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Assessing human urinary clomiphene metabolites after consumption of eggs from clomiphene-treated laying hens using chromatographic-mass spectrometric approaches

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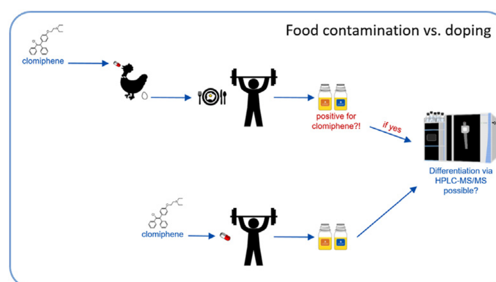
HIGHLIGHTS

- The consumption of eggs obtained from laying hens treated with clomiphene results in adverse analytical findings.
- Various isomers of hydroxylated clomiphene metabolites are traceable.
- Phase-I metabolites are characterized using chromatography-mass spectrometry, ion mobility, and chemical derivatization.
- Metabolic urinary patterns support the differentiation of the intake of egg-derived clomiphene from drug (mis)use.

GRAPHICAL ABSTRACT



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ABSTRACT

The anti-estrogen clomiphene is prohibited in sports at all times. Yet, adverse analytical findings (AAFs) have increased since 2011. This is possibly due to improved analytical sensitivity, but also contamination of food of animal origin needs to be taken into consideration as a potential source of drug exposure. For instance, studies with laying hens that received orally administered clomiphene have shown a significantly increased egg production rate but, as a consequence, eggs were found to incorporate residues of clomiphene.

In order to evaluate if the consumption of clomiphene-contaminated eggs can cause an AAF of a doping control sample, eggs obtained from an animal administration study with clomiphene were consumed by human volunteers. Each volunteer ate two eggs, and urine samples were collected and analyzed using routine doping control procedures. Subsequently, additional volunteers received a microdosed clomiphene capsule to compare the excretion profiles.

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Eggs
Residues

Maximum urinary concentrations of hydroxy-clomiphene (HC) between 80 and 300 pg mL^{-1} were detected following the consumption of clomiphene-containing eggs, which would constitute AAFs if observed in athletes' doping control samples. In order to support the differentiation of potential routes of drug exposure, a method was developed which allows for the chromatographic separation of (*E*)-3-, (*Z*)-3-, (*E*)-4-, and (*Z*)-4-HC using a derivatization step. By comparing the peak areas of these metabolites, characteristic relative distribution patterns were found that assist in identifying AAFs resulting from clomiphene ingested via contaminated eggs and, thus, enable to distinguish clomiphene intake via contaminated eggs from the intake of microdoses or therapeutic dosages, e.g. for doping purposes.
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1. Introduction

The selective estrogen receptor modulator (SERM) clomiphene exists as (*Z*)-clomiphene and (*E*)-clomiphene (Fig. 1). Depending on the isomer, the target tissue, the receptor type and concentration, the drug acts either as an estrogen receptor agonist or as an estrogen receptor antagonist [1,2]. Therapeutically, clomiphene is used to stimulate the release of gonadotropin by inhibiting the estradiol-mediated feedback on the hypothalamus [2]. This stimulation helps to induce ovulation in women with infertility and ovarian dysfunction [1,3]. The metabolism of clomiphene has been investigated in several studies [4–9]. After a single dose of clomiphene, more than 20 metabolites could be detected in urine by high-performance-liquid-chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) [5]. A more recent study examined the stereo-selective quantification of various phase-I and phase-II metabolites [8]. This is particularly important when considering that the human metabolism processes these two forms differently: (*E*)-clomiphene shows a much shorter half-life than (*Z*)-clomiphene due to the different affinity to the phase-I-enzyme cytochrome P450 2D6 [9–11]. Since this enzyme is subject to polymorphism, strong inter-individual variations in metabolism can be expected [10,11]. This is important to both, a better understanding of the efficacy of clomiphene as a drug and to the investigation of the detection windows of different metabolites. The latter, more precisely the detectability of clomiphene and its metabolites in urine, is of particular importance in doping controls.

Clomiphene has been banned in human sports by the World Anti-Doping Agency (WADA) since 2004 [12]. For doping purposes, clomiphene can be used to prevent or mitigate side effects of anabolic steroid abuse such as gynaecomastia or to increase endogenous free testosterone [13–15]. Since 2011, the number of adverse analytical findings (AAFs) of clomiphene in doping control samples analyzed by WADA-accredited laboratories has increased continuously (Fig. 2, [16]). While drug misuse is one explanation,

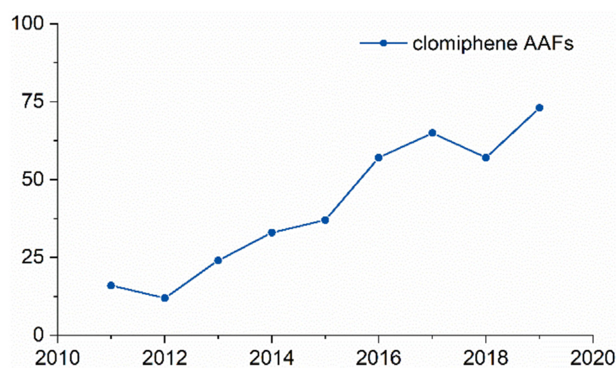

Fig. 2. Course of clomiphene AAFs from 2011 to 2019 [16].

