Detection and metabolic investigations of a novel designer steroid: 3-chloro-17α-methyl-5α-androstan-17β-ol

SHORT TITLE: ANALYSIS OF 3-CHLORO-17α-METHYL-5α-ANDROSTAN-17β-OL

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Abstract

In 2012, seized capsules containing white powder were analyzed to show the presence of unknown steroid-related compounds. Subsequent GC-MS and NMR investigations identified a mixture of 3α- and 3β- isomers of the novel compound; 3-chloro-17α-methyl-5α-androstan-17β-ol. Synthesis of authentic reference materials followed by comparison of NMR, GC-MS and GC-MS-MS data confirmed the finding of a new "designer" steroid. Furthermore, *in vitro* androgen bioassays showed potent activity highlighting the potential for doping using this steroid. Owing to the potential toxicity of the halogenated steroid, *in vitro* metabolic investigations of 3α-chloro-17α-methyl-5α-androstan-17β-ol using equine and human *S9* microsomal liver fractions were performed. For equine, GC-MS-MS analysis identified the diagnostic 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol metabolite. For human, the 17α-methyl-5α-androstane-3α,17β-diol metabolite was found. Results from these studies were used to verify the ability of GC-MS-MS precursor-ion scanning techniques to support untargeted detection strategies for designer steroids in anti-doping analyses.

Keywords

Anti-doping, Designer steroids, Androgen bioassay, Steroid metabolism, Gas Chromatography-tandem Mass Spectrometry.

Introduction

The use of androgens with the aim of increasing performance presents a serious threat to the integrity of animal and human sports, and they are therefore prohibited for use by the International Federation of Horseracing Authorities[1] and World Anti-Doping Agency[2], respectively. Until recently, most abused androgens were marketed synthetic analogs that were approved for therapeutic use for treatment of recognized medical disorders. However, since the turn of the 21st century the illicit production and distribution of novel, never-marketed synthetic androgens selected to evade doping control has been uncovered. Such "designer" steroids feature chemical modifications and may not be detected by existing doping control methods, which rely on knowledge of the chemical structure and metabolism.[3-6] These novel agents are developed using knowledge of structure-activity relationships in the patent literature derived from the thousands of compounds (including androgens) investigated during post-war decades that comprised the "Golden Age" of steroid pharmacology.[7]

The first such designer androgen was norbolethone (13β,17α-diethyl-17β-hydroxygon-4-en-3-one) identified by Catlin *et al.*[8] in 2002. This androgen, synthesized in 1966 and studied clinically but never marketed for anabolic effects, had never been identified in athletes' urine.[8] In 2004, the same group discovered tetrahydrogestrinone (13β,17α-diethyl-17β-hydroxygon-4,9,11-trien-3-one; THG) following analysis of a spent syringe provided anonymously to the United States Anti-Doping Agency (USADA) that had allegedly contained an anabolic steroid undetectable by doping control tests.[9] THG was produced by a facile one-step chemical reduction of gestrinone, a marketed progestin. THG was proven by Death *et al.*[10] to be a highly potent androgen using an *in vitro* androgen bioassay expressing the human androgen receptor linked to a chemical read-out. This provided crucial evidence for the first successful prosecution of THG use in the BALCO scandal. In the absence of toxicological information for THG, Lévesque *et al.*[11] applied *in vitro* studies using human hepatocytes to propose C_{16}- and C_{18}-hydroxylated metabolites as glucuronide conjugates.

Subsequent discovery of desoxymethyltestosterone (17α-methyl-5α-androst-2-en-17β-ol; DMT or Madol)[12] soon after prompted anti-doping laboratories to commit additional resources to the identification of designer androgens,[13-20] with investigations complementing

conventional *in vivo* administration studies with *in vitro* methodologies. Ethical and safety considerations precluded conventional *in vivo* clinical studies as had been used for marketed synthetic androgens. The advantages and limitations of *in vitro* methods specific to equine metabolic studies of anabolic steroids have been described by Wong *et al.*[21]

Concerning the present study, in 2012 red and black capsules that contained white powder were seized by Queensland authorities in Australia and analyzed by forensic chemists to show the presence of unknown steroid-related compounds. We describe herein the identification, synthesis, *in vitro* androgenic bioactivity, *in vitro* metabolic studies using both equine and human liver *S9* fraction, and Gas Chromatography-tandem Mass Spectrometry (GC-MS-MS) methods for detection in routine anti-doping analysis of 3-chloro-17α-methyl-5α-androstan-17β-ol. The workflow presented is proposed as a suitable template for the study of designer steroids in racehorses.

Experimental

Commercial reference materials, chemicals and reagents

Epiandrosterone (3β-hydroxy-5α-androstan-17-one) and androsterone (3α-hydroxy-5α-androstan-17-one) were purchased from Steraloids, Inc. (Newport, RI, USA). d_3-Testosterone was purchased from the National Measurement Institute (Sydney, NSW, Australia). Chemicals and solvents used at the Australian National University for synthesis were purchased from Sigma-Aldrich (Castle Hill, Australia) and used as supplied unless otherwise specified. All general laboratory chemicals and reagents used at the Australian Racing Forensic Laboratory for analysis were of HPLC grade and were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Merck petroleum benzine boiling range 40-60 °C (hexanes) was used as supplied. Dry diethyl ether and tetrahydrofuran (THF) were obtained by distillation from sodium wire as required. Triphenylphosphine was recrystallized from methanol and dried under vacuum before use. *meta*-Chloroperoxybenzoic acid was recrystallized from dichloromethane and dried under vacuum before use. Reactions were monitored by analytical thin layer chromatography (TLC) using Merck Silica gel 60 TLC plates using the specified solvents and were visualized with a solution of concentrated sulfuric acid in methanol (5% v/v) with heating as required. Column chromatography was performed using Merck Silica gel 60 (230-400 mesh) using the specified solvents. *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide was purchased from UCT (Bristol, PA, USA). Ammonium iodide was obtained from BDH (Poole, UK). Water was obtained using a Millipore filtration system (Bedford, MA, USA).

NMR spectroscopy

Analysis of seized material at Griffith University

Spectra were acquired on a Varian 400MHz Unity INOVA spectrometer in d_6-DMSO/$CDCl_3$ solvent at 298K. ^{1}H and $^{13}C\{^{1}H\}$ NMR chemical shifts are referenced to d_6-DMSO solvent residuals, taken as 2.49 ppm and 39.9 ppm respectively. 2D gradient filtered COSY, heteronuclear single quantum correlation (gHSQC) and heteronuclear multiple bond correlations (gHMBC) were acquired using the standard sequences implicit in the VNMRJ 2.1b software package. NOE difference spectra were acquired using the cyclenoe sequence implicit in the VNMRJ software package, with internal data subtraction. Control frequencies were set 1-2 ppm from saturating frequencies where possible. Saturation times were set to 4s,

with mix and tau values both set to 100 ms. 256-800 transients were acquired and 0.5 Hz line broadening functions acquired to the difference spectrum prior to Fourier Transform.

Analysis of synthesized 3α/β-chloro-17a-methyl-5a-androstan-17β-ol at the Australian National University

^{1}H and ^{13}C NMR spectra were recorded using either Varian 400 MHz, Bruker Avance 400 MHz or Bruker Avance 600 MHz spectrometers at 298 K using deuterated chloroform solvent. Data are reported in parts per million (ppm), referenced to residual chloroform or deuterated chloroform solvent respectively (^{1}H: 7.26 ppm, ^{13}C: 77.16 ppm) and multiplicity is assigned as follows: brs = broad singlet, s = singlet, d = doublet, t = triplet, m = multiplet. Coupling constants *J* are reported in Hertz.

GC-MS analysis

Analysis of seized material at Queensland Forensic and Scientific Services

Analysis was performed on an Agilent 5890 GC-MS equipped with Agilent J&W HP5-MS and HP-1 (30m x 0.25mm x 0.25μm) columns. Helium was used as a carrier gas at a constant flow rate of 1.8mL/min. Injection volumes of 1 μL were used in a pulse-splitless mode with an injector temperature of 280 °C. The oven temperature commenced at 65 °C with a hold time of 1 min, followed by a 40°C/min ramp rate to 300 °C and a final hold time of 13 min. Mass spectra were collected over a *m/z* 35-550 scan range with an ionization energy of 70 eV.

Analysis of synthesized 3α/β-chloro-17a-methyl-5a-androstan-17β-ol at the Australian Racing Forensic Laboratory

Stock standards of 200 μg/mL for each isomer were made by dissolving 1 mg of reference material in 5 mL methanol. Aliquots (20 μL) were dried under N_2 at 50 °C before trimethylsilyl (TMS) derivatization using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide/ammonium iodide/dithioerythritol (1000:2:4 v/w/w, 50 μL) at 80 °C for 60 minutes.

