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# Organ-on-a-chip: Determine feasibility of a human liver microphysiological model to assess long-term steroid metabolites in sports drug testing

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## Abstract

A fundamental challenge in preventive doping research is the study of metabolic pathways of substances banned in sport. However, the pharmacological predictions obtained by conventional in vitro or in vivo animal studies are occasionally of limited transferability to humans according to an inability of in vitro models to mimic higher order system physiology or due to various species-specific differences using animal models. A more recently established technology for simulating human physiology is the "organ-on-a-chip" principle. In a multichannel microfluidic cell culture chip, 3-dimensional tissue spheroids, which can constitute artificial and interconnected microscale organs, imitate principles of the human physiology. The objective of this study was to determine if the technology is suitable to adequately predict metabolic profiles of prohibited substances in sport. As model compounds, the frequently misused anabolic steroids, stanozolol and dehydrochloromethyltestosterone (DHCMT) were subjected to human liver spheroids in microfluidic cell culture chips. The metabolite patterns produced and circulating in the chip media were then assessed by LC-HRMS/(MS) at different time points of up to 14 days of incubation at 37°C. The overall profile of observed glucurono-conjugated stanozolol metabolites excellently matched the commonly found urinary pattern of metabolites, including 3′OH-stanozolol-glucuronide and stanozolol-*N*-glucuronides. Similarly, but to a lower extent, the DHCMT metabolic profile was in agreement with phase-I and phase-II biotransformation products regularly seen in postadministration urine specimens. In conclusion, this pilot study indicates that the "organ-on-a-chip" technology provides a high degree of conformity with traditional human oral administration studies, providing a promising approach for metabolic profiling in sports drug testing.

Organ-on-a-chip: Proof-of-concept study in sports drug testing

Christian Görgens and Anja Patricia Ramme contributed equally.

**KEYWORDS**
doping, in vitro metabolism study, liver spheroids/organoids, mass spectrometry, organ-on-a-chip

## 1 | INTRODUCTION

In sports drug testing, the study of metabolism of prohibited substances or newly emerging performance-enhancing compounds is of utmost importance to find efficient markers for their long-term detection in body fluids such as urine or blood. In general, there are two main strategies to investigate drug metabolism: in vitro and in vivo models. Although in vitro techniques utilize human cell cultures as, for example, liver microsomes, occasionally the metabolic pattern deviates substantially from those produced in vivo as current cell culture assays fail to emulate the human tissue microenvironment.[1] Even if different types of cell cultures are co-cultivated, the single cell types are not interconnected and, therefore, are unable to "communicate." Alternatively, experiments performed in the true physiological environment of an animal model are of limited transferability to humans due to various species-specific differences. Therefore, human administration studies currently are the most predictive models for investigating drug metabolism; however, they bear a low but, yet existing, potential health risk for volunteers.

A novel technology for recapitulating human physiology and modeling the complex architecture of the human body is the so-called "organ-on-a-chip" principle. Here, three-dimensional tissue cultures, the so-called "tissue spheroids" or "organoids," which are approximately 100 000-fold smaller than original organs, are located on a chip with the format of a standard microscopic slide.[2–7] As shown in Figure 1, the actual chip consists of three layers: a standard microscopic glass slide, a polydimethylsiloxane (PDMS) layer with channels and culture compartments and an adapter plate made of polycarbonate. The option of heating support allows for experiments to be performed outside of a standard cell culture incubator. The on-chip micropump, which consists of three membranes that are pushed up and down by pressurized air, provides a constant flow rate of a medium, simulating physiological blood flow and a near lifelike nutrient and oxygen supply to the cells.

By interconnection of different tissue spheroids on the same chip, the resulting "multiorgan-chips" are able to mimic higher order system physiology, providing preclinical insight on a systemic level and enabling the direct prediction of effects of chemicals and their metabolism using near real-life models.[8,9] The utilized organ-on-a-chip platform has previously been utilized to the development of pharmaceuticals, chemicals, cosmetics, and food ingredients.[10–12]

The objective of this study was to determine if the organ-on-a-chip technology is suitable to complement or even has the potential to substitute traditional in vitro and in vivo models for the prediction of metabolic profiles of banned performance enhancing substances with unclear excretion profiles. As a proof-of-concept, the well-investigated metabolic profiles of stanozolol and dehydrochloromethyltestosterone (DHCMT, oral turinabol), two frequently misused anabolic androgenic steroids, were investigated by applying the simplest version of an organ-on-a-chip model using liver spheroids derived from human HepaRG and primary human hepatic stellate cells. The metabolic profile was subsequently compared with conventionally obtained urinary excretion profiles after oral administration of either stanozolol or DHCMT.

