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Establishment of a library of fragments for the rapid and reliable determination of anabolic steroids by liquid chromatography-quadrupole time of flight-mass spectrometry

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Abstract: Anabolic steroids have similar structures to testosterone, both of which promote the growth of muscle mass and increase strength. However, the side effects of anabolic steroid use may lead to heart attacks or strokes. Additionally, the excessive use of steroids inhibits the production of the sex hormones in the body via a negative feedback loop, which results in testicular atrophy in males and amenorrhea in females. Currently, the method of choice used to test for the presence of anabolic steroids is GC-MS. However, GC-MS methods require chemical derivatization of the steroid sample to ensure compatibility with the analytical method; therefore, analysis of many different samples is difficult and time consuming. Unlike GC-MS, the liquid chromatography-quadrupole-time of flight mass spectrometry (LC-Q-TOF-MS) method is suitable for many samples. Twenty-two different anabolic steroids were analyzed by LC-Q-TOF-MS with various collision energies (CE). Accurate mass spectral data were obtained using a Q-TOF-MS equipped with an electro-spray ionization source and operated in the positive MS/MS mode for several classes of steroids that are often the targets of testing. Based on the collected data, fragmentation pathways were carefully elucidated. The high selectivity and sensitivity of the LC-Q-TOF-MS instrument combined with these fragmentation pathways offers a new approach for the rapid and accurate screening of anabolic steroids. The obtained data from the 22 different anabolic steroids will be shared with the scientific community in order to establish a library to aid in the screening of illegal anabolic steroids.

Key words: LC-Q-TOF-MS, anabolic steroids, fragmentation, library, dietary supplement

1. Introduction

Anabolic steroids promote the growth of muscle mass and increase strength. They have a similar structure to testosterone, which likewise increases muscle mass development at a much faster rate.

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Unfortunately, the side effects of anabolic steroid use may lead to heart attacks or strokes.[1-3] Additionally, the excessive use of steroids inhibits the production of the sex hormones in the body via a negative feedback loop, which results in testicular atrophy in males and amenorrhea in females. Thus, anabolic steroids are banned by most sporting organizations including the International Olympic Committee (IOC).[4]

The screening and confirmation of anabolic steroids in human urine has been performed by Gas chromatography-Mass spectrometry (GC-MS). However, GC-MS requires chemical derivatization in order to ensure that the steroid samples are compatible for GC analysis, resulting in difficult and time-consuming procedures. Anabolic steroids that have similar structures can be analyzed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) with high selectivity and sensitivity.[6-8] The LC-MS/MS method has only been used for target analysis and has been restricted to a limited number of compounds with known molecular weights; non-target compounds are rarely analyzed using this method. On the other hand, the LC-Q-TOF-MS method has been used for the analysis of unknown, non-target compounds. A high resolution Q-TOF mass spectrometer to quickly and easily analyze samples, determine whether target compounds are present, and at what concentration. Q-TOF MS has strong potential for detection and identification purposes as a consequence of the full-spectrum acquisition with satisfactory sensitivity allowing accurate-mass measurements of the analyte molecule and/or its main fragments, the ability of performing retrospective analysis without the need of additional sample injections, and the feasibility of investigating a large number of contaminants after MS acquisition using a post-target approach.[9] Triple quadrupole instruments, also known as tandem mass spectrometers, are commonly used for steroid analysis. The development of analytical procedures for the determination of anabolic steroids in doped health supplements and functional foods has always been a challenge. The advantage of the time of flight-mass spectrometry (TOF-MS) method for screening is the ability to examine the data for an unlimited number of compounds and the direct availability of the molecular formula of an analyte, from the accurate molecular mass and isotope peak pattern. Analyses of compounds based on TOF-MS spectra have been reported.[10-12] Precursor ion scanning to establish the protonated ion $[M+H]^+$ peak and molecular formula acquisition were carried out in full-scan mode using LC-Q-TOF-MS. Because of the high sensitivity, high resolution, and accurate mass determination, LC-Q-TOF-MS method is a useful analytical tool for the screening of health supplements and functional foods for the presence of illegal anabolic steroids. In addition, the most abused steroids and their derivatives are more suited to analysis than LC-MS/MS, owing to the availability of full-scan mode, which allows for the detection of non-target analytes of unknown molecular weights. Another advantage is the comprehensive data collection, as LC-Q-TOF-MS produces accurate collision-induced dissociation (CID) fragment spectra for all essential components of the sample after isolation of the corresponding parent ions by Q-TOF-MS. In addition, the spectra obtained using the LC-Q-TOF-MS are not disturbed by the matrix and co-eluting substances. Q-TOF-MS/MS experiments are highly useful for elucidating the structures of unknown compounds and are an excellent way to confirm potential positives revealed by TOF-MS.

