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Mass spectrometric characterization of urinary hydrafinil metabolites for routine doping control purposes

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Abstract

Little information on the human metabolism and urinary elimination of hydrafinil (9-fluorenol) exists. In order to support preventive anti-doping activities concerning compounds such as hydrafinil, a pilot elimination study was conducted with three healthy male volunteers receiving a single oral dose of 50 mg of hydrafinil. Urine samples were collected prior to and up to 72-h post-administration and were subjected to both gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry, which allowed for the identification of the intact drug as well as Phase I and Phase II metabolites, primarily hydroxylated and/or glucuronidated or sulfo-conjugated hydrafinil. The identity of these metabolites was corroborated by high-resolution/high-accuracy tandem mass spectrometry, and the applicability of routine doping control workflows for the detection of hydrafinil and its main metabolites was assessed. Therefore, two findings of hydrafinil and its metabolites were recorded, which concerned out-of-competition doping control samples and, hence, were not pursued with confirmatory analyses. Yet, the initial testing procedure results indicate that hydrafinil might require consideration in sports drug testing programs to ensure its detection, if classified as prohibited by the World Anti-Doping Agency (WADA).

KEYWORDS
9-hydroxyfluorene, doping, hydrafinil, mass spectrometry, sport

1 | INTRODUCTION

Containing the misuse of stimulants to illegally enhance attentiveness, concentration, etc. in elite sports has been a major aspect of anti-doping activities for decades.[1–3] Hydrafinil, also known as 9-hydroxyfluorene, is structurally related to modafinil, which is an approved drug for the treatment of narcolepsy or attention deficit hyperactivity disorders (ADHD).[4] Initially patented in 1940 as insecticide,[5] hydrafinil was briefly considered as alternative or successor drug to eugeroic modafinil when in vivo studies with rats demonstrated that the wake-promoting activity of hydrafinil was competitive or even higher than that observed for modafinil.[6] However, the drug candidate was not further developed, and, consequently, preclinical and clinical trials were not pursued. Nevertheless, being considered as unregulated compound in many countries, hydrafinil has appeared on the Internet-based nutritional supplement and nootropics market as cheap and readily available substance,[7] suggesting a considerable potential for misuse in sport. Under the regulation of the World Anti-Doping Agency (WADA), selected stimulants are prohibited in-competition, including structurally as well as

pharmacologically related substances.[8] Whereas modafinil is a well-characterized[9] target compound and included in routine doping controls, hydrafinil has not yet been fully considered, and the urinary metabolic profile of hydrafinil is largely unknown. The analyte itself, that is, 9-hydroxyfluorene, has been employed as urinary biomarker for monitoring the extent of exposures to polycyclic aromatic hydrocarbons (PAHs) in humans, and test methods based on gas chromatography–mass spectrometry (GC-MS) or liquid chromatography–fluorescence detection were used in studies reporting on occupationally caused urinary 9-hydroxyfluorene of a general North American population,[10] roofers,[11] and Mexican miners,[12] with levels reaching up to 340 ng/ml.

In a proactive and preventive anti-doping context, an oral single-dose (50-mg) administration study with hydrafinil was conducted to provide first insights into its in vivo metabolism and elimination in humans. Urine samples were analyzed by high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) to probe for predicted urinary metabolites and to estimate corresponding detection times. As hydrafinil itself is detected by HPLC-HRMS utilizing routinely applied analytical conditions merely with modest sensitivity and to facilitate alternative routine monitoring options, gas chromatography (GC)-HRMS experiments were also conducted. Here, an existing initial testing procedure (ITP) based on enzymatic hydrolysis of glucuronides, liquid–liquid extraction, and subsequent trimethylsilylation was applied, which proved effective by identifying two out-of-competition (OOC) routine doping control samples containing hydrafinil metabolites. These findings were not further pursued or confirmed as stimulants are banned in-competition only.

