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Quantitation of Formoterol, Salbutamol, and Salbutamol-4′-*O*-Sulfate in Human Urine and Serum via UHPLC-MS/MS

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Abstract: The adrenergic beta-2 agonists formoterol and salbutamol are used for the treatment of asthma and COPD but are also misused in sports competitions. Therefore, they are included in WADA regulations. Both drugs are mainly excreted in urine after administration via inhalation. A four-armed, double-blind cross-over clinical trial was conducted involving endurance-trained participants (12 females and 12 males). Inhalation dosages of 36 µg formoterol, 1200 µg salbutamol, a combination of both, or a placebo were administered before exercise. Serum and urine were collected after exercise and 3 and 24 h after administration. Here, we show the successful quantitation of formoterol, salbutamol, and its phase II metabolite salbutamol-4′-*O*-sulfate in all urine and serum samples using ultra-high performance liquid chromatography–tandem mass spectrometry. In the serum analysis, results of up to 14.2 pg/mL formoterol, 10.0 ng/mL salbutamol, and 21.4 ng/mL salbutamol-4′-*O*-sulfate (calculated as salbutamol equivalent) were found. In urine, maximum concentrations (after deglucuronidation) were 17.2 ng/mL formoterol, 948.5 ng/mL salbutamol, and 2738.5 ng/mL salbutamol-4′-*O*-sulfate. Sex-specific differences in serum concentrations as well as in urinary excretion were observed. The results pronounce the importance of the implementation and elucidation of phase II metabolites to quantitation methods in antidoping.

Keywords: bioanalysis; biosynthesis; reference material; sports doping; beta-2 agonists

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1. Introduction

Formoterol and salbutamol are used as therapeutic drugs in the management of airway obstruction in conditions such as bronchial asthma or chronic obstructive airway disease (COPD) [1–6]. As beta-2 sympathomimetic drugs, they lead to the relaxation of smooth muscles via the activation of the beta-2 adrenoceptor [7]. While salbutamol is considered a short-acting drug (SABA) mainly used in acute situations, the long-acting beta-2 agonist (LABA) formoterol is used as a symptom controller due to its extended duration of action [8–11]. Advantageous over several other LABAs, formoterol also provides rapid action when inhaled and is thus used as both a reliever and maintenance medication [12,13]. However, in many cases, asthmatic patients receive combinations of SABAs and LABAs together with further prophylactically acting drugs such as glucocorticoids and/or long-acting muscarinic antagonists [14–16]. Additionally, beta-2 agonists are reported as ergogenic agents with particularly salbutamol and clenbuterol found as anabolic substances in humans [17–20]. While initially only significant overdosing was considered

necessary to achieve anabolic actions of beta-2 agonists, recent studies demonstrated anabolic action even at low doses in animal experiments [21,22]. In a swim ergometer test, Kalsen et al. observed significantly increased sprint performance in the groups treated with salbutamol (inhalation of 1600 µg), formoterol (inhalation of 36 µg), or salmeterol (inhalation of 200 µg) compared to placebo [23].

In sports, beta-2 sympathomimetics are generally prohibited as performance-enhancing drugs according to the regulations of the World Anti-Doping Agency (WADA) [8]. To enable therapeutic options, inhaled doses of salbutamol [10,11] not exceeding 600 µg/8 h and 1600 µg/24 h are not considered as prohibited use and even do not require a therapeutic use exemption (TUE). Similarly, inhaled formoterol < 54 µg/24 h, inhaled salmeterol < 200 µg/24 h, and inhaled vilanterol < 25 µg/24 h are not considered as prohibited use [9]. Unless proven differently, urinary concentrations exceeding 1000 ng/mL for salbutamol and 40 ng/mL for formoterol are considered as adverse analytical findings and related to non-therapeutic use. According to the WADA technical document TD2021DL, the decision limits for salbutamol and formoterol refer to the combination of free substances and their glucuronide-conjugated forms expressed as substance equivalents [9]. However, thresholds considering a combined use of drugs from the same class do not exist, and no studies have been reported so far that investigated concentrations obtained after the combined administration of the two drugs.

While salbutamol is known to be mainly eliminated as an unchanged drug and a sulfoconjugated metabolite [24], formoterol is mainly glucuronidated and excreted as its two glucuronides, phenolic (major) and benzylic, as well as an unchanged drug [25–27]. Further minor metabolites, viz., a sulfoconjugate as well as *O*-desmethylformoterol and its respective glucuronides, are also described [26]. Considering the above-mentioned regulations, doping control analysis targets salbutamol and formoterol aglycones after enzymatic hydrolysis of the urine samples using β-glucuronidase [28–32].

Several publications present the sample preparation and subsequent quantitation of formoterol or salbutamol from urine and serum. Fewer studies were conducted for salbutamol-4′-*O*-sulfate, most probably hampered by the non-availability of a reference substance. While for urine samples an easy dilute-and-inject approach has been shown to be feasible [29,32–35], for the more complex serum samples mostly solid-phase extraction (SPE) [36–46] or liquid–liquid extraction (LLE) [47,48] have been demonstrated to allow for sensitive quantitation. However, sample clean up using SPE and LLE has also been used in the analysis of urine samples [30,41,43–46,48–50]. Apart from a reduction in the matrix burden, the concentration of the analytes and a switch to suitable injection solvents lead to increased sensitivity of the analysis [37,39,50,51]. Studies have been published presenting a systematic development of extraction methods for salbutamol [41,52,53]. Whereas most users decide on either LLE or SPE, both extraction methods can be performed sequentially in the work-up procedure to extract formoterol [25] or salbutamol [54]. Regarding the chemical structures of the analytes (secondary amines) cation exchanger (CX), often provided as a mixed mode with also reversed-phase characteristics (MCX), was widely used to retain salbutamol and formoterol [36,39,43,51,53,55,56]. In this manner, Bozzolino et al. [49] extracted 24 beta-2 agonists inter alia salbutamol and formoterol from urine. Likewise, reversed phase only SPE cartridges were also applied for urine and serum samples [25,26,44]. A sequential SPE method utilizing two different phases was published by Chan et al. [57] for the extraction of salbutamol from porcine urine. Joyce et al. [58] applied a hydrophilic–lipophilic-balanced (HLB) copolymer for the simultaneous extraction of salbutamol and its sulfonated metabolite. LLE of beta-2 agonists is based on the polarity as well as on basic and acidic functional groups of the analytes. Moderately basic buffering is often used to steer the analytes into the organic phase. Ethyl acetate was successfully applied to extract salbutamol [48,59] and formoterol [47,50] from urine and plasma. According to the higher lipophilicity of formoterol, more non-polar organic solvents were also used [30,35]. In doping control analysis, t-butyl methyl ether is widely used in standard procedures for LLE of beta-2 agonists.

Separation and highly sensitive quantitation of salbutamol and formoterol were performed mostly using high-performance liquid chromatography (HPLC) tandem mass spectrometry equipped with an electrospray ionization (ESI) source operated in positive ionization mode [29,30,32,34,35,39,47,49–51,59,60]. In the last quarter century, few publications have been published using gas chromatography mass spectrometry, which was the previous standard method for beta-2 agonists [41,43,54]. Achiral reversed phase HPLC methods mostly apply C8- or C18-based stationary phases in gradient mode [29,34,49], whereas chiral separation most often uses teicoplanin-based columns in isocratic elution mode [37,47,48,50,57,61]. However, proper quantitation of salbutamol and salbutamol-4′-*O*-sulfate in urine and plasma (chiral and achiral) was presented by Joyce et al. [58] achieving limits of quantitation (LOQ) for the sulfated metabolite down to 5 ng/mL in urine and plasma. Mascher et al. performed quantitation of formoterol achieving an LOQ of 0.4 pg/mL by applying MCX-based SPE in the sample clean up and injecting 20 µL of the extract. A LOQ down to 20 pg/mL of salbutamol in serum was achieved by Jacobson et al. [37] using HLB-based SPE for sample work up and chiral chromatography coupled to an orbitrap MS.

This project aims at investigating the serum and urine concentration profiles of formoterol, salbutamol, and salbutamol-4′-*O*-sulfate after single or combined inhalation dosages in endurance athletes. A particular emphasis was placed on the impact of the combined medication on the excretion of the drugs, sex-specific differences, and the proportion of sulfonated salbutamol.

