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RESEARCH ARTICLE

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Multiple incidence of the prescription diuretic hydrochlorothiazide in compounded nutritional supplements

Donata Favretto[1,2] | Sindi Visentin[1,2] | Salvatore Scrivano[1,2] | Emanuele Roselli[1,2] | Fabio Mattiazzi[1,2] | Roberto Pertile[2] | Susanna Vogliardi[1] | Marianna Tucci[1] | Massimo Montisci[1,2]

[1] Department of Cardiac Thoracic and Vascular Sciences and Public Health, University of Padova, Padova, Italy

[2] University Hospital of Padova, via Falloppio 50, 35121, Padova, Italy

Correspondence
Prof. Donata Favretto, University Hospital of Padova, Via Falloppio 50, 35121, Padova, Italy.
Email: donata.favretto@unipd.it.

Abstract

Diuretic agents are prohibited in sports in- and out-of-competition according to the regulations of the World Anti-Doping Agency (WADA) because of their possible masking effects on other doping agents in urine samples, and their ability to produce fast acute weight losses. Despite previous studies reported adverse analytical findings (AAFs) resulting from contaminations at ppm level (μg/g) of medicinal products, and recommended to introduce reporting limits for diuretics in doping controls, these are not adopted in analyses performed by WADA-accredited laboratories. We report the case of an athlete with two AAFs for hydrochlorothiazide (HCTZ) at low urinary concentrations (<10 ng/mL), who declared the use of nutritional supplements prepared in a compounding pharmacy. His nutritional supplements were analyzed revealing HCTZ presence in different concentrations, at the ppm level (μg/g and ng/mL).

With the aim of testing the plausibility of the observed urinary HCTZ concentrations with the nutritional supplement ingestion, a urinary excretion study with three healthy volunteers was performed. HCTZ-contaminated powder (6.4 μg/g of HCTZ) was administered to each subject in different dosages, reproducing the possible ingestion pattern occurred. Urine specimens were collected before and after ingestion of the powder, up to 24 hours, and underwent liquid–liquid extraction and liquid chromatography–tandem mass spectrometry determination.

Post-administration specimens were found to contain HCTZ at concentrations of 5–230 ng/mL, which supported the accidental inadvertent intake of the prohibited substance by the athlete. This study makes the argument that the introduction of reporting limits for diuretics are warranted in doping control samples, in order to protect against inadvertent AAFs due to contaminated products.

KEYWORDS

adverse analytical finding, contamination, doping, hydrochlorothiazide, nutritional supplements

1 | INTRODUCTION

Hydrochlorothiazide (HCTZ) is a diuretic medication often used to treat high blood pressure[1] and is classified, as all diuretics, among the substances prohibited by the World Anti-Doping Agency (WADA) since 1988. Diuretics are considered banned substances because of their possible masking effects on other doping agents in urine samples and their ability to produce fast acute weight losses before competition in sports

with weight categories.[2-8] Their use is prohibited both in-competition and out-of-competition and anti-doping laboratories include diuretics in routine analysis.[2,9] The most common method for diuretic detection in urine samples is liquid chromatography–tandem mass spectrometry (LC–MS/MS), which is capable of detecting substances in urine at picogram level.[9,10] The high sensitivity of the method implies that also minimal concentrations of the drug in urine lead to an adverse clinical finding (AAF). A previous study, in particular, reported an AAF resulting from contaminations at ppm level (μg/g) of over the counter non-steroidal anti-inflammatory drug (NSAID) medications, and recommended the introduction of reporting limits for diuretics in doping controls.[9] Despite this evidence, diuretics have no reporting limit in analyses performed by WADA-accredited laboratories: therefore, any amount of HCTZ detected in urine samples automatically implies an AAF.

We report the case of an athlete with two consecutive AAFs for HCTZ at low urinary concentrations (<10 ng/mL), resulting from contaminated nutritional supplements ingestion, and discuss the urinary concentrations obtained through an elimination study performed in order to identify, if possible, the best reporting limit for HCTZ in analyses performed by WADA-accredited laboratories.

1.1 | Case overview

The case involves a 22-year-old male athlete who was selected for two in-competition doping controls (seven days apart), resulting both in AAFs for HCTZ in low urinary concentrations. The estimated amount of HCTZ was 8 ng/mL in the first sample and 13 ng/mL in the second, with normal urinary specific gravity (first sample SG = 1.015, second sample SG = 1.011).

The athlete declared the use of non-steroidal analgesic medicines on doping control forms, and nutritional supplements prepared in a compounding pharmacy. He denied ever taking HCTZ or any other prohibited substance.

