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SHORT COMMUNICATION

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Elimination profile of low-dose chlortalidone and its detection in hair for doping analysis—Implication for unintentional non-therapeutic exposure

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Abstract

Chlortalidone (CLT) is a thiazide-type diuretic with high affinity for the erythrocyte carbonic anhydrase. Therapeutically, it is mostly used to treat edema and hypertension due to liver cirrhosis, heart insufficiency, or renal dysfunction. Although diuretics and masking agents are prohibited by the World Anti-Doping Agency (WADA) at all times in sports, substances belonging to this category are constantly detected in athlete samples, according to WADA's annual testing figures. Within this group of structurally diverse compounds, a threshold of 20 ng/mL has been introduced for six substances solely due to their presence as contaminants in other permitted drugs because of pharmaceutical production processes. In a recent presumptive doping case with a low urinary CLT concentration, the question of unintentional doping, for example, by contaminated non-steroidal anti-inflammatory drug intake, arose. To examine this potential scenario, a co-elimination of low-dose CLT and hydrochlorothiazide (HCTA; 20 × 50 µg, 0.2 mg/day each) was conducted on five consecutive days in two volunteers. Urine samples were subjected to liquid chromatography-tandem mass spectrometry (LC–MS/MS). Moreover, we examined the incorporation of CLT in scalp hair. HCTA is rapidly excreted renally in comparatively high concentrations. In contrast, the elimination of CLT is considerably slower (terminal elimination half-life extended by a factor of 12) and, consequently, much less concentrated in corresponding urine samples (45 and 53 ng/mL, respectively). Conversely, a higher hair incorporation of chlorthalidone was observed with simultaneous dosing of both. The results suggest that an unintentional intake of sub-therapeutic CLT doses due to contamination might result in an adverse analytical finding.

KEYWORDS
diuretics, hair, LC–MS/MS, urine

1 | INTRODUCTION

The use of diuretics in sports is prohibited because of the significant acceleration of renal excretion of water, electrolytes, and xenobiotics (including doping substances).[1] Potential abusive uses in competitive sports could be intended for short-term body weight reduction in weight category sports disciplines or masking of illicit substances. The former requires the prompt use of high therapeutic doses, which is almost inconceivable in competitions with functioning doping control systems. The use of diuretics to accelerate the excretion of such doping substances, which are only permitted below defined concentrations—so-called threshold substances—has been successfully prevented by the fact that such thresholds are void when diuretics are simultaneously detected. The extent to which acceleration of the complete excretion of doping substances is achievable remains to be investigated.

The retrospective evaluation of adverse diuretic findings in sports does not reveal additional insights into their prevalence, as the increase in absolute diuretic findings appears to be mainly due to improved detection limits (mainly by the introduction of liquid chromatography-mass spectrometry [LC–MS]) (Figure 1). The World Anti-Doping Agency (WADA) statistics were led for decades by the loop diuretic furosemide and hydrochlorothiazide (HCTA; thiazide). From a pharmacological perspective, diuretics were categorized into five groups: loop diuretics, thiazides, potassium-sparing diuretics, osmotic diuretics, and carbonic anhydrase inhibitors (CAIs).[2] Historically, loop and thiazide diuretics dominated in classical doping cases (e.g., HCTA and furosemide; Figure 1), while CAIs, which are preferentially used for the treatment of glaucoma, epilepsy, or high-altitude disorders, should be critically reviewed in particular.[2] However, this classification does not seem to be trivial at all, as the compound chlortalidone (CLT) has been classically categorized as a thiazide-type diuretic in accordance with its chemical structure. On the other hand, CLT possesses characteristic CAI properties, such as the inhibition of carbonic anhydrase and, correspondingly, a pronounced accumulation in erythrocytes (50–100 times the plasma concentration).[3–7] Hence, its categorization as a CAI seems to be more appropriate.[8] This deposit effect resulting in a significantly prolonged plasma half-life has been known for a long time, namely, 40–50 h for CLT versus 6–12 h for HCTA after a single dose.[9,10] In the case of long-term dosing, the plasma half-life is further prolonged up to 60 h for CLT and 15 h for HCTA.[11] CLT was widely used as a classical diuretic until the 1970s but then lost considerable importance.[11] However, its therapeutic use for the treatment of hypertension in the context of liver cirrhosis[12] or renal failure[13,14] led to a significant increase in clinical relevance and sales figures in the last decade.[15]