2. Materials and Methods

2.1. Chemicals and Equipment

Salbutamol hemisulfate (>98.0%) and formic acid (FA, LC-MS grade) were obtained from TCI Europe (Zwijndrecht, Belgium). Formoterol fumarate dihydrate (>98%), d_3-ephedrine (as hydrochloride 1 mg/mL ampoule), β-glucuronidase (generated from *E. coli*), and hydrochloric acid (37%, analytical grade) were obtained from Sigma Aldrich (Taufkirchen, Germany). For use as internal standard, d_3-salbutamol (as hydrochloride, 4-[2-(tert-butylamino)-1-[$^2H_1$]-1-hydroxyethyl]-2-[$^2H_2$]-hydroxymethylphenol) and d_3-testosterone glucuronide (16,16,17-[$^2H_3$]-testosterone glucuronide) from National Measurement Institute (Canberra, Australia) and mefruside from Bayer (Leverkusen, Germany) were provided by Agilent Technologies GmbH (Waldbronn, Germany). Isopropanol was purchased from Carl Roth (Karlruhe, Germany). Methanolic ammonia solution (7 N) and methanol (MeOH, LC-MS grade) were purchased from Thermo Fisher Scientific (Hennigsdorf, Germany). Ammonium formate ($HCOONH_4$, LC-MS grade) was from VWR Chemicals (Damstadt, Germany). Acetonitrile (ACN) was from Fisher Scientific (Geel, Belgium). Ultrapure water was prepared with the Milli-Q water purification system LaboStar 2-DI/UV (SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany). Disposable regenerated cellulose (RC 45/13) filters, solid-phase extraction cartridges Chromabond HR-XC (45 µm, 30 mg, 1 mL) and HLB (30 µm, 30 mg, 1 mL) were purchased from Macherey-Nagel (Düren, Germany). Normal human serum (generated from male person with blood group AB) was obtained from Sigma-Aldrich (Taufkirchen, Germany). d_6-DMSO was purchased from Deutero (Kastellaun, Germany).

Salbutamol-4′-*O*-sulfate, serving as reference for UHPLC-MS/MS analysis, was biosynthesized in house using a method adapted from Sun et al. [62], as presented by Jendretzki et al. [63]. Its amount was determined via quantitative NMR (qNMR) analysis on a Bruker Avance III instrument operating at a frequency of 400 MHz using 1,3,5-trimethoxybenzene (TraceCERT Lot#BCBO5470, 10.05 mg in 0.605 mL d_6-DMSO taking batch assay into account).

2.2. Instrumentation

Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

Quantitation of formoterol, salbutamol, and salbutamol-4′-*O*-sulfate (chemical structures in Figure 1) in serum and urine samples was performed using ultra-high performance

liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS). Unlike in serum analysis, in urine analysis, the β-glucuronides were implemented via hydrolysis with β-glucuronidase as part of the sample preparation. Separation was conducted on a 1290 II Infinity UHPLC System (Agilent Technologies GmbH, Waldbronn, Germany) hyphenated to a 6495 triple quadrupole MS/MS (Agilent Technologies Inc., Santa Clara, CA, USA). Ionization of the analytes was performed using an electrospray ionization (ESI) source equipped with Agilent Jetstream and iFunnel technology. Ionization was conducted in positive mode (ESI+) for all analytes, and data were acquired in multiple reaction monitoring (MRM) mode. Product ions of highest intensity were used as quantifiers. Ionization parameters and chromatographic methods were developed and optimized by one factor at a time approach (OFAT). Details of the chromatographical separation method and the MS/MS operating parameters are presented in Tables 1 and 2.

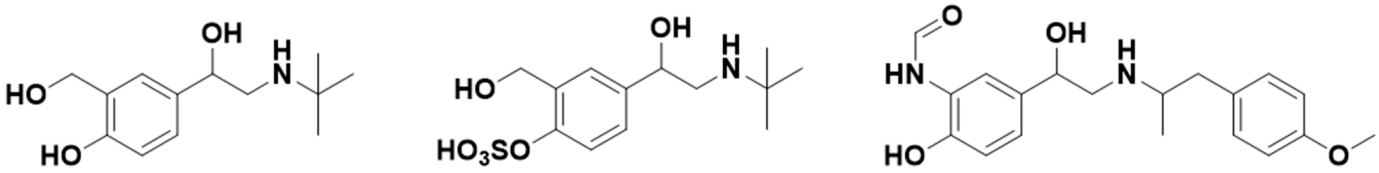

Figure 1. Chemical structures of the targeted analytes. Salbutamol (**left**), salbutamol-4′-*O*-sulfate (**center**), and formoterol (**right**).

Table 1. Chromatographic separation conditions and source parameters for positive electrospray ionization for the analysis of urine or serum samples containing salbutamol, salbutamol-4′-*O*-sulfate, and/or formoterol.

Analytes	Salbutamol and Formoterol	Formoterol	Salbutamol-4′-*O*-Sulfate	Salbutamol and Salbutamol-4′-*O*-Sulfate
Specimen	Urine	Serum	Urine	Serum
Column	Poroshell 120 EC-C18 (2.1 mm I.D. × 50 mm; 1.9 µm)		Poroshell 120 phenyl-hexyl (3.0 mm I.D. × 100 mm; 1.9 µm)	
Flow rate	0.3 mL/min		0.4 mL/min	
Column temperature	35 °C		35 °C	
Injection volume	1 µL (30 µL)	15 µL	2 µL	5 µL
Solvent A	0.1% formic acid and 10 mM ammonium formate in water (1:0.63:1000, *v*:*m*:*v*)		20 mM ammonium formate in water (1.26:1000, *m*:*v*)	
Solvent B	0.1% formic acid and 10 mM ammonium formate in water/ACN (1:0.63:120:900, *v*:*m*:*v*:*v*)		20 mM ammonium formate in methanol (1.26:1000, *m*:*v*)	
Gradient	0 min: 0% B 2 min: 7.5% B 5 min: 40% B 6 min: 95% B 7.4 min: 95% B 7.5 min: 0% B	0 min: 5% B 2 min: 5% B 5 min: 40% B 6 min: 100% B 7.4 min: 100% B 7.5 min: 5% B	0 min: 5% B 1 min: 5% B 5 min: 40% B 6 min: 95% B 7.90 min: 95% B 8 min: 5% B	
Post time	3 min		2.5 min	
Ion source parameters				
Gas temperature	290 °C		170 °C	
Gas flow	20 L/min		17 L/min	
Nebulizer	25 psi		10 psi	
Sheath gas temperature	400 °C		400 °C	
Sheath gas flow	12		12	
Capillary	6000 V		4000 V	
Nozzle voltage	500 V		500 V	

Table 2. Precursors, product ions, and collision energies for multiple reaction monitoring in tandem mass spectrometry. Deuterated analytes are used as internal standards.

Analyte/ISTD	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	Collision Energy (V)
Formoterol	345	327.1	12
	345	149	20
	345	121.1	32
	345	91.1	76
	345	77.1	80
Salbutamol	240	222	8
	240	166	12
	240	147.9	16
	240	91	48
	240	77.1	56
Salbutamol-4′-*O*-sulfate	320	240	4
	320	222	16
	320	166	16
	320	148	32
	320	77	80
d_3-Salbutamol	243.2	225.2	8
	243.2	151	20
d_3-Testosterone-glucuronide	468.3	109	40
	468.3	97	32
	468.3	84.9	84.9
d_3-Testosterone	292.2	109	20
	292.2	97	20

2.3. Study Design and Sampling of Urine and Serum

A balanced four-arm clinical trial was conducted in double-blind cross-over design involving highly endurance-trained human participants (n = 24; 12 females, 12 males). This study was registered at Eudra CT (no. 2015-005598-19) and DRKS (no. 00010574) and approved by the Ethics Committee of Ulm University (number 64/19, issued 20 May 2019). Female participants were aged 22.9 ± 2.7 years and weighted 62.2 ± 5.0 kg (height 170.8 ± 5.6 cm); males were aged 24.4 ± 4.6 years and weighted 73.9 ± 5.5 kg (height 180.5 ± 3.7 cm).

In each study arm, single doses of salbutamol (1200 µg), formoterol (36 µg), a combination of both (1200 µg salbutamol + 36 µg formoterol), or placebo were administered using powder inhalers in double-blinded design. After 20 min post-administration, a cycling exercise performance test (total duration of 35 min, including warm up, a 10 min long high-intensity trial exercise, and cool down) was conducted. Serum and urine samples were collected prior to performance test, at 15-45 min (post), 3 h, and 24 h post-exercise for determination of salbutamol, salbutamol sulfoconjugate, and formoterol concentrations embedded in a larger investigation. Between each study arm, a wash-out period of 5–8 days was ensured. The detailed study design can be found in the published study protocol by Zügel et al. [64].

2.4. Determination of Urinary Concentrations

2.4.1. Sample Preparation

Aliquots of 200 µL urine were added to 10 µL of the internal standard solution (ISTD 1, d_3-salbutamol, mefruside, d_3-ephedrine, and d_3-testosterone glucuronide, each 1 µg/mL in methanol). Hydrolysis was performed using 25 µL β-glucuronidase within one hour at

50 °C while shaking at 1400 rpm. Performance of hydrolysis was proven via hydrolysis of d_3-testosterone glucuronide to the aglycone d_3-testosterone. After hydrolysis, 770 µL of a solvent mixture of high pure water and acetonitrile (50:50, *v*:*v*) were added. This mixture was vortex mixed for three seconds and then centrifuged at 14,100 rcf. The supernatant was transferred into a 1.5 mL glass vial and then directly analyzed or stored at −20 °C until analysis via UHPLC-MS/MS.

