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Separation and confirmation of anabolic steroids with quadrupole ion trap tandem mass spectrometry

Larry D. Bowers*, David J. Borts

Athletic Drug Testing and Toxicology Laboratory, Department of Pathology and Laboratory Medicine, Indiana University Medical Center, Medical Science Building A-128, 635 Barnhill Drive, Indianapolis, IN 46202-5120, USA

Abstract

Gas chromatography–mass spectrometry (GC–MS) is the method of choice for separation and detection of anabolic steroids in urine. Recently, there have been advances in the areas of gas chromatography columns, tandem mass spectrometry using ion traps, and large volume sample injection that have promise for lowering detection limits and extending the utility of GC–MS for steroid analysis. In this work, a Varian Saturn III GC–MS system has been used in its tandem mass spectrometry mode to detect low picogram levels of model steroids in standard solution and the urine matrix. Application of MS–MS–MS provided structurally informative spectra for 3′-hydroxystanozolol at concentrations of 1 ng/ml. In addition, four polysilphenylene–polydimethylsiloxane capillary columns were examined for background and selectivity. The columns had bleed several-fold lower than conventional polysiloxane columns. The columns also exhibited significant differences in selectivity for structurally similar steroids. Finally, a new temperature-programmed split-splitless injector was used to inject as much as 25 μl on column. The resulting limits of detection were 5 pg/ml for norandrosterone.

Keywords: Steroids; Anabolic steroids

1. Introduction

Detection of anabolic steroid misuse continues to be a significant fraction of the workload in athletic drug testing laboratories. Identification of anabolic steroids is a difficult analytical problem due to the large number of potential compounds sought, the similarity of exogenous and endogenous steroid structures and the range of steroid concentrations found in urine. Since the initial reports by Masse et al. [1] and by Donike et al. [2] on the techniques for derivatizing and separating anabolic steroids, many advances in both column technology and mass spectral detectors have occurred. The development of polysilphenylene–polysiloxane bonded phase capillaries would seem to have significant advantages over the first generation polysiloxane capillaries.

Lower limits of detection are desirable for screening and confirmation. Horning and Donike [3] have reported the use of high resolution mass spectrometry (HRMS) to detect lower concentrations of steroid metabolites. We have recently reported on the use of selected ion storage techniques on the quadrupole ion trap (QIT) to improve detection limits in anabolic steroid screening [4]. Both HRMS and MS–MS appear to be well suited for confirmation of presumptive positive results. MS–MS has seen increasing use as an analytical tool [5]. In anabolic steroid testing, de la Torre et al. [6] have reported on a sensitive quadrupole mass filter (QMF) MS–MS

*Corresponding author.

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method for detection of testosterone esters in plasma. The MS–MS fragmentation of trenbolone has been presented by de Boer et al. [7]. In the detection of furans and dioxins in environmental testing, QIT and QMF MS–MS have been compared with HRMS [8]. If the acceptable ion ratio variation is 15% for the HRMS and 25% for the MS–MS, limits of detection for the QMF or QIT are five-fold higher than HRMS when a resolution of 10 000 is used. If the mass resolution is decreased, a decrease in the difference between the techniques is seen. Interestingly, different compounds interfere in the two techniques due to the difference in the mass isolation and collision-induced dissociation (CID) conditions [8]. Although the limits of detection will be a function of the compound analyzed, there would appear to be considerable potential for improving detection limits for anabolic agents with MS–MS in the ion trap. We have investigated the use of QIT MS–MS to lower limits of detection for confirmation of the TMS-enol-TMS ether and *tert.*-butyl-dimethylsilyl derivatives of anabolic steroids. We also report here on a the retention behavior of 37 derivatized steroids on four polysilphenylene–polydimethylsiloxane bonded phase capillary GC columns.