His nutritional supplements included five products:

- A: Liquid in a bottle with dropper cap labeled as vitamin D3 (40000 UI/mL) and vitamin K2 (480 μg/mL).
- B: Capsules in a jar labeled as L-tyrosine (250 mg), chelated selenium (200 μg), and chelated zinc (15 mg).
- C: Powder in sachets (9.5 g each) labeled as creatine monohydrate (5200 mg), beta alanine (1000 mg), hydroxy-beta-methylbutyrate (HMB) (1500 mg), L-carnitine (1000 mg), thiamine (20 mg), riboflavin (30 mg), and nicotinamide (30 mg).
- D: Powder in sachets (9.5 g each) labeled as beta alanine (2000 mg), HMB (1500 mg), ATP (400 mg), ribose (5000 mg), thiamine (20 mg), riboflavin (30 mg), and nicotinamide (30 mg);
- E: Capsules in blister packs labeled as proprietary blend (500 mg) containing vitamin C, vitamin B6, folate, vitamin B1, magnesium, choline, inositol, taurine, L-methionine, betaine HCl; indol-3-carbinol (200 mg), magnesium glycyl glutamine (200 mg), and coenzyme Q10 (100 mg).

Based on the advice of a nutritionist, for about nine months, he had been ingesting five drops of A, one capsule of B, and two capsules of E in the morning (9 am); another two capsules of E in the afternoon (5 pm); and one sachet of C and one of D before each training session.

2 | NUTRITIONAL SUPPLEMENT ANALYSIS

Samples of each compounded supplement were provided by the athlete (unsealed products) and new, identical sealed compounded supplements were purchased at the compounding pharmacy (sealed products), in order to exclude the hypothesis of athlete responsibility in the contamination of the supplements.

2.1 | Materials and methods

Several units were selected and analyzed from both set of supplements, in particular:

- *Unsealed products*: two 0.5 mL samples of A, four capsules of B, two sachets of C, two sachets of D, and 14 capsules of E.
- *Sealed products*: two 0.5 mL samples of A, five capsules of B, one sachet of C, one sachet of D, and six capsules of E.

Before analysis of the supplements the primary packages (blister packs and sachets) were checked for integrity by visual inspection and leak tightness by applying partial vacuum (600 mbar).

2.1.1 | Chemical and reagents

Methanol and acetonitrile (Merck, Darmstadt, Germany) were high-performance liquid chromatography (HPLC) grade. Water was obtained using Milli-Q Plus (Millipore Molsheim, France) system. Formic acid 99% (Carlo Erba Reagents, Italy) was from analytical grade.

Standards of HCTZ at 1.0 mg/mL in methanol and spironolactone to be used as internal standard (IS) at 1.0 mg/mL in acetonitrile were purchased from LCG (LCG Standard, Sesto San Giovanni, MI, Italy).

2.1.2 | Preparation of HCTZ reference test solutions

The reference standard of HCTZ in methanol was diluted with water to yield concentrations of 1–1000 ng/mL for calibration of the ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method.

2.1.3 | Sample preparations

The powder content of each solid supplement (cap or sachet) was mixed to make it homogeneous. To 120 mg of the homogenate, 1 mL of methanol was added. After ultrasonication for 10 minutes, the mixture was centrifuged for 5 minutes at 3200 rpm. The supernatant was membrane-filtered with a 0.2 μm filter. To 100 μL of this solution, 100 μL of Milli-Q water were added. After ultrasonication for 5 minutes, the mixture was ultracentrifuged for 5 minutes at 16000 g. The supernatant was membrane-filtered with a 0.2 μm filter.

For liquid sample A, 0.5 mL of liquid were dried under nitrogen and resuspended in 100 μL of Milli-Q water.

For quantification purposes, three aliquots of a placebo powder (made of microcrystalline cellulose, mono- and di-glycerides of fatty acids, magnesium salts of fatty acids, calcium phosphate and silicon dioxide) were spiked with HCTZ standard solution at 1.3 μg/g, 3.3 μg/g, and 6.6 μg/g, respectively; after dispersion of the solution

into the powder, homogenization, and overnight drying, 120 mg of each spiked powder were prepared as above (Section 2.1.3).

2.1.4 | UPLC–MS/MS analysis of HCTZ in nutritional supplements

UPLC conditions

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was used for separation, applying an Acquity HSS T3 column (2.1 mm ID × 100 mm, 1.8 μm) maintained at 40°C. The LC conditions were as follows: gradient elution with water containing 0.1% (v/v) formic acid as mobile phase A and acetonitrile as mobile phase B. The flow rate was 300 μL/min and the gradient was programmed as follows: 0–1 min at 90% A, 1–3 min to 10% A, 3–5 min hold 10% A, 5–6 min to 90% A and 6–9 min hold 90% A.

MS/MS

An Xevo TQ-S tandem-quadrupole mass spectrometer (Waters, Manchester, UK) equipped with a Z-spray electrospray interface was used. Negative electrospray ionization (ESI) was performed in the multiple reaction monitoring (MRM) mode. The capillary voltage was set to −3.5 kV. The source block temperature was 150°C, and the desolvation gas (nitrogen) was heated to 600°C and delivered at a flow rate of 1000 L/h. The deprotonated molecule of HCTZ was chosen as precursor ion and the following MRM transitions were monitored: m/z 296–269, m/z 296–205, m/z 296–78. System operation and data acquisition were controlled using Mass Lynx 4.1 software (Waters, Manchester, UK). All data were processed with the Target Lynx quantification program (Waters, Manchester, UK).