In this study, we analyzed 22 kinds of synthetic chemicals derived from testosterone (*Fig.* 1) and developed a method for the analysis of illegally doped substances that is desperately needed to build a quick and accurate response system. A user library based on LC-Q-TOF-MS data was obtained by the testing of illegally doped health supplements and functional foods.

2. Experimental

2.1. Standards and reagents

19-Norandrostenedione, 1-androstenedione, bolasterone, boldenone, boldenone (Metabolite, M), boldione, calusterone, clostebol, danazol(M), fluoxymesterone, formebolone(M), methenolone, methandienone(M),

methylnortestosterone, mibolerone, nandrolone, nandrolone(M1), nandrolone(M2), norboletone, norclostebol, norethandrolone, and oral-turinabol(M) were obtained from US Pharmacopeia (Rockville, MD, USA), Sigma-Aldrich Corp. (St. Louis, MO, USA), and steraloids (Newport, RI, USA). Acetonitrile and methanol (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Sigma-Aldrich (St. Louis MO, USA). Deionized water was purified (18.2 m) mΩ using a Milli-Q system (Millipore, USA).

2.2. Preparation of standards and solutions

Individual standard stock solutions (1,000 ppm) were prepared by dissolving each standard in HPLC-grade methanol. The samples were then stored at 4 ºC. Working solutions were prepared from each stock solution by dilution with HPLC-grade methanol.

2.3. LC-Q-TOF-MS analysis

The masses of the anabolic steroids were measured accurately using HPLC (Agilent Technologies, Waldbronn, Germany) coupled with a Q-TOF-MS (Agilent 6530 Accurate-Mass Q-TOF) and equipped with a Jet Stream Technology ESI source (Agilent Technologies, Santa Clara, CA, USA). LC separation was carried out using an Agilent XDB C_{18} column (150 × 2.1 mm, 3.5 μm), which was maintained at 35 ºC in an oven. The mobile phases consisted of distilled water (v/v, A) and ACN (v/v, B) containing 0.1 % formic acid. The gradient elution profile was as follows: 0-3 min (A:B = 80 % : 20 %), 3-13 min (A:B = 80-60 % : 20-40 %) , 13-16 min (A:B = 60 % : 40 %), 16-18 min (A:B = 60-0 % : 40-100 %), 18-21 min (A:B = 0 % : 100 %), 21-22 min (A:B = 0-80 % : 100-20 %), 22-25 min (A:B = 80 % : 20 %). The flow rate of the mobile phases was 0.3 mL/min and the injection volume was 3 μL. The ESI source was operated in the positive ionization mode and the other acquisition conditions for Q-TOF-MS were as follows: gas temperature, 350 ºC; drying gas flow, 8 L/min; nebulizer, 35 psig; sheath gas temperature, 350 ºC; and sheath gas flow, 11 L/min. The scan source parameters were as follows: capillary voltage, 3500 V; nozzle voltage, 1000 V; and fragment voltage, 120 V. The collision energy was set at 15 V and the reference ions for Q-TOF-MS mass calibration were purine (m/z 121.0506, $[M+H]^+$) and HP-921 (hexakis (1H,1H,3H-tetrafluoropropoxy)phosphazine; m/z 922.0098, $[M+H]^+$) (Agilent Technologies, Santa Clara, CA, USA). The mass resolution was 10000-20000 at m/z 100-1000. A hexapole collision cell was used with nitrogen as the collision gas. The data acquisition and processing were conducted using the Mass Hunter Workstation Software (Ver. B.02.01, Agilent Technologies).

3. Results and Discussion

In this study, we analyzed 22 different synthetic compounds derived from testosterone (*Fig*. 1), which all bare the same androgenic core. The anabolic steroids were analyzed by using an LC-Q-TOF-MS instrument equipped with an ESI source operating in the positive ionization mode. The precursor ions of the anabolic steroids were measured in full scan acquisition mode by Q-TOF-MS, which allowed for the detection of the $[M+H]^+$ ions of the analyzed anabolic steroids. To help in the interpretation of the product ion spectra, accurate mass measurements were conducted on the product ions of the anabolic steroids using LC-Q-TOF-MS. Identification of the compounds was based on the retention time, protonated molecular ion, and fragment ion at individually selected collision energies (CE). The CE was set to 5, 10, 15, 20, 25, 30, 35, and 40 eV, and the optimal CE for product ion analysis was determined for each anabolic steroid (*Table* 1). The product ion spectra of the anabolic steroids showed many product ions, suggesting that the precursor $[M+H]^+$ ions underwent fragmentation via multiple competitive pathways. The product ion spectra of the 22 anabolic steroids were studied and compared with their precursor ions. LC-Q-TOF-MS experiments were performed for further confirmation of the steroids detected in the MS/MS data.