## 2 | EXPERIMENTAL

### 2.1 | Material and methods

Stanozolol was obtained from Sigma-Aldrich (Deisendorf, Germany), whereas dehydrochloromethyltestosterone was purchased from the National Measurement Institute, NMI (Sydney, Australia). The isotope-labeled internal standard ($D_3$-testosterone glucuronide) was acquired from Toronto Research Chemicals (North York, Canada). Solid-phase extraction cartridges (OASIS MCX and OASIS HLB, 3 ml, 60-mg resin) were from Waters (Milford, MA, USA), methanol (HPLC grade) was from Merck (Darmstadt, Germany), acetonitrile

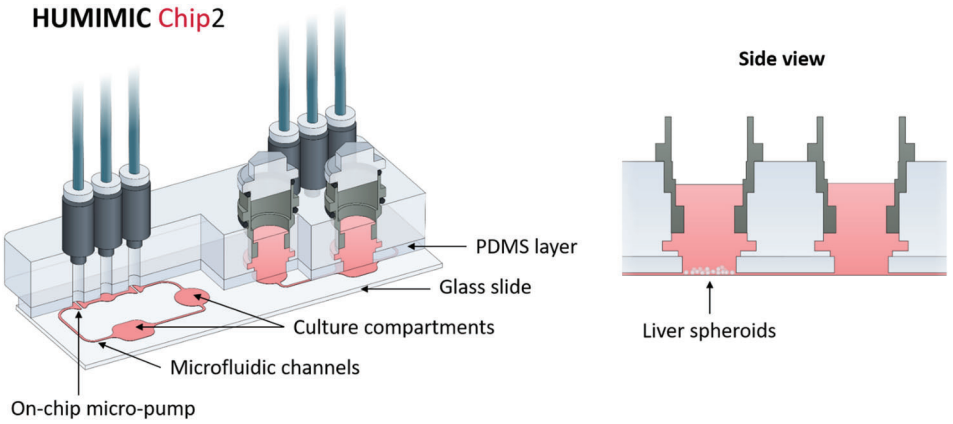


**FIGURE 1** Scheme of the experimental setup of the HUMIMIC Chip2® (copyright by TissUse GmbH). The actual chip consists of three layers: a standard microscopic glass slide, a polydimethylsiloxane (PDMS) layer with channels and culture compartments and an adapter plate made of polycarbonate [Colour figure can be viewed at wileyonlinelibrary.com]

(HPLC grade) was from VWR (Darmstadt, Germany), and deionized water was obtained from a water purification system (Sartorius Stedim Biotech S.A., Aubagne, France).

Cell culture plates and components were purchased from Corning U.S. and were incubated at 37°C and 5% $CO_2$, unless otherwise stated. Differentiated HepaRG cells (HPR116080/#HPR116254-TAO8) were obtained from Biopredic International (Rennes, France), and primary human hepatic stellate cells were purchased from ScienCell (Carlsbad, CA, USA). Standard HepaRG culture medium consisted of William's Medium E (PAN-Biotech, without phenol red, Aidenbach, Germany) supplemented with 10% fetal calf serum (Corning, Lowell, MA, USA), 5 μg/ml human insulin (PAN-Biotech), 2-mM GlutaGrow (Corning), $5 \times 10^{-5}$ M hydrocortisone hemisuccinate (Sigma-Aldrich, H4881-1G, St. Louis, MO, USA), 5 μg/ml Gentamicin sulfate (Corning), and 0.25 μg/ml Amphotericin B (Corning).

## 2.2 | Preparation of human liver spheroids

The differentiated HepaRG cells were thawed according to the manufacturer's instructions in standard HepaRG medium with 0.5% DMSO and seeded with $0.2 \times 10^6$ cells/cm$^2$ 4 days before spheroid formation. On the following day, the medium was renewed with HepaRG medium containing 2% DMSO (VWR Chemicals, PA, USA). The cells were maintained in this medium for 3 days until spheroid formation.

