Mazzarino M, Pernice S, de la Torre X, Botrè F

A screening procedure for synthetic insulins by an HPLC-chip interfaced to a quadrupole time-of-flight mass spectrometer

Laboratorio Antidoping Federazione Medico Sportiva Italiana, Largo Giulio Onesti 1, Rome, Italy

Introduction

Insulin and related synthetic analogues have been prohibited by the World Anti-Doping Agency (WADA) for athletes demonstrably not suffering from diabetes mellitus [1]. Athletes could abuse insulin preparations to artificially improve their performance by increasing the muscle glycogen utilizing hyperinsulinaemic clamps prior to sports events or during recovery phases or by increasing muscle size inhibiting protein breakdown.

A recent method [2] describes an effective method, based on antibody-coated magnetic beads, nano-UPLC and Orbitrap mass spectrometry, for the purification and analysis of synthetic insulin analogs in urine samples. Here we preliminarily present a screening procedure based on the use of an HPLC-Chip interfaced with a quadrupole time-of-flight mass spectrometry system to detect the abuse of synthetic insulins. We tested the effectiveness of this approach on blank urine samples spiked with three synthetic insulins (Novolog®, Apidra® and NovoRapid®).

Experimental

Insulin analogues, Novolog®, Apidra® and NovoRapid® were supplied by Eli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ) and Aventis (Kansas City, MO). All chemicals (acetic acid, acetonitrile, sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate dodecahydrate, sodium chloride and formic acid) were purchased from Carlo Erba (Milan, Italy). Monoclonal anti-insulin antibody was purchased from CER-groupe (Marloie, Belgium). Coated Dynal beads (anti-mouse IgG) were from Invitrogen (Milan, Italy). All buffers and solutions were prepared in ultrapure water Milli-Q-grade (Millipore, Milan, Italy).

The LC-MS/(MS) experiments were performed using Agilent 1200 Rapid Resolution Series nanoHPLC and microHPLC pumps with binary gradient system and automatic injector (Agilent Technologies Milan, Italy). Reversed-phase liquid chromatography was performed using a ProtID-Chip-43 composed by the Zorbaz 300SB-C18 analytical column (75 µm X 43 mm; 5 µm) and enrichment column (4 mm; 40 nL) (see Figure 1 for the scheme). The mobile phases were 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). After injection of 4 µL of the sample, the analytes were pre-concentrated on the enrichment column with 95% of solvent A and a flow rate of 4 µL/min. After 3 min, the flow was diverted to the analytical column at 0.3 µL/min and the gradient started with an isocratic step for 1 min with 95% of solvent A. The rate of organic solvent B increased to 85% in 9 min, followed by a re-equilibration phase at starting conditions for 5 min.

High-resolution/high-accuracy measurements were performed on an Agilent Technologies 6520 orthogonal acceleration time-of-flight mass spectrometer, equipped with an nano-electrospray ionization source operated in positive ion mode. Nitrogen was used as the drying and nebulising gas. The drying gas flow rate and temperature were 3 L/min and 300 °C, respectively. The nebulizer gas pressure was 45 psi. The applied capillary and fragmentor voltage were set at 1750 V and 300 V respectively. Mass spectra data were collected from m/z 100 to 3000 at 9300 transients per second. Targeted MS/MS experiments was carried out at a collision energy of 60 eV using the fivefold protonated precursor ion at *m/z* 1162.4 for human insulin (Humulin®) and insulin lyspro (Humalog®), at *m/z* 1165.3 for insulin glulisine (Apidra®), and at *m/z* 1166.1 for insulin aspart (NovoRapid®). Mass calibration was performed daily before starting the analysis using a calibration solution of provided by the manufacturer. All aspects of instrumental setup (tuning, method setup and parameters, sample injection and sequence operation) were controlled by the Agilent Technologies Mass Hunter software.

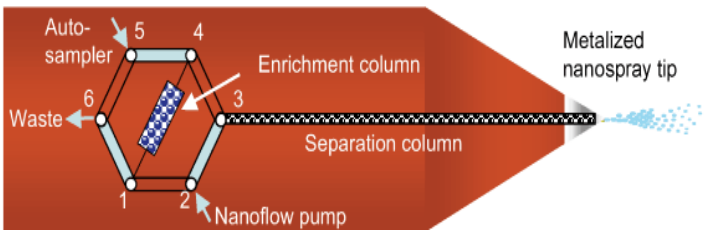

Figure 1: Chip scheme (modified from the manufacturer's technical bulletin, available at http://www.chem.agilent.com/enUS/newsletters/pharmaanalysis/issue25/pages/PN_25_chip_qqq.aspx)

The sample preparation was based on a previously described procedure [2]. Specifically 10 mL of urine were purified and pre-concentrated onto an OASIS HLB cartridge previously preconditioned with 2 mL of acteonitrile and 2 mL of water. After the sample loading, the cartridge was washed with 2 mL of water. Then elution with 1.4 mL of a mixture of acetonitrile/water (80/20, v:v) was performed directly into a polypropylene tube and evaporated in a vacuum centrifuge (40 °C for at least 60 min). The residue was reconstituted in 500 µL of phosphate buffered saline (pH 8), and 5 µL of anti-insulin antibodies and 50 µL of Dynal magnetic beads suspension were added. After incubation for 4 hours at 4 °C, the supernatant was discarded by means of a magnetic separator device and the residual beads were washed twice by consecutive adding, mixing and discarding of 300 µL of phosphate buffered saline buffer. Finally the antigen-antibody aggregate of the target analytes were dissolved by addition of 50 µL of acetic acid (2%). After removing the beads with the magnetic separator, an aliquot of 4 µL was injected into the LC-MS/MS system.