Bolasterone and calusterone are epimers. Though these compounds have the same molecular formula,

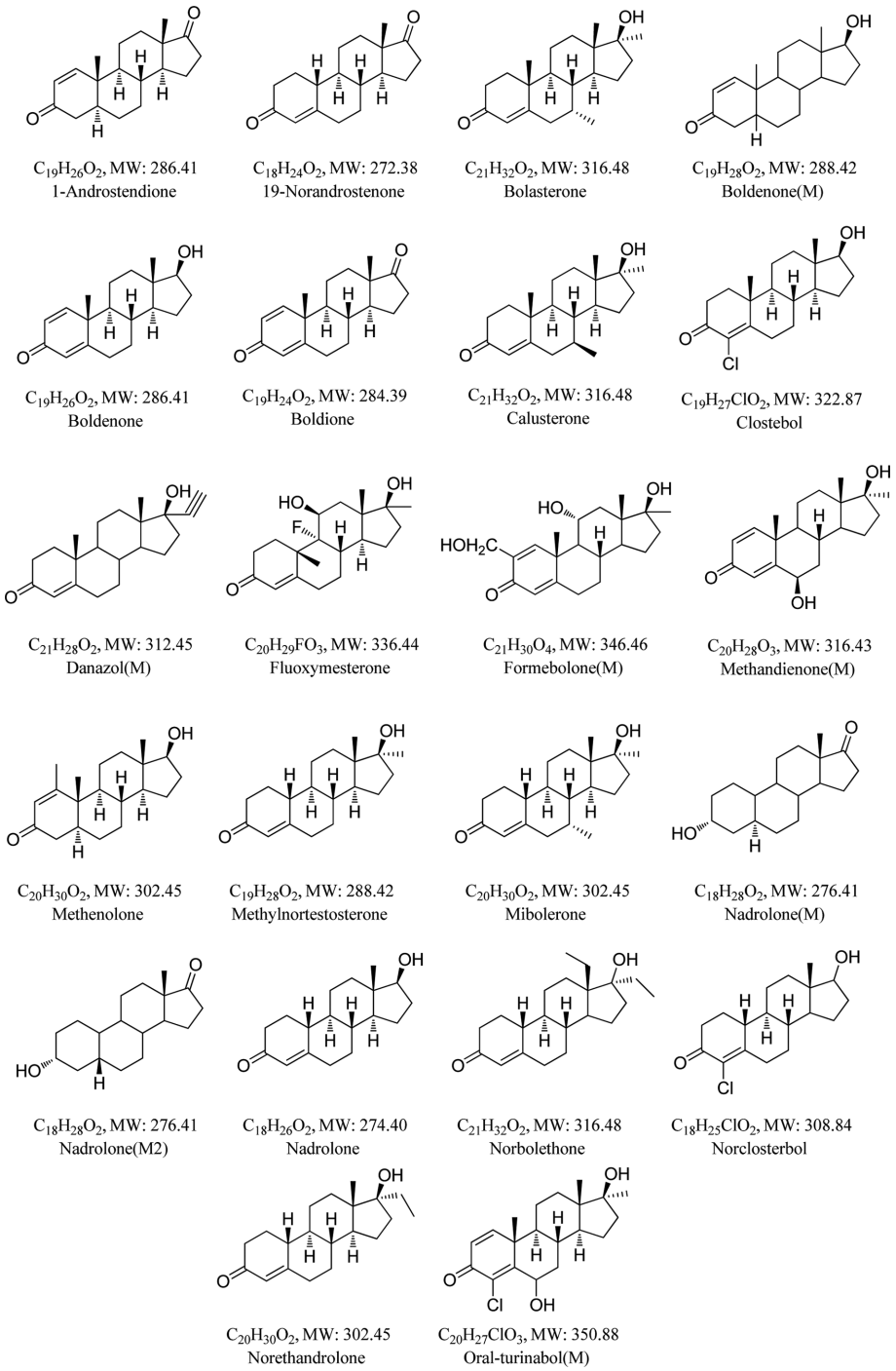

Fig. 1. Chemical structures of the 22 anabolic steroids tested in this study.

