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High-temperature liquid chromatography/Orbitrap mass spectrometry with hypercarb column for selective screening of exogenous anabolic steroids in human urine

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Abstract

The presence of a large number of endogenous steroids and corticosteroids with similar structures in a urinary matrix can hamper the specific detection of exogenous steroids using LC-MS with reversed-phase columns. Therefore, the development of LC-MS methods using alternative columns is of great interest. Porous graphitized carbon is a unique stationary phase for HPLC, with properties differing from traditional silica-based and polymeric stationary phases. Non-derivatized porous graphitic carbon surface allows unique retention and separation of geometric isomers. This study demonstrates the application of a porous graphitized carbon column for selective separation of steroids. A screening method for the detection of 56 exogenous steroids has been developed. The method involves enzymatic hydrolysis, liquid-liquid extraction, and determination by high-temperature liquid chromatography-Orbitrap mass spectrometry with atmospheric pressure photoionization. For all of the analytes, the relative retention times proved to be stable between days, with RSDs below than 0.3%. The matrix effect for the 56 analytes varied between 5% and 11.0%. Of the 56 steroids studied, 53 showed limit of detection smaller than 1 ng/mL⁻. The proposed method meets the general criteria for all methods used to analyze drugs or metabolites in an antidoping laboratory, i.e., sensitivity, selectivity, and specificity.

Introduction

The routine LC-MS methods are applied for the detection of a limited number of steroids, normally those that exhibited poor detection by GC-MS. Recently, an LC-MS/MS method was developed for the detection of 44 anabolic steroids and metabolites [1]. However, the presence in a urinary matrix of a large number of endogenous steroids and corticosteroids with similar structures can hamper the specific detection of the exogenous steroids by LC-MS. Therefore, up to now the development of selective LC-MS methods is of great interest. Because the most important issue in qualitative steroid analysis is the selectivity, we developed in this study a sensitive and selective high-temperature LC-Orbitrap MS (HTLC-Orbitrap MS) method with a porous graphitized carbon column for screening anabolic steroids.

Experimental

Chemicals. Anabolic steroids were purchased from Sigma, LGC Standards (formerly LGC Promochem, Wesel, Germany), and Steraloids (Newport, RI, USA). The β -glucuronidase preparation (from *Escherichia coli*) was purchased from Roche (Mannheim, Germany). Analytical-grade potassium carbonate, sodium hydrogen carbonate, ammonium hydroxide, trifluoroacetic acid, diethyl ether, 2-propanol, ethanol, and methanol were obtained from Merck (Darmstadt, Germany). The HPLC-grade water and acetonitrile were purchased from Biosolve (Valkenswaard, The Netherlands). Standard stock solutions of the analytes (concentration 1 mg/mL) were individually prepared in methanol. For validation purposes, working standard solution were prepared in methanol by subsequent dilution of the stock solution.

Instrumentation. The experiments were performed using an Accela HPLC system interfaced to an Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) with an APPI ion source. The mass spectrometer was operated in the positive ion mode. The desolvation temperature was 230 °C. The mass spectrometer was operated at a resolution of 50000



(FWHM). Data were acquired in the full scan mode from 100 to 2200 Da, with a scan time of 0.5 s, and processed using Xcalibur software (Thermo Scientific, Bremen, Germany). Nitrogen produced by a Peak nitrogen generator system (Peak Scientific, Billerica, MA, USA) was used as the nebulizing gas in the APPI experiments. The nebulizing gas flow rate was 0.5 L/min. The LC separation was carried out at 95 °C on a Thermo Scientific Hypercarb column (100 mm x 1 mm i.d., 3 μ m particle size), with a mobile phase consisting of 0.1% CF₃COOH (A) and a mixture of ethanol/2-propanol (15:85 v/v), containing 0.1% CF₃COOH (B). A gradient elution program was employed at a constant flow rate of 170 μ L/min with solvent B increasing from 20% to 100% in 13 min, where it was held for 7 min before returning to 20% within 4 min. The injection volume was 5 μ L. Sample pretreatment. Urine samples were treated as described elsewhere [2].

Results and Discussion

Most anabolic steroids are ionized to their protonated molecules. For some anabolic steroids, signals resulting from neutral losses $(-H_2O)$ or $(-2H_2O)$ of the protonated molecules were observed. The properties of porous graphitized carbon differ from reversed-phase material, e.g. by giving an unrivaled stereoselective surface with unique ability to resolve isomeric steroids. Although steroids are strongly retained on porous carbon at ambient temperature, they can be eluted with 2-propanol/ ethanol/water by raising the temperature. The retention times were reduced by increasing the flow rate without loss of peak efficiency, and there was no evidence of analyte degradation during the short chromatographic run.

The data in Fig. 1 and 2 show that when working in accurate mass mode we achieved very high selectivity and sensitivity. No interfering compounds were detected at the retention times of the analytes. Detection criteria were established for retention time (tolerance of ± 0.05 min) and a mass accuracy (± 4 ppm) for registered analyte ions. Specificity was verified by analyzing 10 blank urine samples. The specificity was satisfactory; no interfering background was found at the relevant retention times when 10 blank urine samples were analyzed. The ion suppression was established for each tested compound before final method validation. The matrix effect for the studied compounds varied between 5% and 11%. The detection limits were below 10 % of the MRPL levels for all studied compounds [3]. The relative retention times proved to be stable between days, with RSDs smaller than 0.3%. In addition, the interday RSDs of the peak area ratios ranged between 0.7% and 14.5%.

Conclusions

This study demonstrated the effective application of HTLC-Orbitrap MS with APPI, where the use of a porous graphitized carbon column allowed the separation of all studied anabolic steroids.

References

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Poster

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

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Figure 2: Mass chromatograms of blank urine sample fortified with the representative analytes at the MRPL

Poster