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Terbutaline sulfoconjugate: characterization and urinary excretion monitored by LC/ESI-MS/MS

A. K. Orlovius,*[a,b] S. Guddat,[a] M. K. Parr,[a] M. Kohler,[a] M. Gütschow,[b] M. Thevis[a] and W. Schänzer[a]

Terbutaline is a fast-acting β_2-adrenergic agonist used in the treatment of obstructive pulmonary diseases. Doping control for β_2-agonists, which are forbidden in sports by the World Anti-doping Agency (WADA), is performed in screening by liquid chromatography/mass spectrometry after hydrolysis of phase-II metabolites. In this study, the mono-sulfoconjugated phase-II metabolite of terbutaline was synthesized and the chemical structure was characterized by ^{1}H-nuclear magnetic resonance spectrometry and high resolution/high accuracy Orbitrap mass spectrometry. The metabolite was designated as the phenolic esterified compound, which has been mentioned in most literature reports but has not been verified so far. The benzylic esterified compound was also synthesized and characterized by high-resolution/high accuracy Orbitrap mass spectrometry but was not detectable in urine samples from an excretion study performed after a single application of one terbutaline capsule (7.5 mg terbutaline sulfate salt). The phenolic sulfate of terbutaline was detected for two to four days after administration, whereas the unchanged terbutaline was detected for four to five days. A glucuronidated, disulfated or trisulfated phase-II metabolite of terbutaline was not found. The measurement of phase-II metabolites is planned to be incorporated into existing screening procedures to allow a faster sample preparation.

Keywords: beta-2 agonists; doping control; LC/ESI-MS/MS; phase-II metabolism; sulfate conjugate

Introduction

Terbutaline (IUPAC: 5-[2-(tert-butylamino)-1-hydroxyethyl]benzene-1,3-diol) (for structure see Figure 1A) is a selective fast-acting β_2-adrenergic agonist. It has marketing authorization for use by injection, inhalation or oral dosage for the treatment of obstructive pulmonary diseases and as a short-term asthma treatment. Terbutaline acts directly on the bronchial muscle cell. After binding to the receptor, adenylate cyclase is activated to generate 3′–5′-cyclic-adenosine-monophosphate (cAMP) from adenosine-5′-triphosphate (ATP). The increased cAMP concentration induces a bronchodilation directly, by electrolyte adjustment in the bronchial muscle cells. The immediate relaxation of the tracheal and bronchial muscles is most important for the antiasthmatic therapy, as this relaxation is independent of the pathological mechanism of endobronchial obstruction. As a result, lung function normalizes and conditions for improved gas exchange are provided.[1]

Terbutaline can be misused in sports and therefore appears on the 2009 prohibited list of the World Anti-doping Agency (WADA) for use in and out of competition.[2] Due to its chemical structure, terbutaline is stable against the metabolizing enzymes catechol-O-methyl transferase (COMT)[3] and monoamine oxidases (MAO)[4] after uptake into the body. Following oral administration, one-third to one-half of the dose is excreted in the urine, whereas after intravenous and subcutaneous administration more than 90% is eliminated by this route. This indicates a high first-pass metabolism, which occurs preferentially in the gut wall.[5] Metabolism studies of terbutaline in man revealed a mono-sulfoconjugate as the main metabolite in urine beside the unchanged drug[1,3,6,7] and a glucuronidated conjugate was detected as a minor component.[8] The amount of free terbutaline in serum was determined with about 15% after oral application.[7]

Sulfoconjugation in the human body takes place with the co-substrate 3′-phosphoadenosin-5′-phosphosulfate (PAPS), which transfers a sulfate function to a hydroxyl group of an acceptor by a cytosolic sulfotransferase.[9] The sulfate conjugation of racemic terbutaline by human liver cytosol is stereoselective, favouring the (S)-(+)-enantiomer (which is the distomer without physiological properties) over the (R)-(−)-enantiomer (which is the eutomer) by about twofold.[10] Moreover, the oral bioavailability of terbutaline is stereoselective. The (R)-(−)-enantiomer has a twofold higher bioavailability than the (S)-(+)-enantiomer and this was suggested to depend on both, an incomplete absorption of the (S)-(+)-enantiomer and a more efficient presystemic metabolism.[11] The preferential sulfatation of the (S)-(+)-enantiomer may thus explain the enantioselective presystemic metabolism. As medical formulations of terbutaline contain the racemate, the enantioselective pharmacokinetics should be considered in clinical use.[10]

With respect to the conjugation site, the conjugates of terbutaline have not yet been characterized in the literature.