An Agilent 6890 GC coupled to a 5973 MS (Palo Alto, CA, USA) was used. The GC column was a Restek Rtx-5Sil-MS (15 m x 0.25 mm x 0.25 μm) with helium as carrier gas. Sample injections (2 μL) were made in splitless mode with an injector temperature of 260 °C. The

column was initially held 180 °C for 0.2 min, then was increased at 5 °C/min to 235 °C, 15 °C/min to 265 °C, then finally 25 °C/min to 300 °C and held for 10 min. Head pressure was programmed to maintain a constant flow rate of 1.2 mL/min. The MS transfer line was set at 300 °C and the ion source was operated in EI+ mode with an ionization energy of 70 eV. Data was acquired in scan mode (*m/z* 50 to 650) using Agilent ChemStation® software.

Purification of seized material

The combined contents of three seized capsules (1.04 g) were extracted in 10 mL of methanol with sonication. The methanol extract was filtered and evaporated to dryness under nitrogen. The residue was taken up in 1 mL of the eluant solution (10% ethyl acetate/n-hexane) and loaded on to a silica gel (Kieselgel 60, 0.2-0.5mm, Merck) column of approximate dimensions 10 cm x 2 cm (id). Fraction collection commenced after the band had progressed five centimeters. A mixture of compounds (**3**) and (**6**) was recovered (approximately 6 mg) from fractions 29-35, which were evaporated under nitrogen.

Synthesis of reference materials

Synthesis of 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5α-androstane-3β,17β-diol used in this study was described previously by McKinney *et al.*[22] Melting points were determined using a SRS Optimelt MPA 100 melting point apparatus and are uncorrected. Optical rotations were determined using a Perkin-Elmer 241MC polarimeter (sodium D line at 25 °C). Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed using positive electron ionization (+EI) on a Micromass VG Autospec mass spectrometer at the Australian National University Mass spectrometry facility. IR spectra were recorded using a Bruker Alpha FT-IR Spectrometer and peaks are reported in wavenumbers (cm^{-1}) and described as follows: br = broad, s = strong, m = medium, w = weak, with assignment of major bands.

Synthesis of 3-chloro-17α-methyl-5α-androstan-17β-ol reference materials

The target 3-chloro-17α-methyl-5α-androstan-17β-ols had not been previously reported in the literature. They could be accessed by methylation of the corresponding known 3-chloro-5α-androstan-17-ones[23] which in turn could be synthesized from the 3-hydroxy-5α-androstan-17-ones by chlorination with inversion of configuration at the reacting center.[24] To this end epiandrosterone (**1**) underwent chlorination to afford 3α-chloro-5α-androstan-17-one (**2**)[23] in 79% yield (Figure 1). Methylation of this compound provided 3α-chloro-17α-methyl-5α-

androstan-17β-ol (**3**) in 56% yield. The identity and stereochemistry of this compound was confirmed by X-ray crystallography (Supporting Information, Figure S10).[25] In a similar manner, androsterone (**4**) underwent chlorination to afford 3β-chloro-5α-androstan-17-one (**5**)[23] in 27% yield followed by methylation to give 3β-chloro-17α-methyl-5α-androstan-17β-ol (**6**) in 32% yield. Detailed synthesis conditions and results are provided in Supporting Information.

Synthesis of 16α-hydroxylated reference materials

The synthesis of the 16α-hydroxylated reference materials was completed as outlined in Figure 2. Formation of the enol acetate (**7**) from ketone (**2**) followed by a sequence involving epoxidation, rearrangement and acetylation afforded the acetoxy-ketone (**8**). Subjecting this material to Grignard reaction with methylmagnesium bromide gave a separable mixture of 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol (**9**) and 3α-chloro-17β-methyl-5α-androstane-16α,17α-diol (**10**) in a 6.5:1 ratio. Detailed synthesis conditions and results are provided in Supporting Information.

In vitro androgen bioassays

The *in vitro* yeast and mammalian (HuH7 and HEK293) cell androgen bioassays have been previously described by Akram *et al.*[27] For the yeast cell androgen bioassay, early to mid-log phase yeast cells were sub-cultured into 24-well plates (500 µL/well) and androgen receptor (AR) expression activated with 1 µM $CuSO_4$. Cells were then treated with steroids (5 µL/well) over a concentration range to generate a sigmoidal dose curve used to calculate the EC_{50} of the steroid response. Treatment continued overnight at 30 °C with vigorous orbital shaking (300 rpm). Yeast cell growth was stalled by ice treatment for 30 min. The optical density (600 nm) was measured then cells were lysed and assayed for β-galactosidase activity using a standard assay. For the mammalian cell androgen bioassay, HuH7 and HEK293 cells were seeded in 96-well plates (1x105 cells/mL) and allowed to recover overnight in phenol red-free DMEM supplemented with 10% charcoal-stripped FCS, L-glutamine and 5.5 µg/mL puromycin. Cells were then treated with steroids over a concentration range to generate a sigmoidal dose response curve. Cells were heated for 35 min at 65 °C to deactivate endogenous alkaline phosphatase before SEAP activity was measured using a commercially available assay (Clontech Laboratories, CA, USA). The EC_{50}

for both the yeast and mammalian cell-based assays was determined from a sigmoidal curve fit using GraphPad.

In-vitro metabolism studies

A solution containing β-NAD (1.5 mM), magnesium chloride (4.5 mM), glucose-6-phosphate (7.5 mM), glucose-6-phosphate dehydrogenase (1 unit), equine or human liver S9 fraction (1 mg protein) and 3α-chloro-17α-methyl-5α-androstan-17β-ol (250 μg) in sodium phosphate buffer (50 mM, pH 7.4, 1 mL) and methanol (2 μL) was incubated with shaking in an open tube at 37 °C for 3 h. The reaction was quenched with acetonitrile (1 mL) and centrifuged (2000 rpm, 5 min). The resulting supernatant solution was decanted and extracted with ethyl acetate (2 x 1 mL) and the organic layer was dried under a stream of nitrogen. Residues were analyzed by GC-MS-MS after formation of 1.) TMS derivatives as outlined above and 2.) acetonide-TMS derivatives to investigate C_{16}-C_{17} stereochemistry using a modified version of the procedure described by Houghton and Dumasia,[28] specifically the reaction of acetone (0.5 mL) and trifluoroacetic acid (50 μL) at 55 °C for 60 min, drying under N_2 at 60 °C, followed by TMS-ether formation. The final extracts were dried under nitrogen at 60 °C before being reconstituted in *n*-dodecane (50 μL) for GC-MS-MS analysis. Control experiments were performed in parallel in the absence of (a) cofactor regeneration (β-NAD, magnesium chloride, glucose-6-phosphate and glucose-6-phosphate dehydrogenase), (b) equine or human liver S9 fraction and (c) steroid (3α-chloro-17α-methyl-5α-androstan-17β-ol).

Isolation of steroids from urine

Urine (3 mL) aliquots were fortified with d_3T (1 μg/mL, 30 μL) as surrogate before adjustment to pH 6 (±0.2) with the addition of 10% HCl. Enzyme hydrolysis was performed using β-glucuronidase K12 from *E.coli* (20 μL) overnight at 37 °C. Urinary steroids were isolated by solid phase extraction using ABS Elut NEXUS® extraction columns (60 mg, 3 cc, Agilent Technologies, Lake Forest, CA, USA) that were conditioned with methanol (3 mL) and water (3 mL) before loading with sample, washed with 0.1 M NaOH (1 mL) and water (3 mL) before elution with methanol (3 mL). The eluates were evaporated to dryness under nitrogen at 60 °C. Equine urine samples were then subjected to chemical hydrolysis with methanolic HCl (1M, 0.5 mL) at 60 °C for 15 minutes. After cooling to room temperature the reaction was quenched with sodium hydroxide (2M, 3 mL) before extraction with di-

isopropylether (4 mL). The tubes were centrifuged at 2000 rpm before the organic supernatant was transferred to a clean test tube and evaporated to dryness under nitrogen at 60 °C. Equine and human steroid extracts were derivatized to form TMS ethers as described above prior to the final extracts being dried under nitrogen at 60 °C and reconstituted in *n*-dodecane (50 μL) for GC-MS-MS analysis.