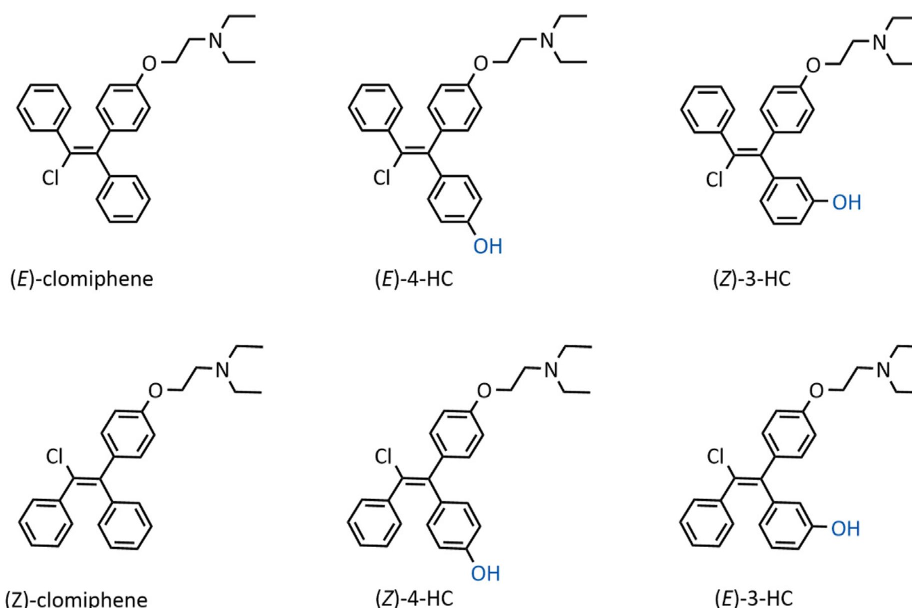

Fig. 1. Structural formulae of clomiphene and HC isomers.

food or supplement contamination may be another one due to the substantial detection time for clomiphene reported in human elimination studies. The possibility of food contamination scenarios in general has been observed before in the past, for example with clenbuterol [17–20].

The potential existence of a contamination issue is supported by the fact that the fertility-enhancing effect of clomiphene was also observed in hens [21–24]. This effect is taken advantage of in studies to increase egg production [23,24]. Also it is reasonable to assume that residues of this drug can occur in poultry meat and especially in eggs, considering that the transfer of hydrophobic veterinary drugs into chicken eggs is a known problem [25].

In a previous study [26], it was shown that the daily administration of 10 mg of clomiphene citrate resulted in a transfer of the drug into eggs and muscle tissue, leading to maximum concentrations of more than 300 μg kg^{-1} in eggs. The eggs obtained during this animal experiment were consumed by human study participants in the follow-up study described in this manuscript. Subsequently, collected urine samples were analyzed for clomiphene and hydroxy-clomiphene (HC) residues using a routine doping control method [27]. Based on the results, a method was developed and characterized to differentiate therapeutic clomiphene intake from clomiphene intake via contaminated eggs.

2. Materials and methods

2.1. Reagents and chemicals

Clomiphene citrate (97%) and (*E*/*Z*)-4-HC citrate were obtained from Toronto Research Chemicals (Toronto, Canada). Clomiphene-d_5 citrate was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). (*E*)-3-HC, (*Z*)-3-HC and (*E*/*Z*)-4-HC-d_6 were synthesized as described previously [8,28]. Clomiphene for the microdose study was extracted and diluted with Ethanol absolute (pharma grade, AppliChem, Darmstadt Germany) from the pharmaceutical product (Clomiphene ratiopharm, ratiopharm, Germany). 5-Dimethylamino-1-naphthalinsulfonyl chloride (dansyl chloride) and formic acid were purchased from Sigma-Aldrich (Saint Lois, MO, USA). Acetonitrile (ACN), methanol, potassium hydrogen carbonate, potassium carbonate and di-sodium hydrogen phosphate anhydrous were procured from VWR (Radnor, PA, USA). Sodium dihydrogen phosphate monohydrate and sodium acetate were obtained from Merck (Darmstadt, Germany) and *tert.*-butyl methyl ether (TBME) was acquired from AppliChem (Darmstadt, Germany). For solid phase extraction (SPE), Chromabond® HLB (60 μm, 3 mL/200 mg) cartridges from Macherey-Nagel (Düren, Germany) were used. For hydrolyzing the glucuronide conjugates, β-glucuronidase from *E.coli* produced by Roche (Basel, Switzerland) was utilized.

Stock solutions of clomiphene, (*E*/*Z*)-4-HC, (*E*)-3-HC, (*Z*)-3-HC, (*E*/*Z*)-4-HC-d_6 (each 1 mg mL^{-1}, compensated for the counter ion if present) and clomiphene-d_5 citrate (1 mg mL^{-1}, not compensated for the counter ion) were prepared in methanol. Working solutions were prepared by diluting the stock solutions in ACN/water to a concentration appropriate for the calibration curves and method validation. All stock solutions and working solutions were stored at −20 °C.

2.2. Ethics statement

Both the consumption of clomiphene-containing eggs and the intake of the clomiphene microdoses as well as the subsequent collection of urine samples was performed in accordance with the ethical standards laid down in the Declaration of Helsinki and followed the approval (#012/2019) by the local ethics committee of the German Sport University Cologne. The human participants were non-athletic, healthy male volunteers who provided written informed consent.

The preceding animal administration study was planned and carried out under conditions in compliance with the animal welfare guidelines outlined in directive 2010/63/EU [29] and was approved by the Belgian Ministry of Small Businesses and Agriculture.

2.3. Hens and egg collection

In the preceding animal administration study [26], 24 laying hens were orally administered 10 mg of clomiphene citrate daily for 28 days. The eggs were collected every morning prior to the first administration, during and up to 15 days after the last administration. The eggs were stored at 4 °C. Immediately before consumption, the eggs were hard-boiled using an electric egg boiler.