2.4.2. Matrix-Matched Calibration of Salbutamol, Formoterol, and Salbutamol-4′-*O*-Sulfate

Matrix-matched calibration was generated by spiking urine free from targeted analytes. The analyte-free urine was an equally combined urine mixture provided by three male and three female participants of the ELSA study. Salbutamol and formoterol were jointly calibrated using nine calibration levels, whereas calibration of salbutamol-4′-*O*-sulfate was separately generated in 11 calibration levels. Considering the WADA threshold and decision limits for salbutamol and formoterol (WADA TD2021DL [9]), salbutamol calibration levels were from 0.4 ng/mL to 1244.9 ng/mL, and formoterol calibration levels were from 41 pg/mL to 122.9 ng/mL. Salbutamol-4′-*O*-sulfate calibration levels were from 10 pg/mL to 207.0 ng/mL. Quality control samples were prepared as matrix-matched calibration level 5. Urine samples, which showed concentrations of salbutamol-4′-*O*-sulfate beyond highest calibration level were diluted (1:5, 1:10, or 1:20) with blank urine before sample preparation. Samples that showed formoterol results between LOQ and LOD were remeasured injecting 30 µL sample volume and an advanced calibration range down to 0.8 pg/mL was applied.

2.4.3. Determination of the Specific Gravity of Urine

Specific gravity of all analyzed urine samples was determined by applying a hand refractometer. Results of urine samples were corrected by following equation, where C represents concentration [ng/mL]; C(adj) represents adjusted concentration [ng/mL]; SG represents specific gravity of the analyzed urine:

$$\mathrm{C(adj)} = \frac{(1.020 - 1)}{(\mathrm{SG} - 1)} \times \mathrm{C}$$

According to the regulations of WADA [9], the decision limit would be adjusted for the specific gravity. Furthermore, the adjustment would only be applied in case of concentrated urines (specific gravity > 1.018). Moreover, the World Anti-Doping Code International Standard Testing and Investigations 2021 (ISTI 2021) [65] requests collecting an additional sample from the athlete in case of unmet requirements for specific gravity, i.e., very diluted urines (specific gravity < 1.005).

2.5. Determination of Serum Concentrations

2.5.1. Development of Sample Preparation Protocol

Due to the expected ultra-low analyte concentrations in serum, pre-concentration and purification of the serum samples were considered necessary. A suitable solid-phase extraction (SPE) method was subsequently developed in multiple steps. Firstly, the performance of hydrophilic–lipophilic-balanced copolymer-based cartridge (HLB) was compared to a cation exchange-based cartridge which also possessed reversed phase properties (HR-XC). Therefore, 10 µL of a methanolic solution of 0.82 µg/mL salbutamol and 82 ng/mL formoterol was filled up to 20.0 mL with a mixture of human norm serum and water (50:50, *v*:*v*) and served as homogenous sample. SPE cartridges were primed with methanol and conditioned with ultra-pure water, and the serum samples (n = 5) were loaded. The HLB-based phase was washed with water and the extract was eluted from the column with pure methanol, whereas the HR-XC cartridges were washed with 0.1 mM HCl in water. For elution, 5% NH_3 in methanol was used. After drying under a gentle stream of nitrogen, the residues were reconstituted with 0.1% formic acid in water. A more detailed protocol is provided in Table A1.

In the second step, the optimum volumes used for priming, conditioning, and elution using the HLB-based cartridge were investigated. For practical reasons, the volume of MeOH to elute the analytes was limited to 1.5 mL, since the extract was eluted into a 1.5 mL conical glass vial. Volumes of 0.5 mL, 1 mL, and 1.5 mL MeOH were tested, and elution performance was compared, whereby priming, conditioning, and washing were kept constant at 3 mL MeOH, 3 mL water, and 1 mL water, respectively. Furthermore, elution with 1.5 mL MeOH was compared to elution with 1.5 mL of a mixture of FA/MeOH (0.1:99.9, *v*:*v*) (n = 3).

The third step focused on the suitability of the injection solvents for UHPLC-MS/MS analysis and included screening of different reconstitution solvents and volumes, liquid–liquid extraction, filtering through a disposable filter, and the volume of washing water in the SPE protocol focusing on pressure curve stability in the UHPLC-MS method. The following experiments were carried out as single experiments but injected four times each. The following injection solvents were evaluated: 50 and 100 µL of FA in water (0.1:100, *v*:*v*), 50 µL of FA in water (0.2:100, *v*:*v*), 50 µL of FA in iPrOH/water (0.1:5:95, *v*:*v*:*v*), 50 µL of FA in iPrOH/water (0.1:50:50, *v*:*v*:*v*), 100 µL of FA in ACN/water (0.1:5:95, *v*:*v*:*v*), and 50 µL of FA in ACN/water (0.1:50:50, *v*:*v*:*v*). For liquid–liquid extractions of the residue after HLB-based SPE 400 µL FA/water (0.2:100, *v*:*v*) and 100 µL of either chloroform or n-hexane were added followed by vortex mix, the organic phase was disposed of, and the step was repeated one more time. The aqueous phase was evaporated under nitrogen at 40 °C and the residue was reconstituted in FA/ACN/water (0.1:5:95, *v*:*v*:*v*). Filtering through a disposable filter was tested and recovery of the filter process was investigated by comparing the mean area values of formoterol and salbutamol of three unfiltered samples to the mean area values of three filtered samples of the same concentration. For the filtering process, 300 µL ACN/water (5:95, *v*:*v*) was added to the dried SPE residue, then it was filtered into a new conical glass vial. This step was repeated two times. The filtered solution was evaporated to dryness under nitrogen at 40 °C, and the residue was reconstituted in 50 µL ACN/water (5:95, *v*:*v*). The volume of water in the washing step of the HLB-based SPE was increased to two times 1 mL and three times 1 mL, whereby all other steps of HLB-based SPE were kept unchanged.

2.5.2. Final Study Sample Preparation Protocol

Aliquots of 600 µL serum and 600 µL water were added to 10 µL internal standard solution (ISTD 2, d_3-salbutamol, mefruside, d_3-ephedrine, and d_3-testosterone glucuronide, each 0.1 µg/mL in MeOH) in a 1.5 mL reaction tube, and the mixture was homogenized using a vortex mixer for three seconds. SPE was carried out utilizing HLB-based SPE cartridges attached to an SPE vacuum trap kit. The HLB cartridges were primed with 3 mL of MeOH and then conditioned with 3 mL high pure water before loading 1000 µL of the sample mixture by aspirating slowly through the column. For washing step, another 1000 µL of water were used. Then, the SPE cartridge was run dry for ten minutes under vacuum. An amount of 1.4 mL MeOH was used for elution from the sorbent into a 1.5 mL conical glass vial. The extract was dried under a gentle stream of nitrogen at 40 °C and then reconstituted with 50 µL FA/water/ACN (0.1:95:5, *v*:*v*:*v*). A volume of 300 µL of ACN/water (5:95, *v*:*v*) was added. The solution was filtered through a disposable filter into a new conical 1.5 mL glass vial. Two times more 300 µL ACN/water (5:95, *v*:*v*) was added and transferred via syringe and disposable filter into the glass vial. The filtered extract was again evaporated to dryness, and the residue was reconstituted in 50 µL ACN/water (5:95, *v*:*v*). The samples were directly analyzed or stored at −20 °C until analysis.

2.5.3. Matrix-Matched Calibration of Salbutamol, Formoterol, and Salbutamol-4′-*O*-Sulfate

Matrix-matched calibration was generated by spiking normal human serum free from targeted analytes. Salbutamol and formoterol were jointly calibrated in nine calibration levels, whereas calibration of salbutamol-4′-*O*-sulfate was separately generated in 11 calibration levels. Salbutamol calibration levels were from 2.1 pg/mL to 207.5 ng/mL. Formoterol

calibration levels were from 0.2 pg/mL to 20.5 ng/mL. Salbutamol-4′-*O*-sulfate calibration levels were generated to be from 1.0 pg/mL to 51.8 ng/mL. Each calibration level was created as triplicates following the sample preparation protocol for serum samples. Quality control samples were prepared as matrix-matched calibration level six containing 0.41 ng/mL formoterol and 4.2 ng/mL salbutamol.

2.6. Method Performance Characterization

2.6.1. Matrix Effect

The effects of the biological matrix on the signal intensities of the target analytes in UHPLC-MS analysis were elucidated. Matrix effect (ME) calculations were carried out according to Matuszewski et al. [66].

$$\text{ME\%} = \frac{\text{Peak area matrix matched calibration}}{\text{Peak area neat solvent calibration}} \times 100$$

For urine samples, three concentration levels for formoterol and salbutamol (level 3, level 5, and level 9) were prepared containing matrix, while, for serum analysis, calibration levels 4, 7, and 9 were used. For salbutamol-4′-*O*-sulfate, calibration levels 3, 6, 8, and 11 (urine) and 5, 8, and 11 (serum) were used. Mean values (n = 3) were compared to the mean values of the same calibration level free from the matrix.

2.6.2. Recovery

The loss of salbutamol and formoterol during the sample preparation process was investigated. Analyte-free pooled urine was used to generate samples, whereby the first group of four samples was spiked before, and the second group of four samples was spiked after sample preparation with 10 µL MeOH containing 0.82 µg/mL salbutamol and 82 ng/mL formoterol. The mean peak areas were compared.