GC-MS-MS analysis

An Agilent 7000B GC-MS-MS (Palo Alto, CA, USA) was used. The GC column was an Agilent J&W HP5-MS (30 m x 0.25 mm x 0.25 μm) with helium as carrier gas. Sample injections (2 μL) were made in pulsed-splitless mode with an injector temperature of 260 °C. The column was initially held 180 °C for 0.2 min, then was increased at 5 °C/min to 235 °C, 15 °C/min to 265 °C, then finally 25 °C/min to 300 °C and held for 10 min. Head pressure was programmed to maintain a constant flow rate of 1.2 mL/min. The MS transfer line was set at 300°C and the ion source was operated in EI+ mode with an ionization energy of 70 eV. Nitrogen was used for the collision gas at 1.5 mL/min. Instrument control and processing was performed using Agilent MassHunter® software. Mass spectra were acquired in full scan (*m/z* 50-650), product ion scan, multiple reaction monitoring (MRM) and precursor ion scan modes using optimized parameters outlined herein. Qualitative confirmation of identity was performed by comparison between sample and reference standard according to *Association of Official Racing Chemists* criteria.[29]

Results

GC-MS

Analysis of seized material at Queensland Forensic and Scientific Services

A methanol extract of the seized capsules showed two major peaks by GCMS at 7.58 and 7.61 min (Figure 3). Both peaks provided similar mass spectra with a molecular ion at *m/z* 324, however slight differences in relative ion ratios were observed. The mass difference of 34 amu and 3:1 chlorine isotope pattern displayed by these peaks in comparison to an earlier peak at 6.90 min suggested a putative chloromethylandrostanol structure. Based on the chlorine position of previously encountered steroids, such as clostebol and 4-chlorodehydromethyltestosterone (oral turinabol), a 4-chlorinated structure was initially theorized, but chlorine substitution was later shown by NMR to be at the C_3-position. Samples of the synthesized compounds (**3**) and (**6**) were analyzed in parallel with the seized material as TMS derivatives (Figure 4). The molecular ion at *m/z* 396 and the presence of *m/z* 130 and 143 supported the formation of *mono*-O-TMS derivatives and a 17-methyl-17-hydroxy structure,[30-32] respectively.

Analysis of synthesized 3α-chloro-17a-methyl-5a-androstan-17β-ol by the Australian Racing Forensic Laboratory

The full scan spectrum revealed *m/z* 73 (TMS, 20%), 130 (15%), 143 (100%), 264 (3%), 291 ($[M\text{-}CH_3\text{-}OTMS]^+$, 5%), 306 ($[M\text{-}OTMS]^+$, 3%), 381 ($[M\text{-}CH_3]^+$, 8%) and 396 ($[M]^{+\cdot}$, 1%). The presence of chlorine was supported by an accompanying $[M+2]^+$ ion at one-third abundance for *m/z* 291, 306, 381 and 396. The diagnostic fragment ions at *m/z* 130 and 143 result from the well characterized D-ring cleavage described for TMS derivatives of C_{17}-methyl/C_{17}-hydroxylated steroids.[30-32] Comparison to the spectra in Figure 4 provided additional agreement between the seized material and synthesized steroid.

NMR

Analysis of seized material at Griffith University

The purified fraction of the seized material was analyzed using ^1H and ^{13}C NMR spectroscopy. The data were consistent with a 2.5:1 mixture of 3α-chloro-17α-methyl-5α-androstan-17β-ol (**3**) and 3β-chloro-17α-methyl-5α-androstan-17β-ol (**6**).

The 400 MHz ^{1}H NMR spectrum of the mixture (Supporting Information, Figure S1) showed a high degree of complexity, with significant overlapping of resonances, due to both the relatively poor dispersion of the aliphatic resonances at the comparatively low field strength and the presence of the two species in the sample. The structural elucidation of the components of the mixture was therefore based on a number of specific diagnostic observations. Expansions of the 400 MHz ^{1}H NMR spectrum of the mixture focusing on the resonances assigned to the protons attached to the carbon bearing the chloro substituent are provided in Figure 5. Qualitative splitting diagrams for the two resonances are illustrated. Both resonances exhibit four doublet couplings; the major component showing four similar couplings (~4-5Hz), the minor component showing two pairs of similar couplings (~4-5Hz and ~12Hz). This suggested substitution at position C_2 or C_3 (and not C_1 or C_4), as the carbons will be flanked by two methylene units given the observed couplings. NOE, ^{13}C chemical shift and gHMBC data obtained for the mixture, confirmed attachment of the chloro substituents to C_3 in both compounds.

The $^{13}C\{^{1}H\}$ NMR spectrum of the seized material is provided in Supporting Information, Figure S2. 2D gHSQC and gHMBC analysis of the sample facilitated assignment of the relevant ^{13}C chemical shifts (Supporting Information, Figures S3 and S5). Confirmation of the attachment of the chloro substituent to the C_3 carbons of the two compounds was obtained via a series of selective NOE experiments (Supporting Information, Figure S6). Selective saturation of the H_3 proton of (**6**; 3.82 ppm) resulted in enhancement of the mutually axial H_1 and H_5 protons. Partial saturation of the broad resonance of the axial methylene proton of the major compound (**3**; 1.84 ppm) resulted in the expected NOE enhancement of the protons of the C_{19} methyl group, with no observable enhancement of the H_5 proton resonance, confirming the methylene's location at C_2 and thus the attachment of the chloro substituent to C_3 of the A-ring.

The chemical shifts of the protons and the carbons of the C- and D-rings of both compounds showed close correspondence, indicating similar substitution in those rings. The stereochemistry of the C_{17} substituents was established by selective irradiation of the C_{17} hydroxyl protons (Supporting Information, Figure S4). Strong NOE enhancement of the resonances of both the C_{18} and C_{20} methyl groups, as well as enhancement of the adjacent β C_{16} and equatorial C_{12} methylene proton resonances, was observed. Enhancement of both

methyl substituents locates the hydroxyl substituents on the top faces of the molecules (i.e. in the β position).

In vitro androgen bioassays

Results from Yeast, HEK293 and HuH7 androgen receptor bioassays are summarized in Table 1. In the yeast androgen bioassay, compared with testosterone (T) and dihydrotestosterone (DHT) over the range of 10^{-4} M to 10^{-10} M, both isomers displayed androgenic bioactivity with the 3α-chloro-17α-methyl-5α-androstan-17β-ol having a similar potency to T but less than DHT whereas the 3β-chloro-17α-methyl-5α-androstan-17β-ol isomer had low relative potency. No cellular toxicity was observed for either isomer.

HEK293 cells, a human embryonic kidney cell line, express limited levels of steroid metabolizing enzymes. Both isomers displayed androgenic bioactivity with the 3α-chloro-17α-methyl-5α-androstan-17β-ol comparable to T but an order of magnitude more potent than the 3β-chloro-17α-methyl-5α-androstan-17β-ol isomer. Neither isomer showed any cellular toxicity.

HuH7, human liver cell line, cells express steroid metabolizing enzymes at higher levels than HEK293 cells. Both 3α-chloro-17α-methyl-5α-androstan-17β-ol and 3β-chloro-17α-methyl-5α-androstan-17β-ol displayed androgenic bioactivity in similar relative proportions to their bioactivity in the HEK293 cell line.

In-vitro metabolic analysis

A predictive MS strategy, based on results from previous studies investigating equine metabolism of 17α-methyltestosterone and mestanolone,[22,33] and human endogenous steroids,[34] was applied to identify candidate products from incubation with equine and human *S9* fractions that required further investigation by GC-MS-MS. Precursor ions selected for TMS derivatives for *mono-*, *bis-* and *tris*-hydroxylated steroids considered to be potentially formed from 3α-chloro-17α-methyl-5α-androstan-17β-ol starting material are provided in Table 2.

Equine S9 fraction

Product ion experiments targeting *m/z* 484 identified a response at Rt=15.46 min that indicated the 3α-chloro substituent of the parent steroid was conserved. Control experiments showed no presence of this response in the absence of co-factor, equine *S9* liver microsomes or parent steroid. Putative identification of 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol was based on 1.) the presence of *m/z* 231 reported by McKinney *et al.*[22] and Schoene *et al.*[31] to be diagnostic of C_{16}-hydroxylated metabolites of 17α-methyltestosterone, and 2.) unsuccessful formation of the *cis*-acetonide derivative supporting 16α-stereochemistry.[28] The identity of 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol was confirmed by comparison of the equine *S9* fraction to the synthesized reference material (Figure 6). The stereochemistry of C_{16}-hydroxyl and C_{17}-hydroxyl/methyl substituents was verified by performing acetonide-TMS derivative experiments on synthesized 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol and 3α-chloro-17β-methyl-5α-androstane-16α,17α-diol reference materials, with the latter used as a control standard to demonstrate the efficiency of *cis*-acetonide derivative formation.

Human S9 fraction

Product ion experiments targeting *m/z* 450 identified a response at Rt=13.92 min. Control experiments showed no presence of this response in the absence of co-factor, human *S9* liver microsomes or parent steroid. The identity of 17α-methyl-5α-androstane-3α,17β-diol was confirmed by comparison of the human *S9* fraction to a certified reference material and verification of C_3 stereochemistry by comparison to 17α-methyl-5α-androstane-3β,17β-diol (Figure 7). The equine metabolite; 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol was not detected.

Translation to routine doping control

Product ion scan experiments were used to optimize collision energies for MRM transitions corresponding to 3α-chloro-17α-methyl-5α-androstan-17β-ol and 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol that could be included with 17α-methyl-5α-androstane-3α,17β-diol into routine anabolic steroid screening (Table 3). The limit of detection (LOD) for each steroid was determined from triplicate analysis of three authentic blank equine and human urine samples together with steroid spikes (n=3) of the same urine samples at concentrations of 0.1, 0.2, 0.5, 1, 2, 5 and 10 ng/mL.