2.4. Study design

Eggs (n = 2) obtained from the study animals at different time points (day 7, day 14, day 21, day 28, day 30 and day 36) were consumed by human study participants (n = 3 each time, see Suppl. Table 1). Urine samples were collected right before and up to 21 days after consumption of the clomiphene-contaminated eggs. During the first 48 h after consumption of the eggs, all urine was sampled, followed by 3 days of sampling 4 times a day (morning, noon, evening and bedtime). After 5 days, only morning urine samples were collected. The clomiphene levels found in the eggs led to absolute amounts between 10 and 20 μg per egg. Hence, a follow-up comparative study was conducted with microdoses of 1, 10 and 50 μg clomiphene following the same sampling scheme (Suppl. Table 1). The clomiphene extract was quantified by HPLC-MS/MS and then diluted with water/ethanol to accomplish 10, 100 and 500 μg mL^{-1} solutions. 100 μL of the solutions were used to fill sugar-filled gelatine capsules.

2.5. Sample preparation

2.5.1. Egg analysis

To investigate the isomer ratio of the clomiphene transferred into the egg, two eggs each, collected every second day, were processed using the previously described sample preparation [26] and then measured with the screening method. Eggs were homogenized by grinding. Five g of homogenized sample were transferred to a disposable 50 mL centrifuge tube and mixed with 50 μL internal standard (ISTD, 10 μg mL^{-1} clomiphene-d_5 citrate). For extraction, 10 mL of ACN were added and shaken mechanically by a head-over-head machine for 30 min. The samples were then centrifuged at 4000*g* and 20 °C for 5 min. The supernatant was diluted 1:10 for analysis with doubly distilled water.

2.5.2. Urine analysis: liquid-liquid extraction (LLE)

To 2 mL of urine, 50 μL of the ISTD (clomiphene-d_5 citrate, 10 ng mL^{-1}), 700 μL of phosphate buffer (0.8 M, pH 7) and 50 μL of β-glucuronidase were added and incubated at 52 °C for 1 h. After hydrolysis, 500 μL of carbonate/bicarbonate buffer (20%, pH 10) were added to alkalinize the sample. The LLE was carried out with 4 mL of TBME. After centrifugation (3 min, 1800*g*), the organic layer was transferred to a clean tube and evaporated to dryness. The residue was reconstituted in 100 μL of the mobile phase and an aliquot of 7 μL was injected to the HPLC-HRMS system (Suppl. Fig. 1a).

2.5.3. Urine analysis: solid-phase extraction (SPE)

For analyzing the phase-II-conjugates, a SPE purification was conducted. First, a HLB cartridge was conditioned with 3 mL of

methanol and 3 mL of water. The cartridge was then loaded with 2 mL of urine spiked with 50 μL ISTD (clomiphene d_5-citrate, 10 ng mL^{-1}) and 1 mL of acetate buffer (0.8 M, pH 5.2). Subsequently, the cartridge was washed with 3 mL of water and then eluted with 3 mL of methanol. The samples were evaporated to dryness, reconstituted in 100 μL of mobile phase and an aliquot of 5 μL was injected into the HPLC-HRMS system (Suppl. Fig. 1b).

2.5.4. Urine analysis: LLE and derivatization

For derivatization of the samples, the LLE-sample preparation mentioned above was carried out (with (*E*/*Z*)-4-HC-d_6 as the ISTD) until the evaporating step. The residue was then reconstituted with 200 μL of ACN and transferred into a reaction tube. A volume of 100 μL of 100 mM Na_2CO_3 in water was added as a catalyst. For derivatization of HC, 100 μL of a 1 mg mL^{-1} solution of dansyl chloride in ACN was used. The reaction took place at 65 °C and 700 rpm for 30 min. The sample was then evaporated to dryness and reconstituted in 100 μL of mobile phase and an aliquot of 5 μL was injected into the HPLC-HRMS system (Suppl. Fig. 1c).

2.6. Analytical methods

Chromatographic separation was achieved using a Vanquish UHPLC system from Thermo Fisher Scientific (Dreieich, Germany) equipped with a Poroshell 120 EC-C18 column (3.0 × 50 mm; 2.7 μm) from Agilent (Santa Clara, CA, USA). The mobile phase consisted of 0.1% formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). The flow was set to 0.4 mL min^{-1}. The HPLC was coupled to a high-resolution Exploris™ 480 mass spectrometer (Thermo Fisher Scientific) with a heated ESI source in positive ion mode. Targeted MS/MS experiments were performed with a resolution of 45 000 and an isolation window of *m/z* 0.4. The considered ion transitions are shown in Table 1. The ionization voltage was set at 3 kV. The vaporizer temperature was 300 °C and the ion transfer tube was heated to 320 °C.

2.6.1. Screening method and glucuronide analysis

The HPLC-gradient started with the initial content of 30% B for 30 s. In 6.5 min, it increased to 56% B and to 100% B in one more minute. The column was rinsed with 100% B for 1 min before it was equilibrated with 30% B for another 2 min. The MS/MS experiments were carried out with a normalized collision energy of 35%. The screening method included the precursor ions of clomiphene, clomiphene-d_5 and HC (Table 1). For glucuronide analysis the ion transitions of hydroxy-clomiphene glucuronide were added.

Table 1
MS/MS method parameters for urine analysis.