Results and Discussion

The data obtained analyzing blank urines spiked with the three insulins selected in this study (Novolog®, Apidra® and NovoRapid®) showed that despite the drastic reduction of the chromatographic run time (18 minutes instead of 35-40 minutes of the reference methods [2-4]) all three analytes were still clearly distinguishable in urine without being interfered. The three analytes are not completely separated but the selected ion transitions, together with the use of accurate mass, allowed to achieve the necessary specificity. Figures 2A-D show the reliability of the method in terms of repeatability of relative retention times (CV% lower than 1). Furthermore, the specificity and sensitivity was similar to the reference procedures [2-4]. The full validation of the newly developed analytical procedure is currently in progress.

References

1. The World Anti-Doping Agency. The 2011 Prohibited List. International Standard, Montreal. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2011_EN.pdf (last accessed: August 26th 2011)
2. Thomas A, Schanzer W, Delahaut P, Thevis M. (2009) Sensitive and fast identification of urinary human, synthetic and animal insulin by means of nano-UPLC coupled with high-resolution/high-accuracy mass spectrometry. *Drug Test. Analysis*. **1**, 219-227.
3. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schanzer W. (2006) Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography-tandem mass spectrometry. *Anal. Chem.* **78**, 1897-1903.
4. Thevis M, Thomas A, Schanzer W. (2008) Mass spectrometric determination of insulins and their degradation products in sports drug testing. *Mass Spectrom. Rev.* **27**, 35-50.

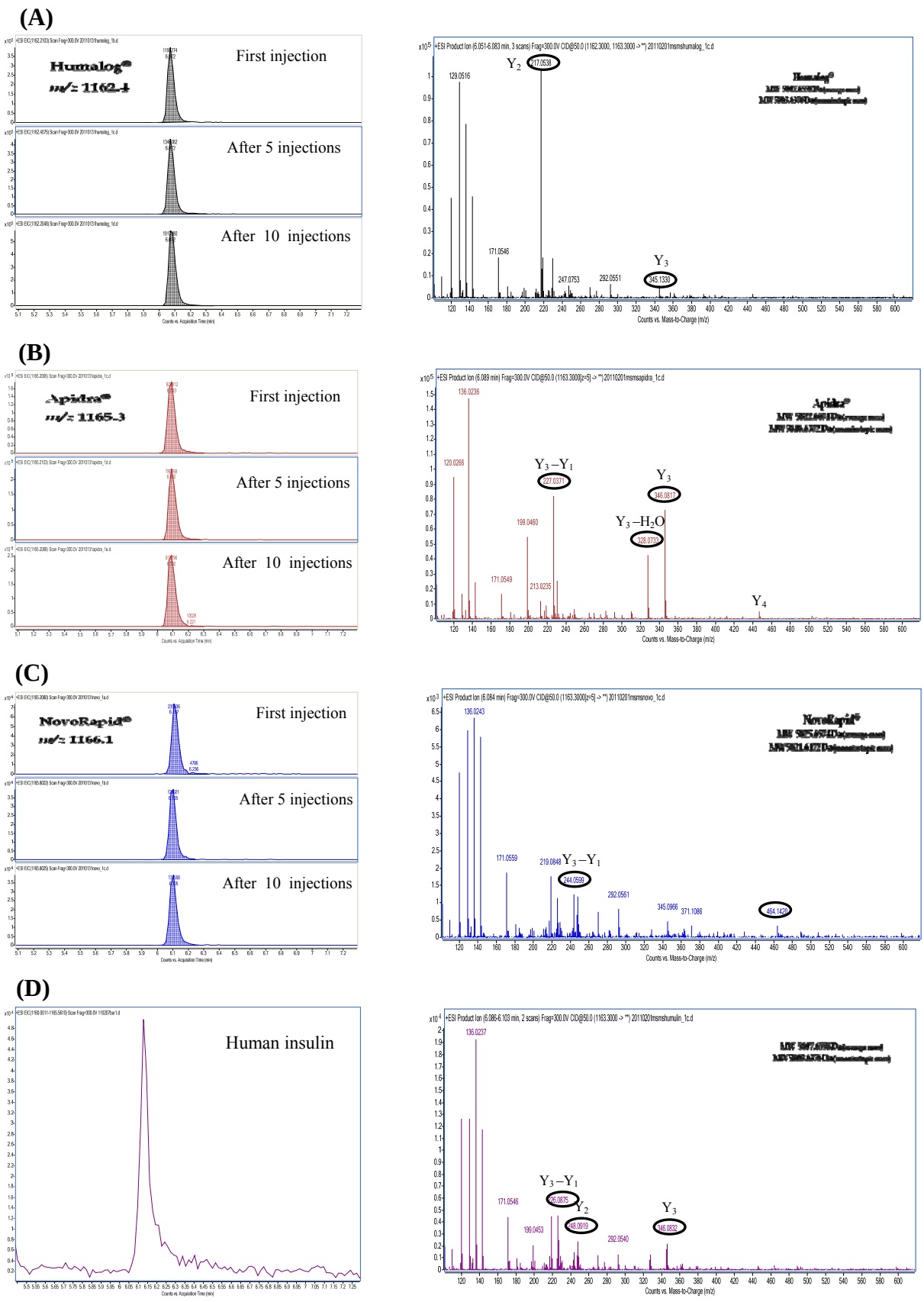

Figure 2: Analysis of a blank urine spiked with Novolog® (A), Apidra® (B) and NovoRapid® (C) at a concentration of 1 ng/mL and of a blank urine sample (D).