Recoveries of formoterol and salbutamol in serum were tested by preparing normal human serum free from analytes following the serum sample preparation protocol. Then, the dried residues were reconstituted with methanol spiked to mimic 100% recovery of salbutamol and formoterol. Triplicates were generated.

2.6.3. Limit of Quantitation and Limit of Detection

Limit of quantitation (LOQ) was set as the lowest calibration level showing a signal-to-noise ratio (S/N) > 10 or higher and providing accuracy between 80% and 100%. Prerequisite for limit of detection (LOD) was S/N > 3.

2.6.4. Carry Over

Carry over was tested via injection of pure methanol after highest calibration standards for serum and urine analyses.

2.6.5. Retention Time Stability

Retention time (RT) stability was monitored over 4 days of analysis time. Relative retention time (RRT) was calculated by dividing the RT of the analyte via the RT of the internal standard.

3. Results

3.1. SPE Method Development

HLB-based SPE outperformed HR-CX-based SPE by giving ten times better recovery for formoterol. Using HR-CX-based SPE recovery of formoterol was 11% and recovery of salbutamol was 92% compared to HLB-based SPE. Experiments on optimization of water and MeOH volumes used for priming, conditioning, and elution showed that priming and conditioning with 3 mL of MeOH and water gave slightly better retention for formoterol (10%) but did not change the retention of salbutamol. The elution from the solid phase became better by increasing the elution volume from 0.5 mL to 1 mL and even better

to 1.5 mL. Furthermore, 1.5 mL MeOH led to better elution performance regarding the variance of the results. Standard deviation decreased from 6.5% or 15.2% for 0.5 mL methanol and from 5.7% or 16.7% for 1 mL MeOH to 4.7% or 4.3% for formoterol and salbutamol, respectively. Filtering the reconstituted extract through a disposable filter was the only successful way to achieve pressure stability in liquid chromatography.

3.2. UHPLC-MS/MS Method Performance Characterization

3.2.1. Limit of Quantitation and Limit of Detection

In the serum analysis, LOQ and LOD found for salbutamol were 8.3 pg/mL and <2.1 pg/mL; for formoterol, 0.8 pg/mL and <0.8 pg/mL; and for salbutamol-4′-*O*-sulfate, 51.8 pg/mL and <1.0 pg/mL.

In the analysis of urine samples, LOQ and LOD were 0.17 ng/mL and <0.17 ng/mL for salbutamol, 81.5 pg/mL and <16.3 pg for formoterol, and 2.1 ng/mL and <1.0 ng/mL for salbutamol-4′-*O*-sulfat. By increasing the injection volume of the samples to 30 µL, performance was improved, resulting in an LOQ of 41.0 pg/mL for formoterol.

3.2.2. Carry Over

Carryover was found to be lower than 0.05% for formoterol, salbutamol, and salbutamol-4′-*O*-sulfate in the urine and serum analyses.

3.2.3. Retention Time Stability

In the analysis of urine samples, RT shifts did not exceed 1% or 0.1 min for formoterol (RT 4.67 min), salbutamol (2.90 min), and salbutamol-4′-*O*-sulfate (RT 3.66 min). In the analysis of the serum samples, RT shifts were observed over 4 days of analysis time for formoterol (RT 4.825 min) at 2.8% (0.13 min), for salbutamol (RT 4.31 min) at 3.1% (0.12 min), and for salbutamol-4′-*O*-sulfate at 7.6% (0.26 min). Therefore, relative retention times (RRT) were considered. RRTs of formoterol related to d_3-testosteron glucuronide were in the range of 0.72 to 0.71. RRTs of salbutamol related to salbutamol-d_3 were from 1.120 to 1.024. RRTs of salbutamol-4′-*O*-sulfate related to salbutamol-d_3 were from 0.84 to 0.86.

3.2.4. Performance of Hydrolysis in Urine Samples by β-Glucuronidase

A proper response of d_3-testosterone was detected in all urine samples. Thus, successful enzymatic hydrolysis was achieved, as confirmed by the cleavage of the internal standard d_3-testosterone glucuronide.

3.2.5. Recovery

Recovery was found at 101% for formoterol, 106% for salbutamol, and 98% for salbutamol-4′-*O*-sulfate for urine sample preparation. In the serum sample preparation, recovery was 83% for formoterol and 113% for salbutamol.

3.2.6. Matrix Effect

Different ME values were observed depending on the concentration of the analytes and the matrix present in the injection solvent. In urine analysis at calibration levels 3, 5, and 9, ME for formoterol was found at 122%, 101%, and 95%, respectively. For the same calibration levels, ME for salbutamol was observed at 138%, 103%, and 108%. At calibration levels 6, 8, and 11, ME for salbutamol-4′-*O*-sulfate was 25%, 26%, and 27% for urine. In the analysis of serum samples, ME was found at 111%, 121%, and 53% for salbutamol-4′-*O*-sulfate at calibration levels 5, 8, and 11. ME for salbutamol was 216%, 147%, and 125% at calibration levels 4, 7, and 9.

3.3. Quantitation of Formoterol, Salbutamol, and Salbutamol-4′-O-Sulfate

Formoterol, salbutamol, and salbutamol-4′-*O*-sulfate were successfully quantified in serum and urine samples, giving a comprehensive pattern of the trial. Out of the four study arms per participant, one was positive for a single dosage of formoterol, one was

positive for a single dosage of salbutamol, one was positive for the combined administered dosages of salbutamol and formoterol, and one was tested negative for all target analytes. Figure 2A exemplifies the determined concentrations (uncorrected for specific gravity) in the urine samples of one participant from the four arms of this study. This shows that the administered drugs were successfully traced back using the applied methodology. Figure 2B presents the results from the same participant, which were adjusted by the specific gravity of the urines. In particular, the adjustment of formoterol concentrations highlighted its necessity in order to compare inter- and intra-individual results.

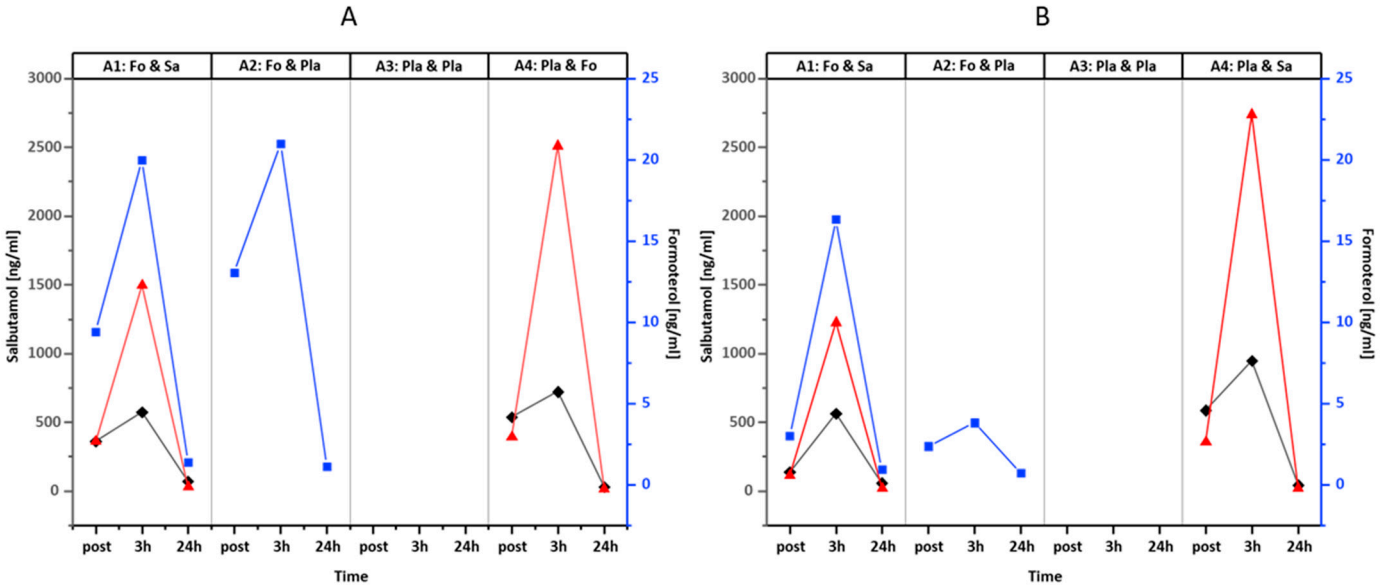

Figure 2. Results of urine analysis of all four arms (A1–A4) for one participant (T22) of this study. (**A**) Determined concentrations (non-adjusted); (**B**) concentrations adjusted for the specific gravity of the urines. Administration regime of formoterol (Fo), salbutamol (Sa), and placebo (Pla) is indicated in the header. Samples were collected 15–45 min after exercise (post), three hours after exercise (3 h), and 24 h after exercise (24 h). Sample preparation included enzymatic cleavage of glucuronides by β-glucuronidase. Concentrations of salbutamol (black rhombs) and salbutamol-4′-O-sulfate (red triangles) relate to the left Y-axes, and concentrations of formoterol (blue squares) relate to the right Y-axes.