Compound	Ion transition
Clomiphene	406.1932 → 100.1126
	406.1932 → 72.0813
	406.1932 → 58.0657
Clomiphene-d_5	411.2046 → 100.1126
Hydroxy-clomiphene	422.1881 → 100.1126
	422.1881 → 72.0813
	422.1881 → 58.0657
Hydroxy-clomiphene glucuronide	598.2202 → 422.1881
	598.2202 → 100.1126
Hydroxy-clomiphene-d_6	428.2258 → 100.1126
Hydroxy-clomiphene-d_6 dansyl derivative	661.2768 → 100.1126
Hydroxy-clomiphene dansyl derivative	655.2392 → 100.1126
only (E/Z)-4-HC	655.2392 → 421.1804
	655.2392 → 72.0813
only (E/Z)-3-HC	655.2392 → 58.0657

2.6.2. Differentiation method

The HPLC-gradient started with the initial content of 30% B for 30 s. In 3 min, it increased to 59% B, and to 65% B in another 6 min. The column was rinsed with 100% B for 1 min before it was equilibrated with 30% B for another 2 min. The MS/MS experiments were carried out with normalized collision energies of 35% and 50% in a stepped collision energy mode. In addition to the precursor ions considered in the screening method, their corresponding derivatives were included in this method (Table 1).

2.6.3. Ion mobility analysis

The available HC reference standards and the AAF samples additionally were examined by HPLC-trapped ion mobility spectrometry (TIMS)-MS. Details on implementation are provided in the Supplementary Information.

2.7. Method characterization

The newly developed method (sample preparation: LLE and derivatization; MS method: differentiation method) was characterized based on the requirements for validation of a confirmation procedure for non-threshold substances approved by WADA, described in the International Standards for Laboratories [30]. Additionally to the required parameters selectivity, limit of identification (LOI) and carry-over, the method was also characterized regarding the parameters LOD, robustness, intra- and inter-day imprecision, recovery and linearity. The determination of the validation parameters is explained in the Supplementary Information.

3. Results and discussion

3.1. Egg analysis

Clomiphene occurs as (*Z*)- and (*E*)-isomer and has been administered as a mixture in the animal administration study. Since the isomers are metabolized differently in humans and (*Z*)-clomiphene is detectable in urine significantly longer than (*E*)-clomiphene, it could be relevant for doping analysis, if one isomer was deposited in the egg preferentially. Hence, clomiphene was extracted from the eggs and analyzed for the isomer distribution. The screening method allowed for the separation of the stereoisomers of both clomiphene and the metabolite 4-HC, as shown in Fig. 3a) and b). The chromatograms of an egg sample were superimposed on the chromatograms of the reference standards. In both cases, the (*Z*)-isomer was found to be much more abundant in the egg sample. Plotting the isomer-specific concentration of clomiphene and HC in the egg against the time of laying revealed that discrimination occurred over the entire period (Fig. 3c) and d)). The (*Z*)-isomer accounted for over 99% of the transferred clomiphene or HC.

3.2. Urine analysis: screening for clomiphene and HC

After the LLE sample preparation and analyzing the urine samples with the screening method, clomiphene residues were found in the urine of all volunteers after egg consumption and after drug intake, respectively. Since the maximum concentrations of excreted HC were higher than those of clomiphene, Fig. 4 illustrates the estimated HC concentrations (sum of isomers) for all study volunteers. These concentrations ranged from 82 to 266 pg mL^{-1} among egg consumers and just above the LOD among the test persons who received the drug. The excreted amounts of HC were thus much higher in the urine of those who had eaten the contaminated eggs than in the urine of those who had ingested the microdose of clomiphene, even though these amounts were

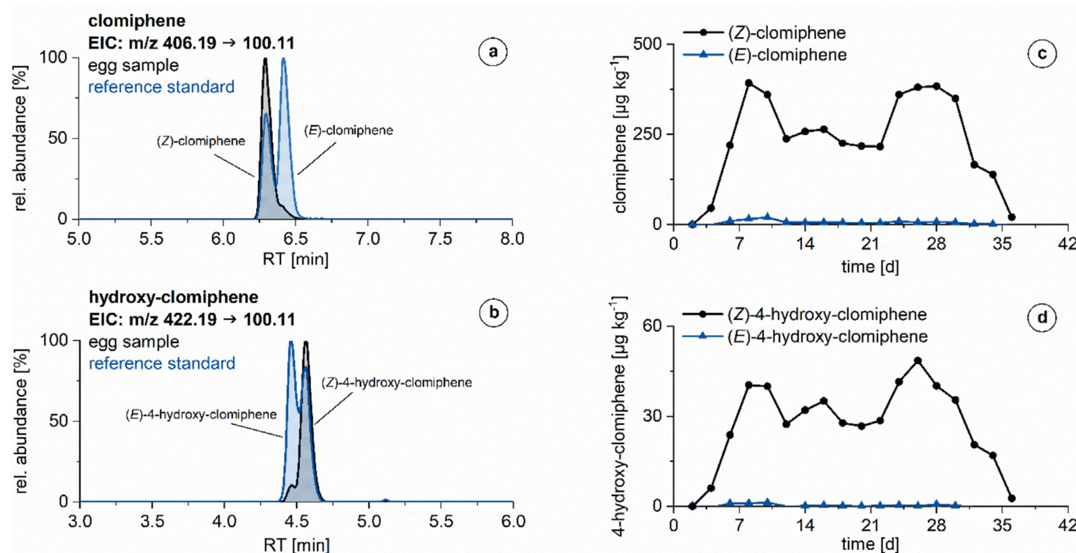

Fig. 3. Stereoselective HPLC-MS/MS chromatograms of clomiphene (a) and 4-HC (b), and the isomer-specific course of clomiphene (c) and 4-HC (d) levels in eggs. Laying hens were administered 10 mg clomiphene citrate per day from day 1 to day 28. Clomiphene and HC concentrations were estimated using the screening method.

