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Sulfoconjugates of Heavy Volatile Nitrogen Containing Doping Substances for Improved LC-MS/MS Screening

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Introduction

In the fight against doping the laboratories are confronted with an increasing number of substances to screen on. Therefore new methods for the screening of those substances have to be implemented. To keep costs for doping control analysis acceptable, to ensure rapid reporting times and to lower the amount of urine needed to screen for all substances, combining 'traditional' screening procedures for different classes of substances is desirable.

The reported study aimed at checking the possibility to combine the screening for beta-2-agonists, heavy volatile stimulants and narcotics with screening methods such as for diuretics. Thus, the phase-II metabolism of beta-2-agonists and heavy volatile stimulants was investigated.

Most of the beta-2-agonists and heavy volatile nitrogen containing doping substances are excreted unconjugated and as sulfoconjugates in different ratios. Some conjugates with glucuronic acid in the human metabolism are also known for these substances. Until now the research was mainly based on the cleavage of phase II-metabolites of the beta-2-agonists and heavy volatile stimulants. The structures for some of these sulfates were proven by LC-MS analysis of the intact substances (e.g. Salbutamol (Joyce *et al.* 1998), p-Hydroxy-methamphetamine (Shima *et al.* 2006a; Shima *et al.* 2006b)). For morphine the excretion of a glucuronide, which is available as reference substance, was confirmed (Benyhe 1994; Coughtrie *et al.* 1989; Peterson *et al.* 1990).

Since reference substances of the conjugates are barely available, the methods used in doping control include the cleavage of the conjugates and use the aglycons for the detection of a possible misuse. Best results for screening purpose of these classes were obtained by chemical hydrolysis with hydrochloric acid (Henze *et al.* 2001; Thevis *et al.* 2003a, b).

However, for a combination of screening procedures it was desired to evade the hydrolysis of the conjugates, which on the one hand is time consuming and on the other hand revealed problems with the stability of diuretics, and to analyse the excreted metabolites (where applicable as intact conjugates) directly as intact molecules.

Experimental

For the synthesis of the sulfoconjugates p-Hydroxyamphetamine, Pholedrine (p-Hydroxymet-amphetamine), p-Hydroxyephedrine, p-Hydroxynorephedrine, Etamivan and Etilefrine were coupled to sulfuric acid by reaction with sulfur trioxide-pyridine complex.
The structures of the relevant mono-sulfates were confirmed by nuclear magnetic resonance (NMR: ^{1}H, ^{13}C). The data were recorded in deuterated dimethylsulfoxide (d_6-DMSO) or deuterium oxide (D_2O) at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) at 298 K on a Bruker Avance DRX 500 spectrometer. Additionally the conjugates were characterised by LC-ESI-MS/MS (conditions in Table 1).

The sulfoconjugate of Salbutamol, obtained by purification from a post-administration (p.a.) urine, was kindly provided by Glaxo-Smith-Kline (GSK). Its structure was confirmed by NMR (^{1}H, H,H COSY, APT, H,C HMQC, H,C HMBC and H,H NOESY). Salbutamol glucuronide was obtained from an administration study in rats, which are known to excrete the glucuronide as phase-II metabolite. Both conjugates were characterised by LC-ESI-MS/MS.

For the preparation of urine samples 2 ml were applied to SPE columns after their conditioning. In method development different sorbents were tested. For the final sample preparation SERDOLIT® PAD I (0.1 - 0.2 mm) was used, filled in glass pipettes. After washing with 2 ml of H_2O the analytes were eluted with 2 ml of methanol. Following evaporation to dryness the residue was reconstituted in 200 µL of ammonium acetate buffer (5 mM in H_2O, pH=3.5). The concentrates were analysed by LC-MS/MS (MRM, Table 2).

Table 1: LC-MS/MS parameters for the measurement of the heavy volatile nitrogen containing doping substances

System	Agilent 1100 Series LC API 3200^{TM} Triple Quadrupole mass spectrometer (Applied Biosystems)
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 acetic acid, B: acetonitrile, 0.8 ml/min
Gradient	0-1 min 100% A, 1-8 min 0% B to 100 % B 2.5 min reequilibration
Injection Volume	10 µl
Ionisation	ESI, negative or positive mode
Interface Temp.	550 °C

Results and Discussion

The sulfoconjugates of p-Hydroxyamphetamine**,** Pholedrine, Etilefrine, p-Hydroxyephedrine, p-Hydroxynorephedrine, and Etamivan were obtained from the synthesis and characterised by LC-ESI-MS/MS. The structures of the relevant mono-sulfates (structures in Figure 1) were confirmed by NMR.

