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Screening for β-blockers in urine with on-line extraction and LC-MSMS detection

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Introduction

The new method has been developed for routine analysis of ß-blockers in urine. The analysis of 23 ß-blockers and some of their metabolites is performed by on-line solid phase extraction, liquid chromatography separation and tandem mass spectrometry detection, so called XLC-MSMS technology – automated from Sample List to MSMS results.
ß-Blockers block the action of epinephrine and norepinephrine on beta-adregenic receptors in the sympathetic nervous system. They are one of the most frequently prescribed heart drugs today, most commonly used for treatment of angina pectoris, to control hypertension, to reduce intraocular pressure and cardio-arrhythmia.
The physiological symptoms of performance anxiety, such as pounding heart, cold/clammy hands, increased respiration, sweating, tremor etc. are significantly reduced by ß-blockers, thus abling anxious athletes and actors to better concentrate on their specific task. ß-Blockers are therefore listed as doping substances by WADA in some sport disciplines [1], for example shooting, archery, ski jump and curling.
There are several methods available to analyse this group of compounds using different techniques and instrumentation which have been published [2, 3]. The gas chromatography mass spectrometry (GC-MS) method used in our laboratory before is extremely time consuming. It was therefore of great interest to develop a new and faster method for screening and confirmation of ß-blockers. A shorter hydrolysis time (ß-glucuronidase from *E.coli*), elimination of the derivatisation step by using liquid chromatography tandem mass spectrometry (LC-MSMS) and a simplified purification with on-line solid phase extraction (SPE) on Symbiosis Pharma instrument.

Materials and Methods

Instrumentation

Symbiosis Pharma instrument from Spark Holland interfaced to the Finnigan TSQ Quantum Discovery mass spectrometer (Thermo Electron Corporation).

Sample Extraction Procedure

400µL of urine sample is mixed with 400µL of 10mM ammonium acetate buffer pH 6.9, 10µL (20µg/mL) internal standard bupranolol and 20µL ß-glucuronidase from *E.coli.*
After hydrolysis (1hour, 50°C) vials with urine samples are placed in a cooled chamber of the automatic injector, so called Reliance of Symbiosis Pharma instrument.
20µL of urine sample is injected in partial loopfill mode. Total sample consumption for one injection is 65µL. Cartridge used is Sorbent HySphere Resin GP (General Phase), 5-15µm, spherical shape, polymer phase (polydivinyl benzene) from Spark Holland.
The cartridge is activated with 1mL of methanol and equilibrated with 1mL of water.
The sample is loaded with 1mL of water. After washing the cartridge with 1mL of water the elution is performed with mobile phase for 7.5 min.

Chromatographic conditions

The analytes are separated on the Phenomenex Luna 3u C18(2) column (100x 2.0 mm). Mobile phase constitutes of water with 1‰ acetic acid (A) and acetonitrile with 1‰ acetic acid (B). Flow used is 0.2mL/min. Gradient is as follows: initial mobile phase composition is 5% B, hold for 15s, followed by linear gradient to 40% B up to 6 min., followed by another increased gradient up to 95% of B up to 7 min., hold for 1min. and then decrease to 5% of organic phase B and hold until the run is completed. Run time is 12min.

Mass spectrometer

Finnigan TSQ Quantum Discovery tandem mass spectrometer with electrospray ionization ESI ion source is operated in the positive ionization mode with SRM monitoring and 0.02s dwell time. All compounds produce $[M+H]^+$ ions (Figure 1). ESI voltage is 4000V, capillary temperature 300°C and collision gas pressure 1.7 mTorr.

