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Identification and monitoring of octopamine sulfoconjugate in urine by LC/(ESI)-MS/MS

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Introduction

Octopamine is listed on "The 2011 Prohibited List" of the World Anti-Doping Agency (WADA) and is classified in section S6 Specified Stimulants [1]. The major metabolic pathway after oral administration of octopamine includes deamination of the side chain to p-hydroxymandelic acid and conjugation of the active drug. This conjugate, to our knowledge, has not been characterized with respect to conjugation site and type of conjugation [2].

Octopamine is discussed to be produced endogenously from tyramine but readily metabolized afterwards [3]. However, after consumption of food with high tyramine content octopamine was not detected in urine samples [4].

Several sympathomimetics (α- and β_2-agonists) are excreted as conjugates [5, 6] and are generally analyzed after chemical hydrolysis in the context of human sports doping control. The degree of conjugation is mostly unknown. For direct determination and characterization of the intact conjugates, reference material is required.

Herein we report the synthesis and characterization of the sulfoconjugate of octopamine and the excretion profile of the sulfoconjugated compound compared to the unchanged drug.

The evaded hydrolysis of the compounds enables combination with other substances in screening procedures; for example direct determination of intact sulfoconjugates in combination with diuretics, stimulants, β_2-agonists, plasma volume expanders and narcotics without any sample preparation except addition of internal standard [7]. In order to investigate the intact conjugate of octopamine as well as the unchanged therapeutic agent and phase-I metabolites, urine samples were extracted by means of solid phase extraction (SPE)

and subsequently analyzed by liquid chromatography tandem mass spectrometry.

The employed assay was validated including intra-day and inter-day precision, limit of detection, specificity and recovery. Further, post-administration urine samples were analyzed according to described sample preparation and evaluated concerning the quantity of octopamine and its sulfoconjugate.

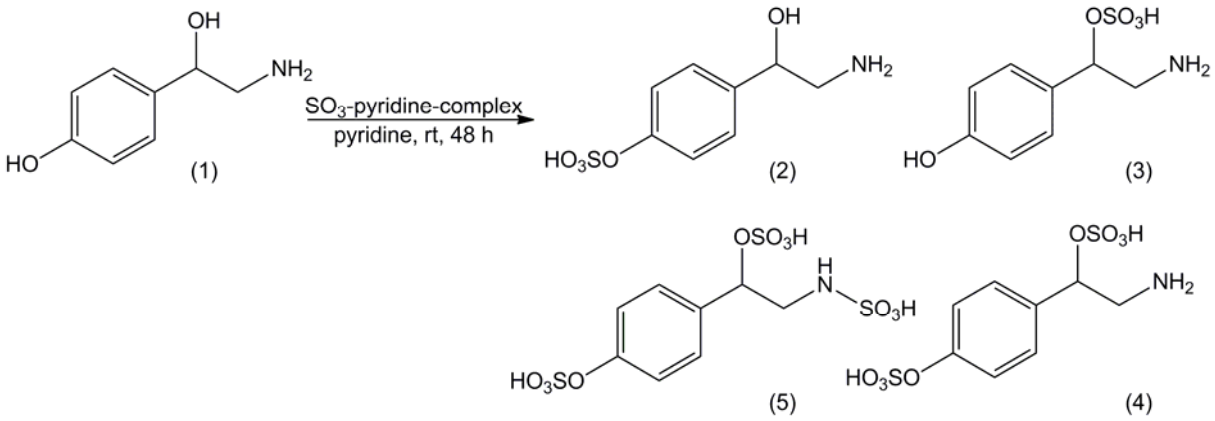

Fig. 1: *Structure of octopamine (1) and octopamine sulfoconjugates (2-5) and scheme of synthesis of octopamine sulfoconjugate including possible side-products*

Materials and Methods

Synthesis:

Synthesis was performed by stirring octopamine with sulfur trioxide pyridine complex in pyridine for 48 hours (Fig. 1) [8]. After consecutive purification steps by preparative liquid chromatography (LC) (parameters see section "Preparative LC/UV"), the obtained sulfoconjugate (Fig. 1, structure 2) was characterized by NMR and high resolution mass spectrometry (HRMS).

Preparative LC/UV:

System	Knauer Smartline (Berlin, Germany): HPLC Pump 1800 UV Detector 2600 Smartline Autosampler 3950 Fraction Collector: Foxy R1 (Teledyne Isco, Lincoln, USA)
Column	Gemini C6-Phenyl (Phenomenex®, 250 x10 mm; particle size 5 µm)

Mobile phase	100 % Ammonium acetate buffer containing 0,1% glacial acetic acid (5 mM; pH 3.5) 5 mL/min
Injection volume	10 µL
Detection wavelength	278 nm
Collected fraction	3.8 – 4.2 min

The collected fraction contained the desired octopamin sulfoconjugate and, additionally, ammonium acetate from the buffer of several LC runs. To yield clean reference material for characterization and validation of a method for direct measurement of the conjugate, SPE was performed. The mixture was solved in water and set to pH 9 and purified via Oasis HLB cartridge (200 mg; 6mL).