the orientation of the methyl group at C-7 is different. The product ions of these compounds were analyzed using LC-Q-TOF-MS analysis. The major ions of bolasterone obtained with a CE of 30 eV correspond

Table 1. LC-Q-TOF-MS result by optimized collision energy of anabolic steroid

No	Compound	RT	Molecular Weight	Exact Mass	Parent Ion	CE (eV)	Product ion
1	1-Androstenedione	13.8	286.41	286.19	287.20	25	251.17, 203.14, 185.13, 143.08
2	19-Norandrostenedione	11.9	272.38	272.18	273.18	25	197.13, 145.10, 109.06
3	Bolasterone	13.2	316.48	316.24	317.25	30	299.23, 281.23, 259.21, 241.19, 191.14, 147.12, 123.08
4	Boldenone(M)	13.0	288.42	288.21	289.22	20	271.21, 253.19, 201.16, 187.15, 121.06
5	Boldenone	10.7	286.41	286.19	287.20	15	269.19, 173.10, 149.13, 135.12, 121.07
6	Boldione	11.6	284.39	284.18	285.18	15	267.17, 151.11, 133.10, 121.07
7	Calusterone	13.6	316.48	316.24	317.25	25	299.25, 241.19, 203.18, 123.08
8	Clostebol	13.8	322.87	322.17	323.18	20	305.17, 269.19, 213.16, 175.15, 143.03
9	Danazol(M)	12.9	312.45	312.21	313.22	20	295.21, 245.19, 199.15, 109.07
10	Fluoxymesterone	10.0	336.44	336.21	337.22	30	317.21, 281.19, 241.16, 223.15, 199.15, 181.10, 131.09
11	Formebolone(M)	7.3	346.46	346.21	347.22	15	329.21, 311.20, 281.19, 173.09, 147.08, 121.10
12	Methandienone(M)	7.2	316.43	316.20	317.21	10	299.20, 281.19, 171.08, 147.08, 121.06
13	Methenolone	12.7	302.45	302.22	303.23	25	267.21, 205.16, 187.15, 145.10, 119.09
14	Methylnortestosterone	11.8	288.42	288.21	289.22	25	271.21, 253.20, 231.17, 213.16, 201.16, 121.10, 109.07
15	Mibolerone	12.5	302.45	302.22	303.23	25	285.22, 267.21, 245.19, 227.18, 177.13, 135.12, 121.10, 107.09
16	Nandrolone(M1)	13.9	276.41	276.21	277.22	10	259.21, 241.20, 185.13, 145.10, 121.10
17	Nandrolone(M2)	13.4	276.41	276.21	277.22	10	259.21, 241.20, 201.17, 159.12, 121.10
18	Nandrolone	11.1	274.40	274.19	275.20	20	257.19, 239.18, 199.15, 145.10
19	Norboletone	15.3	316.48	316.24	317.25	20	299.24, 281.23, 245.20, 227.18, 187.15, 163.11, 135.12
20	Norclostebol	13.2	308.84	308.15	309.16	25	291.15, 273.14, 255.18, 213.16, 187.15, 143.03
21	Norethandrolone	13.8	302.45	302.22	302.23	25	285.22, 267.21, 231.17, 197.13, 173.13, 135.12, 121.10
22	Oral-turinabol(M)	9.4	350.88	350.16	351.17	15	333.15, 315.15, 289.14, 275.11, 259.10, 221.08, 181.05, 155.03, 121.10

to $[M+H-H_2O]^+$, $[M+H-H_2O-C_3H_6O]^+$, $[M+H-H_2O-C_3H_6O-CH_4]^+$, $[M+H-C_{13}H_{22}O]^+$, and $[M+H-C_{13}H_{22}O-O]^+$ at m/z 299.2380, m/z 241.1948, m/z 225.1619, m/z 123.0804 and m/z 107.0855, respectively. The major ions of calusterone obtained with a CE of 25 eV correspond to $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, and $[M+H-H_2O-C_{13}H_{20}]^+$ at m/z 299.2353, m/z 281.2272, and m/z 123.0806, respectively (*Fig.* 2). The different fragmentation patterns allowed for the analysis of what would otherwise be identical compounds as far as mass spectrometry is concerned.