2 | MATERIALS AND METHODS

2.1 | Study design and sample collection

Following approval (#049/2020) by the local ethics committee of the German Sport University Cologne, three healthy male volunteers without any self-reported diseases or medications participated in this excretion study and provided written informed consent. The participants ingested a nutritional supplement (which was tested for its content and compared with 9-fluorenol reference material) according to the manufacturer's recommendations, and a 50-mg single oral dose of hydrafinil was administered in the form of a capsule. In addition to blank urine samples collected before ingestion, spot urine specimens were collected up to 72 h.

2.2 | Chemicals and materials

For all aqueous solutions, deionized water was obtained from a Barnstead GenPure device from Thermo Scientific (Bremen, Germany). Methanol (MeOH), *tert*-butyl methyl ether (TBME), formic acid (FA) of analytical grade, and β-glucuronidase from *Escherichia coli* were purchased from Merck (Darmstadt, Germany). Potassium carbonate (K_2CO_3), potassium bicarbonate ($KHCO_3$), disodium phosphate (Na_2HPO_4), monosodium phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$), ethanethiol (EtSH), and ammonium iodide (NH_4I) were obtained from VWR International GmbH (Bruchsal, Germany), and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Düren, Germany). The nutritional supplement with capsules containing 50-mg hydrafinil (9-hydroxyfluorene, CAS 1689-64-1) was ordered via the Internet, and Oasis HLB (1-cc) solid-phase extraction (SPE) cartridges were from Waters (Eschborn, Germany).

3 | SAMPLE PREPARATION AND ANALYSES

3.1 | LC-HRMS

Urine samples were prepared by SPE using Oasis HLB cartridges for subsequent HPLC-HRMS analysis. Therefore, 1 ml of urine was loaded onto preconditioned (1 ml of MeOH) and equilibrated (1 ml of H_2O) cartridges, which were subsequently washed (1 ml of H_2O) and eluted (1 ml of MeOH) before the obtained eluate was evaporated to dryness in a heated vacuum centrifuge at 40°C. The dry residue was reconstituted in 200 µl of MeOH and subjected to the HPLC-HRMS analysis.

The LC consisted of a Thermo Scientific (Bremen, Germany) VANQUISH UHPLC system equipped with a Poroshell 120 EC-C18 2.7 µm (3.0 mm ID × 50 mm) analytical column from Agilent Technologies (Waldbronn, Germany). Gradient elution was carried out with 0.1% FA (A) and MeOH acidified with 0.1% FA (B). Within the first 7.5 min, the organic proportion (B) was raised from 1% to 99%. After 1 min at 99% B, initial conditions were restored and maintained for 1.5 min, resulting in a total runtime of 10 min. The flow rate was set to 400 µl/min, and the injection volume was 2 µl.

For mass spectrometric detection, a Thermo Scientific Orbitrap Exploris 480 mass spectrometer equipped with a heated electrospray ion source (H-ESI) operating in negative mode (−3000 V, 320°C ion transfer tube, 420°C vaporizer) was used. For identification and characterization of in vivo generated hydrafinil metabolites, high-resolution full MS scans (*m/z* 150–500 at R = 60,000) as well as targeted MS/MS experiments (isolation width 1.2 *m/z*, R = 30,000) were performed using an inclusion list (see Table 1).

3.2 | GC-HRMS

The sample preparation was adapted from existing routine doping control testing procedures utilizing enzymatic hydrolysis for steroid-related urinary Phase II metabolites and subsequent trimethylsilylation of the resulting aglycons before GC-HRMS analysis. Briefly, 2 ml of urine was fortified with 20 µl of ISTD (10 µg/ml 9-fluorenol-d_9 in MeOH) and mixed with 1 ml of 0.8 M phosphate buffer (pH 7) (34.6 g Na_2HPO_4 and 17.6 g $NaH_2PO_4{\cdot}H_2O$ in 460 ml H_2O). Enzymatic hydrolysis was conducted by adding 50 µl of β-glucuronidase before

incubation for 60 min at 50°C. The hydrolysis was stopped by further addition of 750 µl of 20% K_2CO_3/$KHCO_3$ solution (1:1 *v/v*), and liquid–liquid extraction was performed by addition of 5 ml of TBME, shaking (5 min), and centrifugation (5 min). The separated organic phase was evaporated to dryness and reconstituted in 100 µl of a derivatization mix (20 mg NH_4I and 30 µl EtSH in 10 ml MSTFA), and trimethylsilylation was carried out at 60°C for 30 min. The reaction solution was transferred to autosampler glass vials and subjected to GC-HRMS analysis.