Regarding doping analyses, a categorization of diuretics in terms of their potential doping efficacy according to their pharmacological classification would be worth evaluating. In contrast, diuretics are unified in one category of the prohibited list, regardless of their diverse pharmacological, cellular, and molecular mechanisms or dosages.[16] Hence, similar analytical principles are currently applied, such that diuretics are sanctionable at any concentration as non-specific doping substances except for six diuretics (acetazolamide, bumetanide, furosemide, HCTA, torasemide, and triamterene) for which a minimum reporting level of 20 ng/mL has been defined.[17,18] This exception is based on their detection as minimal contaminations in permitted drugs, among them non-steroidal anti-inflammatory drugs (NSAIDs), resulting from the pharmaceutical production processes.[19,20] As the contamination with pharmacologically ineffective diuretic doses

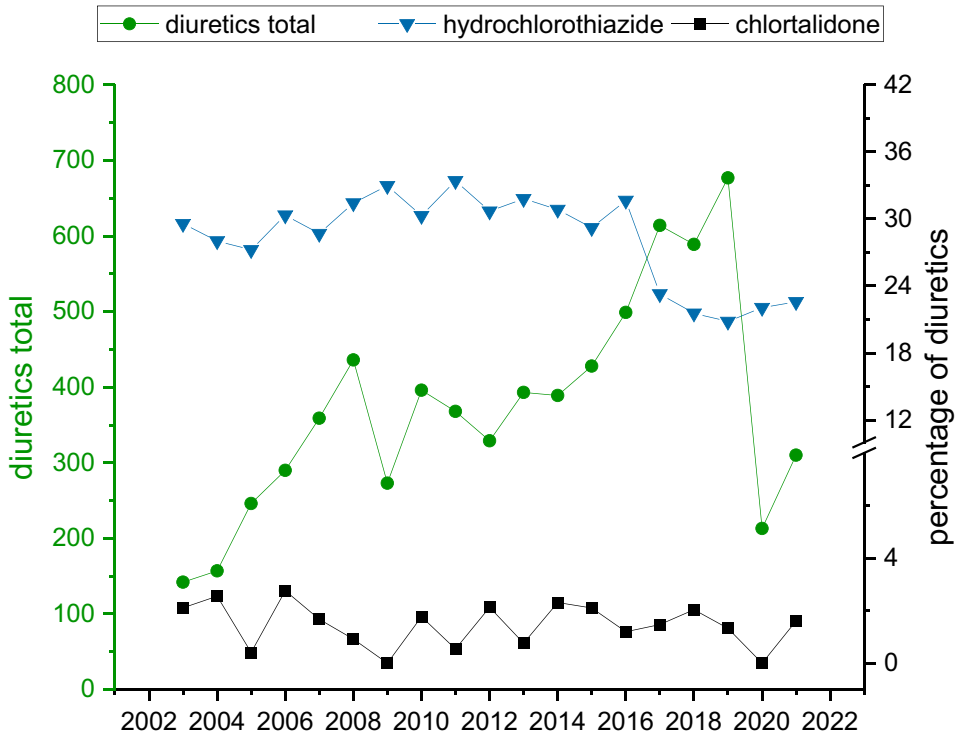

FIGURE 1 Number of diuretics and the respective proportion of hydrochlorothiazide and chlortalidone identified as adverse analytical findings between 2003 and 2021 published as anti-doping testing figures by the World Anti-Doping Agency.

appears unavoidable and proved to be consistent with good manufacturing practice rules in pharmaceutical drug production, the presence of other than the six diuretics specified above can also be expected. Besides, the presence of diuretics in dietary supplements has also been described.[21,22]

Currently, a presumptive doping case of an athlete with low urinary CLT concentrations raises the question of whether repeated ingestion of contaminated NSAIDs may lead to long-term accumulation and increased urinary excretion of CLT. Therefore, several sub-therapeutic administration studies in two male volunteers mimicked an unintended exposure to CLT by the intake of contaminated permitted drugs.

2 | MATERIALS AND METHODS

2.1 | Materials

CLT was supplied by Hexal AG (Holzkirchen, Germany), and HCTA was supplied by ALIUD Pharma GmbH (Laichingen, Germany). Mefruside was purchased from Gepepharm GmbH (Hennef, Germany). Merck (Darmstadt, Germany) supplied ammonium acetate and acetic acid (>98%). Acetonitrile, methanol (both LC–MS grade), and ethanol (EtOH; ≥99.9%, analytical grade purity) were provided by Th. Geyer (Berlin, Germany).

Solvent A (mobile phase A) consisted of 2 mM of ammonium acetate and 0.1% acetic acid (v/v) in water/acetonitrile (95:5, v/v). Mobile phase B consisted of 2 mM of ammonium acetate and 0.1% acetic acid (v/v) in water/acetonitrile (5:95, v/v). For hair analysis, mobile phase A consisted of 0.1% formic acid in water/acetonitrile (95:5, v/v) and B2 of 0.1% formic acid in water/acetonitrile (5:95, v/v).