Results and Discussion

The presented XLC-MSMS on-line solid phase extraction method replaced the GC-MS method used in our laboratory before. The extraction procedure is simplified and small quantities of urine sample and reagents are used. Hydrolysis of conjugated compounds is

performed with ß-glucuronidase from *E.coli* instead for *H.pomatia*. Ammonium acetate buffer is used instead of phosphate buffer which is convenient for ESI source. The sensitivity of the method is very good. ß-Blockers can be detected at urinary concentration lower than 10ng/mL. This is more than 50 times lower than MRPL 500ng/mL required by World Anti Doping Agency WADA in the Technical Document-TD2004MRPL. Limit of detection is below 10ng/mL both for dilution factor 2 (screening method) and 10 (confirmation when a high concentration of β-blockers is found). Calibration curves for all ß-blockers measured were obtained by analyzing spiked urine samples in range 0 – 500ng/mL (dilution factor 2) and 0 – 1000ng/mL (dilution factor 10). Correlation factor for all standard curves was better than 0.99 which means that the linearity was very good.

The method was tested with omitting hydrolysis step as well, using reference urine samples for acebutolol, alprenolol, atenolol, bisoprolol, carteolol, carvedilol, metoprolol, nadolol, oxprenolol, penbutolol, pindolol, propranolol, sotalol and timolol. In all analysed specimens the presence of an unconjugated compound was confirmed.

Furthermore there was possible to find a metabolite of metoprolol and atenolol , (4-[2-hydroxy-3-(isopropylamino)propoxy]phenyl acetic acid. It elutes at RT = 4.4min. and could be found in metoprolol window (16) and metipranolol metabolite window (15).

Conclusions

An extremely sensitive screening method for ß-blockers and some of their metabolites in urine based on XLC-ESI-MSMS was developed;

Confirmation of ß-blockers is performed with the same extraction and chromatography XLC system and mass spectrometry ESI-MSMS method modified for respective compounds;

All compounds produce at least three transitions, which is sufficient for confirmation purposes;

An online extraction system allowes to achieve good chromatography even for compounds like atenolol, practolol and sotalol which elute early in our conditions, at low concentration of organic mobile phase.

References

1. World Anti-Doping Agency. The 2008 Prohibited List. International Standard, Montreal (2008) http://www.wada-ama.org

2. Thevis M., Opferman G., Schänzer W. (2001) High speed determination of beta-receptor blocking agents in humane urine by liquid chromatography/tandem mass spectrometry. *Biomed. Chromatogr.*, **15**, 393-402

3 . Deventer K., Van Eenoo P., Delbeke F.T. (2005) Simultaneous determination of beta-blocking agents and diuretics in doping analysis by liquid chromatography/mass spectrometry with scan-to-scan polarity switching. *Rapid Commun. Mass Spectrom.*, 19, 90-98.