Method validation

Method validation considered the parameters specificity, limit of detection (LOD), limit of quantification (LOQ), linearity and recovery for LC–MS/MS.

2.2 | Results of nutritional supplement analysis

2.2.1 | Method validation

For UPLC–MS/MS analysis, specificity was verified by comparison of the retention time and the ion transitions abundances obtained from a certified reference material containing HCTZ.

The result of the recovery from blank powder spiked with HCTZ reference solution was 85%–90%, calculated by comparing the signal of extracted powder with that of pure reference solution.

The method resulted linear in the range 1–1000 mg/g. The LOD (signal-to-noise = 3/1) and the LOQ (signal-to-noise = 10/1) were respectively 0.3 mg/Kg and 1 mg/Kg in the spiked powder.

For the fully validation of the method it would be necessary to conduct a standard addition method, ie, to spike all 5 matrices of the different supplements with HCTZ to determine the recovery, linearity, and "true" concentration of HCTZ in each supplement.

2.2.2 | Analysis

Both unsealed and sealed batches of nutritional supplements were found to contain HCTZ in different concentrations and quantity depending on the type of supplement and on the different dose units of the same supplements (capsules or sachets), as reported in Table 1. Type C was the unique supplement without HCTZ both in unsealed and sealed products.

In analyzed supplements, HCTZ was identified in A at concentration of 2.1–4.6 ng/mL; in B and E capsules in dosages ranging from n.d. to 384 μg/capsule; and in the powder contained in D sachets in dosages ranging from n.d. to 147 μg/sachet.

3 | EXCRETION STUDY

With the aim of testing the plausibility of the observed urinary HCTZ concentrations with the nutritional supplement ingestion, a urinary excretion study with three volunteers was performed, with the written consent of the study participants.

3.1 | Materials and methods

HCTZ-contaminated powder (6.4 μg/g of HCTZ), residual from the analysis performed on the sachet of nutritional supplement type D (unsealed product), was administered in different dosages to the 3 healthy male volunteers (subject 1: 29 years old, BMI 23.9; subject 2: 26 years old, BMI 25.7; subject 3: 27 years old, BMI 20.6), reproducing the possible pattern of consumption occurred by ingesting contaminated supplements.

In particular:

- Subject 1 and subject 2 ingested a first dose of 6.4 μg of HCTZ (1 g of powder) at 9 am (t^0), and a second dose of 3.2 μg of HCTZ (0.5 g of powder) at 5 pm (t^{+8}).
- Subject 3 ingested a first dose of 12.8 μg of HCTZ (2 g of powder) at 9 am (t^0), and a second dose of 6.4 μg of HCTZ (1 g of powder) at 5 pm (t^{+8}).

A urine sample was collected from each subject prior to the first dose (t^0); and subsequent urine samples were collected for 24 hours after the first administration, reporting the hours passed from the first dose.

For each urine specimen pH, specific gravity and creatinine level were determined.

Blank urine samples were collected to be used as negative controls in the analytical sequence. Blank urine samples were spiked with HCTZ standard in the range 1–500 ng/mL, to be used as calibrators.

3.1.1 | Urine preparation

To 2 mL of urine 1 mL of sodium acetate at pH 5.2 was added; 50 μL of the IS spironolactone at 10 μg/mL were added; liquid–liquid extraction (LLE) with 4 mL of ethyl acetate was carried out. After centrifugation at 4000 rpm for 15 minutes, the organic layer was transferred into a new tube. To the remaining urine, 250 mg of ammonium carbonate was added and a second step of LLE with 4 mL of ethyl acetate was performed. After centrifugation, both organic layers were combined and evaporated until dry under nitrogen stream at 40°C. The remaining residue was dissolved in 100 μL of mobile phase.

TABLE 1 Results of the analysis on nutritional supplements furnished by the athlete (*unsealed products*) and ordered in the compounding pharmacy (*sealed products*)

