QUICK AND AUTOMATED SCREENING METHOD FOR PRIORITY β-AGONISTS IN URINE

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Summary

A quick and simple method for screening of priority β-agonists in urine samples is presented using TurboFlow technology in combination with novel benchtop Orbitrap™ based mass spectrometer.

The presented method gains in simplicity, speed and sensitivity for high-throughput labs when compared with a conventional approach employing molecularly imprinted polymer SPE column clean-up (MIP) and UPLC/Time-of-Flight MS.

Introduction

β-agonists are synthetically produced compounds that, in addition to their bronchodilatory and tocolytic effects, can promote live weight gain in the food producing animals. There have been documented cases when consumption of liver and meat from animals illegally treated with clenbuterol has resulted in serious human intoxication[1]. Due to their adverse effects, the use of clenbuterol and its analogues from the beta-agonists group has been banned by the European Union[2] and other regulatory agencies worldwide. Monitoring programs have shown that β-agonists are still illegally used by food producers and moreover, newly developed analogues with modified structures are being continuously introduced in routine practice. There is a clear need for quick and simple screening methods to routinely and accurately control levels of β-agonists in samples of animal origin (urine, plasma, tissues).

Materials & Methods

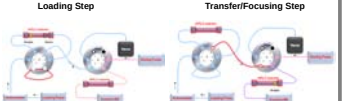
A new benchtop single stage Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) coupled to a TurboFlow system Aria TLX-1 consisting of loading and eluting pumps, switching valve module and autosampler (Thermo Fisher Scientific, Franklin, USA) was used for analysis of a group of priority beta-agonists, see Table 1.

All Exactive MS measurements were performed at resolving power settings of 50,000 FWHM. Separations of analytes was carried out on Zorbax XDB-Phenyl column (Agilent Technologies, Santa Clara, USA).

Table 1. The list of analyzed beta-agonists

Brombuterol	Clenpenterol	Pirbuterol
Carbuterol	Clenproperol	Ractopamin
Chlorbrombuterol	Fenoterol	Ritodrin
Cimaterol	Hydroxymethylclenbuterol	Salbutamol
Cimbuterol	Isoxsuprin	Salmeterol
Clenbuterol	Mabuterol	Terbutalin
Clencyclohexerol	Mapenterol	Tulobuterol
Clenhexerol	Metoprolol	Zilpaterol

FIGURE 1. Schematics of Aria-TLX/Exactive system operation



Results

The optimization of the TurboFlow method was performed using standard solution containing salbutamol, clenbuterol and mabuterol at 100 µg/L levels. These compounds have been selected for optimization due to their amphoteric properties and strong dependency on the pH of the mobile phases.

During the method development following TLX parameters were optimized:

- **TurboFlow column selection** – evaluation of different types of columns with respect to the retention of analytes
- **Sample loading parameters** – optimization of the pH of the loading mobile phase
- **Sample elution parameters** – optimization of the elution mobile phase pH, flow rate and time
- **Analytical column focusing parameters** – optimization of initial gradient composition to effectively trap and focus all compounds of interest

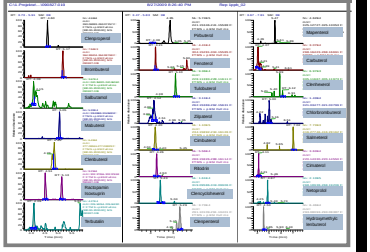
Optimized TurboFlow system conditions are summarized in Figure 2.

FIGURE 2. Optimized TurboFlow system parameters

TLX Column	• CYCLONE-P, 0.5 mm x 50 mm
Loading	• 10 mM NH_4Ac in Water, pH 8, 1.5 mL/min, 30 s
Elution	• methanol/0.1% acetic acid, 50/50, 0.10 mL/min, 120 s
Wash	• methanol/0.1% acetic acid, 80:20, 1.5mL/min, 60s • acetonitrile/acetone/isopropanol; 30/30/40 (v/v/v), 1.5 mL/min, 60 s

Optimized TLX method was then applied to the analysis of spiked and unspiked urine samples and standard solutions. The urine was spiked at different concentration levels, centrifuged for 10 min and diluted with water 1:1; pH was adjusted to 6 with 0.1M HCl. Prepared urine samples were directly injected into TLX/Exactive system. Calibration was performed using urine matrix matched standards at concentration levels of 0.25; 0.5; 1; 5; 10; 15; 20 and 25 µg/L. The typical chromatogram of the spiked urine sample at concentration level of 1 µg/L is shown in Figure 3.

FIGURE 3. Extracted ion chromatograms of beta-agonists spiked in urine at 1 µg/L concentration level



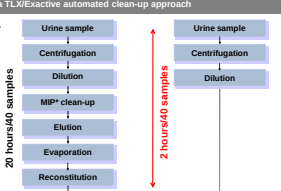
From the chromatogram shown above it can be seen that all analyzed compounds (except for clenhexerol and pirbuterol that were not detected at 1 µg/L level probably due to matrix suppression) could be successfully detected at 1 µg/L concentration levels. In neat standard all compounds are detected easily at 0.5 µg/L. Also, in a few cases coelutions occurred (salbutamol, clenbuterol and zilpaterol) but the peak can be distinguished from the matrix.

Table 2 summarizes detailed performance characteristics of the method evaluated by analyses of urine samples spiked at 0.5; 1 and 10 µg/L. For estimation of precision and accuracy the spiked samples were analyzed in 6 replicates. Carryover sample (blank urine) was analyzed immediately after the spiked samples at 1 and 10 µg/L concentration levels. All calibrations were carried out using matrix matched standard solutions to eliminate matrix effects during the ionization step.

Table 2. Method performance characteristics

Concentration (µg/L urine)	Accuracy (%)	RSDs (n=6) (%)	Carryover (%)	R2 (0.25-25 ug/L)	Estimated LODs (ug/L urine)
10	95 - 122	1.7 - 8.7	0.9	0.952-0.999	0.2-5
1	84 - 138	3.0 - 18.8	0.7		
0.5	48 - 175	4.5 - 21.7	nd.		

FIGURE 4. Comparison of the CVUA β-agonist method[3] workflow (left) and Aria TLX/Exactive automated clean-up approach



Conclusions

The automated TurboFlow clean-up system offers significant **improvement of lab throughput** and **minimization of manual work**. In combination with high resolution provided by Exactive MS it provides unique tool for quick urine sample screening for residues of beta-agonists at regulatory levels.

References

[1] Botsoglou, N.A., Fletouris D.J., *Drug Residues in Food. Pharmacology, Food Safety and Analysis*, Marcel Dekker: New York, 2001

[2] EU COUNCIL DIRECTIVE 96/22/EC of 29 April 1996, OJ L 125, 23.5.1996

[3] Internal SOP, CVUA, Postfach 1980, 48007 Münster, Germany