* *Correspondence to: A. K. Orlovius, Institute of Biochemistry, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany. E-mail: a.orlovius@biochem.dshs-koeln.de*

a *Institute of Biochemistry, German Sport University Cologne, Germany*

b *Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Germany*

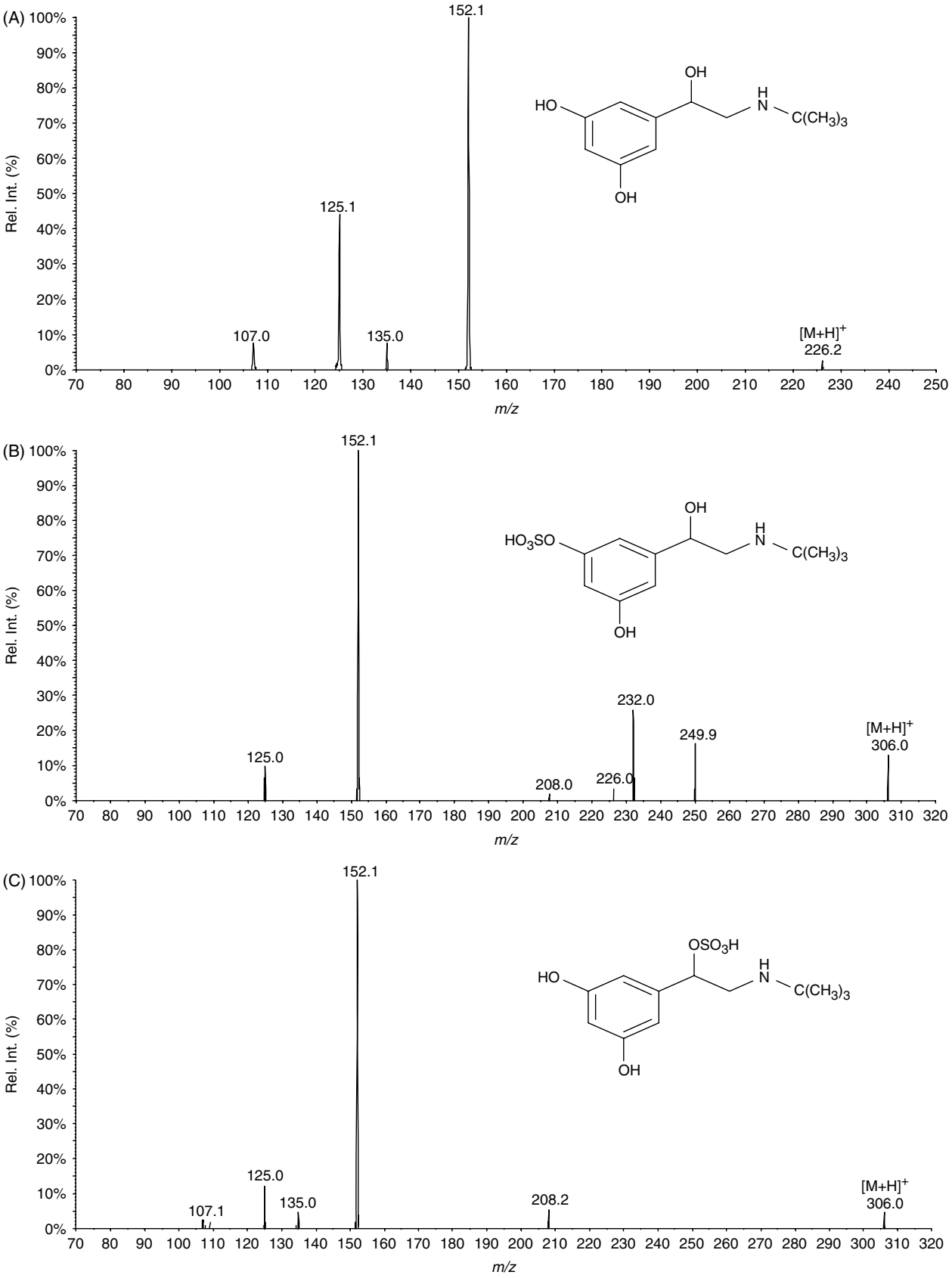

Figure 1. ESI-product ion spectra of protonated molecules $[M+H]^+$ of (A) free terbutaline, (B) sulfoconjugate **1** and (C) sulfoconjugate **2**.

Until now, they have been investigated indirectly by enzymatic or chemical hydrolysis.

Common methods for doping analysis of terbutaline in urine samples are based on liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS)[12] and gas chromatography/mass spectrometry (GC/MS) for minor incidents.[13,14] For LC/ESI-MS/MS analysis, urine samples are prepared by chemical hydrolysis with hydrochloric acid followed by liquid-liquid extraction.[12] For GC/MS analysis, samples are typically prepared by enzymatic hydrolysis with β-glucuronidase/arylsulfatase from *Helix pomatia* followed by liquid-liquid or solid phase extraction and subsequent trimethylsilylation. Acylation, trimethylsilylation and acylation,[13] cyclic methylboronates[15] and reaction with formaldehyde to perform a tetrahydroisoquinoline derivative with subsequent trimethylsilylation[16] are also described as derivatization steps. Analysis of urine specimens by an enzyme-linked immunosorbent assay (ELISA) and capillary electrophoresis is also described in the literature.[17]

The present study was carried out to characterize the sulfoconjugate of terbutaline and to integrate the measurement of the conjugate into existing screening procedures. For the characterization of the sulfate conjugate, reference material was synthesized and the product was characterized by high-resolution/high accuracy Orbitrap mass spectrometry and ^{1}H nuclear magnetic resonance spectrometry (^{1}H NMR) techniques. Hydrolytic sample preparation was unnecessary for the direct measurement of the conjugates from urine, and urine specimens were prepared under less difficult conditions. The excretion of the sulfoconjugated terbutaline as well as the unchanged drug were monitored in urine after an oral dose of 7.5 mg terbutaline.