The hepatic stellate cells (passage 6) were expanded in stellate cell medium, provided by ScienCell (Carlsbad, CA, USA). Preculture of hepatic stellate cells was also started 4 days before spheroid formation. Four days ($t$ = day −4) before starting the experiment ($t$ = day 0), human liver spheroids were formed by combining differentiated HepaRG cells and hepatic stellate cells in Eplasia 96-well plates (Corning 4442) in standard HepaRG culture medium. Briefly, the wells were filled with 100-μl standard HepaRG culture medium, and the Eplasia plate was centrifuged at 200$g$ for 1 min. Afterwards, 100-μl cell suspension containing 240 000 hepatocytes and 10 000 stellate cells was pipetted into each well. Compact spheroids with around 3000 cells per spheroid formed within 3 days in the ∼79 cavities per well.[13]

## 2.3 | Experimental design of the in vitro drug administration study

For the chip experiments, the spheroids of four wells were transferred into the outer compartment of the Chip2. In total, each chip circuit was therefore loaded with 1 000 000 cells. The spheroids attached to the compartment bottom overnight while incubating the chips at 37°C without perfusion. On day 0, the medium in the chips was removed and 200 μl of standard HepaRG culture medium was added to each compartment. Afterwards, the chips were connected to the HUMIMIC Starters control unit, which operated the on-chip micropump with a pressure of 500 mbar and a pump frequency of 0.5 Hz. On day 1, a total medium exchange was performed. Therefore, 200 μl of standard HepaRG culture medium was removed from both compartments and replaced with 200 μl of fresh standard HepaRG culture medium.

On day 3, 200 μl of standard HepaRG culture medium was removed from both compartments and replaced with 250 μl of fresh standard HepaRG culture medium containing either stanozolol, DHCMT, or DMSO. The target doses for stanozolol and DHCMT were 20 and 100 ng/ml dissolved in standard HepaRG culture medium.

In Figure 2, a scheme of the experimental design is illustrated. After 1, 3, and 6 h postadministration of the first dose of the drugs, 20-μl medium was sampled from the outer compartment of the chip. On days 5 and 7, a total medium exchange was performed. Therefore, 200-μl medium was removed from both compartments and replaced with 200 μl of fresh standard HepaRG culture medium containing the substances. On days 10 and 12, a 50% medium exchange was performed. Therefore, 200-μl medium of the liver compartment was removed and replaced with 200-μl fresh standard HepaRG culture medium (without drug). Chip incubation experiments were terminated after 14 days, and medium samples were collected. To determine cell viability, all media samples were analyzed photometrically for the presence of glucose, lactate, albumin, and lactate dehydrogenase (LDH) activity using an Indiko Plus chemical analyzer (Thermo Scientific, Bremen, Germany) according to the manufacturer's instructions. Medium samples for LC-HRMS analysis were collected in 96-well deep-well plates (Brand 701352) and were allowed to dry.

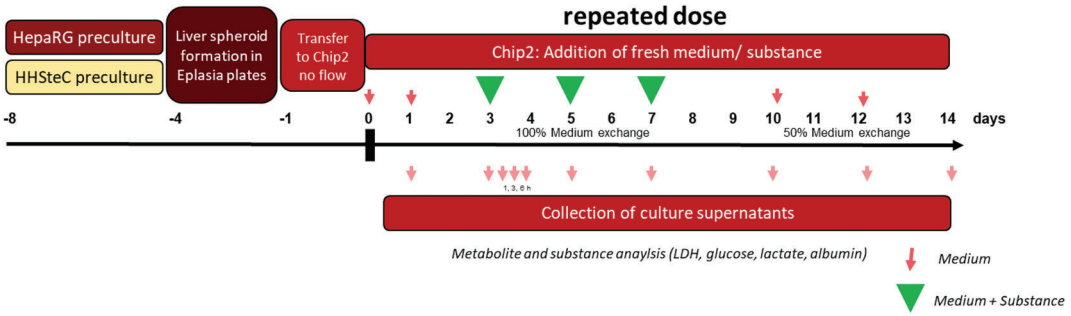


**FIGURE 2** Scheme of the experimental design of the in vitro study using liver spheroids on a HUMIMIC Chip2®. Samples of the culture supernatants were taken before and 1, 3, and 6 h after the first application of the drugs, as well as on days 3, 5, 7, 10, 12, and 14 of the experiment [Colour figure can be viewed at wileyonlinelibrary.com]

Three replicates for 20 ng/ml stanozolol and DHCMT, four replicates for 100 ng/ml stanozolol and DHCMT, and three replicates for the solvent DMSO control were performed. In "empty Chip" experiments, 100 ng/ml stanozolol and DHCMT were applied to the Chip2 circuits (two replicates each) in exactly the same way as that in the main experiments, but no liver spheroids were included.