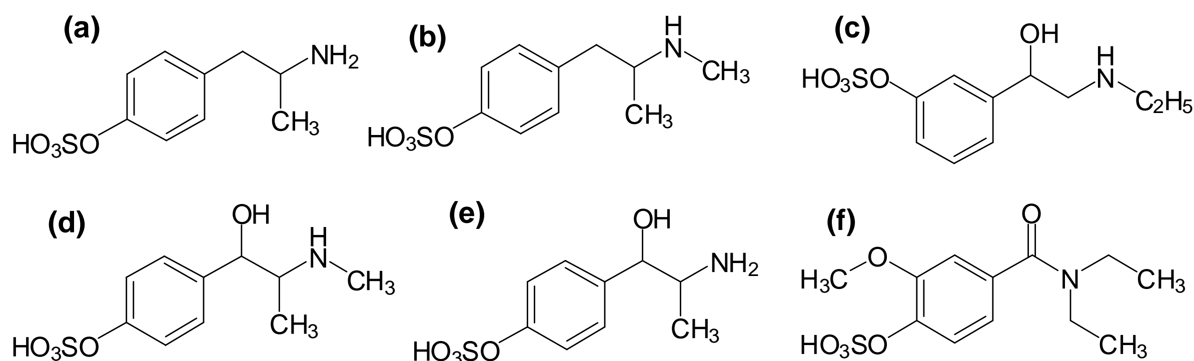

*Figure 1: Chemical structures of the sulfoconjugates of p-Hydroxyamphetamine (**a**), Pholedrine (**b**), Etilefrine (**c**), p-Hydroxyephedrine (**d**), p-Hydroxynorephedrine (**e**), and Etamivan (**f**)*

The sulfoconjugate of Salbutamol was identified as 4-O-sulfate (chemical structure in Figure 2) by NMR and characterised by LC-ESI-MS/MS.

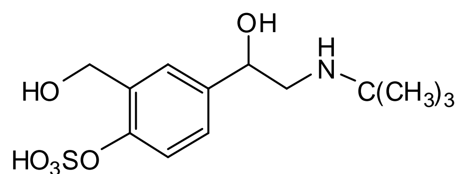

Figure 2: Chemical structure of Salbutamol 4-O-sulfate

As the threshold concentration for Salbutamol in doping control refers to unconjugated plus glucuronide, also Salbutamol glucuronide was characterised by LC-MS/MS to check for the absence of the glucuronide in human urine. The product ion spectra (ESI, positive, CE 20) of both conjugates are shown in Figure 3.

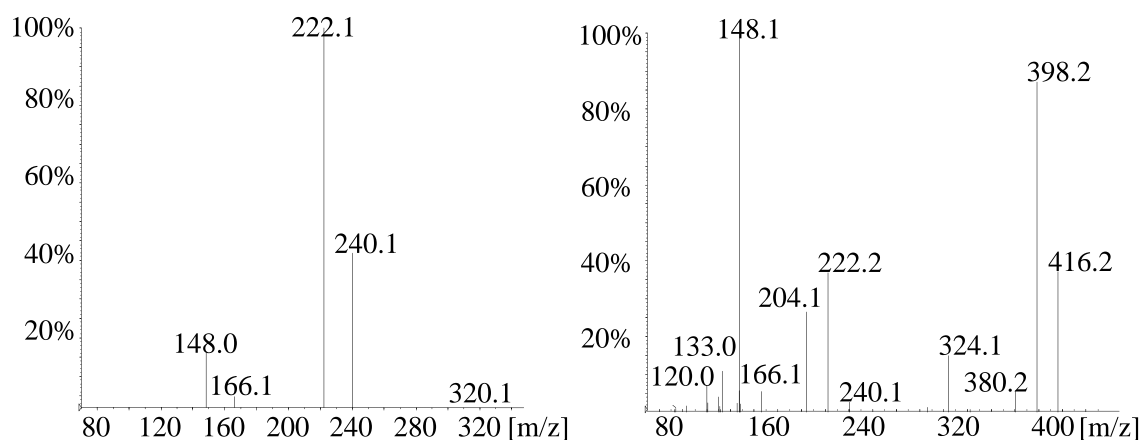

Figure 3: Product ion spectra of Salbutamol sulfate, $[M+H]^+=320$, (left) and glucuronide, $[M+H]^+=416$, (right)

Different materials for SPE were tested and best results for screening purposes were obtained with PAD I. This also allows a simple combination with the procedure for diuretics routinely used in our laboratory, which also implies SPE with PAD I. The retention times and ion traces used to monitor the sulfoconjugates are shown in Table 2.

Table 2: Retention times and ion traces used for the detection of the monosulfates

Substance	**Retention Time [min]**	**$[M+H]^+$**	**ESI**
p-OH-Norephedrine-Sulfate	4.13	248/150	pos
p-OH-Ephedrine-Sulfate	5.87	262/164	pos
Etilefrine-Sulfate (phenolic)	6.15	260/180	neg
p-OH-Amphetamine-Sulfate	6.27	230/150	neg
Salbutamol-Sulfate	6.28	320/240	pos
Pholedrine-Sulfate	6.37	244/164	neg
Etilefrine-Sulfate (benzylic)	6.37	260/ 97	neg
Etamivan-Sulfate	8.04	224/151	pos

Validation for the sulfoconjugates of p-Hydroxyamphetamine, Pholedrine, p-Hydroxy-ephedrine, and Etamivan and application of the whole method to real urine samples (p.a. urines of Salbutamol, Etilefrine, Oxilofrine (p-Hydroxyephedrine), Ephedrine, Pseudoephedrine, Amphetamine, and Etamivan) was successfully performed.

Remarks

The detailed results of the investigations will be published elsewhere.

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