2.2 | Administration

One male healthy volunteer initially self-administered a single dose of 10 mg of CLT resolved in 40% ethanol. Urine samples were collected prior to intake, as well as every excreted urine sample during the first 24 h and one sample per day up to 5 days after intake. Moreover, a hair sample of 2 cm was collected 3 days post-administration (3 mm of remaining hair length).

Moreover, the volunteer for the single dose administration and another male volunteer performed an administration of multiple low doses. The sub-therapeutic dosing included 50 µg of CLT and 50 µg of HCTA per dose orally taken four times per day for five consecutive days to mimic a potential unintended exposure scenario, by contamination of NSAIDs, for instance. For this, a stock solution of 1 mg of CLT and 1 mg of HCTA was prepared in 10 mL of 40% ethanol, of which 0.5 mL was ingested per dose. Blank urine was collected prior to the administration start and a sample of every urine up to the intake of the last low dose, respectively. After that, one urine sample was collected every 24 h in the morning. Besides, hair samples of approximately 2 cm length were collected 3 days post-initial (10 mg single dose of CLT) and 5 weeks after the onset of the study (3 mm of remaining hair length) from Volunteer 1. From Volunteer 2, a 4-cm hair sample was collected 8 weeks after the onset of the sub-therapeutic administration study. Both volunteers signed a written informed consent form before.

2.3 | Sample preparation

Urine samples were diluted by adding 0.2 mL of solvent A containing the internal standard to 0.05 mL of urine and directly injected into an LC–MS/MS system.

For hair sample preparation, 50 mg of each sample was cut, and the internal standard (mefruside) was added. For matrix calibration, increasing amounts of CLT and HCTA (1, 2, 5, 10, or 20 pg/mg, respectively) and the internal standard were added to blank hair samples. Extraction was performed with 3 mL of methanol in an ultrasonic bath at 50°C for 6 h. After centrifugation (3100 rpm, 5 min), the supernatants were evaporated under nitrogen. The reconstituted samples (50 µL of solvent A) were subjected to LC–MS/MS.

2.4 | Mass spectrometric analyses

Analyses were carried out on two LC–MS/MS systems: an Agilent 1290 coupled to either ABSciex 5500+ for urine samples or ABSciex 6500+ for analysis of the hair samples (Agilent Technologies, Waldbronn, Germany; Sciex, Darmstadt, Germany). Chromatographic separation was carried out with a ZORBAX Eclipse XDB-C8 (3.5 µm × 2.1 mm × 100 mm; Agilent Technologies) at a column temperature of 35°C for both urine and hair samples. After 1 min of equilibration at 90% A, the gradient decreased to 0% A and was held for 5 min. After another 1 min at 0% A, the gradient increased to 90% A, which was held for 0.5 min. The flow rate was constant at 0.25 mL/min. The injection volume was 5 µL for both urine and hair samples. For analysis of the hair samples, the following gradient with mobile phases A2 and B2 was used for chromatographic separation after equilibration at 100% A2 for 1 min: the gradient decreased to 10% A2 over the course of 4 min and was hold for 1.5 min at 10% A2, subsequently, the gradient increased to 100% A2 over the course of 0.5 min. The flow rate was constant at 0.25 mL/min. Data analysis was carried out using Analyst TF® 1.71 and MultiQuant 3.0 software (Sciex).

3 | RESULTS AND DISCUSSION

3.1 | Urinary elimination data

A controlled low-dose elimination study was conducted in two volunteers in order to examine the chance of a low CLT adverse analytical finding (AAF), for example, because of unintentional exposure to permitted drugs. The administered total daily dose of 0.2 mg of CLT was

clearly higher than published contaminations and was chosen to model the effects of a possible accumulation over manageable periods of time. In comparison, a therapeutic dose is 125–500 times higher (25–100 mg once per day). Urine samples were collected up to 10 days after oral intake of the last CLT dose and analyzed by the standard anti-doping control LC–MS/MS analysis. In addition, 10 mg of CLT was ingested as a single dose, resulting in a peak urinary concentration of 878 ng/mL 6 h post-administration. The urinary elimination half-life was 59 h (data not shown).

