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Potential of atmospheric pressure chemical ionization source in gas chromatography tandem mass spectrometry for the screening of urinary exogenous androgenic anabolic steroids

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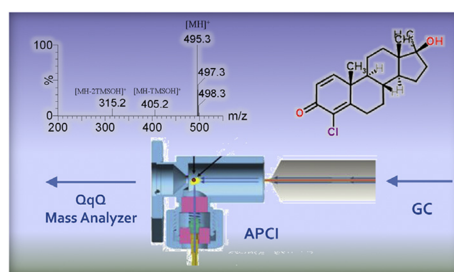
HIGHLIGHTS

- APCI source has been evaluated for the screening of 16 exogenous AAS in urine.
- Suitable precision was observed for APCI although lower than with EI.
- LODs for APCI were commonly lower.
- GC-APCI-MS/MS method increases the period in which the misuse of 4ClMTD is detected.
- APCI source is an adequate alternative to the traditional EI source in GC–MS.

GRAPHICAL ABSTRACT



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ABSTRACT

The atmospheric pressure chemical ionization (APCI) source for gas chromatography-mass spectrometry analysis has been evaluated for the screening of 16 exogenous androgenic anabolic steroids (AAS) in urine. The sample treatment is based on the strategy currently applied in doping control laboratories i.e. enzymatic hydrolysis, liquid–liquid extraction (LLE) and derivatization to form the trimethylsilyl ether-trimethylsilyl *enol ether* (TMS) derivatives. These TMS derivatives are then analyzed by gas chromatography tandem mass spectrometry using a triple quadrupole instrument (GC-QqQ MS/MS) under selected reaction monitoring (SRM) mode. The APCI promotes soft ionization with very little fragmentation resulting, in most cases, in abundant $[M + H]^+$ or $[M + H\text{-}2TMSOH]^+$ ions, which can be chosen as precursor ions for the SRM transitions, improving in this way the selectivity and sensitivity of the method. Specificity of the transitions is also of great relevance, as the presence of endogenous compounds can affect the measurements when using the most abundant ions. The method has been qualitatively validated by spiking six different urine samples at two concentration levels each. Precision was generally satisfactory with RSD values below 25 and 15% at the low and high concentration level, respectively. Most the limits of detection (LOD) were below 0.5 ng mL^{-1}. Validation results were compared with the commonly used method based on the electron ionization (EI) source. EI analysis was found to be slightly more repeatable whereas lower LODs were found for APCI. In addition, the applicability of the developed method has been tested in samples collected after the administration of 4-

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chloromethandienone. The highest sensitivity of the APCI method for this compound, allowed to increase the period in which its administration can be detected.

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1. Introduction

Since 2004, the World Anti-Doping Agency (WADA) publishes a list of prohibited substances and methods in sport which is yearly updated [1]. Among the groups of substances included in the list, androgenic anabolic steroids (AAS) are the most frequently reported ones [2]. AAS are mainly used due to their anabolic effects such as muscle and strength growth among others [3].

AAS are prohibited at all times i.e. in and out of competition. This prohibition makes that any evidence of AAS misuse (e.g. the mere presence of traces of the AAS and/or its metabolites) is sufficient for reporting an adverse analytical finding [4]. The detection of AAS misuse is a constant analytical challenge due to their low concentration in urine, the complexity of the matrix and the similarity between endogenous and exogenous AAS. Thus, sensitivity and selectivity of analytical methods are key factors and requirements for AAS detection have evolved hand in hand with instrumental developments.

AAS have been traditionally determined by gas chromatography mass spectrometry (GC–MS) methods working in selected ion monitoring mode (SIM) using electron ionization (EI) sources [5]. After some preparation steps [6] i.e. hydrolysis with *β*-glucuronidase, liquid–liquid extraction and conversion of both hydroxyl and carbonyl function into the corresponding TMS *ether/enol-TMS* ethers, these methods allowed the detection of most of AAS metabolites at concentrations below 10 ng mL^{-1}. For this reason, the minimum required performance level (MRPL) for most AAS was set at 10 ng mL^{-1}. However, these methods failed for the detection of several AAS at the required MRPL, mainly those with difficulties in the derivatization step. Among them, stanozolol and AAS bearing a 4,9,11-triene nucleus like tetrahydrogestrinone (THG) [7].

