Angelis Y[1], Petropoulou G[2], Athanasiadou I[3], Fragkaki A[1], Archontaki E[2], Lyris E[1]

Detection of synthetic anabolic androgenic steroids and metabolites by LC-Q-TOFMS after derivatization with 1,1'-carbonyldiimidazole

Doping Control Laboratory of Athens, Olympic Athletic Center of Athens, Athens, Greece[1]; Department of Chemistry, University of Athens, Athens, Greece[2]; Anti-Doping Laboratory of Qatar, Doha, Qatar[3]

Abstract

A liquid chromatography/mass spectrometry method that involves a novel derivatization procedure was developed for the sensitive screening analysis of anabolic androgenic steroids. The proposed method aims at the introduction of an easily ionizable moiety into the steroid molecule by converting the hydroxyl groups into imidazole carbamates. The reaction conditions were optimized regarding solvent, derivatization reagent concentration, presence of catalyst, incubation time and temperature. After the sample purification, dry residue of steroids was incubated with 100 µL 1,1'-carbonyldiimidazole solution (CDI 0.5 M) in acetonitrile at 60°C for 30 min and transferred into vials for LC-MS analysis. The proposed method was applied to water and urine samples spiked with exogenous AAS in various concentration levels. Briefly, 2.5 mL of urine was hydrolyzed with β-glucuronidase from *E. coli* followed by extraction with diethyl ether at pH 9-10. After solvent evaporation under nitrogen stream at 50°C, the dry extracts were incubated with CDI solution, as described above. Steroid imidazole carbamate derivatives showed intensive $[M+H]^+$ signals under electrospray ionization and common fragmentation patterns in MS-MS mode with $[M-CO_2+H]^+$ and $[M-ImCO_2+H]^+$ as major ions with low collision energy (e.g. 5 eV). Despite of significant matrix effect, the majority of the steroids were detected at least at the minimum required performance level (MRPL) as established by the technical document of the World Anti-Doping Agency (WADA). The present method is sensitive, requiring a facile preparation procedure which could be easily applied to doping control analysis.

Introduction

Anabolic androgenic steroids (AAS) present limitations regarding their detection using liquid chromatography (LC) coupled to mass spectrometry (MS) via soft ionization of atmospheric pressure interface (e.g. electrospray, ESI). Difficulties in the ionization of AAS result from the absence of acidic or basic groups and the low proton affinity of carbonyl and hydroxyl groups. A variety of derivatization procedures have been proposed in order to introduce an easily ionizable moiety into the steroids' molecule and to improve their LC-MS detectability [1,2]. In the current study, the objective was the development of a sensitive AAS screening method for LC-MS instrumentation that uses 1,1'-carbonyldiimidazole (CDI) as the derivatization agent for the conversion of hydroxyl groups into imidazole carbamates. The method was applied to 34 hydroxysteroids.

Experimental

The sample preparation began with the spiking of urine (2.5 mL) with the examined steroids followed by addition of 1 mL phosphate buffer (pH 7) and 25 µL β-glucuronidase. The sample was hydrolyzed for 1.5 h at 50 °C. Subsequently, the pH was adjusted to 9-10 using a 10:1 (w/w) mixture of sodium carbonate-sodium bicarbonate and the resulting mixture was extracted with 5 mL of diethyl ether using 1.5 g of sodium sulfate as salting out agent. After centrifugation (1800 rpm, 10 min), the organic layer was separated and evaporated to dryness at 50°C under nitrogen. For the derivatization step, 100 µL of freshly prepared CDI solution in dry acetonitrile (0.5 M) was added to the dry extracts and the mixture was incubated for 30 min at 60°C.
LC-QTOF analysis was performed using an Agilent 1200 Series rapid-resolution LC system interfaced to an Agilent 6520 Accurate Mass Q-TOF. Chromatographic separation was achieved using a Zorbax Eclipse Plus C18 column (100×2.1 mm ID,

 ISBN 978-3-86884-040-7

1.8 µm) at 35°C and 5 µL of sample was injected. The mobile phase consisted of 5mM ammonium formate in 0.01% (v/v) formic acid (solvent A) and a mixture of acetonitrile/water (90:10 v/v) containing 5mM ammonium formate and 0.01% (v/v) formic acid (solvent B). Gradient program started at 30% of eluent B, increased linearly to 100% in 35 min, and returned back to 30% within 5 min. Flow rate was 0.3 mL/min. Full scan mass spectral data were acquired using positive mode electrospray ionization and nitrogen as the drying and nebulizing gas. The drying gas flow rate was set at 10 L/min and temperature at 330°C. Other MS parameters were capillary, fragmentor, and skimmer voltages, which were set at 3500 V, 140 V, and 50 V, respectively.

