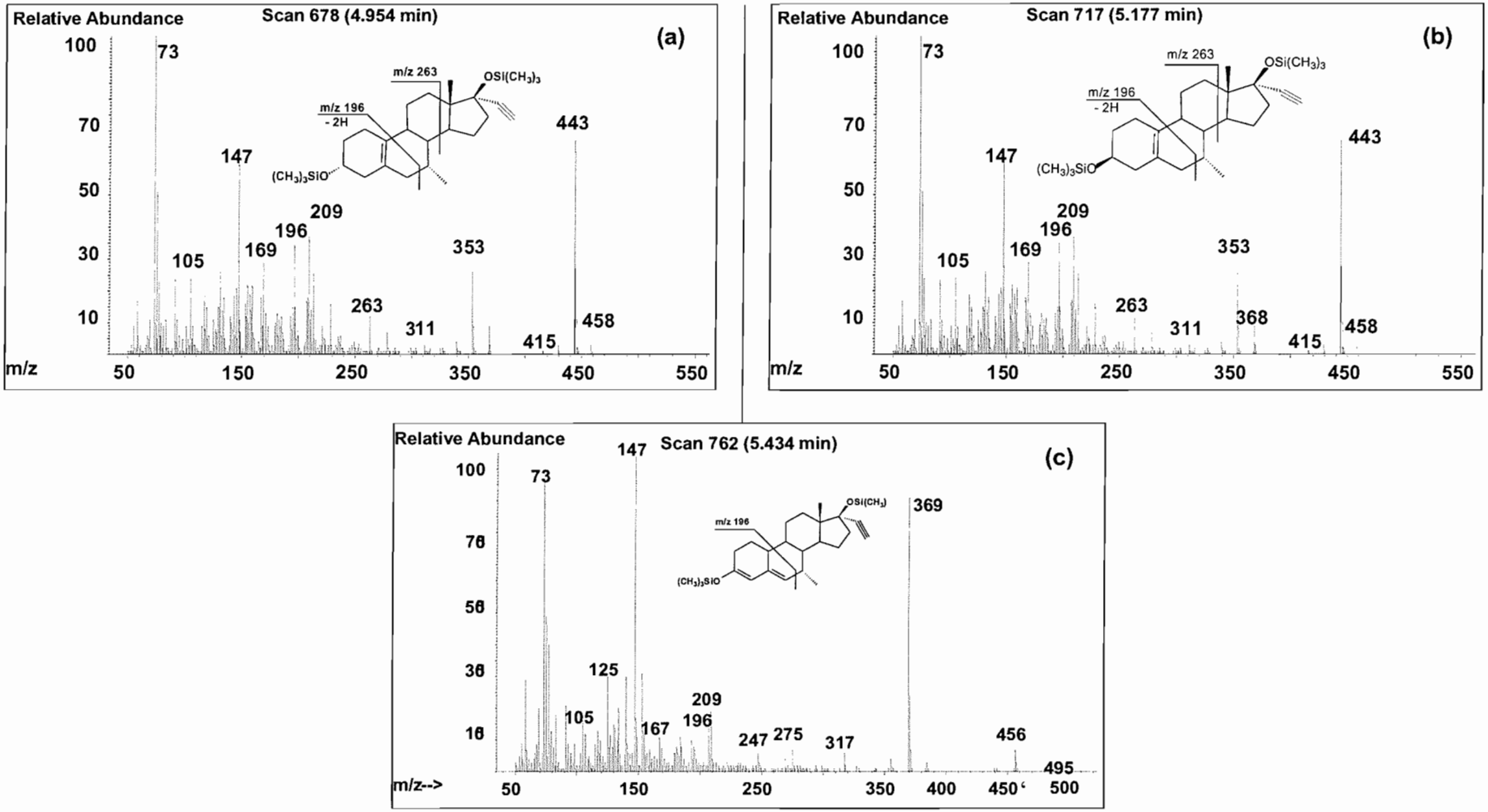


**Fig. 4.** Bis-OTMS of Tibolone metabolites detected in urine (a) 3α-OH-tibolone (b) 3β-OH-tibolone and (c) $\Delta^4$-tibolone by GC-MS. Main fragmentation pattern of 3α-/3β-tibolone: $(M^{+\cdot} - CH_3)$ = m/z 443; $(M^{+\cdot} - Si(CH_3)_3 - OH)$ = m/z 368; $(M^{+\cdot} - CH_3 - Si(CH_3)_3 - OH)$ = m/z 353. $\Delta^4$-tibolone: $(M^{+\cdot} - Si(CH_3)_3 - CH_3)$ = m/z 369.