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International Journal of Mass Spectrometry 247 (2005) 48–54
International Journal of Mass Spectrometry
www.elsevier.com/locate/ijms

Structure and mechanism of formation of an important ion in doping control

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Received 14 June 2005; received in revised form 15 August 2005; accepted 15 August 2005
Available online 7 October 2005

Abstract

An ion with *m*/*z* 143 serves as a biomarker that is often continuously monitored in urine samples undergoing screening by electron ionization gas chromatography/mass spectrometry (EI GC/MS) for banned anabolic agents. The ion is known to arise from trimethylsilyl (TMS)-derivatized synthetic 17-hydroxy, 17-methyl steroids. The purpose of this work was to characterize, in detail, the origin(s), structure(s), and mechanism(s) of formation of such ions with *m*/*z* 143. High resolution mass spectrometry (HRMS) data revealed the elemental composition of the D-ring derived *m*/*z* 143 ion to be $C_7H_{15}OSi$. Analysis of dihydrotestosterone (DHT) and its 2-methyl substituted analog dromostanolone by HRMS revealed that an elementally equivalent ion of *m*/*z* 143 could be derived from the A-ring of TMS-derivatized 3-keto-enol steroids demonstrating that an abnormally intense peak in the *m*/*z* 143 extracted ion chromatogram of urine samples undergoing screening for banned anabolic agents does not necessarily indicate the presence of a 17-hydroxy, 17-methyl steroid. To gain information on ion structure, breakdown curves for the most abundant product ions of the *m*/*z* 143 ion were generated using both native and perdeutero-TMS derivatives, providing structures for second, third, and fourth generation product ions. An EI-mass spectrum of [16,16,17-$^2H_3$]-DHT (DHT-d3) demonstrated that one of the C-16 hydrogen atoms is removed prior to the formation of an ion that is highly analogous to the ion with *m*/*z* 143 strongly suggesting, in accord with all other evidence, one particular fragmentation pathway and resulting product: a resonance stabilized 3-(*O*-trimethylsilyl)but-1-ene ion.

Keywords: *m*/*z* 143; Steroids; Anti-doping; Ion structure

1. Introduction

One of the primary goals of anti-doping research is to define biomarkers of doping, i.e., chemical entities that, when detected at or above certain concentrations, are indicative of a doping offense by the provider of the urine or blood sample under analysis. Although these markers are generally the banned substances or their metabolites, classification as such is not a requisite condition for their establishment as a doping biomarker. In fact, due to the manner in which sporting federation rules are written, ideal biomarkers would be general in nature such that detection of a single marker substance would indicate an anabolic doping offense. Details of the offense could be sorted out after an initial positive report, but the facile and rapid acquisition of a positive result based on a general biomarker would greatly simplify the analytical process. This idyllic scenario may never become possible, but the dramatic simplification of sample screening procedures appears to be within the realm of modern technology.

For over a decade, the technique of choice for screening urine samples for banned anabolic agents has been gas chromatography coupled to electron ionization mass spectrometry (EI GC/MS) [1–3]. Analyses of urine extract samples by GC/MS are typically run in selected ion monitoring (SIM) mode with multiple ion windows to obtain required sensitivity limits [3], but ions produced with a *m*/*z* of 143 are continually monitored in every sample by many world anti-doping agency-accredited laboratories during the course of screening trimethylsilyl (TMS)-

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doi:10.1016/j.ijms.2005.08.013

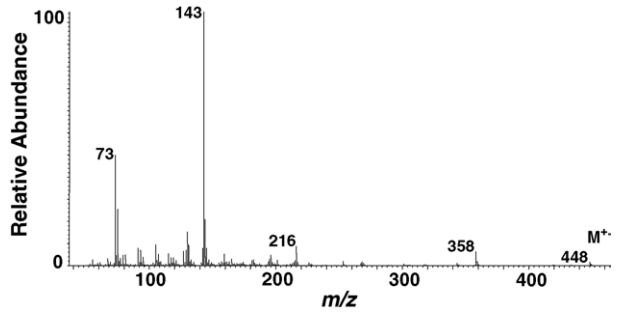
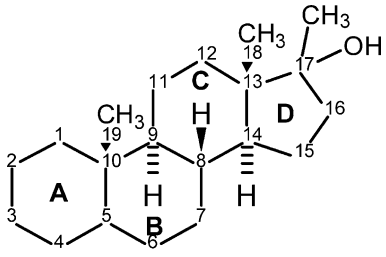


Fig. 1. EI-mass spectrum of TMS-derivatized epimetendiol (**7**)—a representative 17-hydroxy, 17-methyl synthetic steroid. The peak at *m*/*z* 448 represents the molecular ion (M^+). The origin, structure, and mechanism of formation of the ion represented by the base peak at *m*/*z* 143 are the focus of the work presented here.