Extraction recovery was evaluated by comparison of 1 and 10 ng/mL pre- and post-extraction spikes in each of the three equine and human blank urine samples. Relatively poor results were obtained for the three target analytes in equine urine; 3α-chloro-17α-methyl-5α-androstan-17β-ol (10-11%), 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol (30-34%) and 17α-methyl-5α-androstane-3α,17β-diol (21-24%). Recoveries observed from spiking of human urine samples were far superior; 3α-chloro-17α-methyl-5α-androstan-17β-ol (51-56%), 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol (82-85%) and 17α-methyl-5α-androstane-3α,17β-diol (77-81%), indicating acid hydrolysis used in the equine method to be the likely cause.

The estimated LOD of 0.5 ng/mL for the most sensitive transitions of the target analytes provides effective routine screening capability in equine urine. Example chromatograms for 3α-chloro-17α-methyl-5α-androstan-17β-ol and 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol are provided by Figures 8 and 9, respectively. The limit of confirmation, however, is determined by the LOD for the least sensitive of a minimum three MRM transitions required to satisfy AORC criteria.[29] This was estimated at 5 ng/mL for 3α-chloro-17α-methyl-5α-androstan-17β-ol and 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol in equine urine.

GC-MS-MS precursor ion strategies

The work of Thevis and Schänzer[32] has been used to develop "untargeted" steroid analysis methods using precursor ion scanning of fragments arising from diagnostic dissociation mechanisms. In addition to the metabolic and translational foci of the current study, the opportunity was taken to evaluate the application of this detection mode to designer steroids in horses. A strategy based on monitoring *m/z* 143, 218 and 231 was first applied to the analysis of *in vitro* extracts obtained from equine *S9* incubation. This afforded precursor ion scans that displayed high *S/N* (>10) for parent (i.e. unreacted) 3α-chloro-17α-methyl-5α-androstan-17β-ol (*m/z* 143) and 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol (*m/z* 218 and *m/z* 231). These results supported the potential for precursor ion scanning to identify novel products from *in vitro* studies.

In equine urine, however, matrix effects were observed to significantly reduce *S/N* for the target compounds (Table 4) surveyed between 2 ng/mL and 10 ng/mL. The best results were observed for 17α-methyl-5α-androstane-3α,17β-diol by monitoring *m/z* 143. Parent 3α-chloro-17α-methyl-5α-androstan-17β-ol using the same ion was poor, even at 10 ng/mL, thereby requiring further work to evaluate *m/z* 130 as a suitable alternative. Equine metabolite 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol displayed reasonable precursor ion (*m/z* 218) sensitivity at concentrations greater than 5 ng/mL (Figure 10). Inclusion of *m/z* 169 into a precursor ion scan strategy will be important for identifying unknown steroids containing a C_{17}-ketone moiety.[35]

Discussion

The identification of designer steroids by anti-doping laboratories has to date relied on investigative testing of bulk materials seized and submitted by law enforcement agencies or acquired via ad-hoc procurement. The former identified 3α/β-chloro-17α-methyl-5α-androstan-17β-ol using underivatized and TMS full-scan GC-MS, ^{1}H NMR and ^{13}C NMR techniques. These findings were confirmed following underivatized and TMS full-scan GC-MS comparison to synthesized reference materials of both isomers.

Investigations of designer steroids should include an assessment of androgenic bioactivity to assess the likelihood of misuse and to therefore prioritize the investment required to develop detection methods. Using a panel of yeast and mammalian host cell *in vitro* androgen bioassays providing a complementary rapid and sensitive appraisal of androgen activity, it was shown that both 3α-chloro-17α-methyl-5α-androstan-17β-ol and 3β-chloro-17α-methyl-5α-androstan-17β-ol are androgens, with the former having higher intrinsic androgenic activity. In addition, both are likely to be relatively resistant to either activating or inactivating metabolism in the body after ingestion. To explore metabolism effects, both isomers were assessed for bioactivity in the HEK293 and HuH7 androgen bioassays to show expression of steroid metabolizing enzymes, while retaining similar relative potencies and therefore indicating little impact of mammalian cell metabolism. In considering the likely potency of these isomers *in vivo,* other relevant factors including species-specific metabolism (as illustrated by the diagnostic metabolite profiles in horse and man) and circulating half-life, which is largely determined by its binding to sex-hormone binding globulin, need to be assessed but are undefined at present.

Having identified the novel steroid(s) and assessed the potency to support a target likely for misuse, *in vitro* and/or *in vivo* metabolic studies can be performed. Owing to the halogenation of the target steroid in this study, only the former was employed to mitigate any risk to animal and human welfare. This was conducted using the microsomal *S9* fractions for equine and human metabolism of 3α-chloro-17α-methyl-5α-androstan-17β-ol and identified the major metabolites; 3α-chloro-17α-methyl-5α-

androstane-16α,17β-diol and 17α-methyl-5α-androstane-3α,17β-diol, respectively. Conservation of the chlorine substituent highlights the diagnostic potential of the equine metabolite. The discrete equine and human *in vitro* metabolism will require further investigation *in vivo* should the toxicity of 3α-chloro-17α-methyl-5α-androstan-17β-ol prove to be negligible and therefore safe for administration. Indeed, limitations need to be considered in assuming correlation of *in vitro* and *in vivo* experiments.[36] For example, additional hydroxylation may be expected *in vivo*, with the potential to provide longer detection periods. Conceivably, 3α-chloro-17α-methyl-5α-androstan-17β-ol could be metabolized to 17α-methyl-5α-androstan-3α,16α,17β-triol. Furthermore, the excretion of parent steroid *in vivo* cannot be discounted and so has been included in the routine screening method.

While there is merit to the adoption of precursor ion strategies for complementary untargeted detection of "designer" steroids, validation in matrix is imperative to assess performance. The results of this study have highlighted the limitation in sensitivity with precursor ion scanning. Greater sensitivity may be achieved in complex matrices such as equine urine using more conventional selected ion monitoring (SIM) strategies where MS cycle time allows. Further to hardware considerations, the use of differential and *in silico* software applications will likely provide anti-doping laboratories with the capability for *de novo* identification of "designer" steroids in the future.

The importance of high quality organic synthesis should not be underestimated. First, for timely and unambiguous identification of the parent steroid, and second for responsive translation into routine screening of metabolites.

Conclusion

In this study, ^{1}H NMR and ^{13}C NMR experiments provided unequivocal proof of identity and assisted MS interpretation for 3α/β-chloro-17α-methyl-5α-androstan-17β-ol in seized material. To the author's knowledge, the discovery of a C_3-chlorinated steroid presumed to be intended for doping purposes has not been previously reported. Synthesis of 3α-chloro-17α-methyl-5α-androstan-17β-ol enabled an assessment of doping potential using *in vitro* androgen bioassays. With a high potency recorded, GC-MS-MS studies of *in vitro* metabolic extracts produced from incubation of the synthesized steroid with equine *S9* microsomal liver fractions provided evidence of a putative diagnostic equine metabolite (3α-chloro-17α-methyl-5α-androstane-16α,17β-diol) that was subsequently synthesized to confirm its identity. A previously reported metabolite (17α-methyl-5α-androstane-3α,17β-diol) of methyltestosterone and mestanolone was confirmed from *in vitro* experiments with human *S9* microsomal liver fractions. These compounds were incorporated into the routine anabolic steroid screening procedure for equine urine samples.

This research provides an example for anti-doping laboratories to respond to the threat of designer steroids in animal or human sports. The greatest challenge can be to first obtain intelligence as to what illegitimate products may be available and therefore misused. The internet can be useful for this purpose, however, resource limitations preclude comprehensive reviews by the doping control community at any given time. To alleviate this problem, collaboration with forensic drug laboratories performing evidentiary analysis for law-enforcement agencies can provide intelligence of relevant designer drug targets for investigation.

Acknowledgements

Synthesis and *in vitro* metabolic investigations of 3α/β-chloro-17α-methyl-5α-androstan-17β-ol was supported by the Australian Research Council Linkage Grant (LP120200444) *Strategies for the detection of designer steroids in racehorses*. The authors thank Nick Harden, Tony Dunstan and Tony Crocker (Agilent Technologies) for their advice concerning GC-MS-MS applications. Analysis performed at Queensland Health Forensic and Scientific Services was supported by the QHFSS Cabinet Research Fund.