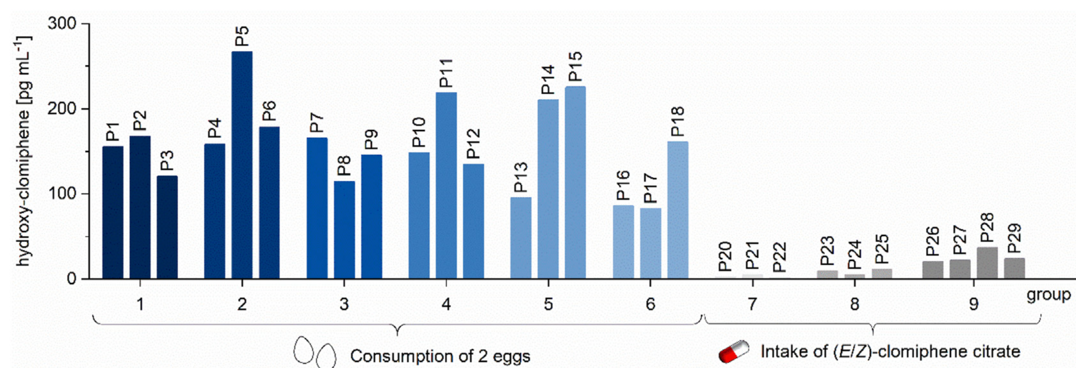

Fig. 4. Maximum HC concentrations found in urine samples of volunteers who consumed two contaminated eggs (group 1–6) or participated in the microdose study (group 7: 1 µg, group 8: 10 µg, group 9: 50 µg). Urine samples were analyzed with the screening method following the LLE sample preparation.

expected to be in the same range. The clomiphene concentrations, in contrast, were found at very low levels in both studies. The elimination profiles (semi-quantitative) of HC and clomiphene of all volunteers can be found in the supplementary Information (Suppl. Fig. 2).

3.3. Differentiation of clomiphene sources

Fig. 5 shows the extracted ion chromatograms (EICs) for clomiphene and HC of a reference standard and a sample from each study after the LLE workup and after analyzing the samples with the screening method. The chromatograms initially indicated that the most abundant hydroxy-metabolite was the same in both samples. For further investigation, the phase-II-metabolites (glucuronides) of the hydroxy-metabolite were investigated. The samples were therefore reprocessed without hydrolysis, but following SPE concentration. The analysis of the glucuronide conjugates indicated a difference between the two sample groups. The retention times of the glucuronide peaks and the corresponding mass spectra exhibited significant differences (Fig. 6a), suggesting different locations of the hydroxy groups in the respective phase-I-metabolites.

Since in a previous study [8], (*Z*)-3-HC was described as the most abundant urinary metabolite after intake of 100 mg of clomiphene, it was postulated that one of the samples contained (*Z*)-3-HC as the most abundant metabolite. Therefore, we established a HPLC-MS/MS method to separate the hydroxy-clomiphene isomers (*E*)-4-HC, (*Z*)-4-HC, (*E*)-3-HC and (*Z*)-3-HC. Here, it is important to note that (*Z*)-3-HC has the trans configuration (derived from (*E*)-clomiphene), while (*Z*)-4-HC has the cis configuration (derived from (*Z*)-clomiphene, Fig. 1).

Via the LLE workup in combination with the screening MS-method, separation of (*E*/*Z*)-3-HC and (*E*/*Z*)-4-HC could not be achieved. Therefore, implementing a derivatization step with dansyl chloride and adapting the HPLC gradient (differentiation method) enabled the separation of these four hydroxy-metabolites (Suppl. Fig. 3). The top chromatogram of Fig. 6b shows the nearly baseline-separated derivatized HC-isomers. Analyzing representative samples with this method showed that the samples after egg consumption contained (*Z*)-4-HC and the sample collected after microdosed clomiphene contained (*Z*)-3-HC as the most abundant hydroxy-metabolite, respectively (Fig. 6b).

Spot urine samples from all volunteers were analyzed following derivatization with dansyl chloride with the differentiation method.