### 2.4 | Sample preparation

For sample preparation, the dried samples were reconstituted in 200 µl of methanol. Before injection into the LC-HRMS system, samples were purified by solid-phase extraction using OASIS® MCX cartridges for stanozolol and OASIS HLB cartridges for DHCMT following the manufacturer's standard protocols. In brief, the SPE cartridges were preconditioned with 1 ml of water and 1 ml of methanol. After sample elution, the resin was washed with 1 ml of water and the analytes eluted with 1 ml of methanol. The organic phase was evaporated to dryness and reconstituted in 100 µl of a mixture of 0.2% formic acid and acetonitrile for LC-MS/MS analysis.

### 2.5 | LC-HRMS conditions

Chromatographic separation was conducted using a Vanquish UHPLC Pump (Thermo Scientific, Bremen, Germany) equipped to a Nucleodur C18 Pyramid column (Macherey-Nagel, Düren, Germany) using a stepped gradient of 0.2% formic acid (solvent A) and acetonitrile (solvent B). The initial mobile phase conditions were 100% of solvent A at a flow rate of 200 µl/min, which was reduced to 60% of solvent A within 5 min and to 10% of solvent A within another 3 min. These conditions were held for 0.5 min before re-equilibration started at 100% of solvent A for 3.5 min. The overall runtime was 12 min injection-to-injection.

Mass spectrometric detection was performed using an Exploris 480 orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) operating in full scan and parallel reaction monitoring (PRM) acquisition mode using positive ionization for stanozolol and negative ionization for DHCMT (+4 kV/−4 kV, vaporizer temperature 300°C, capillary temperature 350°C, NCE: 55%). The instrument was set at a resolution of 60 000 FWHM, AGC target as well as maximum injection time was set to "auto." The collision gas utilized was nitrogen with a purity >99% provided by a CMC nitrogen generator type NGM 22 (CMC Instruments, Eschborn, Germany). Mass calibration of the orbitrap mass spectrometer was performed twice a week using the factory-provided calibration solution.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Tissue viability

As depicted in Figure 3, the morphological analysis of the cells demonstrated stable and controlled growth over the entire 14 days of the experiment. The applied doses of the drugs were chosen with regard to the well-investigated hepatocidic effect of the anabolic steroids on the one hand and highest possible concentrations to obtain a maximum quantitative amount of excreted metabolites on the other hand. In comparison with the DMSO control cultures, the liver microtissues that were exposed to the drugs changed their morphology only slightly while maintaining their 3D spheroid structure. The administration of stanozolol and DHCMT did not negatively affect the growth of the cells, at either concentration of 20 or 100 ng/ml.

Beside the morphological analysis of the cells, the metabolic activity was monitored daily by photometric detection of glucose and lactate in the media supernatants as well as lactate dehydrogenase (LDH) activity and albumin production of the cells. Glucose consumption and lactate production of the liver spheroids on the chips were stable with only minor fluctuations due to medium exchange every 24 h until the end of the culture. Moreover, the constant LDH activity as well as albumin secretion in the medium supernatant indicated a stable tissue turnover and cell viability (Figures S1 and S2).

### 3.2 | Analysis of stanozolol metabolites

In order to verify the applicability of the organ-on-a-chip technique as a suitable model for metabolism studies in sports drug testing, all medium supernatant samples were evaluated regarding the presence of known and well-characterized stanozolol metabolites by comparison with a stanozolol excretion study urine sample, collected after the oral application of 5 mg of the substance. Schänzer et al. demonstrated that stanozolol is mainly excreted as glucuronides of stanozolol and its 17-epimer ($[M + H]^+$ at *m/z* 505.29) as well as several hydroxylated analogs (precursor ion $[M + H]^+$ at *m/z* 521.29), which chromatographically separated yielding a series of signals assigned to stanozolol-*O*-glucuronide, stanozolol-*N*-glucuronide, 17-epistanozolol-*N*-glucuronide, 16$\beta$-OH-stanozolol-*O*-glucuronide, 3′OH-stanozolol-glucuronide, and 3′OH-epistanozolol-glucuronide.[14] Figure 4 compares the extracted ion chromatograms of the aforementioned phase-II metabolites of the stanozolol elimination study urine and one circuit of the HUMIMIC Chip2® experiment from day 5 of the 20 ng/ml setup. The in vitro experiment shows a qualitatively similar metabolic pattern to that of oral administration, confirmed by means of accurate masses of the intact protonated molecules and the respective aglycones obtained via collision activated dissociation.