For the identification of hydrafinil and its metabolites, a GC Q Exactive Orbitrap HRMS system from Thermo Scientific was employed. The GC was equipped with a GC column Ultra (17 m, 0.2 mm ID with a film of 0.11 µm) from Agilent Technologies (Waldbronn, Germany). Chromatographic separation was achieved by a temperature gradient starting at 183°C, which was increased by 3°C/min to 232°C and further by 40°C/min to 310°C which was finally kept constant for another 3 min. Injections were performed under constant pressure (14.4 psi at 183°C) with a 1:12 split, and the injector was heated to 300°C. The system was operating in EI-positive mode and a source temperature of 250°C.

Data were acquired in full MS mode ranging from *m/z* 70 to 700 and in parallel reaction monitoring (PRM) generating targeted

TABLE 1 Properties and chromatographic–mass spectrometric parameters for targeted-MS^2 experiments using HPLC-ESI(−)-HRMS regarding the identified urinary hydrafinil metabolites

Compound	Formula	$[M-H]^-$ *(m/z)*	z	Retention time (min)	HCD (%)
Hydrafinil sulfate	$C_{13}H_{10}O_5S$	277.0176	1	5.1	35
OH-Hydrafinil sulfate	$C_{13}H_{10}O_6S$	293.0125	1	4.8	35
Hydrafinil glucuronide	$C_{19}H_{18}O_7$	357.0980	1	6.2	35
OH-Hydrafinil glucuronide	$C_{19}H_{18}O_8$	373.0929	1	5.2	30
Bis-OH-hydrafinil glucuronide	$C_{19}H_{18}O_9$	389.0878	1	5.1	30

TABLE 2 Properties and chromatographic–mass spectrometric parameters for targeted-MS^2 experiments using GC-EI(+)-HRMS/MS regarding trimethylsilylated hydrafinil and its identified metabolites

Compound	Formula	M^+ *(m/z)*	z	Retention time (min)	HCD (%)
Hydrafinil	$C_{16}H_{18}SiO$	254.1121	1	1.9	20
Hydrafinil-d_9	$C_{16}H_9D_9SiO$	263.1686	1	1.9	20
OH-Hydrafinil	$C_{19}H_{26}Si_2O_2$	342.1466	1	4.2	20
Bis-OH-hydrafinil	$C_{22}H_{32}Si_3O_3$	430.1810	1	8.6	20

