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Development and validation of a general screening method for doping substances in human urine by HPLC/Orbitrap mass spectrometry

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Introduction

The demanding task of doping control laboratories is to screen for a wide variety of banned substances in a short period. Six years ago, Georgakopoulos and co-workers proposed the use of HPLC–OTOF in doping control [1]. HPLC–OTOF operating in full-scan mode offers a high sensitive detection of a very broad variety of compounds in potentially a single analysis and, therefore, has the advantage over MS/MS of being able to detect nontarget compounds without method modification. Recently, we have evaluated HPLC–in-source CID atmospheric pressure chemical ionization (APCI) Orbitrap mass spectrometry with accurate mass measurements for its screening potential for agents with antiestrogenic activity, β_2-agonists, exogenous anabolic steroids and other anabolic agents taking advantage of its high resolution, sensitivity and full scan acquisition [2]. Subsequently, recent review indicated the validity of this methodology for doping control [3]. Unfortunately, two separate HPLC–OTOF runs in different polarity modes are usually required. In the present study, we investigated the application of wrong-way-round ionization to allow the simultaneous detection of multiple doping substances (anabolic steroids, β_2-adrenergic agonists, corticosteroids, agents with antiestrogenic activity, diuretics, stimulants and β-blockers) in one LC-MS run without the need to switch the polarity. This feature can provide a significant advantage when reporting time is critical, when only a small sample volume is available or when a considerable number of samples need to be analyzed.

Materials and Methods

Chemicals. Doping agents were purchased from LGC (formerly Promochem, Wesel, Germany), Steraloids (Newport, RI, USA) and Sigma-Aldrich (Steinheim, Germany). The β-glucuronidase preparation (from *Escherichia coli*) was purchased from Roche (Mannheim, Germany). Analytical grade potassium carbonate, sodium hydrogen carbonate, ammonium

hydroxide and diethyl ether were obtained from Merck (Darmstadt, Germany). All solvents were of HPLC-grade or higher. The HPLC grade methanol used was obtained from Merck (Darmstadt, Germany). Stock standard solutions of the analytes at 1 mg/mL were individually prepared in methanol or other appropriate solvents.

Instrumentation. The experiments were performed using a Surveyor HPLC system interfaced to an LTQ-Orbitrap mass spectrometer (Bremen, Germany) with an HESI-II ion source. The mass spectrometer was operated in the positive ion mode. The desolvation temperature was 200 °C. The instrument was calibrated daily using the manufacturer's calibration mixture. The mass spectrometer was operated at a resolution of 60,000 (FWHM). Data were acquired in the full-scan mode from 100 to 650 Da, with a scan time of 1 s. Nitrogen produced by a Peak nitrogen generator system (Peak Scientific, Billerica, MA) was used as the nebulizing gas in the HESI experiments. The nebulizing gas flow rate was 1 L/min. The LC separation was carried out at 35 ^{o}C on a Waters Acquity BEH-C18 column (50 × 2.1 mm i.d., 1.7 μm particle size), with a mobile phase that consisted of 3 mM NH_4OH (A) and a mixture of methanol/water (90:10 v/v) containing 3 mM NH_4OH (B). A gradient elution program was employed at a constant flow rate of 200 μl/min with solvent A increasing from 30% to 100% within 7 min, where it was held for 8 min before returning to 30% within 2 min. The injection volume was 3 μl.

Sample pretreatment. Urine samples were treated as described elsewhere [1].

Validation. Validation of screening method was performed in accordance with the recommendations of Georgakopoulos [1].

Results and Discussion

Ammonium hydroxide was found to be useful additive for the sensitive detection of doping substances in one positive ESI run due to LTQ Orbitrap limitations (it requires to perform two separate runs to cover both polarities). The use of a mobile phase with high pH (pH 10.3) to perform +ESI may appear to be counterintuitive based on the pK_a values of diuretics and corticosteroids, where these compounds are expected to be negatively charged in solution. However, despite the highly basic mobile phase, sufficient ionization of corticosteroids and diuretics occurred in the gas phase. To our knowledge no study has been reported to date on the detect ability of doping agents in high pH mobile phases. Anabolic agents, β_2-adrenergic agonists, agents with antiestrogenic activity, compounds affecting oxygen transfer (efaproxiral), β-blockers, corticosteroids, diuretics and stimulants are ionized to their protonated molecules. Some corticosteroids, diuretics and anabolic agents were