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Table 1: Relative AR potency of 3α-chloro-17α-methyl-5α-androstan-17β-ol and 3β-chloro-17α-methyl-5α-androstan-17β-ol

Cell type	T		DHT		3α		3β	
	EC_{50} (nM)	Relative potency#	EC_{50} (nM)	Relative potency#	EC_{50} (nM)	Relative potency#	EC_{50} (nM)	Relative potency#
Yeast	$4.812E^{-09}$	1	$1.825E^{-09}$	2.64	$3.278E^{-09}$	1.47	$1.283E^{-07}$	0.04
HEK293	$9.274E^{-10}$	1	$6.128E^{-11}$	15.13	$1.07E^{-09}$	0.87	$4.566E^{-08}$	0.02
HuH7	$5.78E^{-08}$	1	$1.141E^{-08}$	5.07	$5.59E^{-08}$	1.03	$6.697E^{-07}$	0.09

to testosterone (T)

Table 2: Predicted precursor ions of TMS steroids from *in vitro S9* experiments

Precursor ion (*m/z*)	Steroid
360	17α-methyl-5α-androst-2-en-17β-ol (Madol)
396	3α-chloro-17α-methyl-5α-androstan-17β-ol
448	17α-methyl-5α-androstan-17β-ol-3-one (mestanolone)
450	17α-methyl-5α-androstane-3α,17β-diol
484	hydroxylated 3α-chloro-17α-methyl-5α-androstan-17β-ol
536	hydroxylated mestanolone
538	hydroxylated 17α-methyl-5α-androstane-3α,17β-diol

Table 3: Optimized MRM parameters for detection of TMS derivatives by GC-MS-MS

Steroid	Retention time (min)	Relative retention	MRM transition(s)	Collision energy (V)	LOD (ng/mL)
D_3-testosterone (INSTD)	14.25	1.000	435 > 420*	10	
3α-chloro-17α-methyl-5α-androstan-17β-ol	14.25	1.000	381 > 291*	15	0.5
			396 > 143	15	5.0
			396 > 381	15	2.0
3β-chloro-17α-methyl-5α-androstan-17β-ol	14.35	1.007	^	^	
3α-chloro-17α-methyl-5α-androstane-16α,17β-diol	15.46	1.085	484 > 218	15	5.0
			484 > 231*	15	0.5
			484 > 469	15	5.0
17α-methyl-5α-androstane-3α,17β-diol	13.93	0.978	435 > 345*	15	0.5
			450 > 143	15	2.0
			450 > 435	15	1.0
17α-methyl-5α-androstane-3β,17β-diol	14.56	1.022	^	^	

* Transition used for quantification

^ As above

Table 4: Detection capability using GC-MS-MS in precursor ion scan mode.

Steroid	*m/z*	Concentration (ng/mL)	Comment
17α-methyl-5α-androstane-3α,17β-diol	143	2	*S/N* < 3:1
		5	4th most intense peak in chromatogram
		10	2nd most intense peak in chromatogram
3α-chloro-17α-methyl-5α-androstan-17β-ol	143	2	Unable to identify due to matrix interference at RT
		5	^
		10	^
3α-chloro-17α-methyl-5α-androstane-16α,17β-diol	218	2	*S/N* < 3:1
		5	8th most intense peak in chromatogram
		10	6th most intense peak in chromatogram

^ As above

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Figure 1: Synthesis of reference materials used in the investigation of 3α/β-chloro-17α-methyl-5α-androstan-17β-ol.

Figure 2: Synthesis of 16α-hydroxylated reference materials.

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Figure 4: Full scan MS of seized compound identified as (**3**; top) and (**6**; bottom) as TMS derivatives.

Figure 5: (a) Resolution enhanced expansion of the region of the ^{1}H NMR spectrum (400MHz, 298K, d_6-DMSO/$CDCl_3$) of the seized material, centered at δ 4.47ppm. All four observed doublet splittings are small (~4-5Hz) and consistent with the values expected for $^{3}J_{eq,ax}$ and $^{3}J_{eq,eq}$ couplings, placing the H3 proton in the equatorial position. (b) An expansion of the region of the same spectrum centered at δ 3.82ppm. The two larger (~12Hz) *trans*-diaxial doublet splittings are highlighted (bold).

Figure 6: Full scan GC-MS spectrum and structure of *bis*-TMS 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol equine metabolite.

Figure 7: Product ion chromatograms (*m/z* 450) of human *S9* fraction (top), *bis*-TMS 17α-methyl-5α-androstane-3α,17β-diol (middle) and 17α-methyl-5α-androstane-3β,17β-diol (bottom).

Figure 8: MRM (381>291) for *mono*-TMS 3α-chloro-17α-methyl-5α-androstan-17β-ol in blank equine urine (top), 1 ng/mL equine urine spike (middle) and synthesized reference material (bottom).

Figure 9: MRM (484>231) for *bis*-TMS 3α-chloro-17α-methyl-5α-androstane-16α,17β-diol in blank equine urine (top), 1 ng/mL equine urine spike (middle) and synthesized reference material (bottom).

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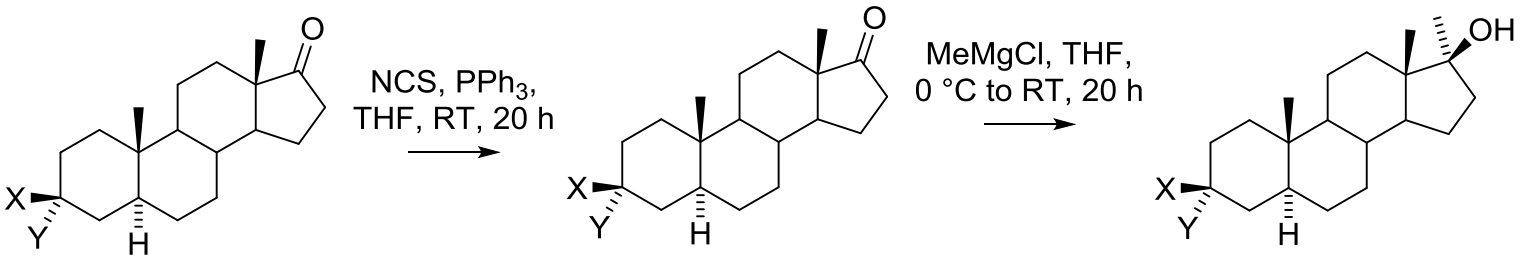

epiandrosterone **1**, X = OH, Y = H
androsterone **4**, X = H, Y = OH

2, X = H, Y = Cl (79%)
5, X = Cl, Y = H (27%)

3, X = H, Y = Cl (56%)
6, X = Cl, Y = H (32%)

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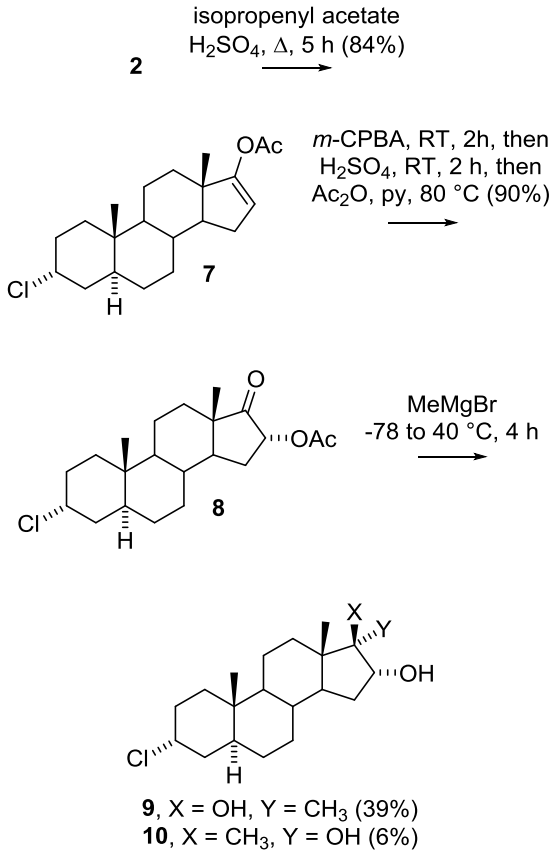