2. Experimental

Hydrocarbon standards (C_{24}–C_{28}; C_{30}–C_{34} in hexane) for determining retention indices were purchased from Alltech (Deerfield, IL, USA) and diluted in hexane prior to injection. 5β-Androst-1-en-17β-methyl-3α,17α-diol, androsta-1,4-diene-2z-hydroxymethyl-17α-methyl-11α,17β-diol-3-one, 6β-hydroxymetandienone, 6β-hydroxydehydrochlorotestosterone, androst-4-en-9α-fluoro-17α-methyl-3α,6β-11β,17β-tetrol, 5β-androstan-7α,17α-dimethyl-3α, 17β-diol and 4-chloroandrost-4-en-3α-ol-17-one were gifts from Professors Donike and Schänzer; and the remainder of the steroid metabolites were purchased from Steraloids (Wilton, NH, USA).

Steroids were extracted from urine using a modification of the method of Donike et al. [2]. Briefly, 3 ml of urine were extracted with SPE, de-conjugated at pH 7.0 using β-glucuronidase from *E. coli* (Boehringer Mannheim, Indianapolis, IN, USA) and extracted with *tert.*-butyl-dimethyl ether. The extract was dried and derivatized with 100 μl of MSTFA–1-propanethiol–NH_4I (1000:3:2, v/v/w) for 15 min at 60°C. The derivatization mixture was injected directly without further treatment. The 1-propanethiol (≥99%, Aldrich, Milwaukee, WI, USA) and N-trimethylsilyltrifluoroacetamide (MSTFA; Sigma, St. Louis, MO, USA) were used as received.

A modification of the method of Anderson and Sjövall [9] was used to prepare *tert.*-butyldimethylsilyl (*t*-BDMS) derivative of tetrahydrobolasterone. Briefly, N-methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide (MTBSTFA; Regis Technologies, Morton Grove, IL, USA) was substituted for MSTFA in the above reaction mixture and allowed to react for 4 h at 60°C. If derivatization of the sterically hindered 17α-hydroxyl group was desired, an additional 100 μl of MSTFA–1-propanethiol–NH_4I (1000:3:2, v/v/w) were added and the reaction continued for 1 h at 60°C.

The GC–MS studies were carried out on a Varian Saturn III equipped with a 3400 GC. The Saturn GC–MS system (Version 5.2 software) was equipped with a Wave~Board and operated using the Ion Trap MS–MS Toolkit for Windows (Version 1.0) software. Injections were made with a Model 8200 autosampler into a Model 1078 temperature programmable split/splitless injector. The injector was operated in the splitless mode, unless otherwise indicated. The split vent was closed for the first 0.7 min after injection. The injection rate was 0.2 μl/s. Polysilphenylene–polydimethylsiloxane bonded phase capillaries contained approximately 5% phenyl (DB-5ms; 30 m×0.25 mm I.D., 0.25 μm d_f; J&W Scientific or BPX5; 30 m×0.22 mm I.D., 0.25 μm d_f; SGE), 12% phenyl (XLB; 30 m×0.25 mm I.D., 0.25 μm d_f; J&W Scientific) and 35% phenyl (BPX35; 30 m×0.22 mm I.D., 0.25 μm d_f; SGE). A DB-1 polydimethylsiloxane capillary column (30 m×0.25 mm, 0.1 μm d_f; J&W Scientific) was also used. The He carrier gas flow-rate was adjusted to 1 ml/min at 250°C. The GC temperature program began at 170°C; ramped to 260°C at 20°C/min; ramped to 305°C at 2.7°C/min; and finally ramped to 320°C at 20°C/min.

Calibration of the mass axis was performed using perfluorotributylamine (PFTBA). The ion trap was operated in either the non-resonant or resonant MS–MS mode. The multiplier voltage was set at 100 V

over autotune parameters. Optimal CID conditions were evaluated using the Automatic Method Development feature that allowed different parameters to be applied during each successive scan. The optimal parameters were then programmed into an ion preparation method (IPM). When multiple compounds were detected in a GC run, the IPMs were time programmed to isolate and fragment the appropriate ions. The most important parameters for optimal CID were the RF storage voltage and the CID voltage. Specific conditions are given in the figure legends.