detected as ammonium adducts. For some anabolic steroids, ions resulting from neutral losses ($-H_2O$) from the protonated molecules were observed. The analytical column used allowed us to work with a basic mobile phase of pH 10.3. It proved to be robust. 950 analyses were conducted without any loss of chromatographic performance. The retention times of the analytes were repeatable: the median RSD (%) of relative RT was 0.27. With the gradient elution program described above, analytes that produce ions with the same accurate masses were separated: for example 3'-hydroxystanozolol/16β-hydroxystanozolol/4β-hydroxystanozolol (*m/z* 345.2537); trenbolone/epitrenbolone; oralturinabol/epioralturinabol (*m/z* 335.1778); boldenone/methyldienolone (*m/z* 287.2011). The detection capability of this method for urine samples was validated for all 139 studied compounds. Figure 1 shows the mass chromatograms of a number of analytes, as examples of the data collected during the validation process for a 2 ppm mass window. A retention time tolerance of ±0.05 min (compared with the control urine samples analyzed in all prepared batch) and a mass accuracy limit of ±3 ppm for registered ions of doping substances were used. No interfering compounds were detected at the retention times of the analytes as shown in Figure 1. The specificity was satisfactory: no interfering substances at the appropriate retention times were found when 10 blank urine samples were analyzed. Up to 64% of compounds had a LOD tenfold lower than the MRPL. Of the 139 analytes studied, 124 showed recovery better than 20%. Other analytes studied could be detected far below the MRPL, despite their low extraction efficiency (<20%). The matrix effect for the 139 analytes varied between 4 and 85.0%. For all the analytes, the relative retention times proved to be stable between days, with RSD (%) lower than 0.4. In addition, the between-day RSD (%) of the peak area ratios ranged between 0.8 and 16.5, with 91 analytes showing RSD (%) <10. This study has demonstrated that the use of ammonium hydroxide allows the detection of majority of doping substances in a single LC–HRMS run, without the need to switch the polarity.

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References

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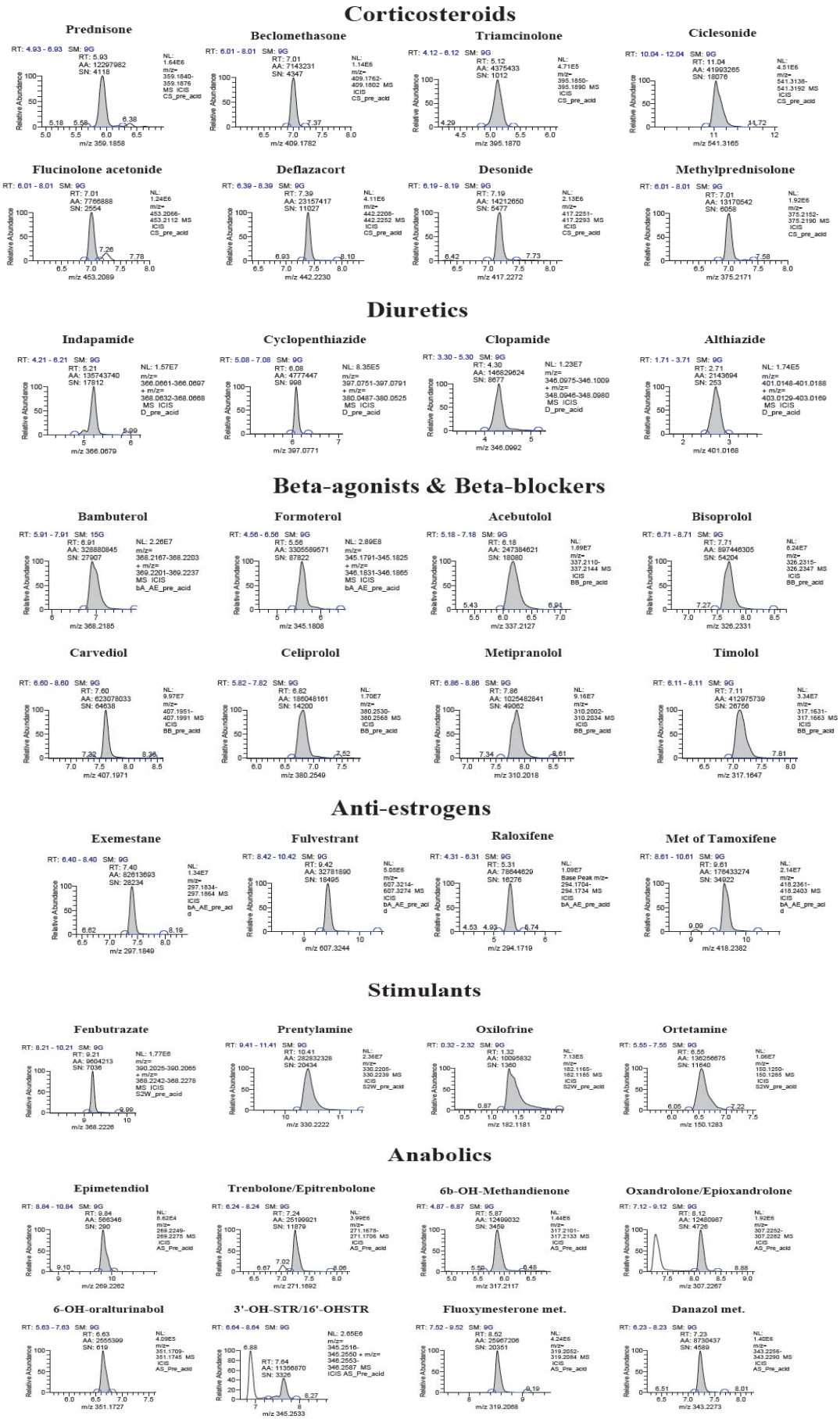

Figure 1 Mass chromatograms of blank urine sample fortified with the representative analytes at the MRPL