Results and Discussion

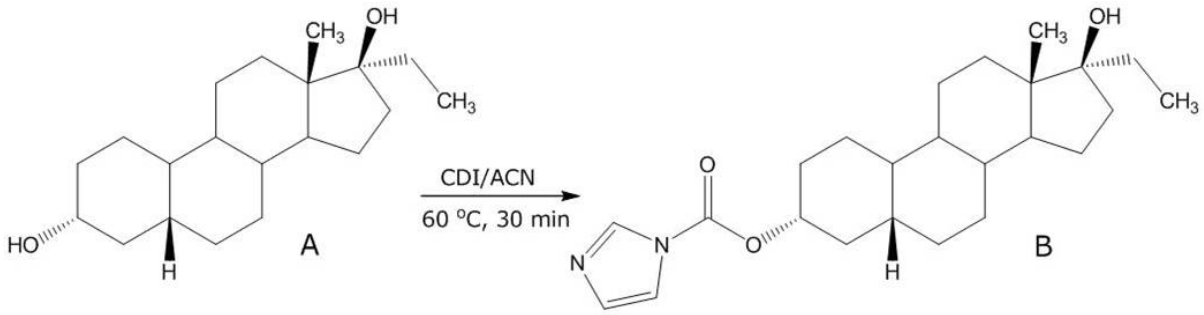

Figure 1: Representative derivatization reaction of Norethandrolone metabolite 2 (A) forming the corresponding imidazole carbamate (B).

The proposed derivatization reaction was optimized for the following parameters: solvent, derivatization reagent concentration, presence of a catalyst, incubation time and temperature. Under the selected conditions primary and secondary hydroxyl groups are derivatized while tertiary hydroxyl groups remain mainly underivatized.
The method was applied to water and drug-free urine samples fortified with 34 steroids at 3 concentration levels (2, 5 and 10 ng/mL). The obtained results demonstrated that while 31 of the examined compounds were detected in water at the required concentration levels, much less were detected in urine samples due to matrix effects.
Various conditions were investigated, both in sample preparation (extraction solvent, dilution) and the chromatographic separation (mobile phase, gradient program, run time, use of two coupled columns), that gave different outcome for the detection of AAS. For example, derivatives of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol were barely detected (Table 1) using standard chromatographic conditions (see Experimental). When two coupled chromatographic columns were employed those analytes were detected at 2 ng/mL while other AAS were detected with decreased sensitivity. Eventually, the conditions selected were those in which the highest number of analytes was detected at their MRPL [3].
Validation of the developed method was performed in ten different urine samples according to the International Standard for Laboratories by WADA [4]. The examined parameters included carryover, specificity, matrix interferences, identification capability and matrix effect.

Substance	Monitoring m/z	LOD (ng/mL)
6α-Hydroxy-androstenedione	353.2224	N.D.
Bolasterone metabolite 1	415.2955	2
Boldenone	381.2173	10
Boldenone metabolite 1	383.2329	N.D.*
Calusterone metabolite	415.2955	5
Clostebol metabolite 2	373.2041	5
Drostanolone metabolite 1	399.2642	5
17-Epimetendiol	355.2744	2
Fluoxymesterone metabolite 1	413.2235	N.D.
Fluoxymesterone metabolite 2	499.2715	N.D.**
Formebolone metabolite 2	535.2551	5
16β-Hydroxy-furazabol	390.2388	N.D.
Mesterolone metabolite 1	399.2642	2
17α-Methyl-5α-androstan-3α,17β-diol	401.2799	5
17α-Methyl-5β-androstan-3α,17β-diol	401.2799	10
Methasterone metabolite	415.2955	2
Methenolone	397.2486	2
Methenolone metabolite	397.2486	5
19-Norandrosterone	371.2329	2
19-Noretiocholanolone	371.2329	2
5α-Tetrahydro-norethisterone	379.2380	2
Norbolethone metabolite 1	415.2955	2
Norbolethone metabolite 2	415.2955	2
Norethandrolone metabolite 1	401.2799	2
Norethandrolone metabolite 2	401.2799	2
16β-Hydroxy-stanozolol	465.2496	N.D.**
3-Hydroxy-stanozolol	533.2871	N.D.**
4-Hydroxy-testosterone	543.2714	2
1-Testosterone metabolite	339.2431	N.D.*
6β-Hydroxy-oral turinabol	401.1990	10
Trenbolone	365.1860	2
17α-Trenbolone	365.1860	2
α-Zearanalol	605.2354	2
β-Zearanalol	605.2354	2

LOD: 10 out of 10 hits
N.D.: Not detected at 10 ng/mL
*Interferences from isobaric endogenous compounds
**More than one derivatives

Table 1: Limit of detection of the examined AAS.