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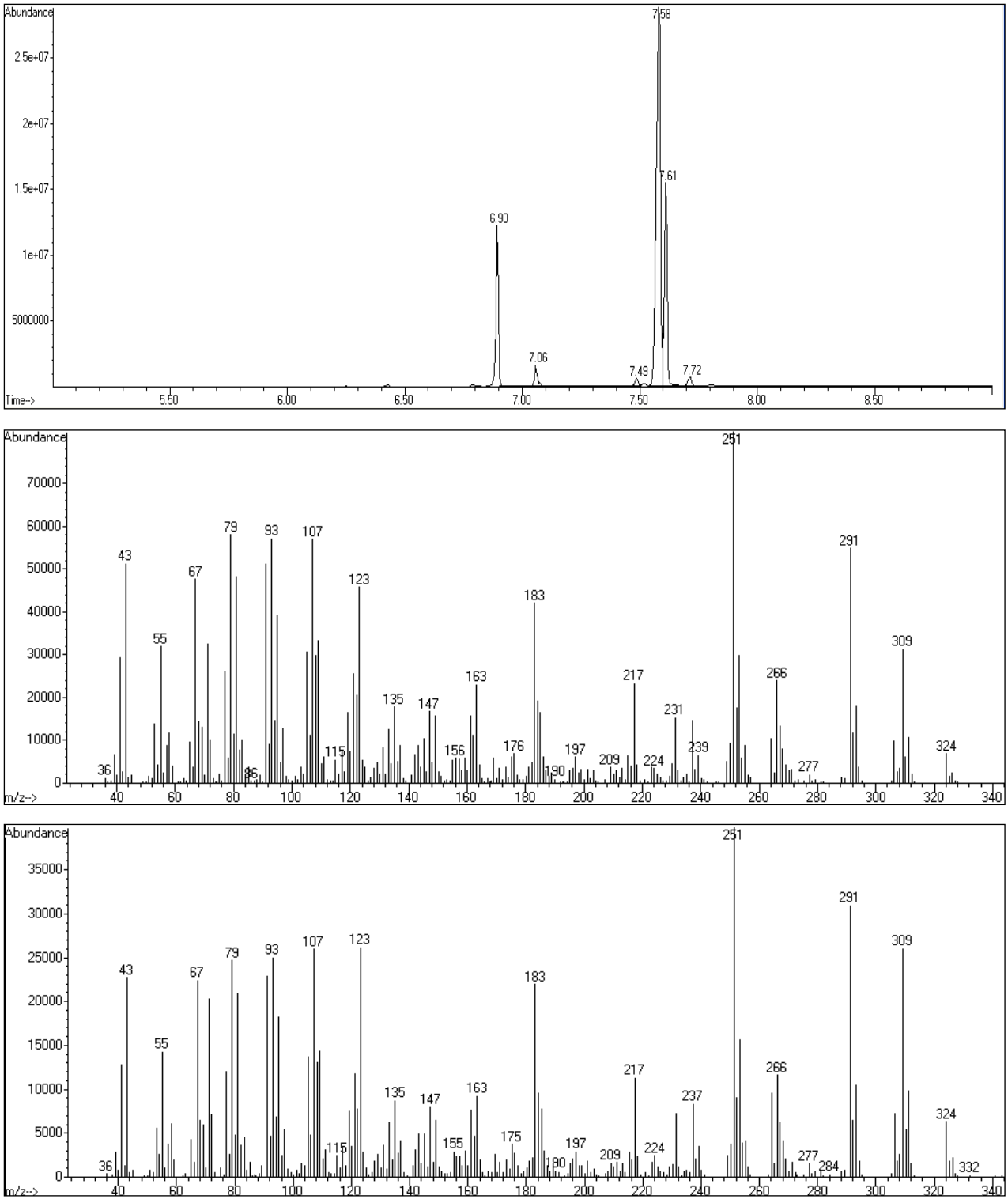

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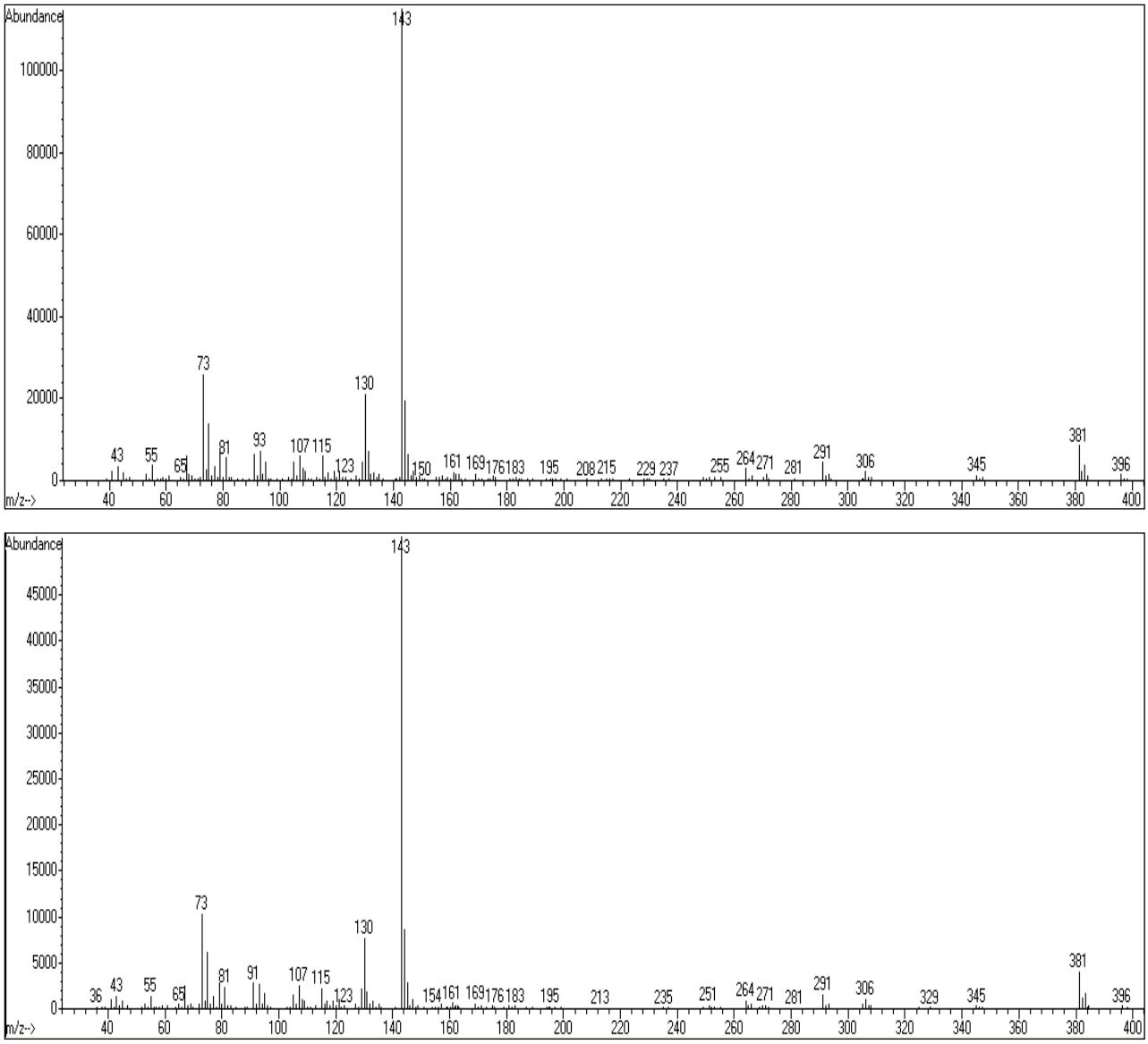

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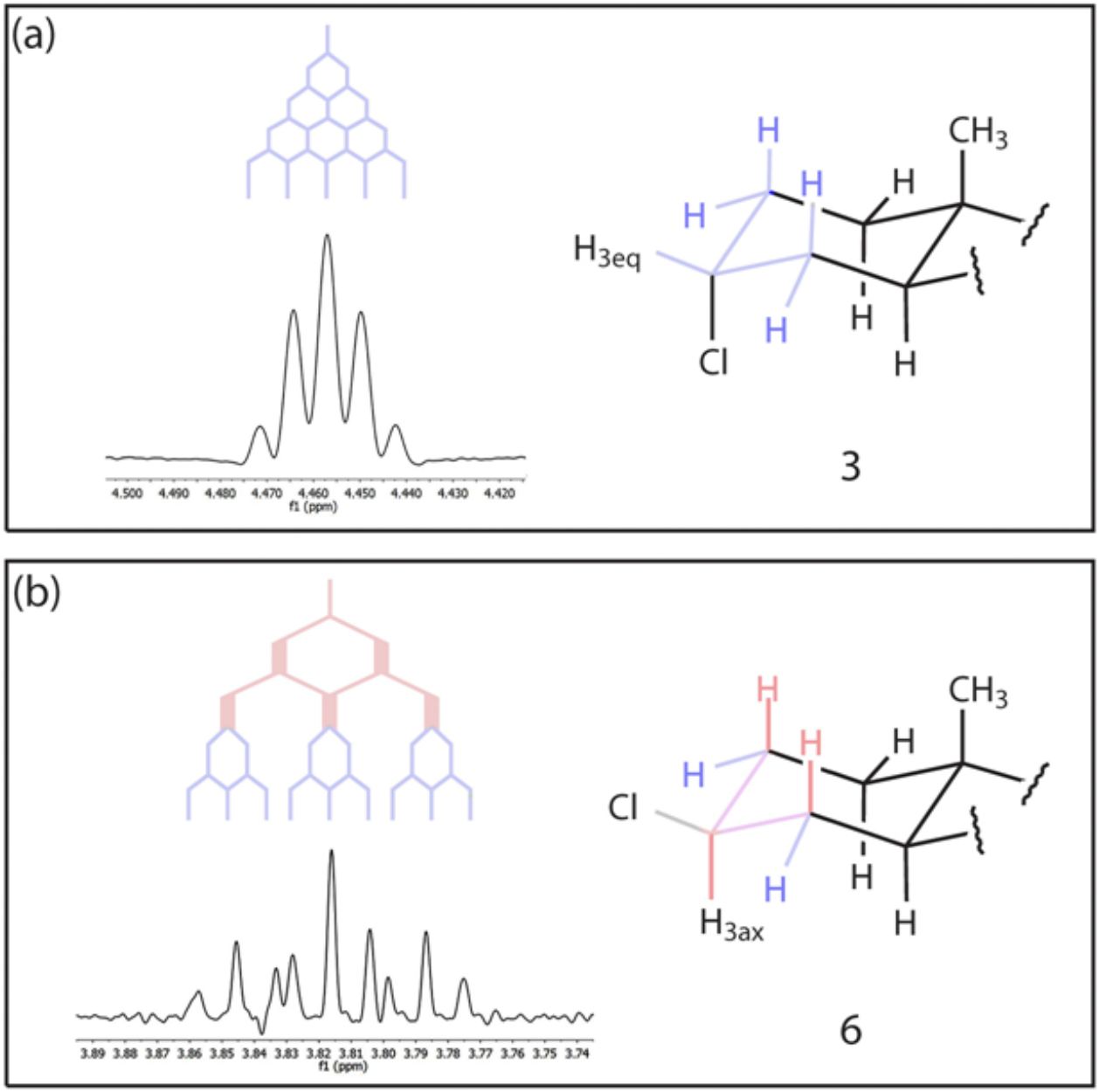