In the multiple dosing approach, the urinary elimination half-life of CLT was 60 h (Volunteer 1) and 86 h (Volunteer 2) (Figure 2a,b). This is in accordance with the elimination half-life described for CLT in the literature (40–89 h).[10] This prolonged elimination phase resulted in urinary concentrations of 4.08 and 4.26 ng/mL CLT (corrected for specific gravity) 10 days after the end of the last low-dose ingestion, that is, the termination of sampling. This half-life determined during the terminal elimination after intake cessation corresponds well with the plasma half-life during administration described in the literature.[9,11] In adherence to the WADA guidelines, a CLT concentration of 4 ng/mL observed in an athlete's urine sample would be reported as AAF.[18] However, the results of the low-dose approach emphasize that an unintentional intake of CLT-contaminated drugs can be responsible for a low CLT urine concentration. Because of the increasing production of CLT in the pharmaceutical industry, an elevated contamination risk is deemed logical. This should be confirmed by the analysis of drugs analogous to the investigation performed for other diuretics, such as HCTA, torasemide, and furosemide.[19,20] Furthermore, internet research revealed partially identical production sites for CLT and HCTA, mostly based in India.[23,24]

In comparison, the elimination of HCTA was significantly faster, which is also in line with other studies. Deventer and colleagues, for instance, showed the elimination profile of one single therapeutic dose (25 mg of HCTA) in six volunteers. Regarding the scenario of potential, unknowingly exposure to sub-therapeutic doses of HCTA due to NSAID intake, one study investigated the elimination after micro-dose administration on three consecutive days up to 76 h after the intake of the first micro-dosage.[19] Although the maximal low dose ingested was lower compared with our approach (10 µg per micro-dose, 30 µg in total on the third day vs. 50 µg of HCTA, 200 µg in total per day in the present study), the elimination behavior is comparable. Moreover, there is a third study, in which the elimination profile was investigated after intake of a single or two sub-therapeutic doses of HCTA over a period of 24 h solely.[25]

3.2 | Hair incorporation

The outstanding advantage of hair analysis is due to its retrospective information on substance administration, which is ideally based on compound incorporation from the circulating blood into the hair root and subsequent linear hair growth. In a few idealized cases, semi-quantitative assessments of hair concentrations are based on a direct proportionality between hair concentrations and the corresponding amount of active substance (area under the curve, AUC) in plasma. This proportionality factor, the so-called incorporation rate,[26] has only been investigated in exceptional cases in application studies and is systematically limited by the structure and biotransformation of the substances of interest. First, there is the possibility of an alternative hair incorporation from sweat, which leads to immediate diffusion into more distant hair segments and excludes kinetic segment analyses.[27] Moreover, the hair incorporation depends on the substance's basicity and hair pigmentation, and a gradual substance washout can take