3.3.1. Serum Sample Analysis

Formoterol was detected in serum samples collected after exercise (post) and in 3 h samples. In four samples of different participants, formoterol could not be quantified in the serum, although it was determined in the corresponding urine samples. Two of these cases were in a combined dosage trial and two in single dosage trials. After 24 h formoterol concentrations dropped below LOD in all serum samples after single or combined administration of formoterol, the highest concentration of formoterol was determined to be 14.2 pg/mL. Out of the 18 highest values, which represent the samples in the upper half of the total range split at 7.15 pg/mL, 13 were found in combined administration trials, and 16 were related to the female sex (Figure A1). Statistically significant differences in the mean values and medians of formoterol concentrations in serum were observed between athletes of different sexes after the combined dosage of formoterol and salbutamol directly after the performance test (7.5 pg/mL vs. 3.3 pg/mL p <0.001). Furthermore, for females, a mean value three times higher than the results from male participants was observed after three hours (5.9 pg/mL vs. 1.8 pg/mL, p <0.001) after combined and 4.0 pg/mL vs. 1.9 pg/mL (p <0.01) in formoterol-only administrations.

Salbutamol and its metabolite salbutamol-4′-*O*-sulfate were successfully quantified in post-exercise, 3 h, and 24 h serum samples of the respective study arms. Out of 144 samples with a potentially positive result, salbutamol could not be quantified in only one. The

highest value in the determination of salbutamol was 9.95 ng/mL, and the maximum concentration of salbutamol-4′-*O*-sulfate calculated as salbutamol was 21.35 ng/mL. Both findings were from male athletes in combined dosage trials and found in post-exercise samples but were not from the same participant.

The highest concentrations of total salbutamol (i.e., parent and sulfoconjugate) were found in post-exercise samples (Figure 3A) with a maximum value of 24.67 ng/mL (calculated as salbutamol). The mean concentrations decreased in all subgroups from post-exercise samples to 3 h samples. The standard deviation values decreased from post to 3 h. In post and 3 h trial arms, female subgroups showed clearly higher average values, except in the combined trial arm post-exercise, in which the male subgroup showed a higher mean concentration (statistically not significant). After 24 h, the highest value for the total salbutamol was 0.80 ng/mL, and the mean value for all 24 h samples was calculated as 0.3 ng/mL.

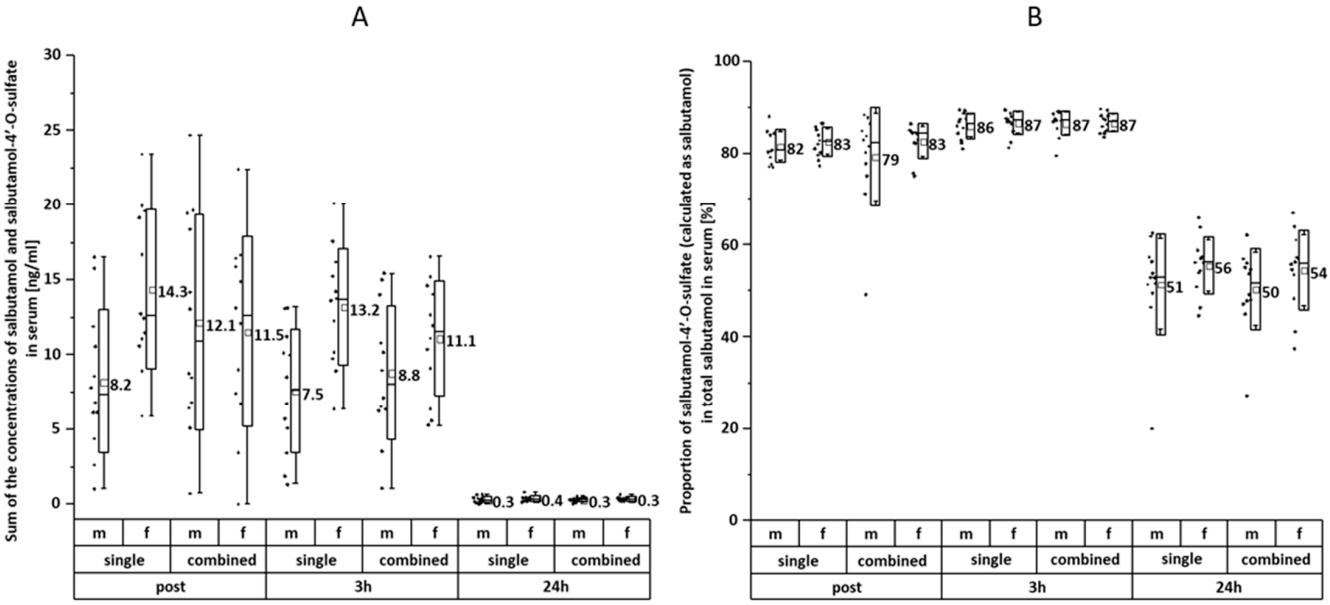

Figure 3. (**A**) Total salbutamol concentrations in serum calculated as sum of parent compound and salbutamol-4′-*O*-sulfate and (**B**) proportion of salbutamol-4′-*O*-sulfate (as % of the total salbutamol) in samples collected directly after exercise (post), after three hours (3 h), and after 24 h (24 h). Subgroups represent data from trials after single dosage of salbutamol (single) or a combined dosage of salbutamol and formoterol (combined). Data from male (m) and female (f) participants are presented separately. Individual data points are shown left to box. Labeled empty squares in the box represent mean values, solid line in the box is median, and upper and lower ends of box represent SD. Whisker shows maximum and minimum values of data range in (**A**) and confidence interval ($p = 0.01$) in (**B**).

The proportion of salbutamol, which was metabolized and then determined as salbutamol-4′-*O*-sulfate, was calculated and varied only a little within the subgroups (Figure 3B). The relative extent of sulfonated salbutamol starts with high values for post-exercise samples. For post-exercise samples, the mean values were calculated from 79% to 83%; for 3 h samples, from 86% to 87%; and for 24 h samples, from 50% to 56%. By dominating the occurrence of salbutamol in serum in post-exercise and 3 h samples, the absolute concentrations of salbutamol-4′-*O*-sulfate in serum show a similar pattern as the combined total amount of salbutamol as described above.

3.3.2. Urine Sample Analysis

In the analysis of formoterol in urine samples, concentrations in 15 out of 40 samples collected after 24 h could be quantified. Regarding the WADA threshold value of 40 ng/mL

and the decision limit of 50 ng/mL formoterol in urine for indicated therapeutic use [9], no urine sample exceeded any limit The observed average formoterol concentrations (unadjusted for specific gravity of the urines) of the whole group of participants were ≤20 ng/mL for single dose formoterol and combined dosages with salbutamol, whereas the highest average value was found for combined drug application after three hours. Mean values at 24 h after the exercise trial were 0.9 ng/mL and 0.4 ng/mL for single and combined formoterol administrations. Regarding the regulations of WADA, an adverse analytical finding is found by exceeding the substance's decision limit. In the case of highly concentrated urines with a specific gravity > 1.018, the decision limit would be adjusted by the urine's specific gravity. Therefore, not any adverse analytical finding was found for formoterol (including β-glucuronide) and salbutamol (including β-glucuronide). Moreover, according to the ISTI 2021 [65], an additional sample will be requested from the athlete in case of unmet requirements for specific gravity, i.e., very diluted urines (specific gravity < 1.005). In this current study, the adjustments of the urine sample concentrations served for a better comprehension and assessment of the excretion kinetics. However, even after the application of correction by specific gravity for all study samples, 50 ng/mL formoterol was exceeded in only three out of 144 samples. These three samples were correlated with specific gravities ≤ 1.003. Apart from this, correcting for specific gravity < 1.019 and especially for lower specific gravities results in an increased combined measurement uncertainty.

After subgrouping the participants into male and female athletes, higher mean concentrations were found for all groups of female participants, and all three samples above the decision limit were related to the female sex. The highest mean value of 29.4 ng/mL and the widest difference in mean values between female and male athletes were found three hours after the combined administration of formoterol and salbutamol with 29.4 ng/mL to 10.6 ng/mL (Figure 4).