In Figure 5, the concentration profiles of the characterized stanozolol phase-II metabolites after organoid exposure to 20 and 100 ng/ml are demonstrated. In general, the abundance-time-curves are similar for all investigated compounds and dosages, providing a linear increase up to 96 h after the first application of stanozolol. However, compared with 20 ng/ml, signal intensities increased by a factor of 5 in the 100 ng/ml study, according to the higher dosage of stanozolol administered to the medium. After the peak concentration at 96 h, concentrations of metabolites decreased, probably due to the exchange with drug-free medium on days 10 and 12 as previously mentioned.

Although stanozolol phase-I metabolites are underrepresented with regard to the glucuronides in the excretion profile from

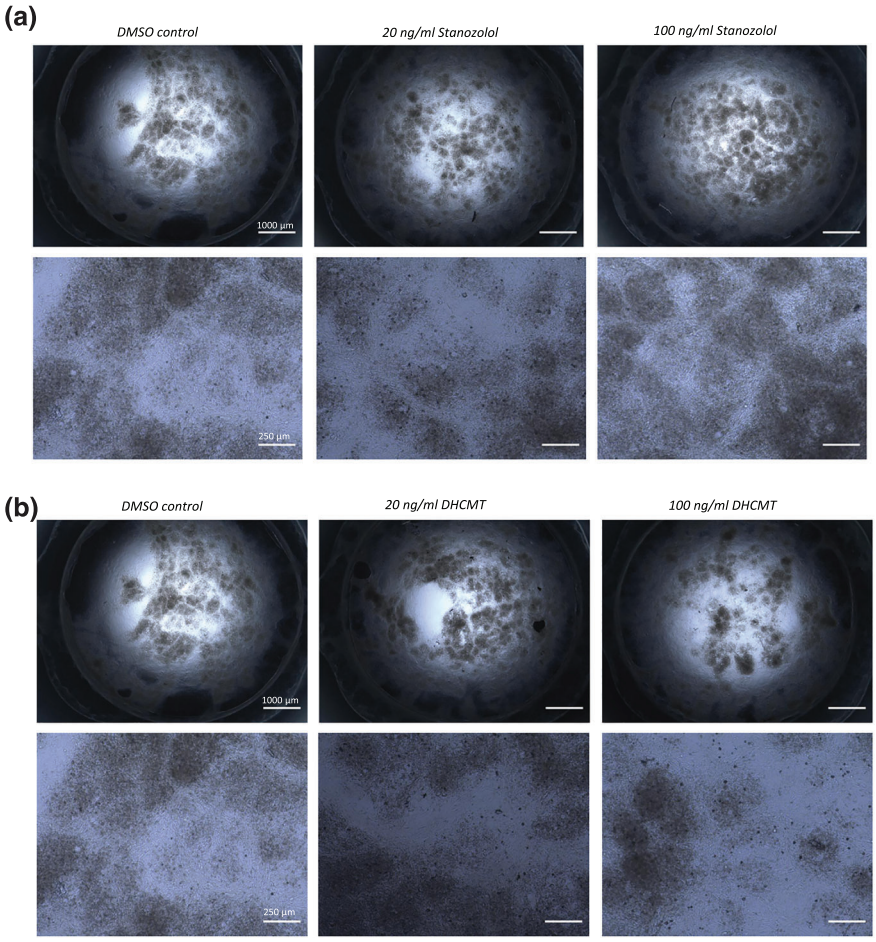


**FIGURE 3** Morphological analysis of the liver spheroids on day 14 of Chip2 culture in stanozolol (a) and DHCMT (b) in concentrations of 20 and 100 ng/ml compared with the DMSO controls. The morphological analysis of the cells demonstrated stable and controlled growth. However, in comparison with the DMSO control cultures, the liver microtissues that were exposed to the drugs changed their morphology only slightly while maintaining their 3D spheroid structure [Colour figure can be viewed at wileyonlinelibrary.com]