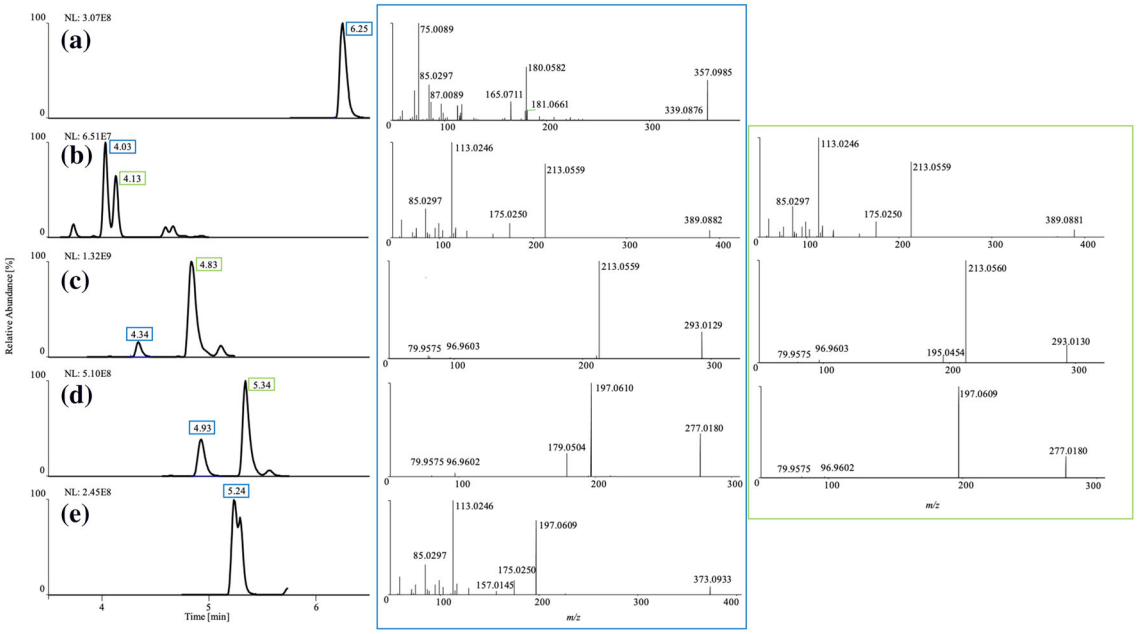

FIGURE 1 Extracted diagnostic product ion traces of hydrafinil metabolites detected in a urine sample 4 h after oral administration of a single 50-mg dose. (a) Hydrafinil glucuronide at *m/z* 357.10 → 180.06 (@HCD 35). (b) Bis-hydroxylated hydrafinil glucuronide at *m/z* 389.09 → 213.06 (@HCD 30). (c) Bis-hydroxylated hydrafinil sulfate at *m/z* 293.01 → 213.06 (@HCD 35). (d) Mono-hydroxylated hydrafinil sulfate at *m/z* 277.02 → 197.06 (@HCD 35). (e) Mono-hydroxylated hydrafinil glucuronide at *m/z* 373.09 → 197.06 (@HCD 30). Corresponding product ion mass spectra are shown next to the chromatograms, assigned by retention times and colored boxes [Colour figure can be viewed at wileyonlinelibrary.com]

MS/MS spectra (isolation window of *m/z* 1.3) using an inclusion list (see Table 2). The resolving power was set to a resolution of R = 60,000, and data were evaluated using Xcalibur (Version 4.0, Thermo Scientific). Mass accuracy of <5 ppm was achieved by daily mass calibration of the instrument.

4 | RESULTS

Five hydrafinil metabolites were detected by HPLC-HRMS analyses in all urine samples of all three volunteers, from the earliest available sampling time point (1.5 and 2 h) up to the last samples collected 72-h post-administration. These hydroxylated glucuronides and sulfates were identified with maximum signal abundances observed within the first 2–4 h in full MS scans and characterized by individual targeted MS^2 experiments (Figure 1).

Hydrafinil as the active substance and its ninefold deuterium-labeled analog (employed as internal standard, ISTD) were not readily applicable to HPLC-ESI-HRMS experiments. Therefore, and to facilitate alternative routine monitoring options, GC-HRMS experiments were conducted in accordance to existing ITPs (Figure 2). This approach further supported the monitoring of urinary hydrafinil elimination profiles as illustrated in Figure 3, which indicates estimated peak concentrations of the active principle with 15–80 µg/ml at approximately 2–4 h after the ingestion of 50 mg of hydrafinil.

Following the implementation of hydrafinil and its metabolites into routine ITPs, two routine doping control samples returned test