			HCTZ	
	Supplement Type	Units	Concentration ppm (µg/g; ng/mL)	Dose in Each Unit (µg)
Unsealed products	**A** Liquid drops *vitamin D3 and vitamin K2*	5 drops	4.6	0.001
	B	1 capsule	10.4	3.0
	Powder in capsules	1 capsule	22.5	6.4
	L-tyrosine, chelated selenium, and chelated zinc	1 capsule	47.9	13.5
		1 capsule	909.0	257.0
	C	1 sachet	n.d.	n.d.
	Powder in sachets *creatine monohydrate, beta alanine, HMB, L-carnitine, thiamine, riboflavin and nicotinamide*	1 sachet	n.d.	n.d.
	D	1 sachet	6.5	61.5
	Powder in sachets *beta alanine, HMB, ATP, ribose, thiamine, riboflavin, and nicotinamide*	1 sachet	15.5	147.1
	E	1 capsule	n.d.	n.d.
	Powder in capsules	1 capsule	n.d.	n.d.
	proprietary blend, indol-3-carbinol, magnesium glycyl glutamine, and coenzyme Q10	1 capsule	1.7	1.0
		1 capsule	3	1.5
		1 capsule	3.4	1.7
		1 capsule	4.4	2.2
		1 capsule	5.7	2.9
		1 capsule	6.3	3.2
		1 capsule	7.2	3.6
		1 capsule	7.9	3.9
		1 capsule	9.8	4.9
		1 capsule	11.8	5.9
		1 capsule	14.5	7.3
		1 capsule	763.0	384.0
Sealed products	A Liquid drops *vitamin D3 and vitamin K2*	5 drops	2.1	0.0005
	B	1 capsule	22.8	5.0
	Powder in capsules	1 capsule	25.8	6.0
	L-tyrosine, chelated selenium, and chelated zinc	1 capsule	38.0	8.7
		1 capsule	44.0	10.7
		1 capsule	199.0	46.0
	C Powder in sachets *creatine monohydrate, beta alanine, HMB, L-carnitine, thiamine, riboflavin and nicotinamide*	1 sachet	n.d.	n.d.
	D Powder in sachets *beta alanine, HMB, ATP, ribose, thiamine, riboflavin, and nicotinamide*	1 sachet	n.d.	n.d.
	E	1 capsule	30.0	11.0
	Powder in capsules	1 capsule	32.0	12.0
	proprietary blend, indol-3-carbinol, magnesium glycyl glutamine, and coenzyme Q10	1 capsule	35.0	13.0
		1 capsule	36.6	13.6
		1 capsule	39.8	15.0
		1 capsule	44.2	16.7

HMB = hydroxy-beta-methylbutyrate; n.d. = not detected (< 0.3 ppm)

Italic have been used for the ingredients of the nutritional supplements, while non-italic is used for the description of the supplement physic form (powder, capsules, etc.)

3.1.2 | UPLC–MS/MS analysis of HCTZ in urine

UPLC conditions

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was used for separation, applying an Acquity HSS T3 column (2.1 mm ID × 100 mm, 1.8 µm) maintained at 40°C. The LC conditions were as follows: gradient elution with water containing 0.1% (v/v) formic acid as mobile phase A and acetonitrile as mobile phase B. The flow rate was 400 µL/min and the gradient was programmed as follows: 0–1 min at 90% A, 1–3 min to 10% A, 3–5 min hold 10% A, 5–6 min to 90% A and 6–9 min hold 90% A.

MS/MS

An Xevo TQ-S tandem-quadrupole mass spectrometer (Waters, Manchester, UK) equipped with a Z-spray electrospray interface was

used. Negative ESI was performed in the MRM mode. The capillary voltage was set to −3.5 kV. The source block temperature was 150°C, and the desolvation gas (nitrogen) was heated to 600°C and delivered at a flow rate of 1000 L/h. The deprotonated molecule of HCTZ was chosen as precursor ion and the following MRM transitions were monitored: m/z 296–269, m/z 296–205, m/z 296–78. System operation and data acquisition were controlled using Mass Lynx 4.1 software (Waters). All data were processed with the Target Lynx quantification program (Waters, Manchester, UK).

Identification and quantification

The analyte was identified by comparison of the retention time and the ion transitions abundances obtained from a spiked positive control urine containing HCTZ. The identification criteria were: retention time deviation less than ±1%, signal-to-noise ratio of all diagnostic ions greater than 3, relative abundance of the diagnostic ions ±20% from the corresponding relative abundances of the spiked positive urine. For quantification, a 5-point calibration curve in the range 1–500 ng/mL was prepared by spiking blank urines with known amounts of HCTZ standard solution.

3.2 | Results of the excretion study

Specific gravity was reported between 1.010 and 1.029 and creatinine level between 71 and 242 mg/dL, in all urine samples. HCTZ concentrations in post-administration urine samples ranged from "not detected" (n.d.) to 230 ng/mL. Peak urinary HCTZ concentrations in subject 1 (95 ng/mL) were measured 7 hours from administration of the first dose, while subject 3 reached the highest peak concentration of 230 ng/mL 6 hours after the first dose administration of 12.8 μg of HCTZ. The maximum urinary concentration in subject 2 (60 ng/mL) was reached 6 hours after the second dose (Table 2, Figure 1).

4 | DISCUSSION

4.1 | Nutritional supplements contamination

The use of nutritional supplements in sports has increased notably in recent years[11-13] and, based on the doping control form data, each athlete declares on average 1.7 dietary supplements.[14]

There is evidence that some of the apparently legitimate dietary supplements on sale contain ingredients that are not declared on the label but that are prohibited by the doping regulations of the International Olympic Committee and of WADA.[15]

A recent review reports a high rate of dietary supplements contamination, between 12% and 58%, with steroids and stimulants being the most frequently prohibited substances identified.[12] Diuretics, even if not included in the most commonly detected contaminants in generic supplements, have been often identified in those marketed for weight loss and body image.[16-18] In particular, HCTZ was found as an adulterant not declared on the label in 14% of herbal formulations for weight loss.[17]