Experimental

Reference compounds and chemicals

Terbutaline hemisulfate salt was obtained from Sigma (Steinheim, Germany). The slow-release capsule of terbutaline (7.5 mg as sulfate salt) was bought from Ratiopharm GmbH (Ulm, Germany). Serdolit® PAD-1 was obtained from Serva (Heidelberg, Germany), *tert*-butyl methyl ether from AppliChem (Darmstadt, Germany), propan-2-ol from LGC Promochem (Wesel, Germany), methanol from VWR (Darmstadt, Germany), and *p*-hydroxyephedrine hydrochloride, pyridine (99%) and silica gel 60 (70–230 mesh) were from Sigma (Steinheim, Germany). Ethyl acetate (analytical grade), ammonia solution (25%, analytical grade), glacial acetic acid and sulfur trioxide pyridine complex (for synthesis) were purchased from Merck (Darmstadt, Germany). Deionized water used for sample preparation and buffer solutions was of MilliQ grade.

Preparation of terbutaline sulfoconjugate

For the preparation of the excreted terbutaline sulfate, 0.5 g of terbutaline hemisulfate salt was stirred with 0.585 g of sulfur trioxide pyridine complex in 41 mL of pyridine at room temperature for 29 h.[18–20] The mixture was then subjected to elaborate purification. Reprocessing by column chromatography (propan-2-ol, ethyl acetate, ammonia solution (17.5%) 40 : 40 : 20 (v : v : v); on silica gel; column dimension: inner width 3.5 cm, bed height 38 cm; fraction size 50 mL) no pure product could be obtained. The fraction with the highest content of mono-sulfoconjugates (fraction after 600 mL) was purified again (propan-2-ol, ethyl acetate, ammonia solution (17.5%) 40 : 50 : 10 (v : v : v); on silica gel; column dimension: inner width 1.7 cm, bed height 38 cm; fraction size 50 mL). This procedure achieved one pure fraction with approximately 4 mg of the phenolic sulfoconjugated terbutaline (after 800 mL of the eluent on the second column) and one pure fraction with approximately 1 mg of the benzylic esterified sulfoconjugated terbutaline (after 500 mL of the eluent on the second column). The fractions were monitored for the sulfoconjugates by LC/ESI-MS/MS (conditions see section LC/ESI-MS/MS).

The ^{1}H NMR spectra of the phenolic esterified sulfoconjugate and free terbutaline were acquired on a Bruker Avance DRX 500 spectrometer (Bruker, Karlsruhe, Germany) operating at 500 MHz. Chemical shifts δ are given in ppm referring to the signal centre using the solvent peaks for reference DMSO-d_6 2.49 ppm. ^{1}H NMR (d_6-DMSO): phenolic esterified sulfoconjugate: δ 1.02 (s, 9 H), 4.35 (dd, 1 H), 6.45 (t, 1 H), 6.52 (t, 1 H), 6.57 (m, 1H); free terbutaline: δ 1.21 (s, 9 H), 2.70 (m, 1 H), 2.85 (dd, 1 H), 4.66 (dd, 1 H), 6.10 (t, 1 H), 6.26 (d, 2 H).

Preparation of p-hydroxyephedrine sulfoconjugate

For the preparation of *p*-hydroxyephedrine sulfate (for structure see Figure 2) as an internal standard (ISTD), 0.1 g of *p*-hydroxyephedrine hydrochloride was stirred with 0.082 g of sulfur trioxide pyridine complex in 2 mL of pyridine at room temperature for six days. Evaporation to dryness and reconstitution in 10 mL of water were followed by extraction with *tert*-butyl methyl ether (3 × 10 mL) for purification. The organic layers were discarded and the aqueous layer was subjected to column chromatography (*tert*-butyl methyl ether, methanol, ammonia solution (25%) 70 : 29 : 1 (v : v : v); on silica gel; column dimension: inner width 3.0 cm, bed height 50 cm; fraction size 100 mL). Pure fractions of the product (after 4400 mL of the eluent) were combined, concentrated in vacuum and recrystallized from methanol: ammonia solution (25%) 5 : 2 (v : v) and yielded 11.6 mg of the desired compound.

High resolution/high accuracy mass spectrometry

HRMS-characterization of the synthesized mono-sulfoconjugates and determination of elemental compositions was performed on a LTQ Orbitrap mass spectrometer (Thermo, Bremen, Germany). The instrument was operated in positive electrospray ionization mode and calibrated using the manufacturer's calibration mixture (consisting of caffeine, MRFA and ultramark). Mass accuracies $<$3 ppm (calculated from 30 averaged spectra) were accomplished for the period of the analysis. Analytes were dissolved in acetonitrile/water (1 : 1, v : v) containing 2% acetic acid at concentrations of approximately 10 µg/mL and introduced into the mass spectrometer using a syringe pump at a flow rate of 5 µL/min. The ionization voltage was 4200 V, and the capillary temperature was set to 290 °C. For MS^n experiments, the protonated precursor ions were isolated using a width of 1.5 Da and protonated species were dissociated at normalized collision energies between 15 and 25 (arbitrary units, Xcalibur software version 2.0, Thermo, Bremen, Germany). The damping gas in the linear ion trap was helium (purity grade 5.0) and the gas supplied to the curved linear ion trap (CLT) was nitrogen obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany).