For large volume injections, the Model 1078 temperature programmed injector was operated in the splitless mode. The liner was 0.5 mm I.D. containing a small plug of glass wool. The split flow was 50 ml/min. The initial injector temperature was 130°C. The injection rate was 1.7 μl/s. After a 0.85-min hold, the injector temperature was ramped to 310°C at 180°C/min. The split vent was open for 0.85 min, closed until 2.35 min, then re-opened for the duration of the run. The column temperature program was 150°C for the first 2.35 min, ramped to 260°C at 20°C/min, then to 305°C at 2.7°C/min and held at 305°C for 5 min.

3. Results and discussion

3.1. Evaluation of polysilphenylene–polydimethylsiloxane columns

We hypothesized that inclusion of the phenyl moieties of the stationary phase in the backbone would increase the stereoselectivity of the phase. There are five areas of isomerization on steroids that require sufficient selectivity from the stationary phase. Separation of the epimers of the 17-hydroxyl group, exemplified by testosterone and epitestosterone, is crucial. Steroids with stereochemical differences at the ring junctures, such as androsterone (5α) and etiocholanolone (5β), or in methyl substituent orientation, such as bolasterone (7α-methyl) and calusterone (7β-methyl), must also be separated. Epimers at the 3-hydroxyl position, illustrated by androsterone and epiandrosterone, should also be separated. In addition to natural epimers, the preparation of TMS-enol-TMS ether derivatives creates isobaric structures, such as 5α-androstandione and androst-4-en-17α-ol-3-one (epitestosterone), that differ by the position of the double bond(s) within the ring structure and require separation. We have included representative examples from each of these epimeric sets in the group of model compounds.

Retention indices for 37 anabolic steroids and their metabolites on the four polysilphenylene–polysiloxane columns and the dimethylpolysiloxane column are given in Table 1. Although three of the columns have a common backbone, significant differences in both retentivity and in column bleed were observed. The DB-5ms showed the expected pattern of *m/z* 207, 73 (23%), 147 (6%), 281 (14%) and other cyclosiloxane ions up to *m/z* 625, although at a level significantly less than a standard DB-5 column. The XLB column had about two-fold less bleed than the DB-5ms, based on the total ion current, with *m/z* 147 as the dominant ion below 270°C. Above 270°C, the *m/z* 207 and 147 ion currents were about equal, with ions through *m/z* 625. The BPX35 showed the least bleed at 270°C, with a total ion current of 320 counts, nearly ten-fold less than the XLB column. Even more interesting was the fact that the bleed above *m/z* 300 was very low. This has obvious advantages on the QIT, which experiences space-charging effects when an excessive number of ions is stored. It should be noted, however, that the major background observed for urine extracts contained ions that appear to originate from trace amounts of steroids and other matrix components.

The DB-5ms and XLB columns were able to resolve the 3α/3β-hydroxyl and 17α/17β-hydroxyl functions, but were unable to resolve 5α/5β-androstane epimers such as androsterone/etiocholanolone or 5α/5β-androstan-3β,17β-diol. This has been observed on 5% phenyl–polydimethylsiloxane columns for androsterone/etiocholanolone [10]. Interestingly, both the DB-5ms and XLB columns were able to separate 5α/5β-estrane epimers, norandrosterone and noretiocholanolone. The DB-5ms column was able to resolve the 5α/5β pair 11β-hydroxyandrosterone/11β-hydroxyetiocholanolone, while the XLB column was not. The XLB column was able to separate boldenone from androstendione and epitestosterone from androstandione with a resolution of 0.9.