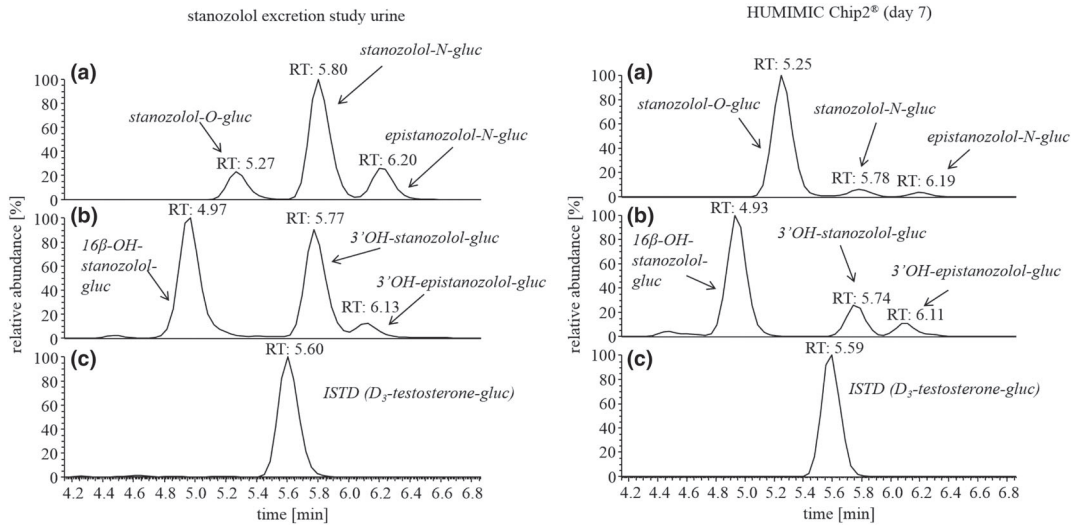


**FIGURE 4** Extracted ion chromatograms of a human stanozolol administration study urine and the metabolic outcome provided by the HUMIMIC Chip2® (100 ng/ml). (a) *m/z* 505.25 → 329.25 (NCE: 55%), (b) *m/z* 521.25 → 345.25 (NCE: 55%), (c) *m/z* 468.25 → 109.07 (ISTD, NCE: 30%)

urine, these metabolites were also investigated in the organoid metabolism profile. Here, 16$\beta$-OH-stanozolol, 3′OH-stanozolol, and 4$\beta$-OH-stanozolol were secreted by the HUMIMIC Chip2®. In keeping with what has been observed for in vivo stanozolol excretion profile from urine and adverse analytical findings (AAF) in sports drug testing, the concentrations of the phase-I metabolites were significantly lower compared with the excreted phase-II metabolites.