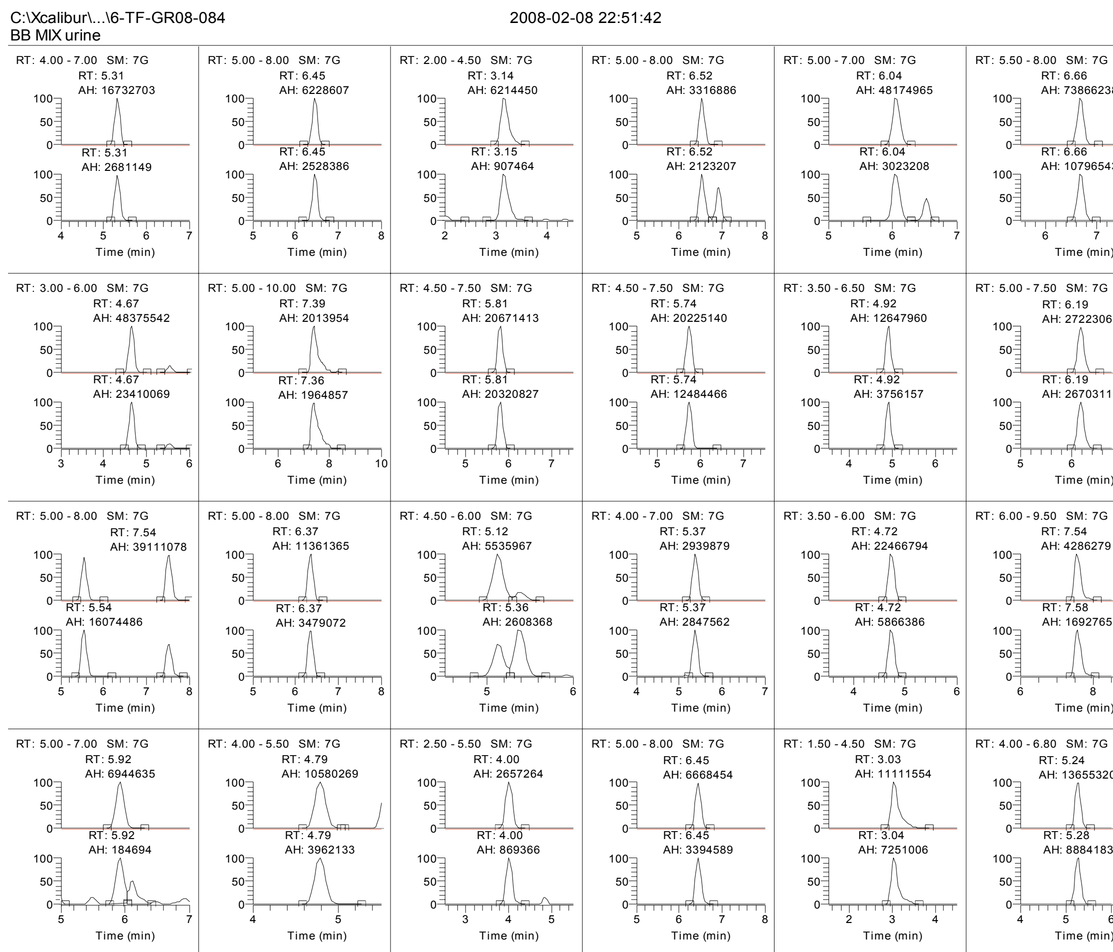

Figure 1 *– Screening of β-blockers – Negative Control urine spiked with 500ng/mL of each compound described below and presented in the chromatogram from upper left corner to right:*
Nr. Compound Name *(RT min.): Precursor Ion – Product Ion 1 (CE), Product Ion 2 (CE);*

1. Acebutolol (5.31): 337-116(20), 218(22); **2. Alprenolol** (6.45): 250- 91(33), 145(16); **3. Atenolol** (3.14): 267-145(19), 208(10); **4. Betaxolol** (6.52): 308-121(26), 91(38); **5. Bisoprolol** (6.04): 326-116(17), 107(44); **6. Bufuralol** (6.66): 262-188(14), 105(32); **7. Carteolol** (4.67): 293-237(14), 202(20); **8. Carvedilol** (7.39): 407-224(20), 222(22); **9. Celiprolol** (5.81): 380-251(20), 307(19); **10. Esmolol** (5.74): 296-145(26), 219(17); **11. Esmolol metabolite**[*1] (4.92): 282-145(21), 205(10); **12. Labetalol** (6.19): 329-162(24), 311(10); **13. Levobunolol** (5.54) & **Penbutolol** (7.54): 292-236(12), 201(18); **14. Metipranolol** (6.37): 310-191(15), 165(21); **15. Metipranolol metabolite**[*2] (5.12): 268-165(20), 91(47); **16. Metoprolol** (5.37): 268-133(19), 103(37); **17. Nadolol** (4.72): 310-254(16), 201(18); **18. Nebivolol** (7.54): 406-151(27), 123(32); **19. Oxprenolol** (5.92): 266-225(8), 248(5); **20. Pindolol** (4.79): 249-116 (14), 172 (15); **21. Practolol** (4.00): 267-148(17), 120(19); **22. Propranolol** (6.45): 260-183(13), 155(19); **23. Sotalol** (3.03): 273-133(22), 213(11); **24. Timolol** (5.24): 317-261 (9), 244 (15);
ISTD Bupranolol (6.52): 272-216 (7) (not presented in the chromatogram)

[*1] 11. Esmolol metabolite: 4-[2-Hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl propanoic acid
[*2] 15. Metipranolol metabolite: Desacetylmetipranolol