Fragmentation of $[M+H]^+$ ions generated under ESI conditions is generally initiated by a positive charge. For example, the precursor ion of 19-norandrostenedione has an exact mass at m/z 272.1776 and an observed mass at m/z 273.1849 owing to the addition of a proton. The accurate masses obtained for the 22 compounds are for the $[M+H]^+$ ions (*Table* 1). Thereafter, acquisitions in MS/MS mode were carried out with different collision energies to study the CID and fragmentation of the $[M+H]^+$ ion. The possible fragmentation pathways and routes were reviewed and selected in accordance with the following criteria: (1) inductive cleavage occurs at the carbon atom with the most branches or strains; (2) the stability of the transition-state carbonium ions

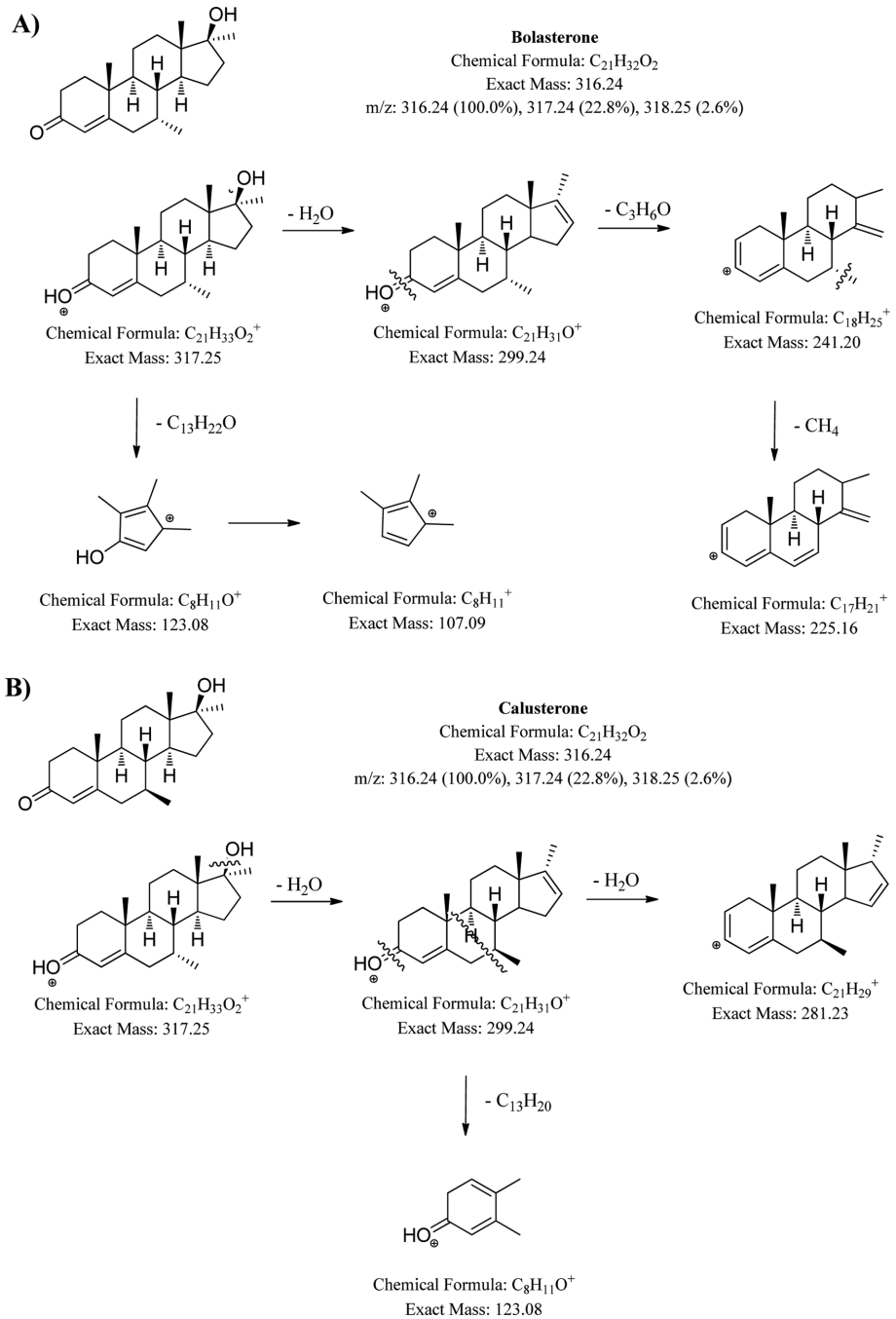

Fig. 2. Fragmentation pathway of A) bolasterone proposed for the generation of the major product ions of m/z 299.24, m/z 241.20, m/z 225.16, m/z 123.0804 and m/z 107.0855 and B) calusterone proposed for the generation of the major product ions of m/z 299.24, m/z 281.23, and m/z 123.08.