Even if there is some evidence of deliberate adulteration of products,[15] the presence of banned substances in nutritional supplements can be the result of non-intentional cross-contamination during manufacturing, processing, or packaging,[12] and despite ongoing improvements to regulatory and manufacturing guidelines, the potential for contaminated nutritional supplements to cause an AAF for an athlete remains a concern.[9,15,19]

TABLE 2 Dosing protocol and results of urine samples analysis of the HCTZ excretion study **performed on three subjects**

	Dosing Protocol		Urine Samples				
Subject	1st Dose (t^0)	2nd Dose (t^{+8})	Time of Collection	pH	SG	Creatinine (mg/dL)	[C] HCTZ (ng/mL)
Subject 1	6.4 μg	3.2 μg	t^0	5.5	1.024	198.1	n.d.
			t^{+1}	6.0	1.027	229.8	18.24
			t^{+7}	5.0	1.024	168.5	95.71
			t^{+15}	6.0	1.020	163.7	67.00
			t^{+19}	6.0	1.022	162.7	24.81
			t^{+24}	6.0	1.024	165,2	8.21
Subject 2	6.4 μg	3.2 μg	t^0	6.5	1.021	151.2	n.d.
			t^{+5}	6.0	1.024	194.6	23.75
			t^{+7}	6.0	1.029	242.3	5.16
			t^{+14}	5.0	1.026	200.6	60.73
			t^{+19}	6.0	1.015	101.9	15.50
			t^{+24}	6.0	1.022	152.3	5.26
Subject 3	12.8 μg	6.4 μg	t^0	7.5	1.017	159.0	n.d.
			t^{+2}	6.5	1.008	71.4	50.42
			t^{+5}	7.0	1.012	99.7	230.75
			t^{+7}	7.5	1.016	142.2	168.92
			t^{+11}	6.0	1.010	78.6	132.14
			t^{+14}	6.0	1.014	106.9	73.99
			t^{+19}	6.0	1.010	100.5	6.25
			t^{+24}	6.5	1.017	131.5	n.d.

SG = specific gravity

[C] = concentration

n.d. = not detected (< 1 ng/mL)

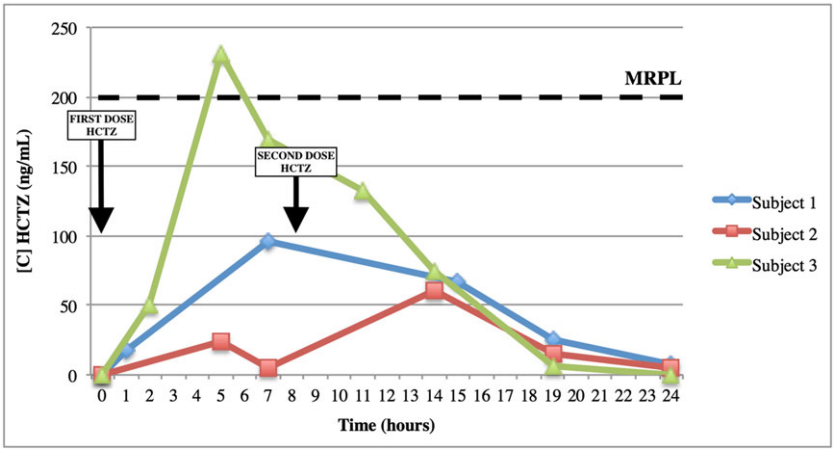

FIGURE 1 Urinary HCTZ concentrations following repeated oral administration of HCTZ contaminated powder in three healthy subjects. The long arrow indicates the time point of first dose of drug administration (subjects 1 and 2 = 6.4 μg of HCTZ; subject 3: 12.8 μg of HCTZ). The short arrow indicates the time point of the second dose of drug administration (subjects 1 and 2 = 3.2 μg of HCTZ; subject 3 = 6.4 μg of HCTZ). Dashed line represents the actual "minimum required performance limit" (MRPL), proposed as the reporting limit to be introduced for diuretics in all WADA-accredited laboratories [Colour figure can be viewed at wileyonlinelibrary.com]

In the present case, the identification of HCTZ in capsules, powder, and liquid drops in sealed supplements was certainly the result of an accidental contamination, probably during product manufacturing and packaging. It is in fact extremely unlikely that HCTZ was deliberately placed in the supplements in order to produce a diuretic/masking effect, since the absolute quantities detected in the examined products, although extremely variable, were always <384 μg per unit (unsealed: min n.d. – max 384 μg per unit; sealed: min n.d. – max 46 μg per unit), far from the therapeutic dosage (≥12.5 mg). Considering an average concentration of 37 μg of HCTZ in each capsule and 52 μg in each sachet, it would be necessary to take 338 capsules or 240 sachets to obtain a 12.5 mg therapeutic dosage.

Moreover, lot-to-lot and unit-to-unit variability are typical features encountered in contaminated products. Capsules and powders, in particular, for their type of manufacturing and packaging in laboratories, are among the pharmaceutical forms most suitable for contamination.[20,21]

4.2 | Regulatory framework of nutritional supplements and pharmaceutical compounding

The need of compounded medicinal products is recognized in both Europe and the United States, with both dedicating specific chapters to the argument in their Pharmacopoeias; however laws and policies vary significantly between different countries.