NMR:

For characterization of the conjugated and the unconjugated compound, ^{1}H NMR was acquired on a Bruker Avance DRX 500 spectrometer (Bruker, Karlsruhe, Germany) operating at 500 MHz. Chemical shifts δ-values are given in ppm referring to the signal centre using the solvent peaks of D_2O at 4.78 ppm for reference. ^{1}H NMR (D_2O): phenolic esterified sulfoconjugate (2): δ 3.22-3.35 (m, 2 H), 5.04 (dd, 1 H), 7.36-7.37 (m, 2 H), 7.48-7.50 (m, 2 H); octopamine (1): δ 3.20-3.29 (m, 2 H), 4.92 (dd, 1 H), 6.93-6.94 (m, 2 H), 7.31-7.33 (m, 2 H).

HRMS:

HRMS-characterization of the synthesized mono-sulfoconjugate (2) and determination of elemental compositions was performed on a LTQ Orbitrap mass spectrometer (Thermo, Bremen, Germany). The instrument was operated in positive electrospray ionisation 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 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.

Application study:

One volunteer ingested 150 mg of octopamine and urine was collected 2 days followed by one additional morning urine. Several urine sample preparations were tested, such as dilute and inject, direct injection and SPE, the latter one turned out to be most suitable for the sulfoconjugate. PAD-1, weak cationic exchange resins (strata X-CW) and Oasis HLB were tested and the Oasis HLB was most promising.

Sample preparation used for the excretion study: Conditioning of the SPE-cartridge (Oasis HLB (60 mg)) with 2 mL methanol is followed by equilibration of the SPE with 2 mL water. 2 mL of the urine sample are fortified with 500 ng d_3-octopamine as internal standard. The cartridge is washed with 2 mL of water and the analytes are eluted with 2 mL methanol. After evaporation to dryness, the sample is reconstituted with 100 µL ammonium acetate buffer (pH 3.5; 5 mM)/acetonitrile 80/20 (v/v), and subsequent injection into the LC-MS system.

Chromatographic parameters:

HPLC system	Agilent 1260 Infinity Series LC
Column	Gemini C6-Phenyl (Phenomenex®, 150 x 4.6 mm; particle size 3 µm)
Mobile phase	A: ammonium acetate buffer (5 mmol/L in H_2O; pH 3.5), 1 mL/L glacial acetic acid, B: acetonitrile 0.8 mL/min
Gradient	0 min 100% A 0% B 1 min 100% A 0% B 8 min 0% A 100% B 5 min re-equilibration
Injection volume	20 µL

Mass spectrometric parameters:

System	AB Sciex 5500™ Q *Trap* mass spectrometer (AB Sciex, Darmstadt, Germany)
Ionisation	ESI, positive
Interface temperature	475 °C

Tab.1: *MRM experiment for urine analysis (positive mode;*this ion transition was used for quantification); for 2nd and 3rd ion transition of octopamine and octopamine sulfoconjugate the in-source generated precursor ion at m/z 136 Da and 216 Da respectively was used*

compound	$[M+H]^+$	Ion transitions			Retention time (min)
octopamine	154	154-119*	136-119	136-91	2.42
octopamine-sulfoconjugate	234	234-136*	216-136	216-91	3.07
d_3-octopamine	157	157-139*	157-93	157-121	2.40

Limit of detection (LOD) was defined as the lowest content that can be measured with reasonable statistically certainty at a signal to noise ratio ≥3. Six urine samples were fortified with 400 ng/mL urine of the sulfoconjugate and free octopamine respectively. The samples were prepared and analyzed according to the established protocol using multiple reaction monitoring mode (MRM; Ion transitions see Tab. 1). Specificity was checked by analyzing 20 different blank urine samples (10 male; 10 female). They were prepared as described, in order to test for interfering signals in the selected ion chromatograms at the expected retention times. To test for recovery, six urine samples were spiked with 2 µg/mL urine of the two analytes and prepared as described. Another set of six samples was prepared with addition of the internal standard only, and the two analytes (2 µg/mL) were added after the SPE and before the evaporation of the sample. Precision was performed by preparing and analysing fortified urine sample in six replicates at 0.5, 2 and 4 µg/mL.