ISBN 978-3-86884-040-7

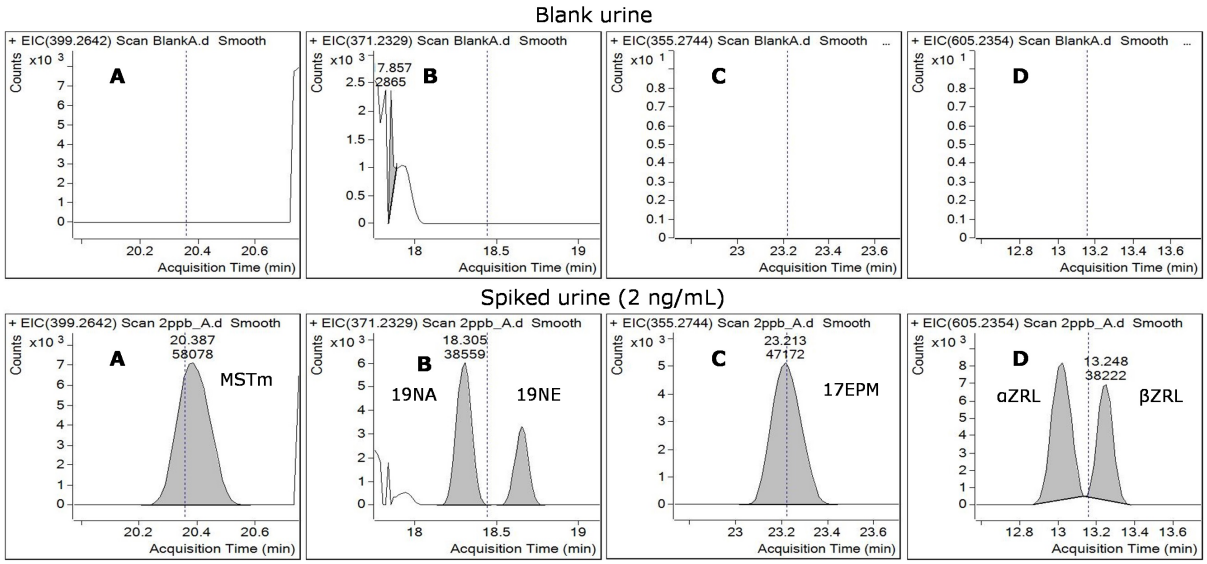

Figure 2: Extracted ion chromatograms of a blank (above) and a spiked urine sample at 2 ng/mL (below) for various derivatives.
[A: Mesterolone metabolite 1 (MSTm), B: 19-Norandrosterone (19NA) and 19-Noretiocholanolone (19NE), C: 17-Epimetendiol (17EPM), D: α-Zearanalol (αZRL) and β-Zearanalol (βZRL)]

In addition to development of a full scan screening method, the MS-MS fragmentation of the derivatives was also investigated. Results demonstrated that the fragmentation of all steroid imidazole carbamates is characterized by abundant ions due to the loss of CO_2 ($[M\text{-}CO_2+H]^+$) and the loss of CO_2 and imidazole ($[M\text{-}ImCO_2+H]^+$) from the protonated molecular ion usually under low collision energy (5-10 eV).

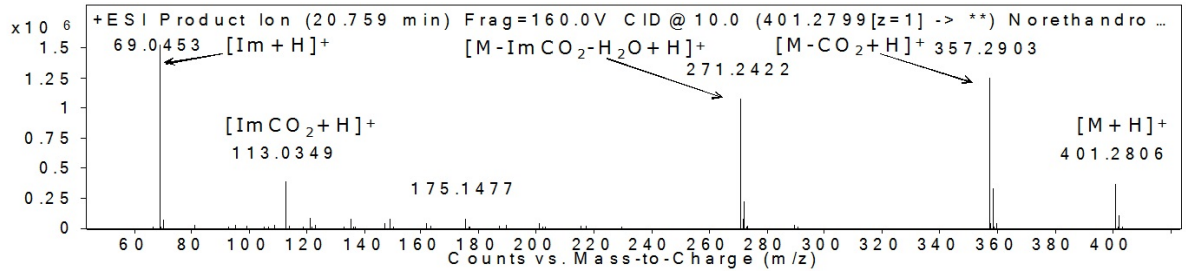

Figure 3: A typical product ion spectrum of Norethandrolone metabolite 2 derivative.

Conclusions

A novel 1,1'-carbonyldiimidazole derivatization method was developed for the LC-MS screening analysis of hydroxysteroids. The imidazole moiety introduced in the molecule enhances the ionization efficiencies, and out of 34 synthetic anabolic agents of the study, 22 were detected at MRPL-concentrations or below. A key advantage of the method is the simplicity of the derivatization reaction that does not require any clean-up or reconstitution step before analysis. Furthermore, the examined derivatives demonstrated interesting fragmentation patterns with structrural information indicating their potential use in confirmatory analysis.

 ISBN 978-3-86884-040-7

References

Athanasiadou I, Angelis Y.S, Lyris E, Georgakopoulos C. (2013) Chemical derivatization to enhance ionization of anabolic steroids in LC-MS for doping-control analysis. *Trends Anal. Chem.* **42**, 137-156.

Athanasiadou I, Angelis Y.S, Lyris E, Vonaparti A, Thomaidis N.S, Koupparis M.A. Georgakopoulos C. (2012) Two-step derivatization procedures for the ionization enhancement of anabolic steroids in LC-ESI-MS for doping control analysis. *Bioanalysis* **4**, 167-175.

World Anti-Doping Agency. Minimum Required Performance Levels for detection and identification of Non-Threshold Substances, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013MRPL-Minimum-Required-Performance-Levels-v1-2012-EN.pdf (access date 22.07.2014)

World Anti-Doping Agency. International Standard for Laboratories, Version 7.0 Montreal (2012), http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/ISL/WADA_Int_Standard_Laboratories_2012_EN.pdf (access date 22.07.2014)

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