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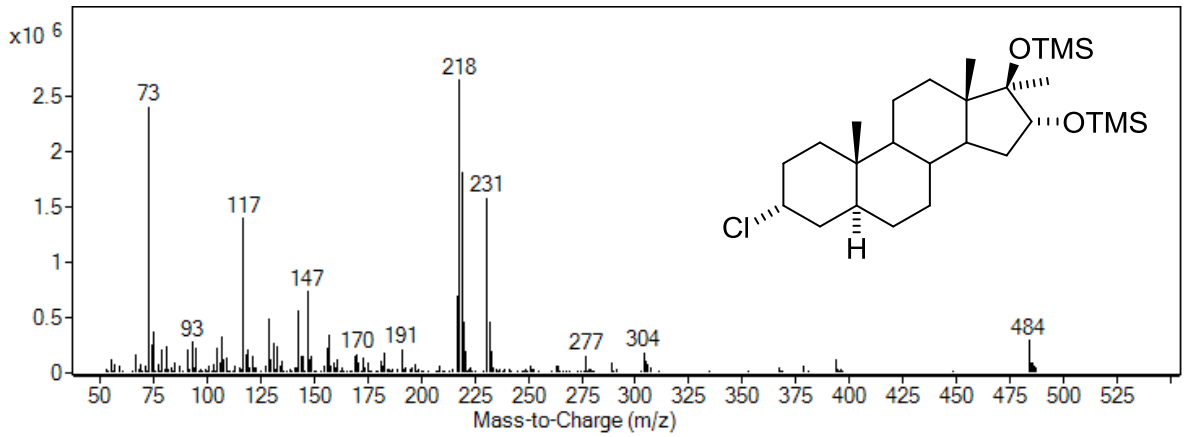

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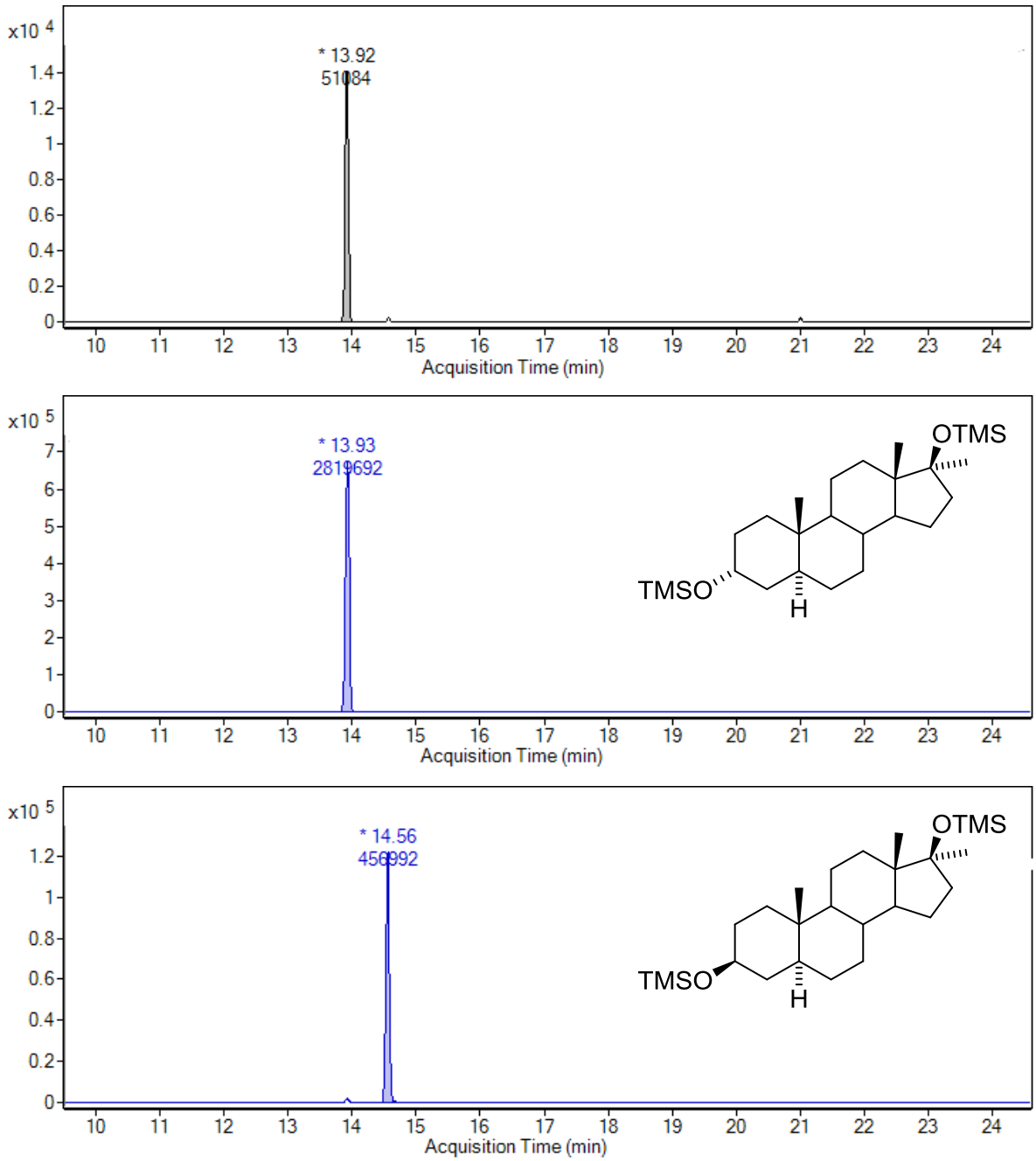

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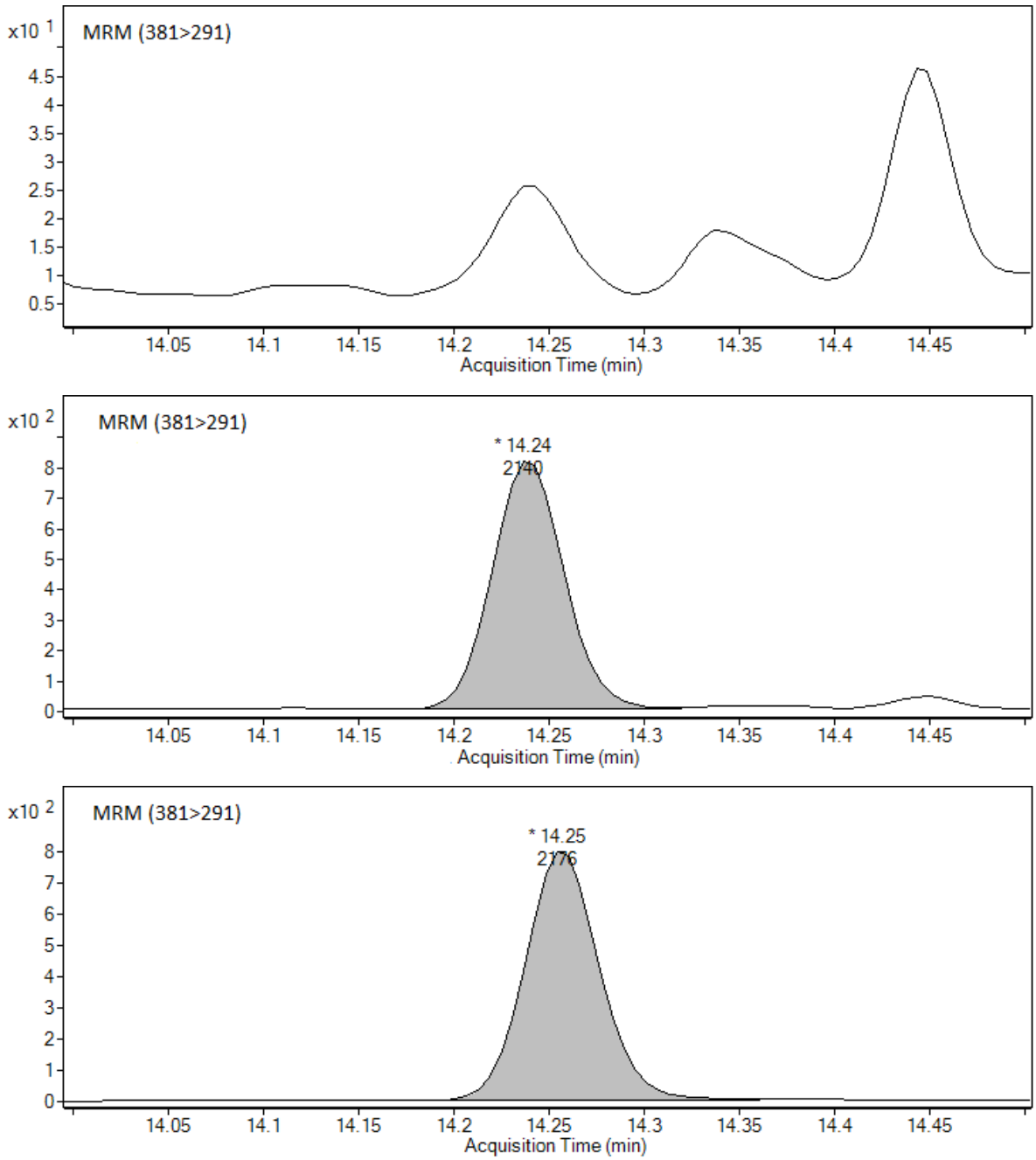