Despite its common backbone, the BPX35 column

Table 1
Temperature programmed retention indices for selected trimethylsilyl derivatives of anabolic steroids

Steroids		Retention index				
		DB-1	BPX5	DB5ms	XLB	BPX35
Norandrosterone	5α-Estran-3α-ol-17-one	2462	2460	2530	2492	2513
Metandienone metabolite	5β-Androst-1-en-17β-methyl-3α,17α-diol	2476	2497	2562	2512	2565
Boldenone metabolite	5β-Androst-1-en-17β-ol-3-one	2474	2493	2562	2518	2563
Noretiocholanolone	5β-Estran-3α-ol-17-one	2510	2518	2587	2547	2578
Epiandrosterone	5α-Androstan-3β-ol-17-one	2535	2531	2617	2560	2582
Norepiandrosterone	5α-Estran-3β-ol-17-one	2535	2531	2601	2572	2594
Etiocholanolone	5β-Androstan-3α-ol-17-one	2548	2556	2622	2579	2610
Androsterone	5α-Androstan-3α-ol-17-one	2545	2548	2601	2581	2603
11-Ketoandrosterone	5α-Androstan-3α-ol-11,17-dione	2611	2569	2647	2587	2605
Androstanediol	5α-Androstan-3α,17β-diol	2567	2569	2637	2606	2607
Epitestosterone precursor	Androst-5-en-3β,17α-diol	2593	2593	2661	2623	2647
Dehydroepiandrosterone	Androst-5-en-3β-ol-17-one	2625	2622	2686	2656	2695
Androstenediol	Androst-5-en-3β,17β-diol	2643	2637	2703	2676	2696
Methylandrostanediol	5α-Androstan-17α-methyl-3α,17β-diol	2643	2653	2718	2678	2704
Methyltestosterone metabolite	5β-Androstan-17α-methyl-3α,17β-diol	2643	2653	2719	2678	2701
Epitestosterone	Androst-4-en-17α-ol-3-one	2645	2652	2718	2685	2738
5α-Androstanedione	5α-Androstan-3,17-dione	2645	2649	2713	2691	2734
11β-Hydroxyandrosterone	5α-Androstan-3α,11β-diol-17-one	2696	2662	2738	2696	2680
11β-Hydroxyetiocholanolone	5β-Androstan-3α,11β-diol-17-one	2696	2672	2747	2696	2687
Stanolone	5α-Androstan-17β-ol-3-one	2667	2667	2733	2712	2736
17-Epimethandienone	Androst-1,4-dien-17β-methyl-17α-ol-3-one	2668	2684	2751	2715	2769
Boldenone	Androst-1,4-dien-17β-ol-3-one	2678	2684	2746	2724	2783
Androstenedione	Androst-4-en-3,17-dione	2678	2684	2751	2731	2783
Testosterone	Androst-4-en-17β-ol-3-one	2696	2699	2764	2743	2786
Bolasterone metabolite	5β-Androstan-7α,17α-dimethyl-3α,17β-diol	2725	2725	2792	2753	2775
Clostebol metabolite	Androst-4-en-4-chloro-3α-ol-17-one	2720	2736	2798	2784	2845
Zeranol	2,4-Dihydroxy-6-[6α,10-dihydroxy-*trans*-1-undecenylbenzoic acid] μ-lactone	2842	2821	2896	2831	2928
Methyltestosterone	Androst-4-en-17α-methyl-17β-ol-3-one	2791	2797	--	2842	2883
Methenolone	5α-androst-1-en-1-methyl-17β-ol-3-one	2797	2841	2893	2899	3020
6β-Hydroxymetandienone	Androst-1,4-dien-17α-methyl-6β,17β-diol-3-one	2915	2876	2951	2964	2935
Fluoxymesterone metabolite	Androst-4-en-9α-fluoro-17α-methyl-3α,6β,11β,17β-tetraol	2858	2785	2968	2787	2778
Oxymesterone	Anrost-4-en-17α-methyl-4,17β-diol-3-one	2984	2951	–	2986	2999
Dehydrochlorotestosterone	Androst-1,4-dien-17α-methyl-4-chloro-17β-ol-3-one	2977	2990	–	3047	3113
Formebelone metabolite	Androst-1,4-dien-2z-hydroxymethyl-17α-methyl-11α,17β-diol-3-one	3096	3027	–	3049	3067
Danazol	2,4-Pregnadien-(2,3-d)isoxazol-17α-ethynyl-17β-ol	3111	3132	–	3195	3261
3′-Hydroxystanozolol	5α-Androstan-17α-methyl-3′,17β-diol-3,2c-pyrazol	3221	3207	–	3234	3264
4-Hydroxystanozolol	5α-Androstan-17α-methyl-4β,17β-diol-3,2c-pyrazol	3233	3216	–	3242	3278