Excretion study and urine sample preparation

For the excretion study, one slow-release capsule of terbutaline (7.5 mg as sulfate salt) was administered to three healthy

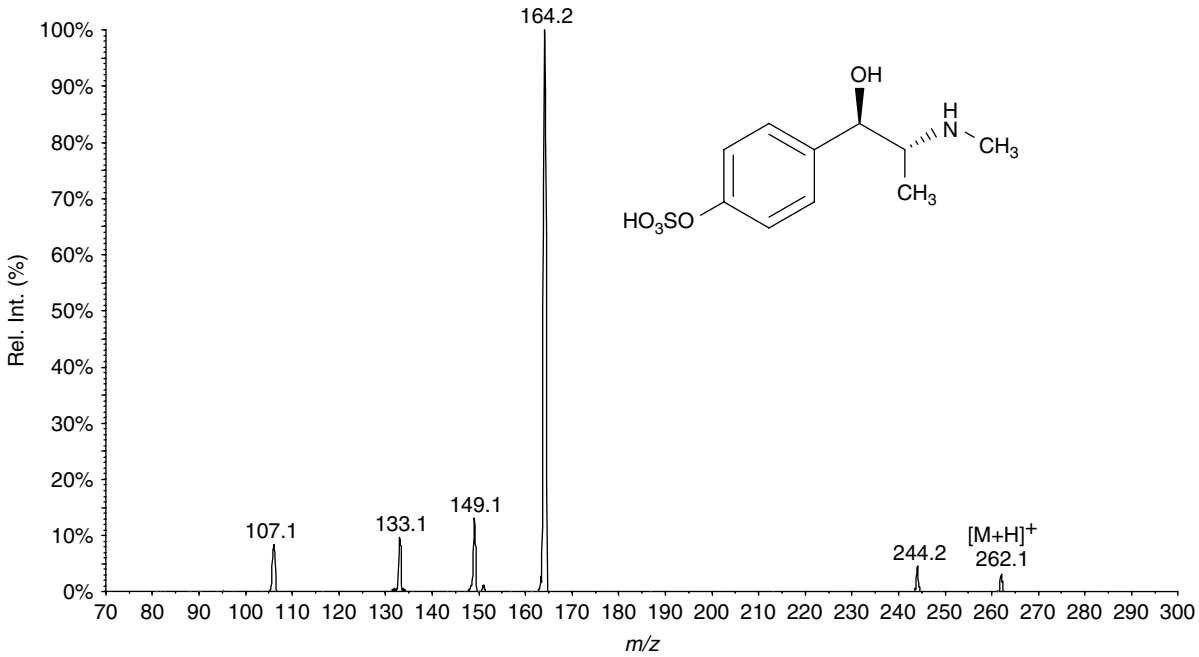

Figure 2. ESI-product ion spectrum of protonated sulfoconjugated *p*-hydroxyephedrine $[M+H]^+$ (ISTD; RT= 4.72 min).

volunteers (26–28 years; weight: 60–91 kg; two females, one male) and urine was continuously collected for five days followed by morning urine specimens taken on the four subsequent days. The volunteers gave their written consent to participate in the study and ethical approval was obtained from the local ethical committee. Urine samples were prepared by solid phase extraction with PAD-1 according to sample preparation of doping analysis of urine samples for diuretics.[21,22] Columns with a bed height of ca. 2.7 cm PAD-1 were conditioned with 2 mL H_2O and then charged with 2 mL of urine specimen. After the addition of 20 µL of *p*-hydroxyephedrine sulfoconjugate solution (1 mg/mL) as ISTD, columns were washed with 2 mL of H_2O and analytes were eluted with 2 mL of methanol. After evaporation to dryness, samples were reconstituted in 200 µL of ammonium acetate buffer (5 mM, pH 3.5, 1 mL/L glacial acetic acid)/acetonitrile (80 : 20) and applied to LC/ESI-MS/MS screening. For quantification of the unconjugated terbutaline calibrant concentrations between 0.2 µg/mL and 10 µg/mL were run. The limit of detection (LOD) for terbutaline with this sample preparation was 2.9 ng/mL and the limit of quantification (LOQ) was 9.8 ng/mL.

LC/ESI-MS/MS

Urine samples and synthesis products were monitored using an Agilent 1200 Series liquid chromatograph (Waldbronn, Germany) coupled to an Applied Biosystems API 4000 Qtrap mass spectrometer (Darmstadt, Germany). The LC was equipped with a Phenomenex Gemini C_6-phenyl column (150 × 4.6 mm, 3 µm particle size), and the eluents used were 5 mM ammonium acetate containing 0.1% acetic acid (mobile phase A) and acetonitrile (mobile phase B). A gradient was employed starting at 0% B for 1 min; increasing to 100% B within 7 min and followed by re-equilibration at 0% B for 4.5 min. The flow rate was set to 800 µL/ min and the injection volume was 5 µL. The effluent was introduced into the mass spectrometer by means of electrospray ionization (ESI) in positive mode at 450 °C using a spray voltage of 5500 V. Nitrogen was employed as curtain and collision gas (5×10^{-3}Pa) delivered from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany). Curtain gas flow was set to 20 arbitrary units. Collision energy (30 eV) was optimized to obtain adequate fragmentation and characteristic product ion spectra of the conjugates with approximately 10% of the relative intensity of the quasi molecular ions.