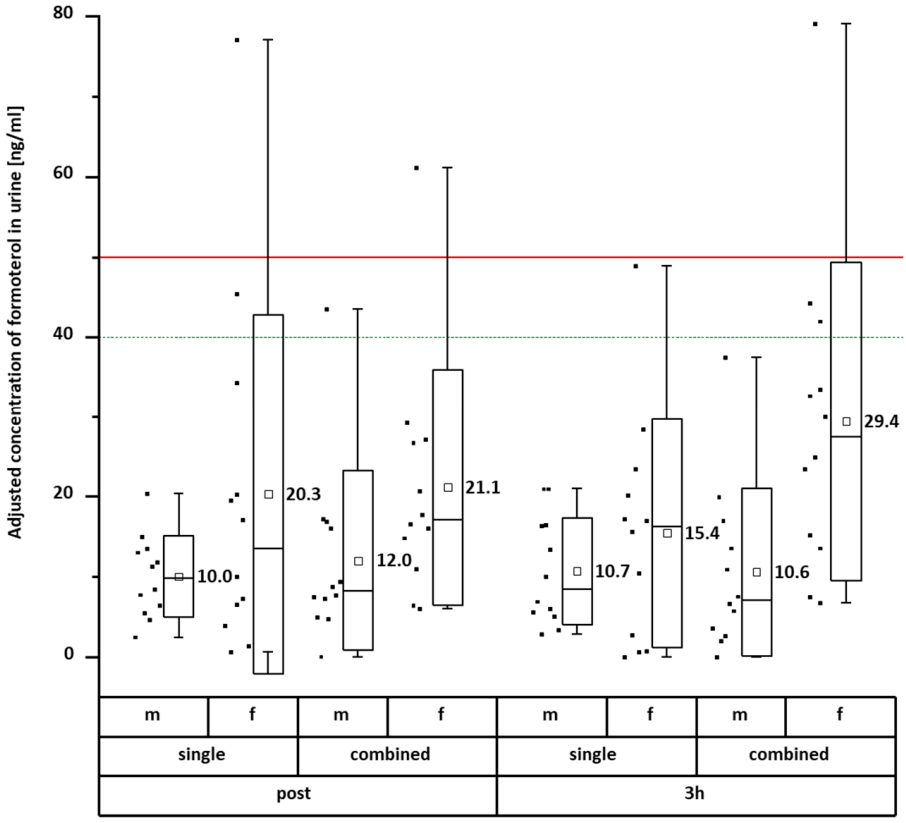

Figure 4. Adjusted urinary concentrations of formoterol in urine samples collected directly after stress (post) and after three hours (3 h). Note that 24 h results are not shown. Sample preparation included enzymatic cleavage of glucuronides by β-glucuronidase. Results are shown after correction

for specific gravity of the urine (adjusted concentration). Subgroups represent data from trial arms after single dosage of formoterol (single) or a combined dosage of formoterol and salbutamol (combined). Data from male (m) and female (f) participants are presented separately. Individual data points are presented from left to box. Labeled empty square in the box represents mean value, solid line in the box is median, upper and lower end of box represent SD, and whiskers shows maximum and minimum values of data range. Green dotted line is WADA threshold (40 ng/mL), and red solid line is WADA decision limit (50 ng/mL) for formoterol in urine after indicated and permitted use in athletes.

Urinary concentrations of salbutamol and salbutamol-4′-*O*-sulfate were successfully determined in all relevant samples. Out of 144 samples after administration of salbutamol (either alone or in combination with formoterol), three samples collected 24 h post-administration were found below LOQ for salbutamol-4′-*O*-sulfate and one out of these samples additionally below LOQ for salbutamol. The highest concentration for salbutamol was 949 ng/mL at three hours and was below the decision limit of 1200 ng/mL (threshold: 1000 ng/mL). Considering the sulfoconjugated metabolite in addition, the maximum value of total salbutamol was determined at a urinary concentration of 3687 ng/mL.

After correction of the concentrations for the specific gravity of the analyzed urines, 19 samples showed salbutamol concentrations exceeding 1000 ng/mL, with 10 above 1200 ng/mL. The measured specific gravities of these samples were ≤1.008. Since specific gravities were <1.019, the WADA rules would not allow an adjustment in the doping control analysis, or an additional sample would be requested from the athlete in case of a specific gravity < 1.005 in the initial samples. Out of the 19 samples beyond 1000 ng/mL, four samples were related to male participants, and nine values were from combined dosage trials (Figure 5A). On average, total salbutamol concentrations increased from post-exercise samples (721–1818 ng/mL) to 3 h samples (1450–3152 ng/mL, Figure 5B) At both collection times, mean values in female athletes were higher than in male participants. The highest concentrations of both salbutamol and total salbutamol were found 3 h after salbutamol-only administration in the urine sample of a male volunteer (Figure 5A,B). After 24 h, total salbutamol mean values from 57 ng/mL to 118 ng/mL were observed (Figure 5B).

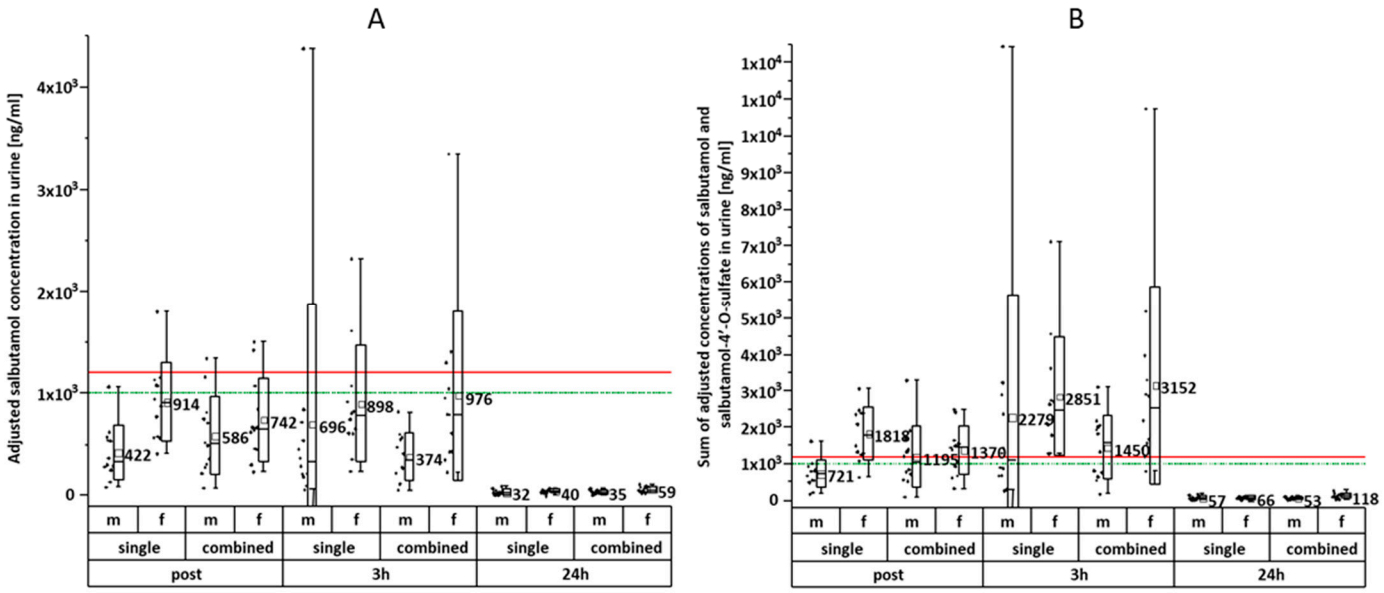

Figure 5. *Cont.*

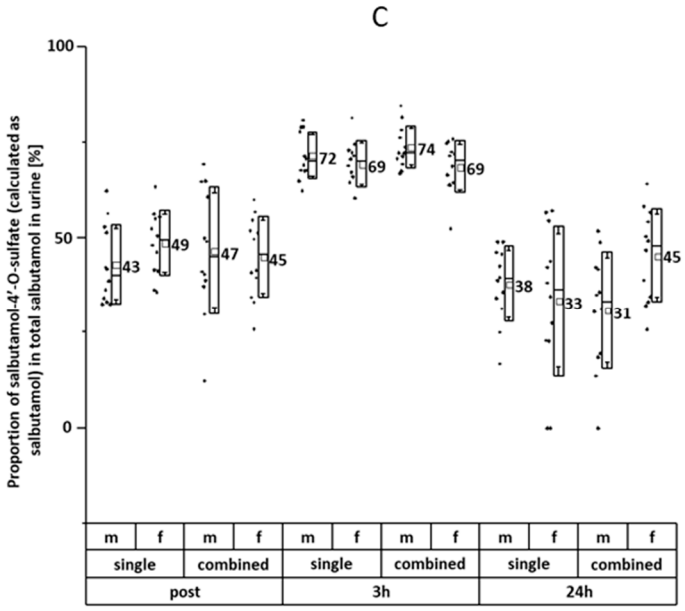

Figure 5. (**A**) Adjusted urinary concentrations of salbutamol, (**B**) total salbutamol concentration (joined salbutamol and salbutamol-4′-*O*-sulfate calculated as salbutamol equivalent), and (**C**) proportion of salbutamol-4′-*O*-sulfate (as salbutamol equivalent) in total salbutamol in urine. Sample preparation included enzymatic cleavage of glucuronides by β-glucuronidase. Results are shown after correction for specific gravity of the urine (adjusted concentration). Samples were collected directly after exercise (post), after three hours (3 h), and after 24 h (24 h). Subgroups represent data from trials after single dosage of salbutamol (single) or a combined dosage of salbutamol and formoterol (combined). Data from male (m) and female (f) participants are presented separately. Individual data points are shown left to box. Labeled empty squares in the box represent mean values, solid line in the box is median, upper and lower ends of box represent SD. Whisker in (**A**,**B**) shows maximum and minimum values of data range and, in (**C**), confidence interval ($p = 0.01$). In (**A**,**B**), the red solid line is decision limit (1200 ng/mL), and the green dotted line is threshold (1000 ng/mL) based on WADA regulations.