In European countries, according with the *Resolution CM/Res(2016)1 on quality and safety assurance requirements for medicinal products prepared in pharmacies for the special needs of patients*,[22] all pharmacy-prepared medicinal products should be made using an appropriate quality assurance system, whose level depends on a risk assessment that distinguishes between two risk levels: "high-risk preparations" and "low-risk preparations." The resolution recommends to follow *Good Manufacturing Practices* (GMP) – defined in the European Commission Directive 2003/94/EC[23] – as a reference for an appropriate quality control system for high-risk preparations and the *Good Preparation Practices* – defined in the Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S GPP) Guide PE 010[24] – for low-risk preparations.

The PIC/S GPP Guide contains the basic requirements that apply to the preparation of medicinal products normally performed by healthcare establishments for direct supply to patients, including the warning about hygiene and prevention of cross-contamination.

Compounded medicinal products, in fact, are generally prepared for the immediate use and cannot await the long and severe trials that the manufactured ones have to follow; moreover pharmacies have fewer automated equipment than the industries and compounded products are directly prepared by the pharmacist or a technician under the pharmacist's supervision.[25]

In the United States, the Drug Quality and Security Act,[26] enacted on November 27, 2013, established a new category of compounders known as outsourcing facilities, which unlike other compounders, are subject to GMP requirements.

Anti-doping organizations (ADOs) recognize that compounding pharmacies may play an important role in athletes' health care needs; however, they also warn athletes about the risks they need to be aware of when considering whether to use a compounded medication.[27] These risks include the possibility that compounded pharmacies may inadvertently purchase contaminated or low-quality raw ingredients, or may not properly clean equipment between making products, thus inadvertently contaminating a medication that an athlete purchases causing the athlete to test positive.

All the regulation set out herein is addressed to regulate the production of compounded medicinal products. In the case of dietary supplements, which are classified as a subcategory of food, manufacturers are not required to provide evidence of product safety and efficacy,[28] even if supplements are regulated on a national level and regulations vary between countries.[21]

In fact, while medications and drugs are governed by pharmaceutical regulation as already mentioned, dietary supplements can be produced and sold without any premarketing authorization or end-product control, as laid down by the Federal Food, Drug, and Cosmetic Act[29] in the United States or the Directive 2002/46/EC[30] in Europe. Only post-marketing surveillance is foreseen for those products. It is therefore important that studies like the present one are published and widely disseminated, in order to focus needed attention on quality control and safety issues in these products.

4.3 | AAFs for diuretics and unintentional doping cases

Diuretics represented 12% of the AAFs at WADA laboratories in 2016, with HCTZ the most frequently identified substance (158 cases,

32% of diuretics positive cases).[31] These data include cases with therapeutic use exemption (TUE) and multiple findings on the same athlete; therefore they are not to be confused with adjudicated or sanctioned anti-doping rule violations (ADRVs).

The analytical performance in anti-doping laboratories and the instrument sensitivity have increased significantly in the past years,[32] and it has been demonstrated that even medicinal products produced according to the GMP guidelines for industrial production may cause an AAF due to contamination.[9] This could lead to an increase of AAFs, particularly for non-threshold substances, with non-distinguishability of intentional and unintentional intake.

There should be an interplay between ADOs and laboratories performing analysis in order to investigate and clarify the circumstances of an AAF. According to WADA rules, to be cleared of an involuntary AAF, the athlete has to prove the way the prohibited substance came into their system and that they bore no significant fault or negligence in relation to the violation, and in this context, the analytical support of certified laboratories may have a crucial task.

Some cases of AAFs for diuretics and other masking agents with subsequent finding of no fault from the United States Anti-Doping Agency (USADA) have been reported in recent years.

A volleyball player, in 2017, had an AAF for HCTZ and triamterene that was ascribed to the ingestion of prescribed medication tablets contaminated with the drugs.[33]

Similarly, in the same year, a Paralympic athlete had an AAF for torsemide as the result of the ingestion of a contaminated permitted medication.[34]

In both these cases the contamination was confirmed by detailed laboratory analysis.

In another case a gymnastic athlete had an AAF for HCTZ that was attributed to contamination of tap water obtained from the municipal water supply.[35] In this case, detailed laboratory analysis could not be conducted. Together with the circumstances of ingestion and scientific evidence provided by published literature and expert reports, The USADA concluded on a balance of probabilities that the athlete unknowingly ingested the hydrochlorothiazide through tap water obtained from the municipal water supply.

In another case, in which a cyclist received an AAF for probenecid, the athlete was cleared of any fault or negligence on his part, proving through receipts that he ingested the drug through capsules contaminated by the pharmacist who had the probenecid on his hands from dealing with a prior customer while issuing him the capsules.[36]

However, the time between the urine sample collection and the AAF notification can pose several challenges to athletes needing to demonstrate the inadvertent ingestion of a drug. Distinguishing intentional vs unintentional doping, in fact, is challenging and up to now there are no reliable markers to discriminate a recent ingestion of minimal dosages of the drug from a distant consumption of an effective dose. In fact, if an athlete intentionally ingests a prohibited substance and the sample collection occurs during the late elimination phase, its concentration value would be the same obtained few hours after unintentionally ingesting a small amount of the drug.