Product ion spectra of the protonated molecules were examined to detect and exclude the compounds in urine and to observe the synthesis products. To monitor the excretion profile from urine samples, the analytes as well as the ISTD were detected by means of characteristic product ions formed from protonated molecules by collision-induced dissociation (CID) using the multiple reaction monitoring mode (terbutaline parent: *m/z* 226-152; sulfoconjugated terbutaline: *m/z* 306-232; glucuronidated terbutaline *m/z* 402-226; disulfated terbutaline *m/z* 386-312 and *m/z* 386-232; trisulfated terbutaline *m/z* 466-312; sulfoconjugated *p*-hydroxyephedrine (ISTD): *m/z* 262-164).

Results and Discussion

Preparation

The preparation of terbutaline sulfoconjugate yielded all possible sulfated products, i.e. two monosulfates (retention time (RT) of sulfoconjugate **1**: 6.32 min, RT of sulfoconjugate **2**: 6.52 min), two disulfates (RT 8.67 min and 9.28 min) and one trisulfate (RT 11.04 min). The extended purification by repeated column chromatography achieved a pure fraction with a small amount of the sulfoconjugates **1** and **2**, respectively. Product ion spectra of terbutaline and all synthesis products are depicted in Figure 1 and Figure 3. Mono-conjugation of both phenolic groups yields the same product because of the free rotation of the bond connecting the phenyl rest with the aliphatic chain. The elucidation of the conjugation site of both monosulfates (phenolic hydroxyl group or benzylic hydroxyl group) is described below.

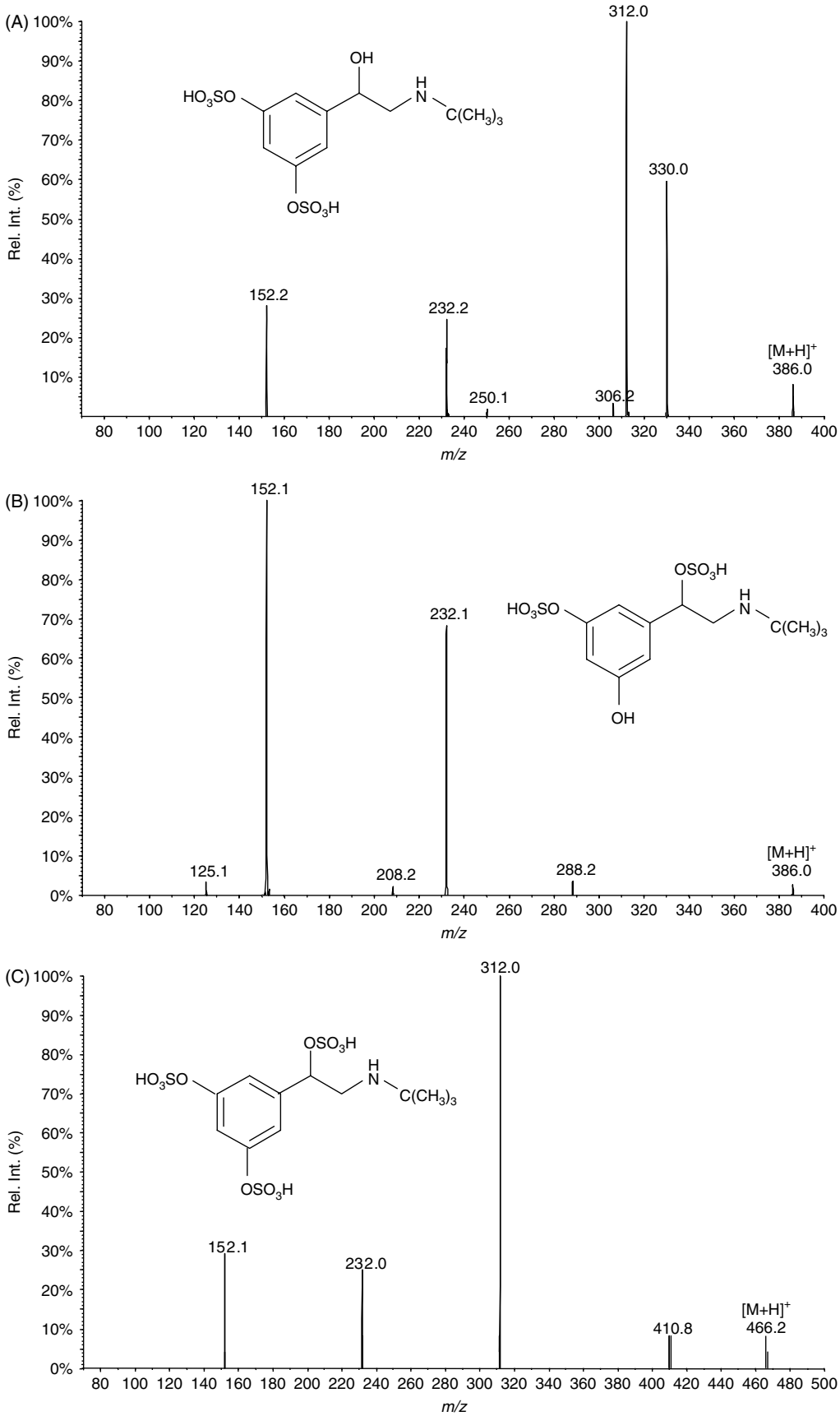

Figure 3. ESI-product ion spectra of protonated molecules $[M + H]^+$ of (A) terbutaline disulfate (RT = 8.67 min), (B) terbutaline disulfate (RT = 9.28 min), (C) terbutaline trisulfate (RT = 11.04 min).