derivatized urine sample extracts for banned anabolic substances (personal communication with Larry Bowers, United States Anti-Doping Agency Senior Managing Director of Technical and Information Resources).[1] A thorough investigation of the structure, molecular origin(s), and mechanism of formation of this ion, however, has not been reported even though work closely related to some aspects of that described here has been carried out [4,5]. It is understood that the ion consistently arises, frequently as the base mass spectral peak (Fig. 1), from the D-ring of 17-hydroxy, 17-methyl steroids (**1**) [5,6] a group of steroids that are predominately synthetic in origin. All the steroidal sources of this unique ion, however, have yet to be established. Thus, the ion at *m*/*z* 143 has served as a (imperfect) biomarker for the presence of synthetic steroids: abnormal and intense chromatographic peak(s) in the *m*/*z* 143 extracted ion chromatogram of a urine sample are typically subjected to further investigation. An example of the utility of this practice is illustrated in Fig. 2. A better understanding of the origin, structure, and mechanism of formation of this ion may provide a greater insight into its utility as a urinary biomarker of synthetic steroid use.



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2. Materials and methods

2.1. Materials

Ethylestrenol (4-estren-17α-ethyl-17β-ol), dromostanolone (5α-androstan-2α-methyl-17β-ol-3-one), mestanolone (5α-androstan-17α-methyl-17β-ol-3-one), and dihydrotestosterone (DHT) (5α-androstan-17β-ol-3-one) were obtained from Steraloids (Newport, RI, USA). Epimetendiol (17β-methyl-5β-androst-1-ene-3α,17α-diol), 3′-hydroxystanozolol (3′,17β-dihydroxy-17α-methyl-5α-androstan [3,2-c] pyrazole), 13β,17α-diethyl-3α-17β-dihydroxy-5α-gonane, and [16,16,17-$^2H_3$]-DHT (DHT-d3) were obtained from the National Analytical Reference Laboratory (New South Wales, Australia). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and bis(trimethylsilyl)acetamide (BSA) were purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). Ammonium iodide and ethanethiol were acquired from Sigma (St. Louis, MO, USA). Bis(trimethylsilyl)acetamide-d18 (BSA-d18) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Perfluorokerosene-H (PFK) was obtained from Lancaster Synthesis Inc. (Pelham, NH, USA).

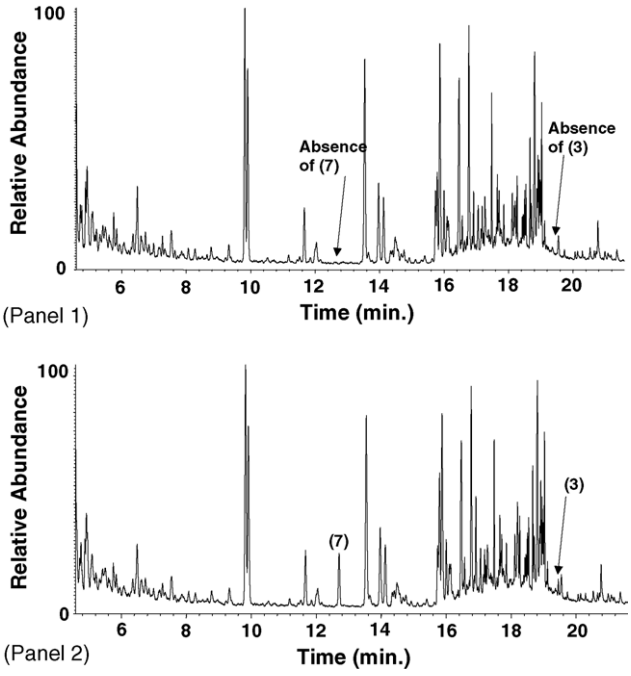

Fig. 2. Extracted ion chromatograms of *m*/*z* 143 from urine samples analyzed by GC/MS. Panel 1 shows a chromatogram from a blank urine sample. Panel 2 shows a chromatogram from a urine sample spiked with 20 ng/ml of epimetendiol (**7**) and 3′-hydroxystanozolol (**3**). Samples were prepared and extracted as described elsewhere [21], but derivatized with MSTFA/ammonium iodide/ethanethiol (1000:2:4, v/w/v), and analyzed by GC/MS as described in Section 2.

2.2. Sample preparation

All steroid samples derivatized with MSTFA were prepared at a final concentration of 300 ng/μl in MSTFA/NH_4I/ethanethiol (1000:2:4, v/w/v) by heating at 75 °C for 30 min. The NH_4I and ethanethiol ensure near-complete keto-enol trimethylsilylation (at the low risk of ethyl thio-incorporation into silylated steroid structures) [7,8], and therefore eliminates the need for methyl oxime derivatives. Following derivatiza-

[1] There are no published reports of this practice, however, it is a well known, well established practice within the anti-doping community.