follows the order CR_3^+ (tertiary) > CHR_2^+ (secondary) > CH_2R^+ (primary)[13]; (3) formation of a product ion is highly dependent on its stability, which depends on whether a positive charge on the product ion is stabilized by resonance or inductive effects.[14]

Anabolic steroids are structurally similar to

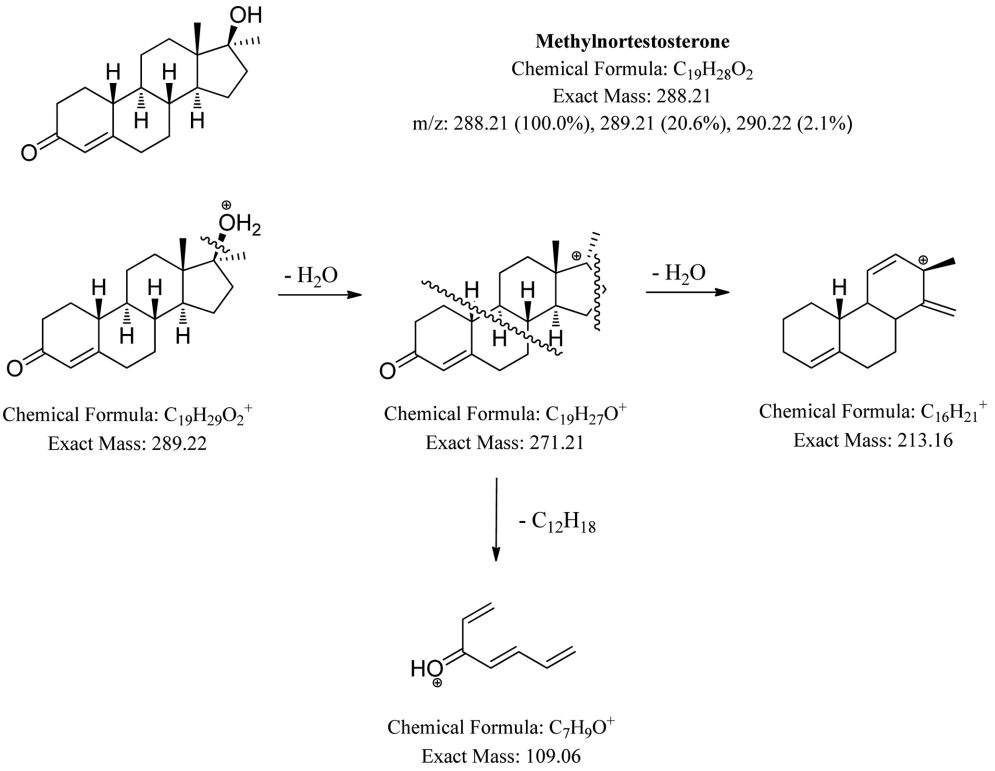

Fig. 3. Fragmentation pathway of methylnortestosterone proposed for the generation of the major product ions of m/z 272.21, m/z 213.16, and m/z 109.06.

testosterone. The generated product ions contain both common ions and characteristic ions. For instance, 19-norandrosterone, danazol(M), methylnortestosterone, nandrolone, norboletone, and norethandrolone have common product ions at m/z 109.06; for example, at CE 25eV, 19-norandrosterone generated an ion at m/z 109.0651. This product ion at m/z 109.0651 was formed by the loss of $C_{11}H_{16}O$ from the 19-norandrostenone $[M+H]^+$ ion. The major product ions of danazol(M) obtained with a CE of 25 eV correspond to $[M+H-H_2O]^+$, and $[M+H-H_2O-C_{14}H_{18}]^+$ at m/z 295.2106 and m/z 109.0652, respectively. The major product ions of methylnortestosterone obtained with a CE of 30 eV correspond to $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, and $[M+H-H_2O-C_{12}H_{18}]^+$ at m/z 271.2050, m/z 213.1646, and m/z 109.0649, respectively (*Fig.* 3). The major product ions of nandrolone obtained with a CE of 25 eV correspond to $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-H_2O-C_7H_{10}]^+$, and $[M+H-C_{11}H_{18}O]^+$ at m/z 257.1906, m/z 239.1799, m/z 145.1014, and m/z 109.0655, respectively. The major product ions of norboletone obtained with a CE of 25 eV correspond to $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-H_2O-C_4H_6]^+$, and $[M+H-C_{14}H_{24}O]^+$ at m/z 299.2397, m/z 281.2378, m/z 245.1916, and m/z 109.0657, respectively. The major product ions of norethandrolone obtained with a CE of 25 eV correspond to $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-2H_2O-C_2H_4]^+$, $[M+H-2H_2O-C_2H_4-C_8H_8]^+$, $[M+H-2H_2O-C_2H_4-C_8H_8-CH_2]^+$, and $[M+H-C_{13}H_{22}O]^+$ at m/z 285.2217, m/z 267.2115, m/z 239.1791, m/z 135.1168, m/z 121.1020, and m/z 109.0658, respectively. For these six anabolic steroids, the suitable energy for MS analysis was CE 25 eV because at this energy all six types produced ions of similar chemical structure and generated the common product ion at m/z 109.06 (*Fig.* 4).