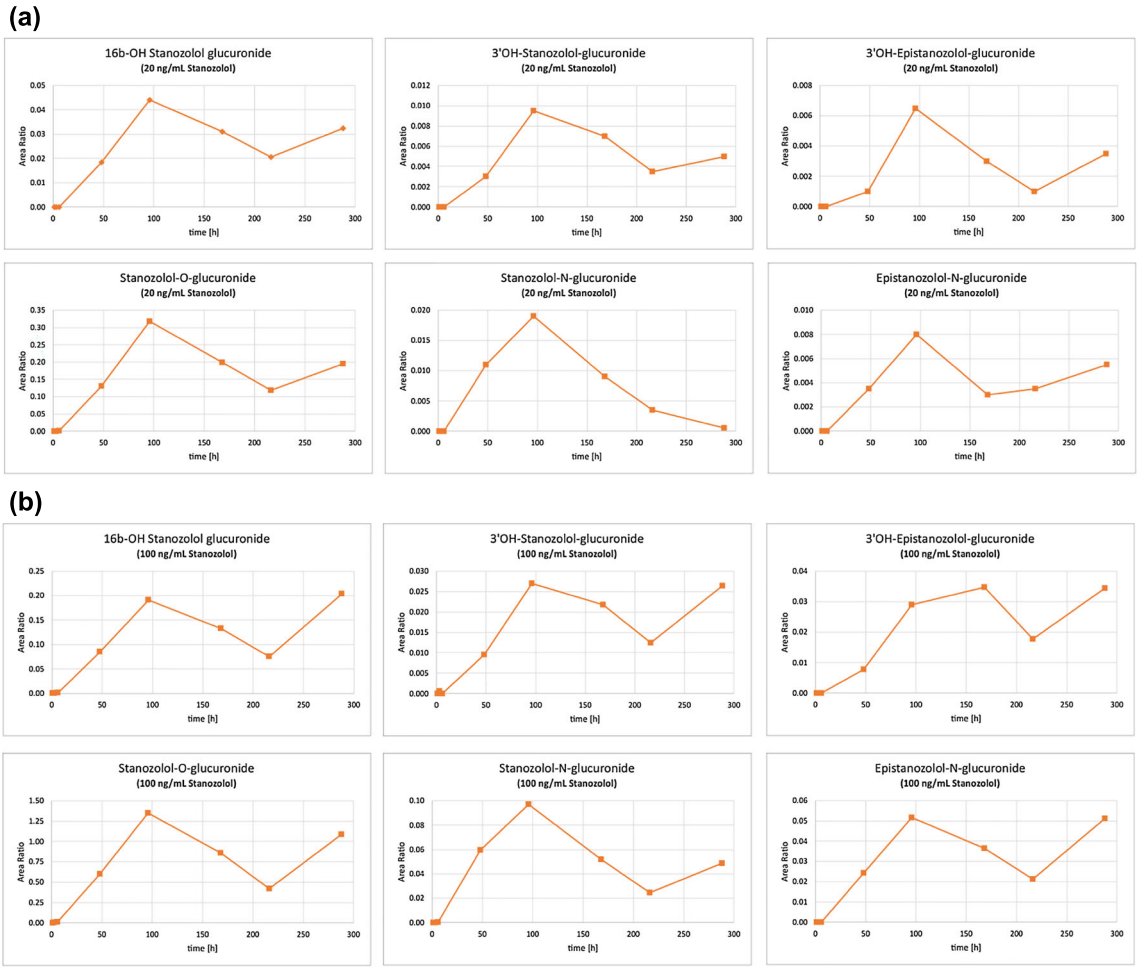


**FIGURE 5** Excretion profiles of the main phase-II metabolites of stanozolol after (a) 20 ng/ml and (b) 100 ng/ml administration using the HUMIMIC Chip2 [Colour figure can be viewed at wileyonlinelibrary.com]

### 3.3 | Analysis of DHCMT metabolites

DHCMT, also known as 4-chloromethandienone or oral turinabol, is routinely detected using GC-MS/MS after enzymatic hydrolysis, liquid–liquid extraction, and derivatization using MSTFA. As the present study focused on both phase-I and phase-II metabolites, the routinely used GC-MS approach was considered less suitable. Therefore, an approach based on liquid chromatography and high-resolution mass spectrometry (LC-HRMS/MS) was chosen.

In general, DHCMT is eliminated rapidly from the body by renal excretion of its main short-term metabolite DHCMT glucuronide. In addition to the glucuronidated metabolite, several hydroxylated and dihydroxylated metabolites including 6β-hydroxy-, 16β-hydroxy-, 6β,16β-dihydroxy-, and 6β,12-dihydroxy-DHCMT were characterized as further short-term metabolites with detection windows of up to 5 days after oral administration of 5 mg DHCMT.[15–17] In recent years, several studies demonstrated the high complexity of the DHCMT metabolism.[18–21] Beside a trihydroxylated metabolite (4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one), which is detectable in urine for up to 14 days using GC-HRMS, the long-term metabolites bearing a 17β-hydroxymethyl-17α-methyl-18-norandrost-13-ene core structure resulting from a Wagner–Meerwein rearrangement demonstrated their applicability for long-term detection of 17α-methyl steroids.[18] For DHCMT, Parr et al. identified 4-chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one, providing a prolonged detection window of up to 22 days after oral application of 15 mg of the drug, which was later confirmed by Parr et al.[19] and Fernandez-Alvarez et al.[20] In 2012, Sobolevsky et al. identified additional DHCMT long-term metabolites after HPLC fractionation of a pooled urinary concentrate and analysis using GC-MS/(MS). Here, the most promising metabolite for doping control analysis was characterized as 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androst-13-en-3α-ol and its 17α-epimer providing excellent retrospectivity.[21]

According to the complex metabolism of DHCMT and the low amount of sample volumes (20 µl), the chosen analytical strategy was to pool samples with potentially high concentrations of DHCMT metabolites (from days 3, 5, and 7 post administration). In general, the extracted ion chromatograms of the HUMIMIC Chip2® demonstrated very low background noise with no interfering matrix components