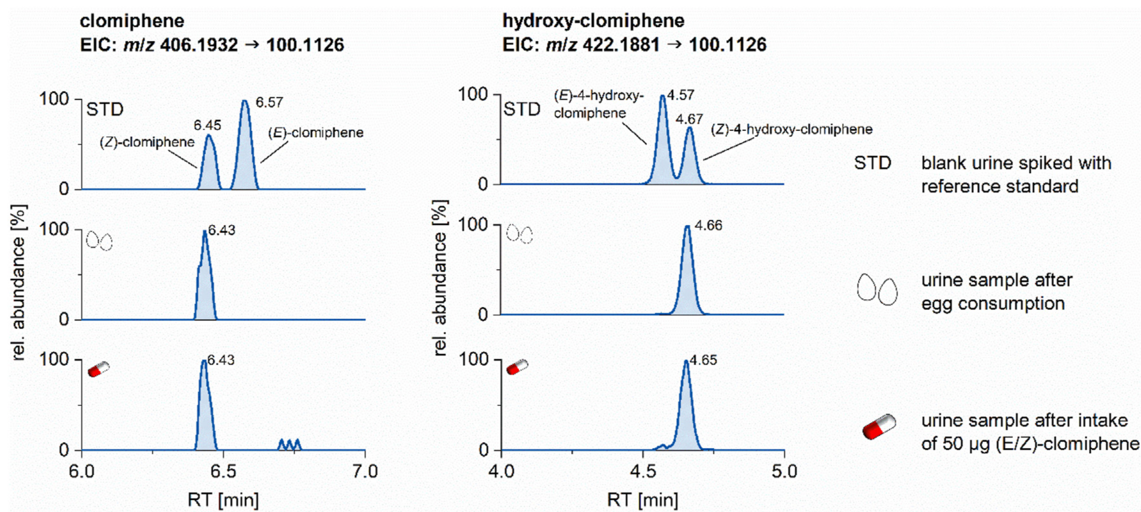

Fig. 5. HPLC-MS/MS chromatograms of clomiphene (EIC: m/z 406.19 → 100.11) and HC (EIC: m/z 422.19 → 100.11) in urine spiked with the reference standards (top), after egg consumption (middle) and after intake of 50 μg clomiphene (bottom). Urine samples were analyzed with the screening method following the LLE sample preparation.

To compare the pattern of hydroxylated clomiphene metabolites in samples from subjects after clomiphene intake from different sources, the relative peak areas of the different isomers are shown in Fig. 7a. Based on the peak areas of all HC-isomers, all volunteers who had eaten clomiphene-containing eggs excreted over 90% (*Z*)-4-HC. The distribution in the urine of those volunteers who had received the clomiphene microdoses differed significantly from the first group (Fig. 7a). The patterns of hydroxy isomers did not change during up to 10 days after intake (Suppl. Fig. 4).

The new method revealed a characteristic urinary HC pattern after consumption of contaminated eggs, which significantly differed from the pattern obtained following the uptake of a microdose of (*E*/*Z*)-clomiphene in this study.

3.4. Method characterization

The newly developed method for differentiation of HC isomers was characterized for (*E*)-3-HC, (*Z*)-3-HC, (*E*)-4-HC and (*Z*)-4-HC along the parameters selectivity, linearity, recovery, intra- and inter-day imprecision, carry-over, LOD, LOI and robustness. Table 2 summarizes the characterization results. No interfering signals occurred in any of the 10 blank urine samples collected from 5 female and 5 male volunteers. Therefore, the method was considered as selective. The imprecision was determined as variation of coefficients from 2.9% to 8.9% for the same day and from 3.3% to 11.5% for different days, depending on the concentration and the compound. The recovery (extraction) ranged from 63% to 80%. To test the robustness, the samples were frozen during the sample preparation (after LLE and before separating the organic phase) to

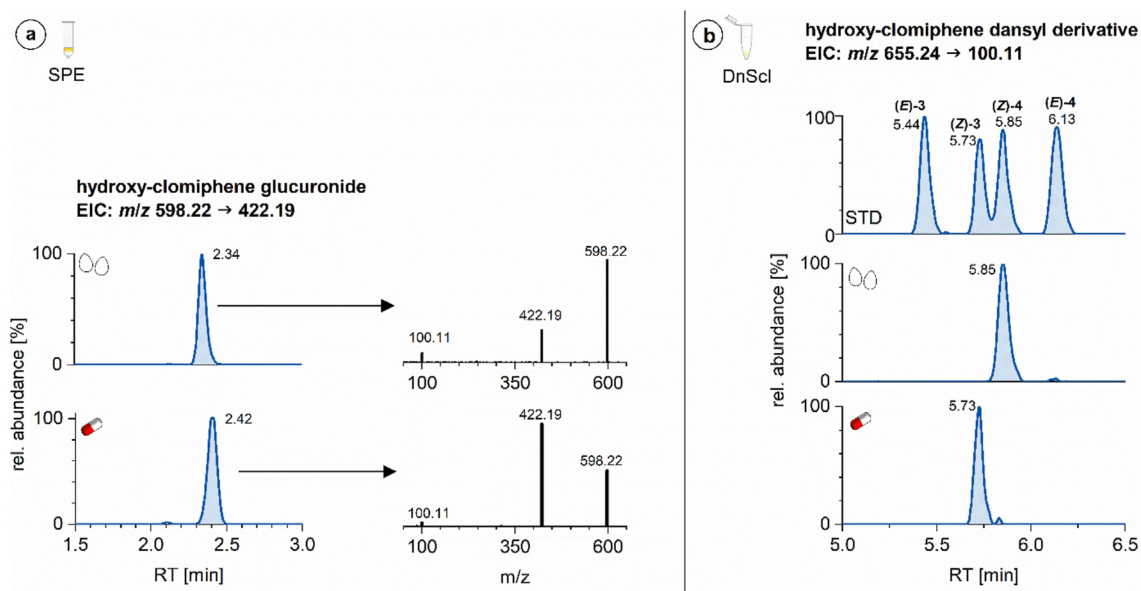

Fig. 6. a) HPLC-MS/MS chromatograms of HC-glucuronide (EIC: m/z 598.22 → 422.19) and the mass spectra of the corresponding peaks after the SPE sample preparation. b) HPLC-MS/MS chromatograms of HC dansyl derivatives (EIC: m/z 655.24 → 100.11) after LLE sample preparation including the derivatization step. Each chromatogram of a urine sample after consumption of clomiphene-containing eggs and after intake of a microdose of clomiphene.