Additionally, boldenone, boldione, and formebolone all have a common product ion at m/z 121.06. The

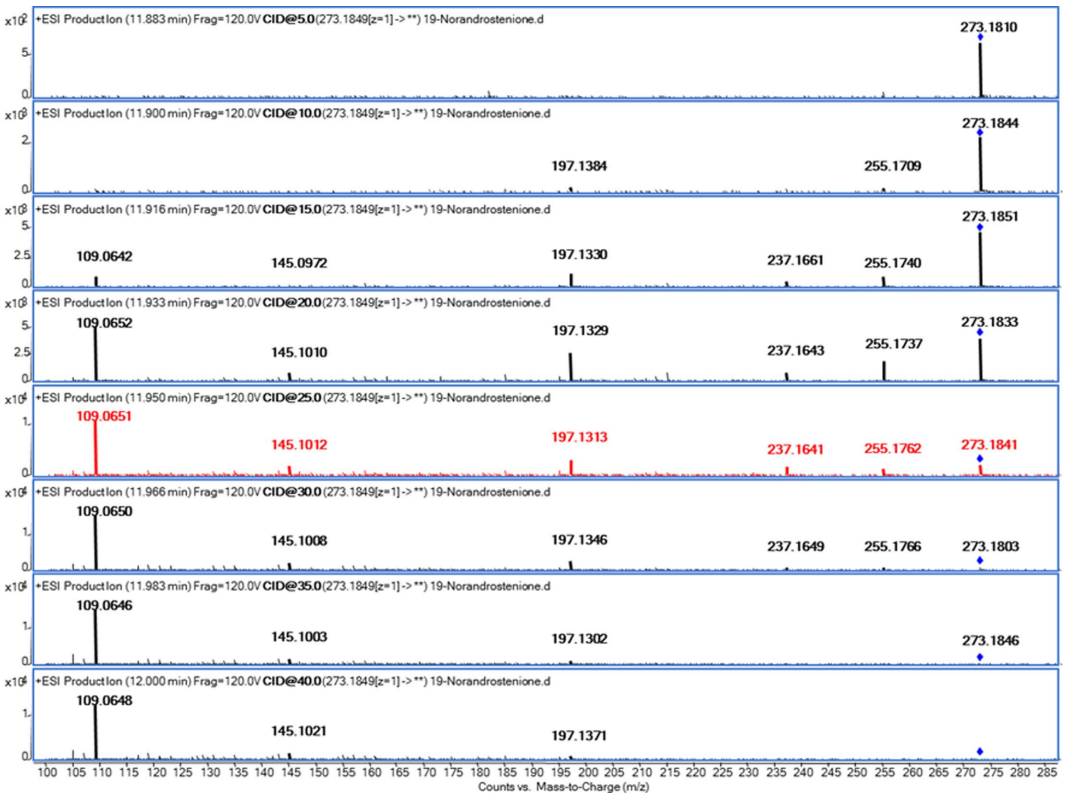

Fig. 4. MS/MS spectra of 19-norandrosterione at various CE level.