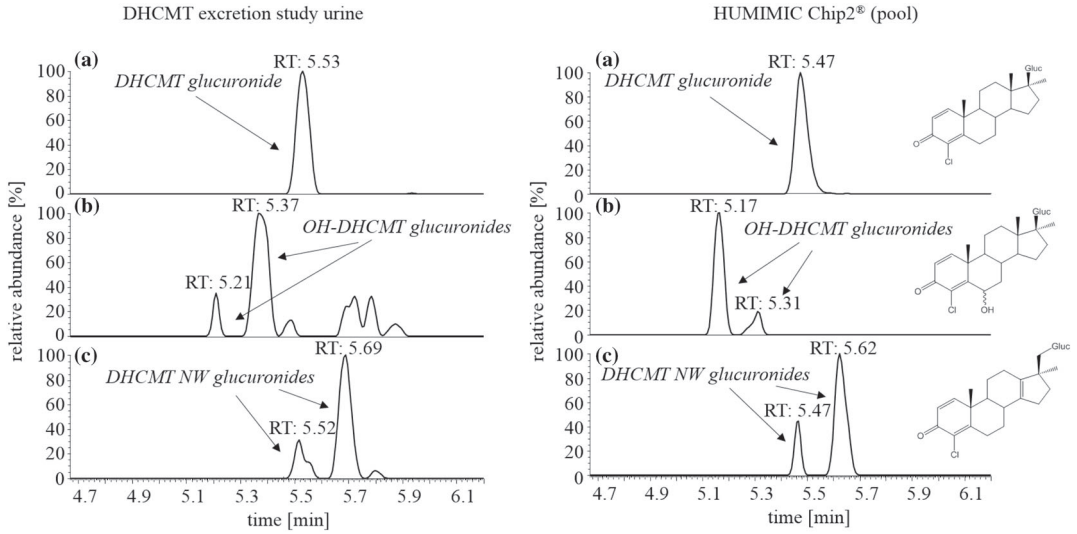


**FIGURE 6** Extracted ion chromatograms of a human DHCMT administration study urine and the metabolic outcome provided by pooled medium supernatant samples of the HUMIMIC Chip2 (100 ng/ml). A: Full MS *m/z* 509.19, B: Full MS *m/z* 521.19, C: Full MS *m/z* 507.18

(Figure 6). As expected, the main metabolite after organoid exposure to DHCMT was DHCMT glucuronide ($[M\text{-}H]^-$ at *m/z* 509.19), which is in accordance with the analyzed DHCMT excretion profile for urine. Besides the aforementioned glucuronidated metabolite, several hydroxylated phase-II glucuronic acid conjugated metabolites ($[M\text{-}H]^-$ at *m/z* 525.19) were observed, which most likely include the already described 6β-hydroxy- and 16β-hydroxy-DHCMT. However, the second peak (Figure 6; (B) RT: 5.37 and 5.31 min) is not appropriately resolved, indicating the presence of additional isomeric compounds. Furthermore, the long-term metabolite 4-chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one and its epimer ($[M\text{-}H]^-$ at *m/z* 507.18) were also found in the organ-on-a-chip medium supernatant samples in keeping with what is found for the excretion profile for urine samples.

As already observed for stanozolol, the organ-on-a-chip principle corroborates its usability by providing a metabolic pattern that is closely related to what is known from human in vivo administration studies. However, multihydroxylated metabolites as described in the literature and the long-term metabolite 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androst-13-en-3α-ol identified by Sobolevsky et al. were not found in the presented approach.[21] Probably the complexity of a one-organ chip representing a simple one-compartment pharmacokinetic model is insufficient here or the amount of spheroids on the chip, the maximum dosage, and exposure time of the drugs has to be further optimized to produce higher amounts of the expected, but not detected, metabolites.

## 4 | CONCLUSION

In this proof-of-concept study, the organ-on-a-chip technique provides a high degree of conformity with traditional oral administration studies in humans with regard to the main excreted metabolites of stanozolol and DHCMT. However, to more completely model the complex metabolism of anabolic androgenic agents and other prohibited compounds from different classes (e.g., selective androgen receptor modulators [SARMS], hypoxia-inducible factor [HIF] activating agents, and metabolic modulators), the complexity of the model has to be further increased. Therefore, a current limitation of the utilized one-organ-chip approach is the inability of the model to replicate the complexity of living organisms. However, multiorgan chips, formed by combination of different organoids on one chip, mimic higher order system physiology more accurate than any previous in vitro model. For example, these chips would be able to simulate oral or topical applications of drugs by combining liver cells with intestinal cells or skin tissues, respectively. Even the analysis of an artificial urinary matrix seems possible using a combined liver and kidney model.

Another limitation with regard to traditional in vivo administration studies is the rather complex and laborious preparation of the cell cultures and the spheroid formation in sterile environment, which requires highly trained personnel. However, the increased laboratory work is incommensurate with animal welfare, potential health risks for volunteers, and the requirement for ethical approval.

In general, with its basic idea to emulate the smallest physiological unit of an organ-on-a-chip, the platform has great potential to become a powerful alternative to substance testing in animal trials and a promising tool for metabolic profiling of prohibited compounds or newly emerging performing enhancing substances in sports drug testing.

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Additional use of Figure 1 requires the permission of the copyright owner TissUse GmbH.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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