Results and Discussion

Synthesis and characterization yielded the phenolic conjugated sulfoconjugate. Elemental composition of protonated molecules of the sulfoconjugate (2) and the unconjugated parent compound (1) and resulting product ions using high-resolution/high accuracy MS^n experiments were determined. Octopamine (1) yielded an elemental composition of $C_8H_{10}ON$ and a mass of *m/z* 136.0755 (error: 1.4431 ppm) which can be elucidated by an elimination of water (18 Da). As due to in-source fragmentation the intact octopamine ($[M+H]^+$, *m/z* 154, $C_8H_{11}O_2N$) could not isolated in this MS-experiment. The synthesized octopamine sulfoconjugate showed a mass of *m/z* 234.0430 (error: 0.2479 ppm) with an elemental composition of $C_8H_{12}O_5NS$.

Figure 2 shows the enhanced product ion spectrum (EPI) of unconjuagted octopamin (1) with relatively low collision energy. This is due to a fast in-source fragmentation by a loss of a water molecule (-18 Da) generating the product ion of *m/z* 136 and the cleavage of the amino function (-17 Da) yields the product ion at *m/z* 119, which was proven by HRMS experiments. Figure 3 shows the EPI spectrum of the synthesized conjugate (2). Elimination of water (-18 Da) generates the product ion at *m/z* at 216. This is a hint for the sulfation site, because the loss of water is known to occur mainly at the free benzylic hydroxyl functions. The subsequent loss of 80 Da, which is typical for protonated quasi-molecular ions of sulfate esters after collision-induced dissociation, yields the product ion at *m/z* 136. Again, the cleavage of the amino function (-17 Da) yields the product ion *m/z* 119. These compositions of the fragments were also proven by HRMS experiments.

^{1}H NMR analysis confirmed the structure of the phenolic sulfoconjugated octopamine.

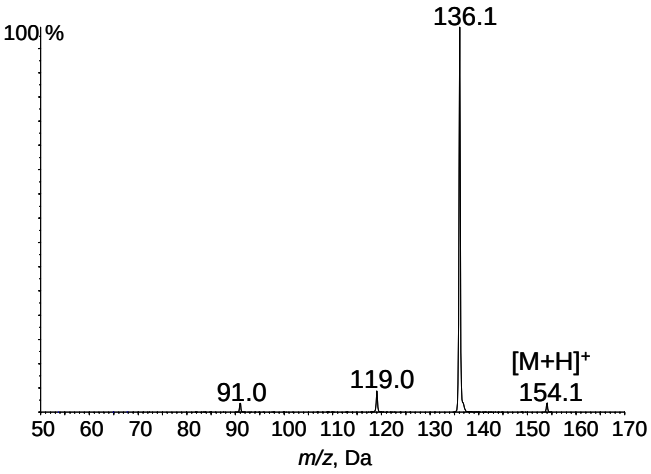

Fig.2: EPI spectrum of octopamine ([M+H]$^{+}$ = m/z 154, CE 5 V)

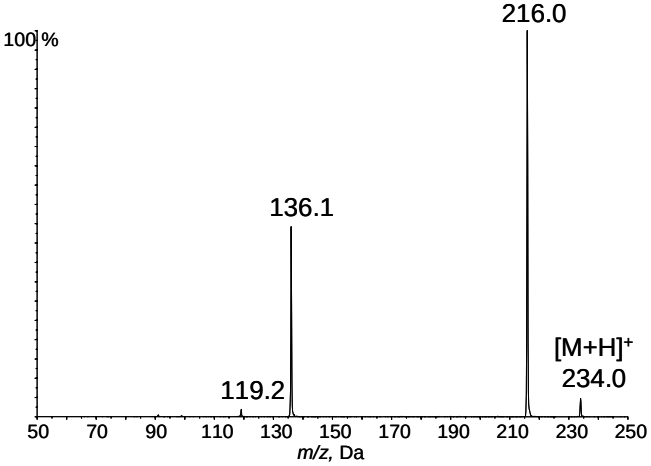

Fig.3: *EPI spectrum of octopamine sulfoconjugate ([M+H]$^{+}$ = m/z 234, CE 25 V)*

Validation results are summarized in Tab. 2. Determination of the peak heights for S/N-ratios showed LODs at 300 ng/mL urine for the sulfoconjugate and 10 ng/mL for free octopamine. The recoveries showed good results for the unchanged octopamine (93 %) but unsatisfactory results for the conjugate (3 %). Samples tested for precision yielded relative standard deviations less than 20 % except for the low concentration of the sulfoconjugate. These data show that an improved screening procedure is needed for screening of the quite polar substance octopamine sulfoconjugate. At least a more suitable internal standard like a sulfoconjugated phenylethylamine is desired. Nevertheless, to get an idea of the excretion of the sulfoconjugate of octopamine, this urine sample preparation was used of the excretion study.