Urine concentration of drugs is influenced by several factor related either to the athlete (age, sex, urine pH, metabolism, renal function, hydration, etc.) or the drug pharmacokinetics (molecular weight, ionization, water soluble, lipophilic and hydrophilic, etc.), and is therefore more variable and much less informative than the blood concentration. On the other hand, urine has the advantage of being a non-invasive sample, which allows identification of multiple substances over a long period of time.

Specifically regarding HCTZ, a previous study[37] showed that 4-amino-6-chloro-1,3-benzenedisulphonamide (ACB), one of the hydrolysis product of HCTZ degradation, is detectable in urine up to 120 h after an oral administration of 25 mg of HCTZ, with concentrations at least 10 times higher than the parent drug, therefore appearing to be an important target compound for the long time detection of the thiazide diuretic in urine. To the best of our knowledge, however, no studies have been performed to investigate ACB concentrations at different HCTZ administered dosages, in order to analyze the usefulness of this marker in distinguishing between a recent intake (less than 10 hours) of a small quantity of HCTZ, as for contamination of medical products, and a less recent intake (between about 2 and 5 days) of an active dosage of HCTZ. In the hypothesis of a direct correlation between HCTZ urinary peak concentration, and ACB concentration in the following 4–5 days, this marker would certainly be an interesting tool for distinguishing intentional doping from inadvertent intake of HCTZ through contaminated products.

4.4 | Reporting limits of non-threshold substances

MRPLs, reporting limits, and decision limits are all concentration values, however they underlie different concepts.

The MRPL is the concentration value of a prohibited substance (or of its metabolite or marker) that the laboratories should be able to routinely detect and identify, representing therefore an analytical parameter of technical performance. MRPL values are established taking into account the metabolism, stability, pharmacokinetics, and pharmacodynamics of the prohibited substance, and are relevant for the detection and identification of non-threshold substances, even if AAFs may result from concentrations below the established MRPL values.[38] A confirmed identification of a non-threshold substance, in fact, is reported as an AAF at any concentration, with the exception of few substances, which should not be reported at levels below a defined concentration (ie, reporting limit).

The reporting limits can therefore be defined as the concentration values of non-threshold substances above which an AAF shall be reported; in the WADA Technical Document on MRPL[39]some reporting limits are available for the following cases:

- Non-threshold substances in classes S6, S7, S8, and P1, which are prohibited in-competition only, should not be reported below 50% of the MRPL.
- Glucocorticoids (S9) should not be reported at levels below the MRPL of 30 ng/mL.
- Salmeterol and higenamine should not be reported at levels below 10 ng/mL (ie, 50% of the MRPL for beta-2 agonists).
- Meldonium should not be reported at levels below 100 ng/mL (ie, 50% of the MRPL).
- Octopamine should not be reported at levels below the MRPL of 1000 ng/mL.

TABLE 3 HCTZ excretion studies performed and concentrations obtained in urine samples

Study	Number of Subjects	Number of Administration	Dose of HCTZ Administered (μg)	Time of Urine Collection	Time Between Administration and Peak Concentration	HCTZ Urinary Concentration (ng/mL)	
						Min	Max
Deventer et al, 2009[37]	6	Single	25000 therapeutic	120 h	3-6 h	4–20	11900–17600
Helmin et al, 2016[9]	1	Single	2.5 sub-therapeutic	24 h	4 h	n.d.	4
Helmin et al, 2016[9]	1	Multiple*	max 10 (x 3)* sub-therapeutic	76 h	3h#	1	16
Present study	1	Single§	12.8 sub-therapeutic	24 h	5 h	5	230
Present study	1	Single§	6.4 sub-therapeutic	24 h	7 h	8	95
Present study	1	Multiple**	6.4 + 3.2** sub-therapeutic	24 h	6h#	n.d.	60

*Multiple administration of different dosages of HCTZ on three consecutive days (2.5 μg at t^0, t^{+6}, t^{+12}; 5 μg at t^{+24}, t^{+30}, t^{+36}; and 10 μg at t^{+50}, t^{+55}, t^{+59}).

#Hours from the last administration.

§The maximum urinary concentration was reached after a single dose of HCTZ even if multiple dosages were administered.

**6.4 μg at t^0, 3.2 μg at t^{+8}.

n.d. = not detected

Reporting limits have been adopted for the aforementioned substances because of the high risk of entering the body inadvertently. For examples, higenamine, which is documented to be a constituent of different plants, can be found unlabeled in some dietary supplements, particularly those related to weight loss.[40] Similarly, AAFs concerning octopamine in doping controls commonly result from surreptitious applications.[41]

The introduction of reporting limits has pros and cons. The main argument in favor of the reporting limits introduction is certainly the exclusion of most of the AAFs due to contamination of foods and legitimate products. On the other hand, the adoption of such limits can reduce the power of doping controls in identifying doped athletes.