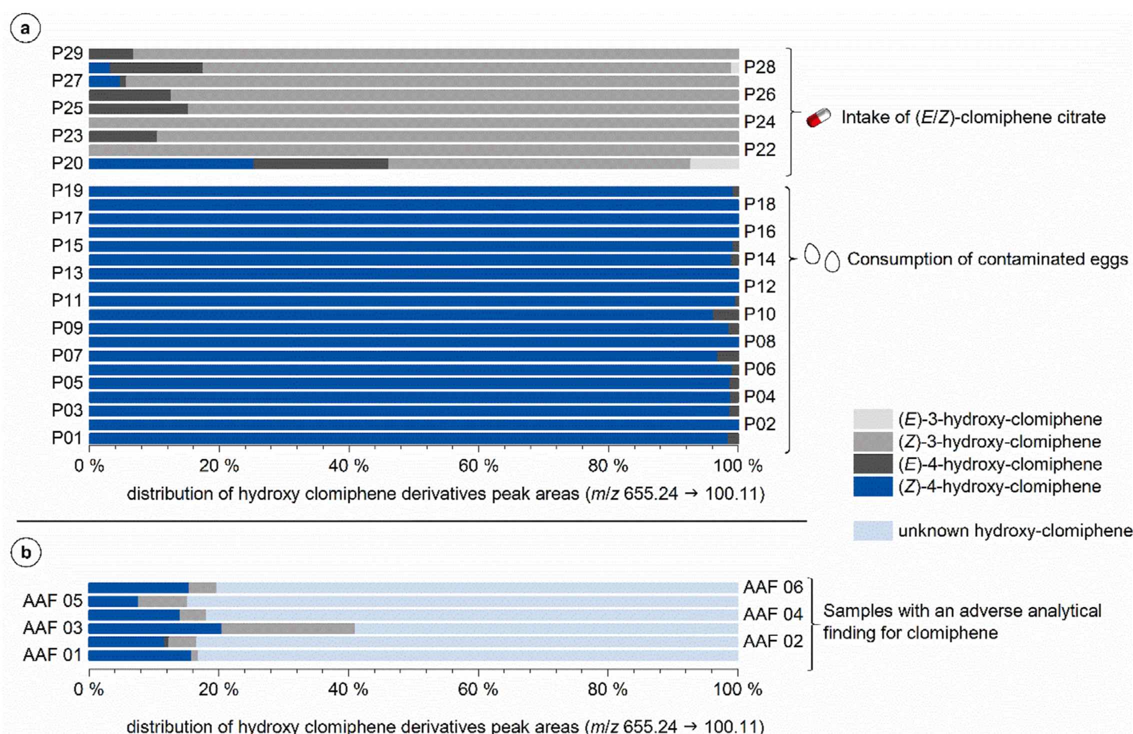

Fig. 7. Normalized peak area distribution of all measured HC dansyl derivatives (EIC: m/z 655.24 → 100.11) in urine samples from all study volunteers (a) and in AAF-samples from 2020 (b).

Table 2
HPLC-HRMS/MS-related characteristics and categories of hydroxy-clomiphene isomers in urine.

		E-3-HC	*Z*-3-HC	*E*-4-HC	*Z*-4-HC	
Intra-day Imprecision	at 500 pg mL^{-1}	7.7%	6.0%	3.4%	2.9%	n = 6
	at 100 pg mL^{-1}	6.3%	8.5%	7.5%	5.5%	n = 6
	at 50 pg mL^{-1}	7.1%	7.8%	8.9%	5.8%	n = 6
Inter-day Imprecision	at 500 pg mL^{-1}	7.0%	7.2%	4.1%	3.3%	n = 18
	at 100 pg mL^{-1}	8.9%	10.1%	5.7%	6.8%	n = 18
	at 50 pg mL^{-1}	10.2%	11.5%	6.6%	6.9%	n = 18
Selectivity	–	yes	yes	yes	yes	n = 10
LOD		4 pg mL^{-1}	4 pg mL^{-1}	4 pg mL^{-1}	3 pg mL^{-1}	n = 6
LOI		140 pg mL^{-1}	140 pg mL^{-1}	80 pg mL^{-1}	120 pg mL^{-1}	n = 6
Recovery	at 100 pg mL^{-1}	64.3–80.4%	63.1–72.9%	65.3–75.5%	64.5–73.0%	n = 6
Robustness	at 100 pg mL^{-1}	8.3%	9.4%	3.4%	5.8%	n = 12
Carry-over	at 500 pg mL^{-1}	0%	0%	0%	0%	n = 1
Linearity (R^2)	at 50–300 pg mL^{-1}	0.992	0.996	0.998	0.997	n = 6 per level

continue with the sample preparation the following day. Since the coefficients of variations were similar to those of the samples prepared on different days, the method was considered as robust regarding the sample preparation. There was no carry-over after the highest sample measured (500 pg mL^{-1}). The LOD (detection rate of 95%) was estimated by plotting the concentrations against the detection rate. For all tested metabolites, the LOD was between 3 and 4 pg mL^{-1}. The LOI (identification rate of 95%) was estimated by plotting the tested concentrations against the identification rate. The lowest LOI was found at 80 pg mL^{-1} for (*E*)-4-HC, at 120 pg mL^{-1} for (*Z*)-4-HC and at 140 pg mL^{-1} for both 3-HC metabolites. The linearity was tested in the range of 50 and 300 pg mL^{-1}. For all compounds, R^2 was higher than 0.99.