Tab.2: *Validation parameters*

	Recovery	LOD (S/N >3) [ng/mL]	Precision (relative standard deviation)		
			low (0.5 µg/mL)	middle (2 µg/mL)	high (4 µg/mL)
octopamine-sulfoconjugate	3%	300	30%	11%	20%
octopamine	93%	10	4%	3%	4%

Urine samples tested for specificity showed in 10 % of the urine samples an interfering signal for the ion transitions of octopamine sulfoconjugate. The occurrence of endogenously produced octopamine sulfoconjugate can be excluded by calculating the ratios of the ion transitions and also by analyzing the samples after hydrolysis and determination of the deconjugated compound ($[M+H]^+$, *m/z* 154). The retention time of the originated deconjugated compound is considerably different to that of free octopamine.

Figure 4 shows an extracted ion chromatogram of a urine sample collected 3 hours after administration of 150 mg octopamine. Apparently, octopamine sulfoconjugate is excreted in a higher amount than free octopamine. The same pattern is visible in the excretion profile of octopamine and its sulfoconjugate (Figure 5). The highest excretion rate of octopamine sulfoconjugate (530 µg/min) is achieved after 70 minutes, whereas free octopamine is only excreted in a minor rate (maximum 2 µg/min after 2 hours).

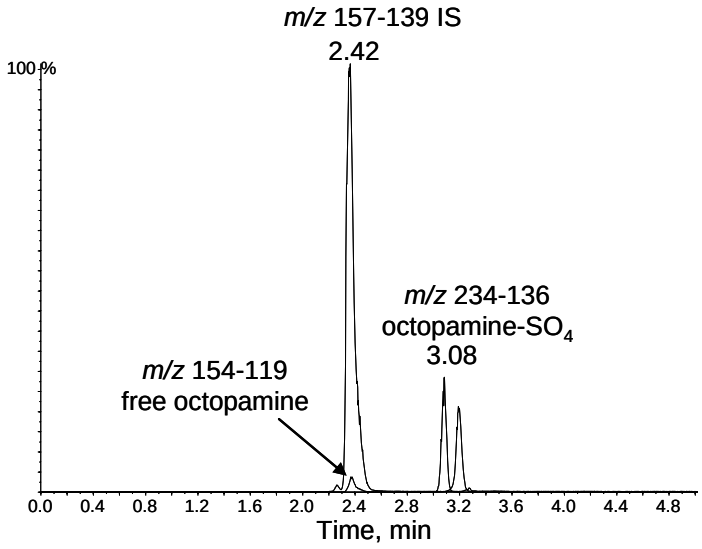

Fig.4: *Combined extracted ion chromatogram of an urine sample after oral administration of octopamine (collection period = 0-3h)*

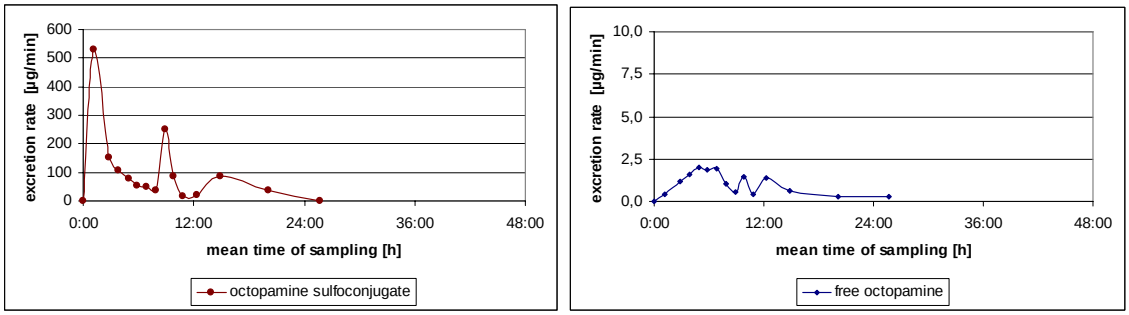

Fig.5: *Excretion profile of octopamine sulfoconjugate (left) and free octopamine (right) after oral uptake of 150 mg octopamine*

Conclusion

In conclusion, sulfoconjugates are metabolites with potential to improve screening procedures. Herein we described a suitable method for the determination of urinary excretion of sulfoconjugate after oral application of octopamine, using synthesized phenolic sulfoconjugated octopamine as reference material. Additionally, we were capable of confirming that neither endogenous octopamine nor sulfoconjugate are excreted.

Due to the limitations of the study (recovery of the sulfoconjugate 3 %) these results are only a first hint for an excretion profile of octopamine sulfoconjugate. Nevertheless, the use of a sulfoconjugated phenylethylamine as internal standard, and an enhancement of sample preparation might improve accuracy and precision of the described method. With this in hands, a combination with screening procedures for other substance groups is possible.

Acknowledgements

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