Table 1. Elemental compositions of protonated molecules of the sulfoconjugates and resulting product ions using high-resolution/high accuracy MS^n experiments

Compound	Precursor ion (*m/z*) MS^2	Precursor ion (*m/z*) MS^3	Elemental comp. (exp.)	Error (ppm)	CE (arb. units)	Product ion (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Cleaved species
sulfoconjugate **1**	306.1003		$C_{12}H_{20}O_6NS$	0.8	15	250.0376	$C_8H_{12}O_6NS$	1.7	C_4H_8
						232.0270	$C_8H_{10}O_5NS$	1.7	C_4H_8, H_2O
						226.1434	$C_{12}H_{20}O_3N$	1.7	SO_3
						152.0702	$C_8H_{10}O_2N$	2.7	C_4H_8, H_2O, SO_3
		226.1433	$C_{12}H_{20}O_3N$	2.0	15	208.1327	$C_{12}H_{18}O_2N$	2.3	H_2O
						152.0702	$C_8H_{10}O_2N$	2.9	C_4H_8, H_2O
sulfoconjugate **2**	306.1005		$C_{12}H_{20}O_6NS$	0.3	17	208.1330	$C_{12}H_{18}O_2N$	1.2	H_2SO_4
						152.0703	$C_8H_{10}O_2N$	2.2	C_4H_8, H_2SO_4
		208.1329	$C_{12}H_{18}O_2N$	1.3	23	152.0703	$C_8H_{10}O_2N$	2.2	C_4H_8

The preparation of *p*-hydroxyephedrine sulfoconjugate yielded the desired compound. LC/ESI-MS/MS, ^{1}H-NMR and ^{13}C-NMR affirmed the structure to be the phenolic esterified sulfoconjugate of *p*-hydroxyephedrine (NMR-data not shown). The product ion spectrum is shown in Figure 2.

Characterization and identification of sulfatation sites

ESI-MS/MS

The product ion spectra of terbutaline and its two synthesized monosulfates are displayed in Figure 1. Elemental composition of protonated molecules of sulfoconjugate **1** and sulfoconjugate **2** and resulting product ions using high-resolution/high accuracy MS^n experiments are summarized in Table 1.

The product ion spectrum of the sulfoconjugate **1** (Figure 1 B) shows a loss of 80 Da, which is typical for protonated quasi-molecular ions of sulfate esters after CID,[23] yielding the product ion at *m/z* 226 with minor abundance. This route of fragmentation was confirmed using HRMS, as depicted in Table 1. Subsequent eliminations of water (−18 Da) and isobutene (−56 Da) generate the product ions at *m/z* 208 (minor abundance) and *m/z* 152, which were already described in the literature for the fragmentation of free terbutaline (product ion spectrum see Figure 1 A) and *m/z* 152 corresponds to $C_8H_{10}O_2N$.[12] Prominent product ions of the sulfoconjugate are at *m/z* 250 and *m/z* 232. These product ions are generated after elimination of isobutene (−56 Da) and the loss of a water molecule (−18 Da), which is a hint of the sulfatation site, because the loss of water can only occur at free aliphatic hydroxyl functions.

On the other hand, the product ion spectrum of sulfoconjugate **2** (Figure 1 C) shows no loss of 80 Da and subsequent elimination of water (−18 Da) and isobutene (−56 Da). Here, the loss of H_2SO_4 (−98 Da) generated a product ion at *m/z* 208 as proven by HRMS. The absence of the loss of a water molecule from the parent compound (*m/z* 306) confirms the proposal that the sulfoconjugate **1** is conjugated at the phenolic function and sulfoconjugate **2** at the benzylic hydroxyl function. Beside the elimination of H_2SO_4, the product ion spectrum shows the loss of isobutene (−56 Da), which yields the prominent product ion at *m/z* 152.

The disulfates are also clearly distinguishable by the fragmentation pattern. Disulfate with RT 8.67 min (Figure 3 A) shows the neutral loss of isobutene (−56 Da) from the protonated molecule (*m/z* 386) to yield the product ion at *m/z* 330, which further eliminates water (−18 Da) to generate *m/z* 312. This indicates an unconjugated benzylic hydroxyl group and suggests that the disulfate with RT 8.67 min is esterified at both phenolic groups. Product ions at *m/z* 232 and *m/z* 152 are generated from *m/z* 312 by stepwise elimination of SO_3 (each with −80 Da). The disulfate with RT 9.28 min (Figure 3 B) shows a loss of H_2SO_4 (−98 Da) and produces the product ion at *m/z* 288, which indicates that the benzylic hydroxyl function is esterified, again because of the absence of the loss of a water molecule. This suggests that the disulfate with RT 9.28 min is esterified at one of the phenolic functions and at the benzylic hydroxyl function. Additional elimination of SO_3 (−80 Da) generates *m/z* 208, and the loss of isobutene (−56 Da) leads to the product ion at *m/z* 232, which generates *m/z* 152 by elimination of SO_3 (−80 Da). The protonated molecule of the trisulfate (*m/z* 466) generates *m/z* 410 by elimination of isobutene (−56 Da), which then eliminates H_2SO_4 (−98 Da) and twice SO_3 (each with −80 Da) to consecutively produce the fragment ions at *m/z* 312, *m/z* 232 and *m/z* 152 (Figure 3 C).