major product ions of boldenone obtained with a CE of 15 eV correspond to $[M+H-H_2O]^+$, $[M+H-H_2O-C_9H_{10}O]^+$, and $[M+H-C_{11}H_{18}O]^+$at m/z 269.1929, m/z 135.1164, and m/z 121.0652, respectively. The major product ions of boldione obtained with a CE of 15 eV correspond to $[M+H-H_2O]^+$, $[M+H-H_2O-C_9H_8]^+$, $[M+H-H_2O-C_9H_8-C_2H_6]^+$, and $[M+H-C_{11}H_{12}O]^+$ at m/z 267.1746, m/z 151.1111, m/z 121.0650, m/z 107.0859, respectively (*Fig.* 5). The major product ions of formebolone obtained with a CE of 15 eV correspond to $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-2H_2O-CH_2O]^+$, $[M+H-2H_2O-CH_2O-C_2H_4]^+$, and $[M+H-2H_2O-CH_2O-C_2H_4-C_{10}H_{12}]^+$ at m/z 329.2126, m/z 311.2024, m/z 281.1912, m/z 253.1602, and m/z 121.1008, respectively. For these three anabolic steroids, the suitable energy for MS analysis was CE 15 eV because at this energy all three steroids produced ions having similar chemical structures and generated common product ions at m/z 121.06. Of the remaining anabolic steroids, three were analyzed at CE 10 eV, one at CE 15 eV, three at CE 20 eV, three at CE 25 eV, and three at CE 30 eV.

Analysis using LC-Q-TOF-MS in various CE conditions for structurally similar anabolic steroids produced good MS spectra. These spectral data can be used as a library to elucidate the anabolic steroid structure.

4. Conclusions

Mass spectrometry analysis of 22 different synthetic steroids was performed by the LC-Q-TOF method. For all 22 samples, the parent ion was $[M+H]^+$. Different collision energies were used to collect the mass spectrum of the parent ion. Five different collision energies (10, 15, 20, 25 and 30 eV) were used depending on the parent structure. The retention times of all samples were short, from 7.2 to 13.9

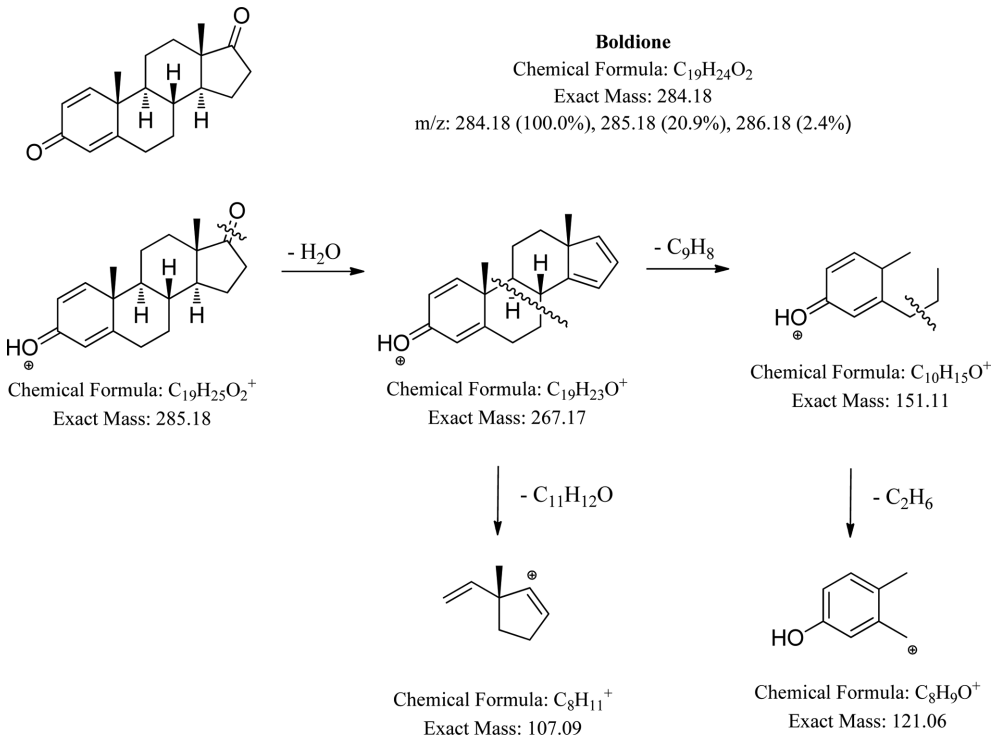

Fig. 5. Fragmentation pathway of boldione proposed for the generation of the major product ions of m/z 267.17, m/z 151.11, m/z 121.06, and m/z 107.09.

min, which corroborates that the analytical conditions and flow rates were suitable for each chemical compound. The obtained data will be shared with the scientific community in order to establish a library to aid in the screening of illegal anabolic steroids.

Acknowledgements

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