The highest proportion of salbutamol-4′-*O*-sulfate excreted in urine was 85% of the total salbutamol observed at 3 h post-exercise (Figure 5C). Post-exercise mean values ranged from 43 to 49%. Herein, the highest SD was 17% within the subgroups. After 3 h, the average proportion of salbutamol-4′-*O*-sulfate excretion increased to 69–74%, with a maximum SD of 8%. The proportion of salbutamol-4′-*O*-sulfate decreased again after 24 h to mean values from 31 to 45% with SDs up to 20%.

4. Discussion

Serum levels of formoterol, salbutamol, and its phase II metabolite salbutamol-4′-*O*-sulfate as well as urinary concentrations of formoterol (including glucuronides), salbutamol (including glucuronides), and salbutamol-4′-*O*-sulfate were determined using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS).

Due to the concentrations expected to be at ultra-trace levels [51,67], a triple quadrupole mass analyzer was utilized. While urine analysis was possible using the dilute-and-inject method, analyte preconcentration and purification via solid phase extraction (SPE) prior to UHPLC-MS/MS was necessary for the serum sample analysis. All medication combinations were reliably traced back from the urine samples meeting WADA standards for identification [68]. Maximum tolerance windows for retention times or relative retention times and relative abundances of at least two ion transitions matched the recommendation for all samples. In serum, concentrations were found considerably lower. Thus, identification of the analytes required preanalytical sample processing.

As mentioned in a note of WADA's 2023 list of prohibited substances and methods [8], "the presence in the urine of salbutamol in excess of 1000 ng/mL or formoterol in excess of 40 ng/mL is not consistent with the therapeutic use of the substance [. . .]". None of the urine samples collected after the application of *verum* medication resulted in concentrations exceeding the threshold concentrations set for doping control analysis in any of the participants of this study.

4.1. UHPLC-MS/MS and Sample Preparation

The applied methodology for sample preparation and UHPLC-MS/MS successfully allowed us to prove the medication regime and, furthermore, to quantitate the analytes in urine and serum. A simple dilute-and-inject approach after enzymatic cleavage of glucuronides in urine samples was suitable to investigate an exceedance of the WADA set decision limits. By increasing the injection volume to 30 µL and adjusting the injection solvent to close to the starting conditions of the mobile phase, an LOQ of 41 pg/mL formoterol in urine was achieved. Keeping the gradient for 2 min at very low proportions of eluent A resulted in focusing of the analytes on the column head before being eluted by increasing eluent B. In this manner, also very low concentrations of formoterol were determined successfully. The quantitation of salbutamol, administered in much higher dosages, was less challenging in urine and serum samples. Switching to a phenyl-hexyl-equipped RP column enabled better retention of salbutamol-4′-*O*-sulfate and separation from salbutamol. While, in urine samples, dilute-and-inject (after enzymatic cleavage of glucuronides) was sufficient for the determination of all concentrations in this study, preconcentration and clean up of the serum samples were achieved utilizing HLB-based SPE cartridges, which were already proven suitable for the simultaneous retention of salbutamol and salbutamol-4′-*O*-sulfate [58]. In this study, the SPE covered the polarity range from the very polar salbutamol metabolite to the more non-polar formoterol. As mentioned above, in many publications, CX or MCX cartridges were used. In this study, method development led to the use of HLB-based SPE in serum sample analysis. The use of CX cartridges requires pH switches between acidic and basic values for retention and then elution of the basic analytes. Therefore, utilizing HLB-based SPE also avoided potential instabilities of the analytes. All UHPLC-MS/MS methods had a turnaround time of 10.5 min and, therefore, achieved fast separation and quantitation of the analytes.

4.2. Urine Analysis

The highest urinary concentration (Cmax) of formoterol (after enzymatic hydrolysis with β-glucuronidase) was observed three hours after the performance test with Cmax = 17.2 ng/mL and did not exceed WADA's threshold (which is currently based on the sum of the glucuronide conjugate and free drug concentration, WADA TD2021DL [9]). However, when adjusting the results for the specific gravity of the urines, the highest value was 79.1 ng/mL of formoterol in urine three hours post-exercise trial. The specific gravity of the urine sample was determined as 1.001. In the doping control analysis, a further sample would be demanded from the athlete to match a minimum required specific gravity of 1.005. The correction for specific gravity accounts for potential differences in the urinary flow and, thereby, for potentially related differences in the concentrations of urine samples. In this present study, the highest values were found in female athletes. A sex-specific difference was clearly observed in urinary excretion patterns. Aberrantly, no differences in relation to sex or ethnic origin were found by Dickinson et al. [55] in a group of 20 participants after a single dose of 1600 µg salbutamol.

Ventura et al. [43] reported maximum formoterol concentrations of up to 20 ng/mL urine after hydrolysis with β-glucuronidase from *Helix pomatia* following a single dose inhalation of 18 µg of formoterol (administered as fumarate). Even more in line with our findings of unadjusted urinary concentrations up to 17.2 ng/mL (after enzymatic hydrolysis) after 36 µg inhaled formoterol, Deventer et al. [30] found maximum concentrations of 8.5 ng/mL without and 11.4 ng/mL following enzymatic hydrolysis after the inhalation

of 18 µg of formoterol. Mazzarino et al. determined maximum urinary concentrations of 15 ng/mL (free + glucuronide) after repeated doses of 12 µg or 36 µg of formoterol fumarate [29]. A considerable difference in sample preparation by Ventura et al. [43] was the application of β-glucuronidase from *Helix pomatia*, which reportedly also shows weak sulfatase activity. Thus, potentially sulfoconjugated formoterol may be included in the quantitation.

In the study arms related to salbutamol or combined administration, no urinary concentration of salbutamol exceeded the WADA threshold after the inhalation of 1200 µg salbutamol. According to the Technical Document on Decision Limits (WADA TD2021DL [9]), the currently applied "threshold concentration is based on the sum of the glucuronide conjugate (expressed as the free drug) and free drug concentrations" in urine. Thus, the deconjugation of potential glucuronides was performed utilizing β-glucuronidase prior to dilution of the urine samples. Although salbutamol glucuronides were reported to be of minor occurrence in humans [69], and the main metabolite excreted next to the parent drug salbutamol was proven and reported as salbutamol-4′-*O*-sulfate [56,70,71], the sulfoconjugated drug is not considered for WADA's threshold and decision limit. According to Ventura et al. [54], using a cut-off concentration of 500 ng/mL for non-sulfated salbutamol for the selection of suspicious samples would result in 4.3% false-positive results.

After inhalation of 800 µg of salbutamol, Elers et al. [45] reported median urinary concentrations (Cmax) of 260.9 ng/mL in the first four hours after administration. In a second manuscript of the same group, the highest urinary salbutamol concentration was 1057 ng/mL four hours after inhalation of 800 µg salbutamol (no correction for urine specific gravity) [38]. Furthermore, Sporer et al. [72] reported the peak urinary excretion as dose independently (200, 400, or 800 µg) at 60 min (maximum concentration of 904 ng/mL). Haase et al. [36] pointed out the influence of exercise in hydrated or dehydrated conditions on the mean salbutamol concentration in urine after the inhalation of the maximum permitted dosage of 1600 µg (per 24 h) but as a single dose of salbutamol. It is worth noting that in 2017, the maximum dosage of 1600 µg per 24 h was complimented by the maximum allowance of 800 µg per 12 h; since 2022, the rules have been tightened to 1600 µg per 24 h and a maximum of 600 µg salbutamol per 8 h. Most comparable to our study is the study arm with exercise in hydrated conditions, in which after 1.5 h and, likewise, after 4 h, 4 out of 13 participants (31%) exceeded WADA's decision limit (maximum concentration 3315 ng/mL), while results were unadjusted for the specific gravity of the urines. Adjusting the concentration still led to 31% adverse analytical findings (maximum concentration 2404 ng/mL) after 1.5 h and to one less (3 out of 13; 23%) adverse analytical findings after 4 h.

In our study, the highest value was found 3 h after administration (949 ng/mL unadjusted by the specific gravity of the urine). In line with the reports from Elers et al. and Haase et al. [36,45,51], the highest urinary salbutamol concentrations were found 3–4 h after inhalation. Yet, in between the sample draw timepoints "post" and "3 h", an even higher concentration might be observable if samples were collected in this period. Similar to an earlier report by Berges et al. [45], urinary concentrations of salbutamol after single-dose inhalation showed high individual variability. To be specific, the observed range at 3 h after the trial exercise was from 106 ng/mL to 949 ng/mL without and 54 ng/mL to 4373 ng/mL with adjustment for the specific gravity of the urines. While the correction by specific gravity in the doping control analysis is only performed if in favor of the athlete, it was used in this study in both directions to cope with the differences in the urinary flow of the participants to obtain comparable results. Regarding the results of this current study, it should be considered that 1200 µg salbutamol was administered as a single dose, whereas 600 µg/8 h and 1600 µg/24 h are allowed in sports without a therapeutic use exemption. Thus, in this present study, 19 samples with specific gravities $\leq$ 1.008 showed salbutamol concentrations exceeding 1000 ng/mL, with 10 samples above 1200 ng/mL after correction of the concentrations for the specific gravity. It should be noted that this adjustment would not be performed in the doping control analysis according to WADA regulations [9].