The diagnostic ion transition, which was chosen for the ISTD (RT 4.72 min; *m/z* 264-164) is due to a neutral loss of H_2O and SO_3 (−18 Da and −80 Da) from the sulfoconjugated *p*-hydroxyephedrine. The resulting product ion at *m/z* 164 generates fragment ions, which were described earlier for *p*-hydroxyephedrine.[24] Elimination of a methyl radical (−15 Da) results in the product ion at *m/z* 149 and the neutral loss of methylamine (−31 Da) produces the product ion at *m/z* 133 (product ion mass spectrum see Figure 2).

NMR

The aromatic protons of the unconjugated terbutaline gave two signals, which can be assigned to the *para* proton (δ = 6.10 ppm, 1H, *triplett*) and the two (magnetically equivalent) *ortho* protons (δ = 6.25 ppm, 2H, *doublet*), respectively.

Besides deshielding the aromatic protons, sulfatation of one of the two phenolic functions results in magnetically distinguishable *ortho* protons. Thus, three aromatic signals (one *multiplett* at δ = 6.57 ppm and two *tripletts* at δ = 6.45 ppm and δ = 6.52 ppm, respectively) are observed for the sulfoconjugate **1**. In combination with the Orbitrap results, NMR data confirm that the sulfatation occurs at one of the two phenolic functions, because a derivatization at the benzylic hydroxyl group would not influence the aromatic coupling pattern.

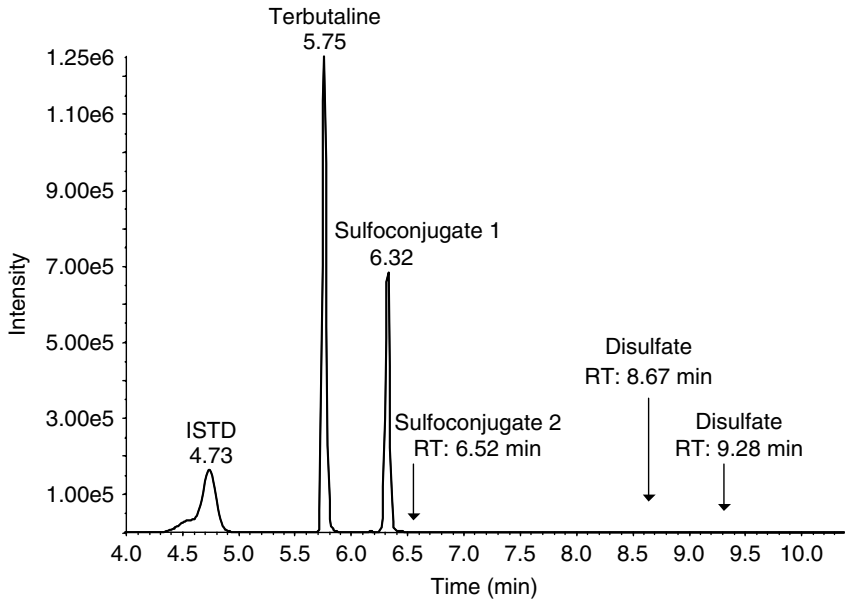

Figure 4. Combined extracted ion chromatogram of a urine sample of one volunteer (female, 26 years; mean time of sampling 4 h) after oral administration of terbutaline. Analytes were determined using ion transition employing multiple reaction monitoring: sulfoconjugated *p*-hydroxyephedrine as ISTD (262–164); terbutaline (226–152); sulfoconjugate **1** (306–232); sulfoconjugate **2** (306-152); disulfates (386-312 and 386-232); trisulfate (466-312). Expected RTs for sulfoconjugate **2**, the disulfates and trisulfate were assigned from the synthesis experiments.

Excretion study

In the excretion study, terbutaline and one mono-sulfoconjugated metabolite were detected. The product ion spectrum of the excreted sulfoconjugate is identical with the synthesized sulfoconjugate **1**, which was concluded to be the phenolic esterified conjugate. As all the product ions of sulfoconjugate **2** are also present in the product ion spectrum of sulfoconjugate **1**, the RT of the two sulfoconjugates was considered as additional decision parameter to exclude the existence of sulfoconjugate **2**. Only one sulfoconjugate with the RT of 6.32 min (which belongs to sulfoconjugate **1** contrarily to RT of 6.52 min for sulfoconjugate **2**) was detected, which excludes sulfoconjugate **2** to be in the urine specimen (Figure 4). The presence of a phenolic esterified sulfoconjugate as metabolite of terbutaline, as opposed to a benzylic esterified sulfoconjugate, is the most advanced view in previous studies, but was not directly proven up to now.

The elimination kinetics are displayed in Figure 5. Sulfoconjugate **1** was detected for two to four days and the unchanged parent compound for approximately four to five days in urine specimen. In the tail end of Figure 5 A the concentration of unconjugated terbutaline declines to average 0.06 µg/mL, which is above the LOQ. Between 24 h and the end of detection of unconjugated terbutaline (inlet of Figure 5 A), the concentration reaches from average 0.91 µg/mL down to average 0.06 µg/mL. In the literature no elimination kinetics from urine for terbutaline are described up to now, but free terbutaline was determined in plasma for 24 h after uptake of 0.1 mg tritium-labelled terbutaline. Total terbutaline (free and conjugated terbutaline) was determined for 72 h.[3] The excretion profile of the sulfoconjugated terbutaline is displayed as an excretion rate calculated with the relative peak area due to the lack of sufficient reference material for quantification.