The occurrence of high resolution mass spectrometry opened new possibilities for the detection of stanozolol [8], although the scenario drastically changed after the introduction of liquid chromatography tandem mass spectrometry (LC-MS(/MS)) in doping control laboratories [9,10]. Several methods have been developed for the LC-MS/MS detection of AAS with poor derivatization properties like stanozolol and THG [11–13]. Thus, both GC–MS(/MS) and LC-MS(/MS) have been employed as complementary techniques in doping control laboratories in order to reach the required MRPLs. Qualitative methods for the detection of exogenous AAS in urine by LC-MS/MS with triple quadrupole (QqQ) analyzers and electrospray ionization source (ESI) [11] have been reported, as well as GC–MS/MS methods with EI [14,15] or chemical ionization (CI) sources [16].

In the last years, the commercialization of triple quadrupole instruments coupled to GC has allowed for increasing the sensitivity of the previous GC–MS methods. Thus, several GC-EI-MS/MS methods in selected reaction monitoring mode (SRM) have been published either for the detection of target analytes [14,15] or for metabolic studies [17–19]. Nowadays, this technique has become the gold-standard in AAS analysis for doping control purposes. Due to the sensitivity improvement, the MRPL for AAS has been recently reduced to 2–5 ng mL^{-1} for most analytes [4]. This fact illustrates the impact of new analytical technologies in the detection of AAS. Therefore, it is valuable to test the performance of emerging analytical tools in this field.

As an alternative to EI, different "soft" ionization sources for GC have been tested for the detection of AAS in doping analysis, i.e. CI [16], heated nebulizer microchip atmospheric pressure photoionization (μAPPI) [20,21] or atmospheric pressure chemical ionization (APCI) [22,23]. The recently commercialized APCI source for GC represents an attractive alternative in several application fields [24–26]. APCI promotes soft ionization for the generation of $[M+H]^+$ or $M^{+\cdot}$ ions as the base peak of the spectrum, by means of protonation or charge transfer mechanisms, deeper explained in literature [22,27]. This soft ionization presents an advantage in the selection of specific precursor ions in MS/MS based methods.

In the present work, the potential of APCI source using GC–MS/MS was evaluated for the development of a screening method for the detection of selected exogenous AAS in urine. After validation, the performance of the GC-APCI-MS/MS method has been compared with the conventional GC-EI-MS/MS, by analyzing a group of samples prepared under the same conditions. The applicability of the method was also evaluated in a set of samples collected at different times after the administration of 4-chloromethandienone (4Cl-MTD).

2. Experimental

2.1. Chemical and reagents

The structures of the selected AAS are shown in Fig. 1. Boldenone (BD) was obtained from Sigma (St. Louis, MO, USA). 17β-hydroxy-5β-androstan-1-ene-3-one (Boldenone metabolite, BDmet), 17β-methyl-5β-androst-1-en-3α,17α-diol (Methandienone metabolite, MTDmet3), 1-testosterone (1-T), 5α-androstan-17α-methyl-3α,17β-diol (Methyltestosterone metabolite, MeTmet1), 5β-androstan-17α-methyl-3α,17β-diol (Methyltestosterone metabolite, MeTmet2), 5β-androstan-7β,17α-dimethyl-3α,17β-diol (Calusterone metabolite, CALUSmet), 17α-metyl-1-testosterone (Me-1-T), 5β-androstan-7α,17α-dimethyl-3α,17β-diol (Bolasterone metabolite, BOLASmet), 13β,17α-diethyl-5β-gonane-3α,17β-diol (Norbolethone metabolite, NORBOLmet2) 6β-hydroxy-4-chloromethandienone (6OH-4Cl-MTD) and 4-hydroxy-testosterone (4OH-T) were purchased from NMI (Pymble, Australia). Fluoxymesterone (FLU) was obtained from Steraloids (Newport, RI, USA). 5α-Androstan-2α,17α-dimethyl-3α,17β-diol (Methasterone metabolite, METHASmet) was a kind gift from the World Association of Anti-Doping Scientists (WAADS). Oxymesterone (OXY) and madol (MADOL) were provided by the Toronto Research Chemicals (Toronto, Canada).

AAS stock standard solutions at 10 and 100 μg mL^{-1} in methanol were stored at −20 °C. Working MIX solutions at appropriate concentration levels for validation were prepared in acetone and also stored at −20 °C, whereas individual standard solutions were employed for the transition optimization step and for potential cross-talk evaluation.

β-glucuronidase solution (*Escherichia coli*, type K12) was purchased from Roche Diagnostics (Mannheim, Germany). Analytical grade potassium carbonate, potassium hydroxide pellets, sodium hydrogen phosphate, di-sodium hydrogen phosphate, *tert*-butylmethyl ether and ammonium iodide were acquired from Merck (Darmstadt, Germany). The derivatization reagent preparation *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Karl Bucher Chemische Fabrik GmbH (Waldstetten,

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