4.3. Serum Analysis

In this present study, the highest concentration (14.2 pg/mL of formoterol in serum) was detected in a post-exercise sample 70 to 100 min after inhalation of 36 µg of formoterol. This is in line with the data of Mascher et al. [39], who reported similar concentrations and a very fast increase and then a continuous decrease in formoterol concentration in serum after inhalation of 36 µg formoterol.

After inhalation of salbutamol, the highest mean concentration in serum was found in post-exercise samples with 2.1 ng/mL (median 2.0 ng/mL). This mean concentration (observed after inhalation of 1200 µg salbutamol) ranged between the reported findings of 1.75 ng/mL (after 800 µg) [45] and 3.8 ng/mL (after 1600 µg) [36], with both studies including exercise prior to sample collection. As reported by Haase et al., serum salbutamol (Cmax) was lower for resting volunteers (3.0 ± 0.7 ng/mL) than exercising (3.8 ± 0.8 ng/mL) and exercise and dehydration (3.6 ± 0.8 ng/mL) [36]. It may be hypothesized that exercise increases circulation and, thereby, absorption, which may lead to earlier and higher peak concentrations.

Overall, higher mean concentrations were found in the female subgroups for both salbutamol and formoterol. Exceptions from this observation were seen for formoterol and total salbutamol concentrations after the combined administration of formoterol and salbutamol in the post-exercise serum samples. Other peculiarities in the post-exercise samples were observed after the combined administration of formoterol and salbutamol. In male athletes, it led to a higher salbutamol mean value in serum, and in female athletes, it led to a higher mean concentration of formoterol in serum. Similar to urine, an unexpected observation was found in the same subgroup (female, combined dosages) but in the three hours urine samples as follows: the average of the adjusted concentrations of formoterol was twice as high compared to the adjusted concentrations after formoterol-only administration (29.4 ng/mL vs. 15.4 ng/mL). A competing excretion mechanism or an impact of combined dosages on the retention in the female body may potentially serve as an explanation. Considering the difference in weight of male and female volunteers, the smaller distribution volume in the female participants might lead to higher concentrations in serum and urine. Another aspect that should be considered is the sampling times: The study design did not allow us to precisely determine the Cmax in serum or a peak in urinary excretion rate.

4.4. Phase II Metabolites

As observed for salbutamol and formoterol for both specimens, serum and urine, higher concentrations in female participants were also found for salbutamol-4′-*O*-sulfate. The proportion of salbutamol present as salbutamol-4′-*O*-sulfate in serum showed no significant difference between the four subgroups at draw times post (79% to 83%) and 3 h (86% to 87%). An exception was observed in the average variation, which gave a very narrow pattern of results for all subgroups at the post and 3 h timepoints, but in the subgroup male plus combined dosage, the standard deviation was noticeably higher. As a result, a relatively higher standard deviation for that subgroup was observed, whereas, at the highest observed salbutamol-4′-*O*-sulfate proportion (3 h after exercise), the mean variations were small. After 24 h, the total salbutamol concentrations in serum and urine were small, as the proportion of sulfoconjugate decreased to 50–56%. The described divergencies may result from shifted sulfonation kinetics and urinary excretion curves. A later t(max) for salbutamol-4′-*O*-sulfate compared to the parent drug was reported before [40]. In accordance with that observation, in this present study, total salbutamol (not subgrouped) concentrations decreased slightly from post to three hours samples, but absolute salbutamol concentrations in serum decreased relatively stronger than salbutamol-4′-*O*-sulfate concentrations. An explanation for a crucial decline of the proportion of sulfonated metabolite after 24 h (from 87% at 3 h to 53% at 24 h, not subgrouped) may be a faster elimination of the more polar metabolite in combination with an enantiomerically selective sulfonation. Following this suggestion, the preferably sulfonated (*R*)-salbutamol [40] would also be

eliminated faster. Thus, after 24 h, the remaining circulating salbutamol would be dominated by the (*S*)-enantiomer. A lower sulfonation rate would be the result of a lower affinity of (*S*)-salbutamol to the sulfotransferase and/or the relatively lower amount of (*R*)-salbutamol available. To prove or refuse this, speculation chiral analysis must be applied. Since, in this study, a continuous collection of urine and serum samples was not performed, a comprehensive excretion kinetics that may support or refute this hypothesis was not generated.

The phase II enzyme SULT1A3 was reported to be the major isoenzyme responsible for salbutamol sulfonation [73]. Genetic polymorphism in SULT1A3 was reported to have an impact on the affinity and catalytic activity toward salbutamol [74]. However, contrary reports are also published [37].

Further investigations are needed to elucidate the impact of genetic polymorphism or drug–drug interactions on the pharmacokinetics of salbutamol sulfonation and, therefore, on its pharmacodynamics.

Especially for salbutamol, there is still contradictory information on the relevance of the excretion of the glucuronidated metabolite in humans [69]. Considering formoterol, metabolism seems to be undervalued in doping control analyses [31]. Only very few studies report the detection of metabolites of formoterol [26,29]. Thus, investigations on the detection of phase I and phase II metabolites besides the unmodified and deglucuronidated parent drug may add additional value. However, method development and optimization are currently hampered by the lack of availability of the respective metabolites as reference material. Our study indicates differences in the metabolism and excretion of formoterol and salbutamol related to the sex of the athletes as well as to the co-administration of both drugs. Further investigations on the pharmacodynamics and pharmacokinetics with a focus on the co-medication of drugs that are legally or illegally misused in doping are highly desirable.

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Informed Consent Statement: Informed consent was obtained in writing from all subjects involved in this study.

Data Availability Statement: The data that support the findings of this study are openly available in OPARU at http://dx.doi.org/10.18725/OPARU-47213 (accessed on 20 June 2023).

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Appendix A

Table A1. Protocols used to compare recovery for salbutamol and formoterol applicating the SPE phases HLB and HR-XC.

Step	HLB	HR-XC
Priming	3 mL MeOH	3 mL MeOH
Conditioning	3 mL H_2O	3 mL H_2O
Samples application (slowly)	1.0 mL	1.0 mL
Washing	1 mL water	1 mL 0.1 M HCl in water, then 1 mL of MeOH
	Drying SPE for 10 min under vacuum	Do not let run dry
Elution into a conical vial	1 mL MeOH	5% NH_3 in 1 mL MeOH
Drying under a stream of nitrogen at 40 °C		
Reconstitution in the same conical vial	50 µL 0.1% formic acid in water (0.1:100, *v*:*v*)	50 µL 0.1% formic acid in water (0.1:100, *v*:*v*)

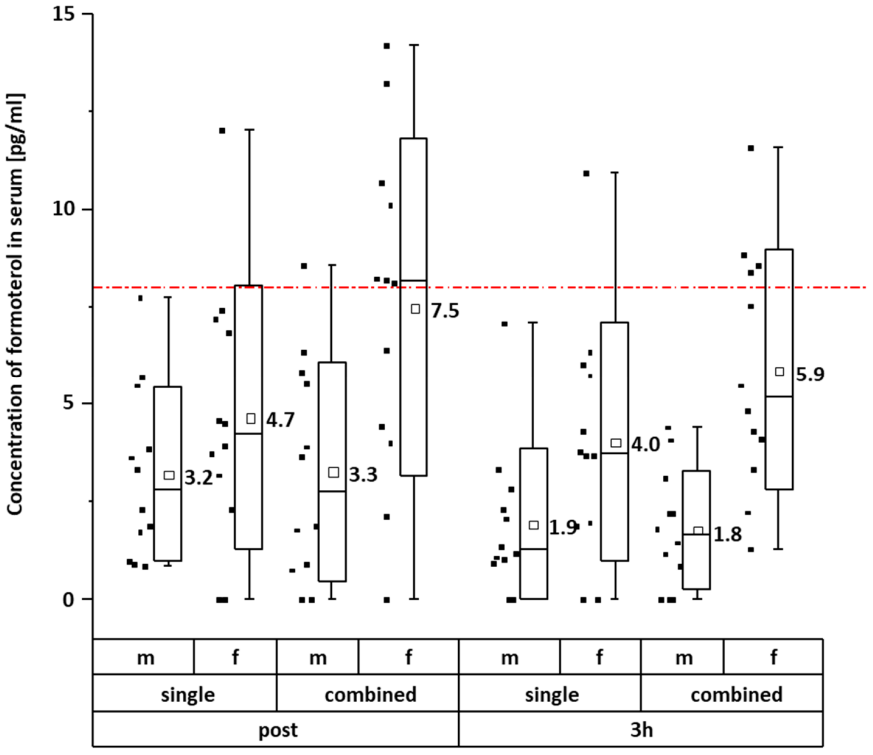

Figure A1. Concentration of formoterol in serum samples collected directly after time trial performance test (post) and after three hours (3 h). In the 24 h post samples, concentrations in all samples were below the LOQ. Subgroups represent data from trials after single dosage of formoterol (single) or a combined dosage of formoterol and salbutamol (combined). Data from male (m) and female (f) participants are presented separately. Individual data points are shown left in the respective box. Labeled empty squares in the box represent mean values, solid line in the box is median, upper and lower end of box represent SD, and whisker show maximum and minimum values of data range. Red dash-dotted line splits detected range at 7.15 pg/mL.

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