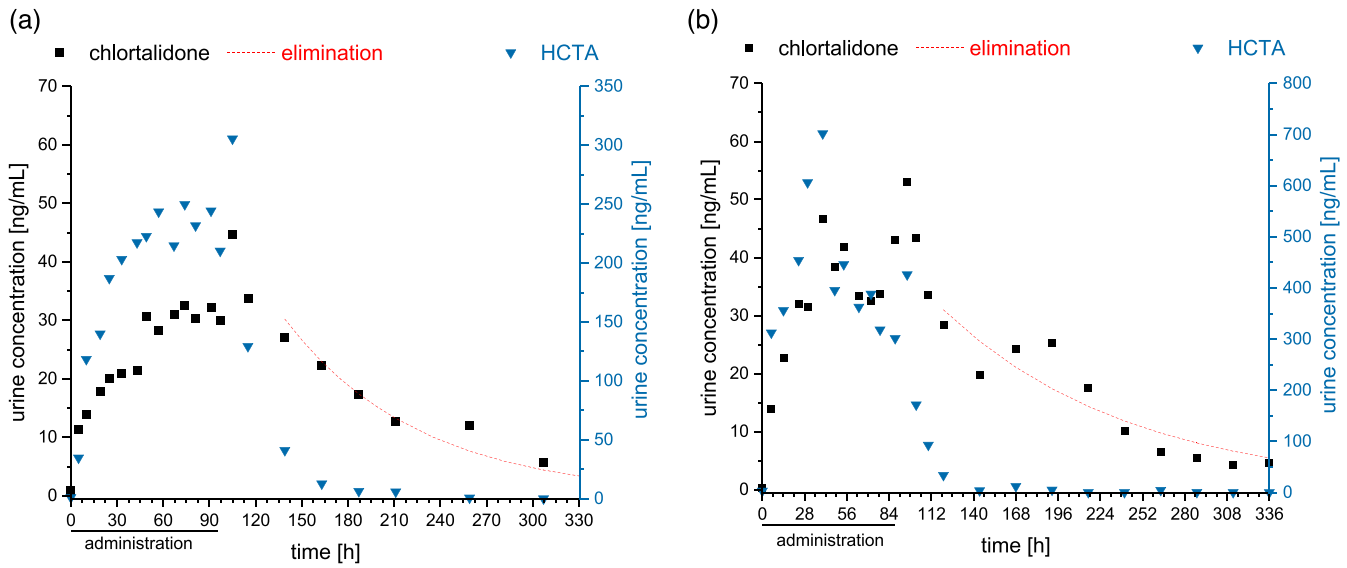

FIGURE 2 Elimination profile of chlortalidone and hydrochlorothiazide (HCTA) after the intake of 20 × 50 µg doses of each compound over a period of 5 days (total dose of 200 µg/day). Urine concentrations are shown for chlortalidone (■) and hydrochlorothiazide (▼) during the low-dose intake period, as well as up to 330 and 336 h after the start of the intake for Volunteer 1 (a) and Volunteer 2 (b). The exponential models for chlortalidone elimination after the last sub-therapeutic dose are shown for both volunteers (–).

TABLE 1 Hair concentration of chlortalidone and hydrochlorothiazide after one single dosage (10 mg) or after 0.2 mg of each substance per day for five consecutive days (four times 50 µg/day, low dose).

	Chlortalidone (pg/mg)	Hydrochlorothiazide (pg/mg)
Single dosage (10 mg)		
Volunteer 1 (after 3 days; 2 cm hair length)	9.5	/
Multiple low dose (1.0 mg in total, 0.2 mg/day)		
Volunteer 1 (after 5 weeks; 2 cm hair length)	4.2	0.39
Volunteer 2 (after 8 weeks; 4 cm hair length)	0.78	0.23

place depending on the polarity of the incorporated substance, the hair structure, and cosmetic treatments. Finally, the presence of approximately 10% non-growing (telogen) hairs preserves older findings over a longer period of time. Due to the large number of potential influencing factors, quantitative decision limits (cut-offs) have only been published for a few substances. The Society of Hair Testing has recently published consensus threshold values in hair for eight drug classes (including 17 target compounds) and stated that "the scientific literature is insufficient to establish cut-off values for any other drugs not listed".[28] To examine the incorporation of CLT in the hair, an extraction of hair samples from both volunteers was performed. Three days after the intake of the 10 mg single dose of CLT in Volunteer 1, CLT was detectable, which is due to sweat or sebum (Table 1). For the same volunteer, the quantification of both compounds 5 weeks after the low-dose approach revealed concentrations for CLT and HCTA of 4.2 and 0.39 pg/mg, respectively (Table 1). In comparison, the hair levels quantified for Volunteer 2 8 weeks after the low dose corresponding to the invasion and elimination period were lower (0.78 pg/mg CLT and 0.23 pg/mg HCTA; Table 1). Those hair levels in the sub-picogram per milligram concentration range are below typical limit of detections (LODs) for relevant hair analyses. They could be measured under ideal model conditions for the collection of scalp hair samples; that is, optimal sampling of the relevant segments was made possible. This demonstrates the diagnostic limitations of hair tests for the quantitative assessment of late stage and/or low-dose administration. The difference between the two volunteers is due to the initial 10 mg single dose 8 weeks before hair sampling, as the 2-cm hair sample represents both single and multiple low dosages. The observation of higher CLT concentrations compared with HCTA can be attributed to the accumulation of CLT in the erythrocytes. So far, literature on the incorporation of diuretics into hair is scarce, as only two publications deal with this topic. One study described the incorporation and detectability of HCTA in hair in the context of a doping case.[29] Another study showed the incorporation of three CAIs (dorzolamide, brinzolamide, and acetazolamide) in the hair of patients under therapeutic treatment.[30]

4 | CONCLUSION

In a current presumptive doping case, potential sources for a low CLT concentration in an athlete urine sample were to be evaluated. To investigate an unintentional doping scenario, a controlled low-dose administration of multiple dosages of CLT together with HCTA was conducted in two male volunteers, and urine and hair samples were analyzed by LC–MS/MS. The elimination profile of CLT was determined in direct comparison to HCTA for both volunteers, and a prolonged elimination of CLT was observed, resulting in a urinary CLT concentration of approximately 4 ng/mL 10 days after the last subtherapeutic dose. The results of this study support the assumption that frequent ingestion of low dosages over a longer period might accumulate to relevant urinary concentrations compatible with CLT AAFs in an athlete's urine sample. Moreover, this study provides the first evidence of the incorporation of CLT into hair as a supplementary matrix, which is most likely mediated by sweat or sebum incorporation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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