We must be aware that increase in instrumental sensitivity makes a low concentration of prohibited substances more likely to be detected in athlete samples. This does not mean that the LODs should be increased or set at the MRPL levels, but that reasonable interpretations should be applied to those cases where tiny amounts of prohibited substances and circumstances clearly point towards the occurrence of a contamination issue. Using low LODs allows the laboratories to thoroughly investigate potential doping cases and to perform a due, correlated research; laboratory activity should not be blind or limited because the detection of one or more substances below the MRPL can make WADA focus on suspect positive cases, without automatically issuing an AAF.

Aiming at guaranteeing the same analytical accuracy in reporting an AAF, a quantitative analysis should be performed in case of concentration estimation above the reporting limits, in analogy to what implemented with threshold substances and decision limits, with consequent increase of the analytical burden of the laboratory.

Balancing all these arguments, the authors suggest, consistent with previous studies, that it is justified to introduce reporting limits for diuretics in the range of the current MRPL (200 ng/mL). [9,39]

This proposal is extended by Helmlin et al to all specified substances, which are more likely than the "non-specified" substances to enter an athlete's body inadvertently, and can be challenging in proving the athletes non-liability in case of unintended doping.

A different approach should be instead maintained for contamination with non-specified substances, such as clenbuterol, an anabolic agent that is prohibited both in- and out-of-competition. Clenbuterol was the issue of known cases of unintentional doping and the risk of its inadvertent ingestion via contaminated meat has been reported in the recent past;[42] it was responsible of 349 AAFs in 2016 anti-doping tests.[31] However, clenbuterol is not likely to enter an athlete's body accidentally because of its legal therapeutic use, but because of its use in animal husbandry, which is prohibited in the entire European Union and meat exporting countries. At present, in fact, WADA does not plan to introduce any threshold level for clenbuterol, but is working closely with specific countries, international federations and event organizers to help minimize the risk of meat contamination.

4.5 | HCTZ excretion studies

Few HCTZ excretion study have been published.[9,37] A previous excretion study after administration of a *therapeutic* dose of HCTZ (25 mg = 25000 μg), in combination with spironolactone (25 mg), in six volunteers reported peak concentrations between 3 and 6 hours that ranged between 11.900 and 17.600 ng/mL.[37] According to the results, HCTZ were detectable in urine even five days post-administration (4–20 ng/mL). [37]

Another two elimination studies of HCTZ were performed with *sub-therapeutic* dosages in order to probe the plausibility of an AAF caused by over-the-counter NSAID medications contamination.[9] The first study revealed urinary peak concentration of 4.6 ng/mL within the first 5 hours of administration of a spiked placebo-tablet containing 2.5 μg of HCTZ. In the second study, the administration of repetitive dosages of the same spiked placebo-tablet, for a maximum equivalent dose of 10 μg of HCTZ administered three times in less than 10 hours, were associated with maximum 16 ng/mL urinary HCTZ concentrations. According to these results, the MRPL of 200 ng/mL[39] for diuretics seemed to be an excellent cut-off to help distinguish the use of the substance for masking or weight loss purposes, achieved with therapeutic dosages, from the positive results due to contaminations.

In the present study, however, the urinary concentrations are much higher than the results presented by Helmlin et al, although nearly the same amounts of HCTZ was administered. This can be due to inter-individual variability, among which different morphometric features. In subject 3, who had the lowest BMI in our study, in fact, a single oral administration of 12.8 μg of HCTZ was responsible for the highest HCTZ urine peak concentration of 230 ng/mL. This value, being above the value (200 ng/mL) proposed by Helmin et al[9] as the reporting limit to be introduced for diuretics in all WADA-accredited laboratories (and corresponding to the MRPL for diuretics), would imply an AAF in an eventual doping control (Table 3).

Considering that the concentration of 230 ng/mL was in the present study obtained with a dose about 2000 times lower than the therapeutic doses, a reporting limit could be set even at higher level, such as the MRPL adopted by WADA before 2013 (250 ng/mL).[43]

4.6 | Limitation of the excretion study

The main limits of our excretion study is that it was performed in only three human volunteers, without recording food and beverage intake during the study, and the peculiar dosing protocol was chosen according to the possible intake of HCTZ through the contaminated nutritional supplements by the athlete. Furthermore, the HCTZ concentration in the contaminated powder used for the study was estimated with calibrators obtained by spiking a blank matrix that *was not identical* to that specific supplement, and the recovery parameter was not adequately studied.

The great subject-to-subject variability suggests the need of further studies.

5 | CONCLUSION

The case reported, in line with previous studies, confirms the necessity of introducing reporting limits for diuretics in doping controls, in order to avoid AAFs due to contaminated medicinal drugs or nutritional supplements. However, it has to be considered that if the actual MRPL of

diuretics would be used as reporting limit, it may nonetheless be exceeded by use of some contaminated supplements at µg/g levels, as demonstrated in our excretion study.

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ORCID

Sindi Visentin http://orcid.org/0000-0002-5913-0120

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