showed significantly different selectivity. Most of the 3α/3β-hydroxyl,17α/17β-hydroxyl, 5α/5β-androstane and 5α/5β-estrane epimers were separated. Unfortunately, neither epitestosterone, 5α-androstandione and 5α-dihydrotestosterone nor boldenone and androstenedione were resolved on this column.

It appears that a factor other than simply the polymer backbone affects the partitioning of the steroid into the stationary phase. There appeared to be more commonality among the three J&W columns than between the polysilphenylene columns. None of the five columns tested provided resolution of all of the steroids. Other than the decreased bleed associated with the polysilphenylene columns, there appeared to be no significant advantages for steroid analysis. It should be stated that all of the columns were not exhaustively optimized with respect to the temperature program. Nevertheless, from the peak shifts observed on two of the columns, it is unlikely that further optimization would have resulted in resolution of significant numbers of steroid derivatives.

3.2. Ion trap GC–MS–MS detection

There are a number of approaches to selectively storing precursor ions and fragmenting them through collisional-induced dissociation (CID) on the ion trap [11,12]. The two major forms of CID involve non-resonant or resonant excitation of the precursor ion. Resonant excitation takes advantage of the fact that ions of different mass have specific secular frequencies that depend on operational parameters of the trap. Energy is most efficiently absorbed by an ion when the excitation energy applied to the end cap electrodes is at the same frequency as the secular frequency. Non-ideal performance in ion traps has required that some form of modulation of the secular or excitation frequency be used to achieve best performance. Non-resonant excitation is the result of a broad band waveform excitation of all ions remaining in the trap after the isolation step. We evaluated both resonant and non-resonant excitation of several steroid derivatives. Resonant excitation tended to produce more intense product ions in the higher mass range, but no consistent advantage relative to non-resonant excitation was observed. We thus report both resonant and non-resonant excitation results.

The observed limit of detection in MS–MS depends on a number of factors including intensity of the precursor ion, CID efficiency, fragmentation efficiency and collection efficiency [13]. Johnson et al. [14] reported a 15-fold advantage of an ion trap over a quadrupole mass filter when all of these parameters were considered, due in large measure to the collection efficiency of the ion trap. Thomson and co-workers [13] were able to achieve similar efficiency with a quadrupole mass filter when a "high pressure" collision cell design was used. The RF storage voltage and CID voltage are critical parameters in ion trap MS–MS operation. If the RF