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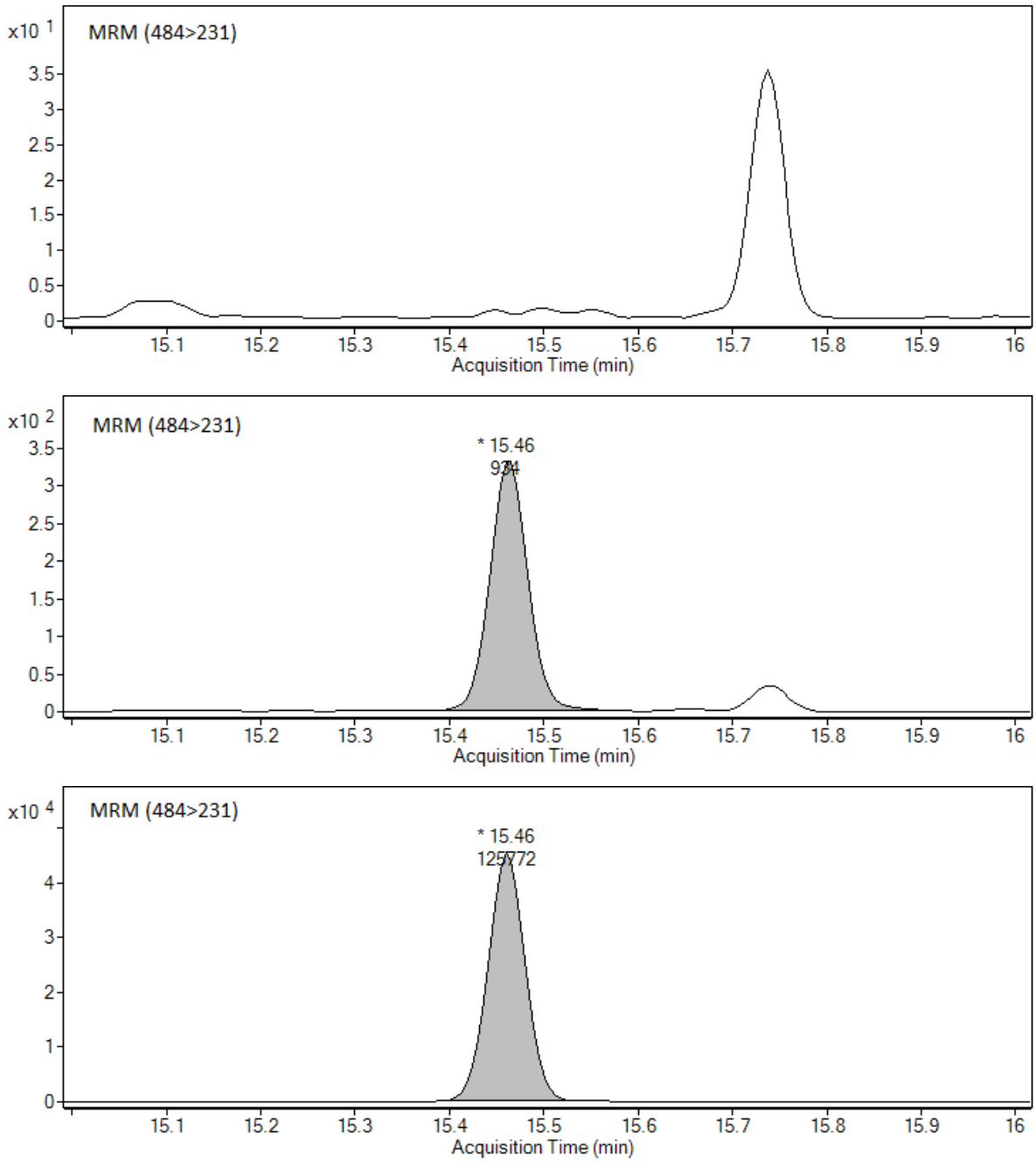

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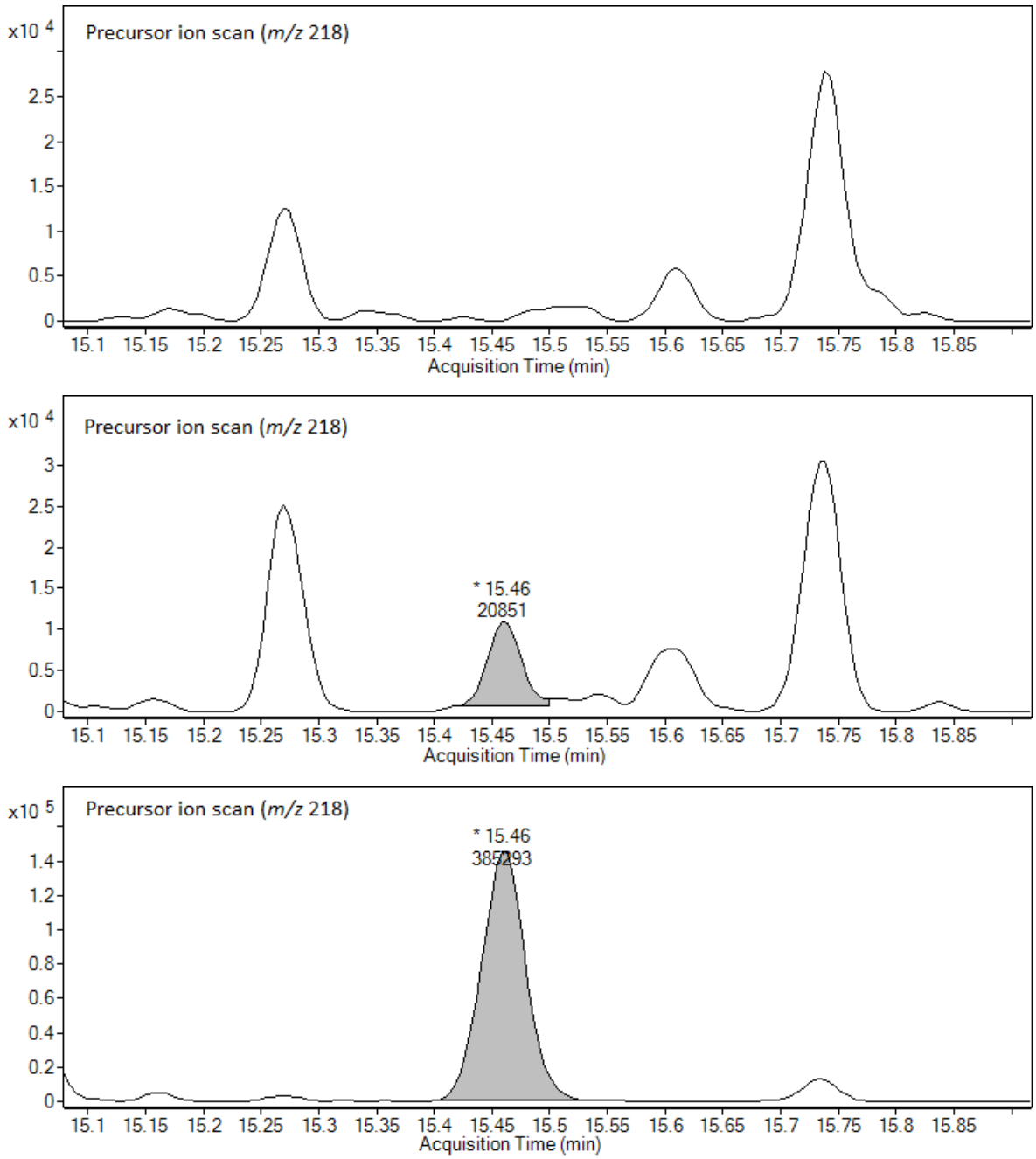

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