



Coupling of gas chromatography and electrospray ionization high resolution mass spectrometry for the analysis of anabolic steroids as trimethylsilyl derivatives in human urine



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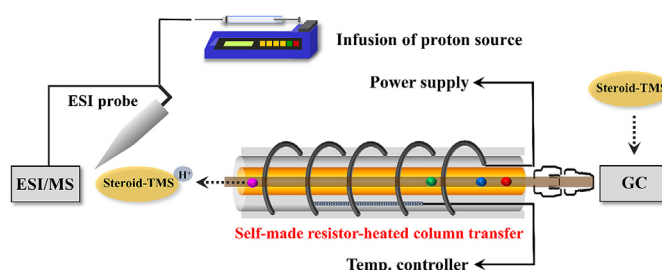
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HIGHLIGHTS

- A GC-ESI/HRMS was developed and optimized to enhance the ionization efficiency.
- Key parameters were investigated and GC-ESI/HRMS was applied to steroids analysis.
- Regardless of analyte phase or derivatization, steroids showed similar ionization profile.
- GC-ESI/HRMS with TMS derivatives showed narrow peak width and good sensitivity.
- This method has potential as novel ionization tool for steroids analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, gas chromatography (GC) was interfaced with high resolution mass spectrometry (HRMS) with electrospray ionization source (ESI) and the relevant parameters were investigated to enhance the ionization efficiency. In GC-ESI, the distances (x-, y- and z) and angle between the ESI needle, GC capillary column and MS orifice were set to 7 (x-distance), 4 (y-distance), and 1 mm (z-distance). The ESI spray solvent, acid modifier and nebulizer gas flow were methanol, 0.1% formic acid and 5 arbitrary units, respectively. Based on these results, analytical conditions for GC-ESI/HRMS were established. In particular, the results of spray solvent flow indicated a concentration-dependent mechanism (peak dilution effect), and other parameters also greatly influenced the ionization performance. The developed GC-ESI/HRMS was then applied to the analysis of anabolic steroids as trimethylsilyl (TMS) derivatives in human urine to demonstrate its application. The ionization profiles of TMS-derivatized steroids were investigated and compared with those of underivatized steroids obtained from gas chromatography-electrospray ionization/mass spectrometry (GC-ESI/MS) and liquid chromatography-electrospray ionization/mass spectrometry (LC-ESI/MS). The steroids exhibited ionization profiles based on their structural characteristics, regardless of the analyte phase or derivatization. Groups I and II with conjugated or unconjugated keto functional groups at C3 generated the $[M+H]^+$ and $[M+H-TMS]^+$ ions, respectively. On the other hand, Groups III and IV gave rise to the characteristic fragment ions $[M+H-TMS-H_2O]^+$ and $[M+H-2TMS-H_2O]^+$, corresponding to loss of a neutral TMS·H₂O moiety from the protonated molecular ion by in-source dissociation. To the best of our knowledge, this is the first study to successfully ionize and analyze steroids as TMS derivatives using ESI coupled with GC. The present system has enabled the

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ionization of TMS derivatives under ESI conditions and this method has potential as a novel ionization tool. It is also useful for the simultaneous analysis of steroids as TMS derivatives.

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1. Introduction

Anabolic steroids are synthetic analogues of the male hormone testosterone and these substances promote muscle growth. Anabolic steroids are widely used to enhance athletic performance in sports. In doping control, the abuse of anabolic steroids is banned by the World Anti-Doping Agency [1], and anabolic steroids are the most frequently detected class of prohibited substances. Moreover, the number of illegal steroids is constantly increasing. Thus, the detection of steroids is a continuous challenge in doping control analysis.

Anabolic steroids are organic compounds with four rings arranged in a specific configuration. The structures and molecular weights of steroids are very similar and endogenous steroids are present in human biological matrices. For these reasons, the chromatographic resolution is important and essential for steroids analysis. Analytical methods for the analysis of anabolic steroids have been developed on the basis of gas chromatography-electron ionization/mass spectrometry (GC-EI/MS). GC-EI/MS methods are well-established and widely used for steroids because of their high chromatographic resolving power and the wide ionization range of the EI ionization source [2,3]. Although GC-EI/MS methods are powerful and have some advantages for steroids analysis, the EI ionization can cause extensive fragmentation owing to the energetic ionization process with excessive ionization energies of 70 eV, resulting in the absence of the molecular ion.