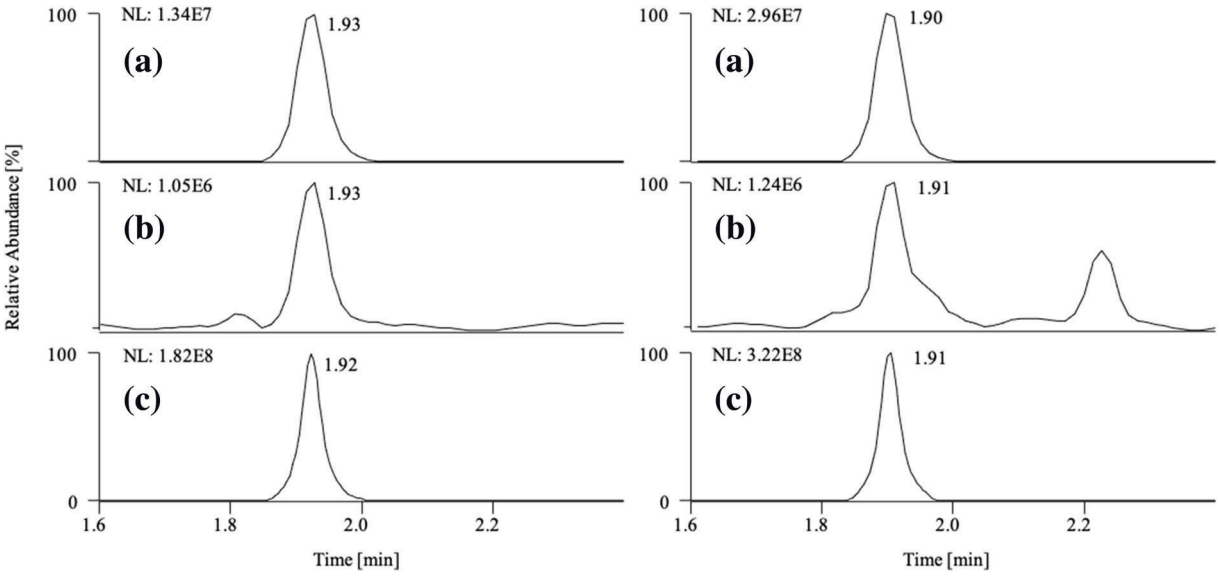

FIGURE 2 Extracted diagnostic product ion traces (a,b) and full MS data (c) of hydrafinil (left) and hydrafinil-d_9 (right) in a spiked blank urine at 100 ng/ml. (a) *m/z* 254.00 → 165.07 (@HCD 20) and *m/z* 263.17 → 174.12 (@HCD 20). (b) *m/z* 254.00 → 91.05 (@HCD 20) and *m/z* 263.17 → 91.05 (@HCD 20). (c) Full MS *m/z* 254.1114–254.1135 and *m/z* 263.1676–263.1696

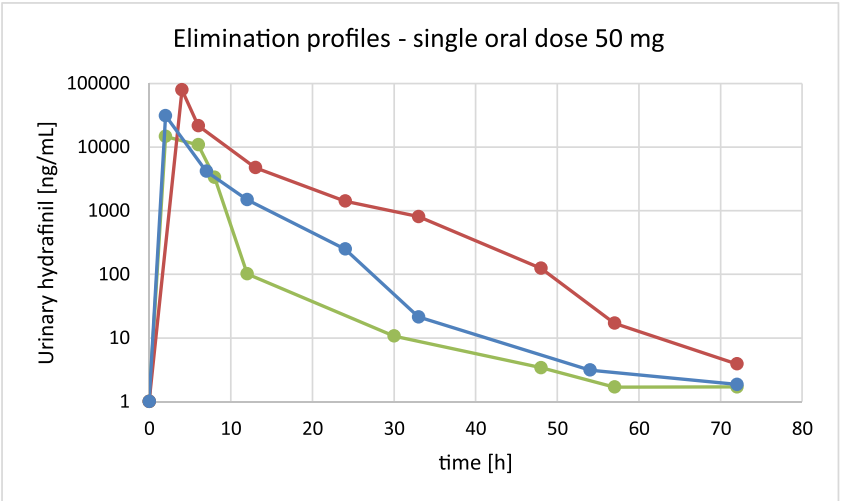

FIGURE 3 Urinary elimination profiles of hydrafinil after consumption of single doses of 50 mg of hydrafinil by three volunteers. Samples were collected up to 72 h post-administration and analyzed by GC-HRMS after enzymatic hydrolysis using β-glucuronidase (*Escherichia coli*) [Colour figure can be viewed at wileyonlinelibrary.com]