3.5. Application to routine doping control samples

Data concerning clomiphene AAFs found in the WADA-accredited anti-doping laboratory in Cologne in 2020 were re-evaluated in light of the newly generated information described in this paper. The metabolite patterns in these samples also differed markedly from the results in urine after contaminated egg consumption (Fig. 7b). Moreover, another hydroxy-metabolite, hitherto unknown and found neither in the microdose samples nor in the egg consumption samples, appeared as the most abundant in these samples. As this hydroxy-metabolite with a retention time of 4.3 min (screening method) elutes earlier than the other HCs, a more polar structure can be assumed. The product ion known to originate from the unaltered side chain (m/z 100.11) was observed in the product ion mass spectrum of this analyte, indicating that the hydroxy-group must be located on one of the two remaining aromatic rings (Suppl. Fig. 5). This tentative assignment was supported by ion mobility experiments, which were conducted to contribute additional information to the assessment of the metabolite structure in question. The measured ion mobilities were used to calculate the collision cross sections (CCS) of the targeted metabolite and the available HC reference standards. Subsequently, the CCS values

for all possible structures were computed and contrasted with the calculated ones in order to estimate the probability for the different structures. Unfortunately, the data did not lead to unequivocal results and several structures are worth considering. The determination of ion mobilities and the simulation of CCS values are described in the Supplementary Information.

For further evaluation of the metabolite, also to exclude that it is formed after multiple consumption of contaminated eggs, a controlled excretion study with therapeutic doses (for instance in the context of clomiphene treatment) should be considered to monitor and further characterize the formation of the new metabolite.

4. Conclusion

In this study, it was investigated whether traces of clomiphene can be found in human urine samples after consumption of clomiphene-contaminated eggs, motivated by earlier studies that demonstrated clomiphene-stimulated increases in egg production rates in hens [22–24] and the retention of clomiphene residues in eggs [26].

Healthy male volunteers consumed the eggs produced in a previous animal administration study. A single consumption of two clomiphene-containing eggs led to maximum HC concentrations between 82 and 266 pg mL^{-1} in the tested urine. Discrimination of the isomers occurred in the hen and eggs, accordingly, causing the almost exclusive detection of (*Z*)-clomiphene and its metabolites in the study volunteers' urine samples. Hence, the findings indicated that it was possible to generate an adverse analytical finding by consuming clomiphene-containing eggs. Consequently, a more detailed study of the metabolites was conducted.

The intake of the substance differed between the two studies: While the microdose study volunteers took a preparation containing 42% of (*Z*)- and 58% of (*E*)-clomiphene, the egg consumers received almost only (*Z*)-clomiphene. In addition to (*Z*)-clomiphene, (*Z*)-4-HC was already ingested by the participants with the contaminated eggs. Therefore, a change in the metabolic pattern was hypothesized. The analysis of phase-II-metabolites showed different glucuronides with respect to retention time and product ion spectra in samples from participants consuming contaminated eggs or a microdose of (*E*/*Z*)-clomiphene citrate, respectively, pointing to differences in the HC isomers present. This was confirmed when analyzing the samples with the method for differentiation of the hydroxy-metabolites of clomiphene by derivatization with dansyl chloride to achieve chromatographic separation. After consumption of clomiphene-containing eggs, (*Z*)-4-HC accounted for over 90% of the hydroxy-metabolites. In contrast, in urine from 8 out of 9 participants who received a microdose of (*E*/*Z*)-clomiphene citrate (*Z*)-3-HC, a metabolite of (*E*)-clomiphene, accounted for >80% of HC metabolites.

In all samples with clomiphene AAFs, the major HC isomer could not be assigned to one of the reference compounds available in this study. The comparison to the microdose of commercial (*E*/*Z*)-clomiphene indicated that this hydroxy-metabolite might be a long-term metabolite. Due to the extremely long half-life of (*Z*)-clomiphene compared to (*E*)-clomiphene [28,31] this so far unidentified metabolite is more likely to derive from (*Z*)-clomiphene. Ion mobility experiments were performed to provide preliminary information on the structure of this metabolite, but further experiments including the synthesis of putative reference compounds are necessary to identify this metabolite.

In conclusion, a method was developed to assist in distinguishing between clomiphene intake via contaminated eggs, recently taken microdose of clomiphene and previous clomiphene intake at therapeutic dosage e.g. for doping purposes. The ion mobility experiments further demonstrated that the separation of the studied HCs is possible not only by liquid chromatography following derivatization but also by ion mobility, which allows for a complementary and fast differentiation method if the required IMS-MS instrument is available.

Authors' contributions

Conceptualization: AT, MT; Methodology: LE, AT, MT; Formal analysis and investigation: LE; Writing - original draft preparation: LE; Writing - review and editing: NG, PD, GP, TM, MS, GD, AT, MT; Funding acquisition: MT; Resources: NG, PD, GP, TM, MS, GD; Supervision: MT.

Consent for publication

All authors have approved this version to be published.

Ethics approval

The consumption of clomiphene-containing eggs and the subsequent collection of urine samples were performed in accordance with the ethical standards laid down in the Declaration of Helsinki and followed the approval (#012/2019) by the local ethical committee of the German Sport University Cologne.

The preceding animal administration study was planned and carried out under conditions in compliance with animal welfare guidelines outlined in directive 2010/63/EU and was approved by the Belgian Ministry of Small Businesses and Agriculture.

Consent to participate

The human participants were non-athlete, healthy male volunteers who provided written informed consent.

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Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2022.339661.

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Abbreviations

AAF: adverse analytical finding
ACN: acetonitrile
CCS: collision cross section
EIC: extracted ion chromatogram
ESI: electrospray ionization
HC: hydroxy-clomiphene
HPLC: high performance liquid chromatography
ISTD: internal standard
LLE: liquid-liquid extraction
LOD: limit of detection
LOI: limit of identification
MS: mass spectrometry
MS/MS: tandem mass spectrometry
SERM: selective estrogen receptor modulator
TBME: *tert.*-butyl methyl ether
TIMS: trapped ion mobility spectrometry
WADA: World Anti-Doping Agency