Recently, various analytical methods based on liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI/MS/MS) have been developed and applied to steroids analysis [4–6]. LC-ESI/MS/MS is suitable for determining the molecular ion owing to its softer ionization by ESI. Unfortunately, however, LC-ESI/MS/MS faces serious problems for steroid analysis. This method provides poor chromatographic separation, compared with GC-EI/MS. Therefore, GC-ESI/MS combining the advantages of GC-MS and LC-MS has potential for steroid analysis. Such gas chromatography-electrospray ionization/high resolution mass spectrometry (GC-ESI/HRMS) can provide efficient chromatographic separation by GC with a capillary column, and it can lead to enhanced selectivity and sensitivity by ESI/HRMS. When considering these points, GC-ESI/HRMS seems to be an attractive instrument. However, GC-ESI/HRMS is not commercially available to date. A few publications regarding the ionization of gaseous analytes by ESI have been reported [7–9]. Among these, Kostianen et al. reported a simple method for coupling GC to ESI/MS and this is the only publication of a practical GC-ESI/MS instrument [9]. They investigated and proposed the *x*, *y*, and *z* distances between the ESI needle, the orifice, and the GC column, as well as the effect of the ESI solvent on the ionization efficiency. Furthermore, the sensitivity of GC-ESI/MS, GC-atmospheric-pressure chemical ionization (APCI)/MS, and GC-EI/MS were investigated, and the mass spectra produced by these instruments were compared. As a result, they successfully ionized and analyzed several analytes using their GC-ESI/MS system. Nevertheless, various studies of the availability and potential of GC-ESI/MS systems as alternative analytical tools are not sufficient to make progress in this field.

Based on previous reports [7–9], the aims of this study are (1) to develop and establish more sensitive and practical GC-ESI/HRMS

instrumentation, and (2) to subsequently investigate and evaluate GC-ESI/HRMS as a novel ionization tool for the simultaneous analysis of anabolic steroids as trimethylsilyl (TMS) derivatives. For this purpose, a self-made resistor-heated column transfer line was prepared, and the optimum distances between the ESI needle, orifice and GC column outlet were established. Furthermore, the ESI solvent, acid modifier, flow rate of ESI solvent, and flow rate of nebulizing gas were investigated to improve the sensitivity. Based on the result, ionization profiles of gas-phase steroids as TMS derivatives were investigated using GC-ESI/HRMS, and the results were compared with gas-phase steroids and liquid-phase underivatized steroids under ESI conditions. Finally, the present GC-ESI/HRMS method was successfully applied to the simultaneous qualitative analysis of anabolic steroids as TMS derivatives in human urine.

2. Materials and methods

2.1. Standards and reagents

Anabolic steroids and chemicals were purchased from Akzo-nobel (BM Arnhem, Netherlands), NMI (Pymble, Australia), Steraloids (Newport, RI, USA), Sigma (St. Louis, MO, USA), and NARL (Pymble, Australia). β -Glucuronidase was purchased from Roche (Mannheim, Germany). SeRDOLIT[®] PAD-1 was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). The HPLC grade methanol (MeOH), acetonitrile (ACN), isopropyl alcohol (IPA), diethyl ether and ethyl acetate were purchased from Burdick & Jackson (Ulsan, Korea). Analytical grade formic acid, acetic acid, sodium phosphate and potassium carbonate were purchased from Sigma (St. Louis, MO, USA). Deionized water (DW) was generated from an in-house water purification system (Milli-Q, Bedford, MA, USA). For derivatization, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), ammonium iodide (NH₄I), and dithioerythritol (DTE) were purchased from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

Twelve anabolic steroids were used for the establishment of operating parameters, and these compounds are summarized in Table 1. To compare their ionization profiles according to analyte phase, underivatized steroids were dried and reconstituted using diethyl ether and 45% formic acid in MeOH for GC-ESI/HRMS and LC-ESI/MS/MS, respectively. For analysis of TMS steroids derivatives, 79 steroids and deuterium labeled internal standard such as 16,16,17-*d*₃-testosterone were spiked in human urine at 10 ng mL⁻¹ and 20 ng mL⁻¹, respectively, and then pretreated for determination. Targeted steroids were classified into four different categories [10,11]: conjugated- or unconjugated-keto functional groups at C3 (Groups I and II) and conjugated- or unconjugated-hydroxyl functional groups at C3 (Groups III and IV). Sample preparation was based on our previous report [11]. Urine (2 mL) samples were loaded onto a PAD-1 column that had been washed with 2 mL of DW. After loading, the column was washed with 2 mL of DW and eluted with 4 mL of MeOH. The eluent was dried by evaporation in a rotary evaporator. The dry residue was incubated with β -glucuronidase (50 μ L) in 1 mL of phosphate buffer (pH 7.2) at 55°C for 1 h. The pH of the reaction mixture was adjusted with

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