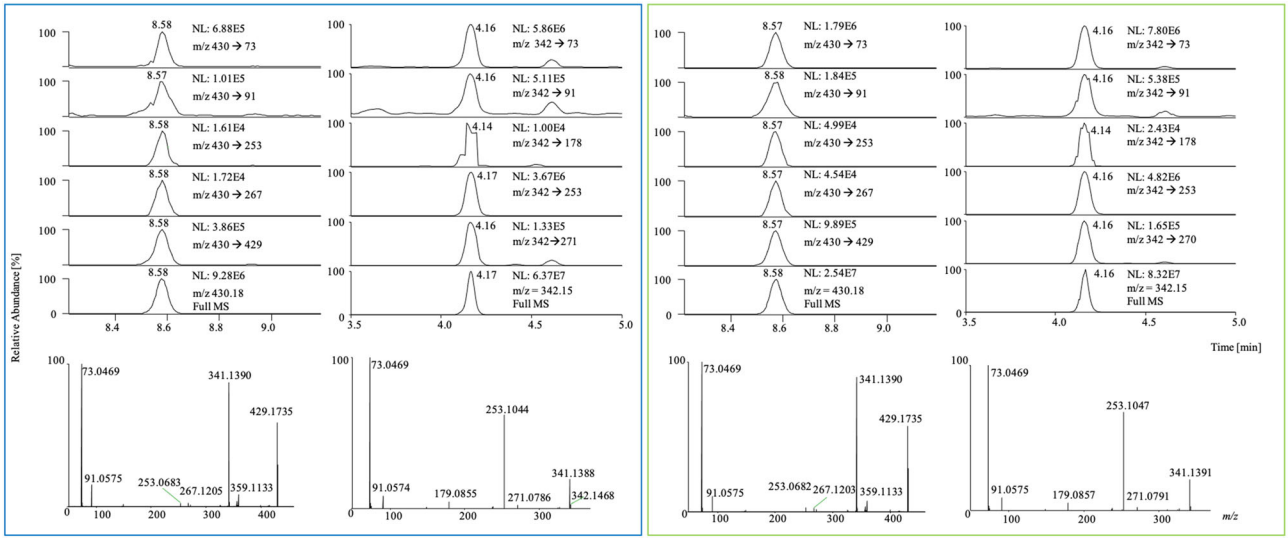

FIGURE 4 Detection of two hydrafinil metabolites in two OOC doping control urine samples (Sample 1: left box; Sample 2: right box). For each sample GC-HRMS-derived extracted diagnostic product ion traces of bis-hydroxylated (left row) and mono-hydroxylated (right row) hydrafinil are depicted, with the bottom chromatograms showing the precursor ions extracted from full MS spectra. Corresponding product ion mass spectra using the ions at *m/z* 430 and 342 as precursor ions, respectively, are illustrated below the chromatograms [Colour figure can be viewed at wileyonlinelibrary.com]

results that suggested the presence of the target analytes (Figure 4) in early 2021. These findings were however not further pursued or confirmed as they occurred in OOC samples. For both urine specimens, mono- and bis-hydroxylated hydrafinil aglycons were detected by monitoring corresponding diagnostic product ion transitions of related precursors (*m/z* 342 and *m/z* 430, respectively).

5 | DISCUSSION AND CONCLUSION

Using the herein obtained pilot study urine samples, collected after the oral administration of 50 mg of hydrafinil as suggested by the Internet-based supplement provider, five abundant phase-II metabolites were identified by HPLC-HRMS experiments. After the implementation of those into existing routine ITPs, hydrafinil in the form of hydroxylated metabolites was detected in two OOC doping control urine samples.

Both HPLC-HRMS and GC-HRMS analyses of these two urine specimens allowed for the unambiguous identification of hydrafinil mono- and bis-hydroxylated (and conjugated) metabolites, which were chromatographically and mass spectrometrically separated from structural analogs such as 2- and 3-OH fluorenols (data not shown). In the light of a constantly increasing availability of so-called neuroenhancers, it appears warranted that anti-doping testing methods are assessed regarding their capability of detecting the (mis)use of such compounds in sport, if hydrafinil and analogs are considered relevant in future in-competition anti-doping testing programs. Also, environmental and occupational factors might necessitate consideration when comparably low concentrations of hydrafinil (and its hydroxylated metabolites) are detected in doping control urine samples, and further research and/or monitoring appears warranted.

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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.

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