For this reason, the ratio between conjugated and free terbutaline was not determined. There are no literature data on the direct measurement of the sulfoconjugate that could be used for comparison of the excretion profile but the excretion

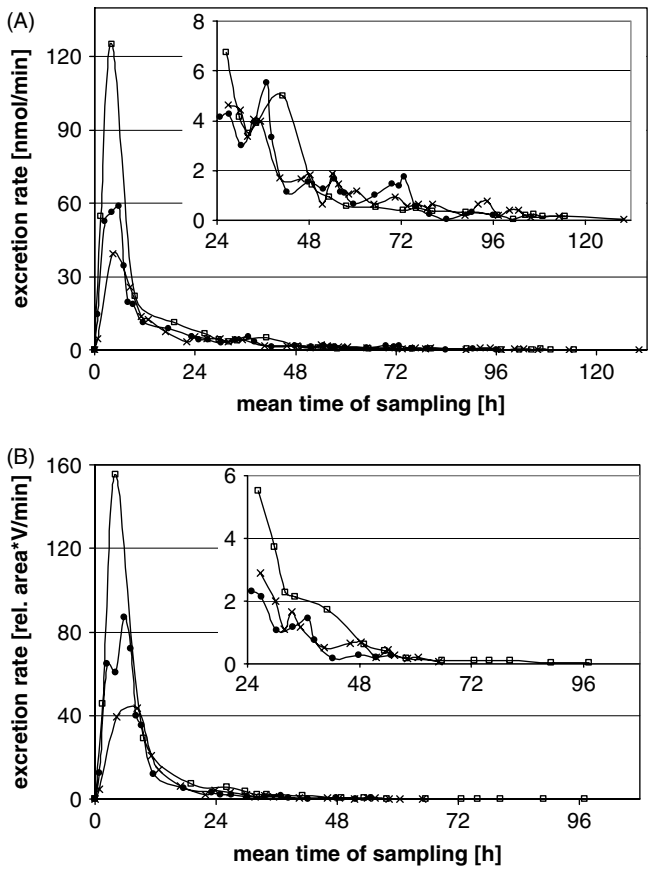

Figure 5. Excretion profile of (A) unconjugated terbutaline (displayed as excretion rate in nmol/min) and (B) sulfoconjugate **1** (displayed as excretion rate in relative peak area*V/min) of volunteers 01 (square); 02 (cross); 03 (circle). Inlets show rescaled excretion profiles from 24 h to end of excretion.

profile of free terbutaline is consistent with literature data. As most kinetic data are determined in plasma or serum, conductive comparison is not possible, but curve progression is similar to blood levels. The peak serum level is obtained 1–3 h after oral administration,[7] whereas the maximum concentration in urine in this study appeared after 4 h.

Figure 4 shows the extracted ion chromatograms with the ion transitions of all synthesized sulfoconjugates and the ISTD. A disulfated or trisulfated terbutaline conjugate in urine after uptake of terbutaline were not detected. For this reason, a closer characterization of these conjugates via HRMS and NMR techniques was omitted.

A glucuronidated conjugate, which was described earlier, was not detected in the urine specimens in this study. The ion transition (elimination of 176 Da, which corresponds to anhydroglucuronic acid), which was chosen for monitoring the glucuronide in this study, is typical for glucuronides of phenolic compounds using CID of protonated precursor ions.[23] The detection of a glucuronidated terbutaline in a previous literature report[8] may be due to a cross-reactivity of the β-glucuronidase used for sulfoconjugates and the less sensitive detection methods. Closer examination of the cleavage of terbutaline conjugates by different enzymes of different origin[7] emphasizes this point of view.

Conclusions

The successful preparation of sulfoconjugated terbutaline resulted in low yields of both potential mono-sulfoconjugates (phenolic esterified or benzylic esterified terbutaline). Advanced synthesis and purification methods should be used in future to avoid elaborate purification and to increase the yield. The excreted sulfoconjugate of the β_2-agonist terbutaline was identified as the phenolic esterified sulfoconjugate, which was verified by characterization with high resolution/high accuracy Orbitrap mass spectrometry and additional ^{1}H-NMR measurement for the sulfoconjugate **1,** as well as the RT and mass spectra of LC/MS experiments. It was monitored and detected for two to four days beside the unchanged terbutaline, which was detected for four to five days. For further investigations on the extent of sulfoconjugation of terbutaline in the human body, more urine samples from different volunteers should be included for exact quantification of the sulfoconjugate. A disulfated, trisulfated or glucuronidated compound was not detected in the excretion study.

The availability of reference material for the sulfoconjugate **1**, as reported in this study for the first time, allows its use in doping control screening procedures. It allows the detection of the analyte directly from urine, omitting the need for hydrolytic sample preparation and can be used as supportive information in addition to the detection of free terbutaline in the screening procedures and as proof of the passage of terbutaline through the body. In a future project, sulfoconjugates and glucuronides of further β_2-agonists will be synthesized and can then be used in a combined screening method.

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