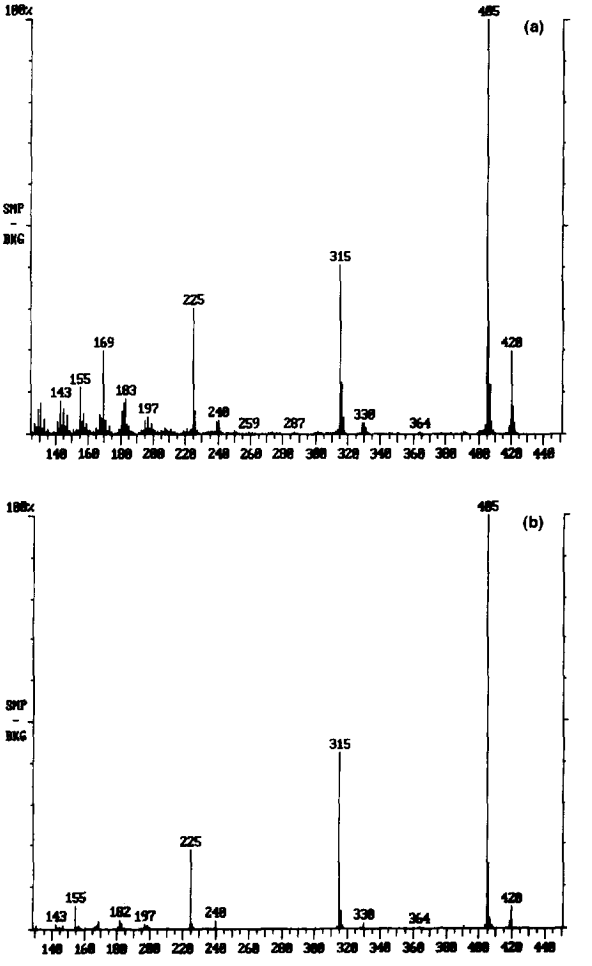

Fig. 1. MS (a) and MS–MS (b) spectra of norandrosterone. (Precursor, m/z 420; CID RF storage: 90 m/z; Non-resonant CID amplitude 45 V; Target 5000.)

storage voltage is too low, energetic precursor and/or product ions are lost from the trap. Conversely, higher RF storage levels limit the low mass limit that can be acquired.

We chose several anabolic steroids as model compounds for these studies based on their incidence of use and their structural features, which relate to their fragmentation. Nandrolone is metabolized to norandrosterone and noretiocholanaolone [15]. The main features of both the MS and MS–MS spectra of norandrosterone are the loss of methyl and TMS groups (Fig. 1). The limit of detection, defined as five times the signal-to-noise ratio, for norandrosterone standards was 1 pg on-column. The result for nandrolone metabolites from a urine matrix is shown in Fig. 2. A positive urine sample containing 26 ng/ml norandrosterone and 4 ng/ml noretiocholanolone respectively, as determined by GC–MS, was diluted 10-fold with normal urine prior to extraction. As shown in the figure, the limits of detection for norandrosterone can be estimated at 20 pg on-column, which corresponds to 0.7 ng/ml using our present sample preparation method. Norepiandrosterone was not detected by GC–MS or GC–MS–MS.

One approach to decrease detection limits further is to inject a larger fraction of the derivatization mixture onto the column. Using the temperature programmed splitless injector function, we were able to inject 25 μl of derivatization mixture onto the capillary column. When 1 ng/μl concentration was injected, the expected 25-fold increase in signal was observed. As the concentration of steroid was decreased, a diminution of the enhancement was observed. We postulate that this was due to adsorptive loss in the injection system. Nevertheless, using a 25-μl injection volume, a detection limit of 100 fg/μl was achieved. This would correspond to a concentration of about 5 pg/ml of urine if 3 ml were extracted, concentrated and derivatized in 100 μl of reagent.

The mass spectrum of the fluoxymesterone metab-

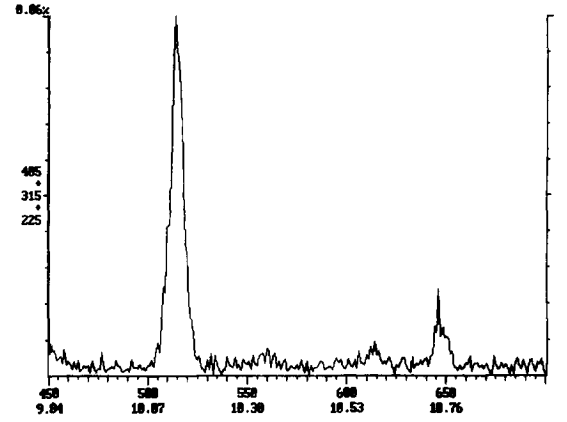

Fig. 2. GC–MS–MS analysis of a urine sample positive for norandrosterone (10.15 min) and noretiocholanolone (10.74 min). Approximately 80 pg of norandrosterone and 13 pg of noretiocholanolone were injected on-column.

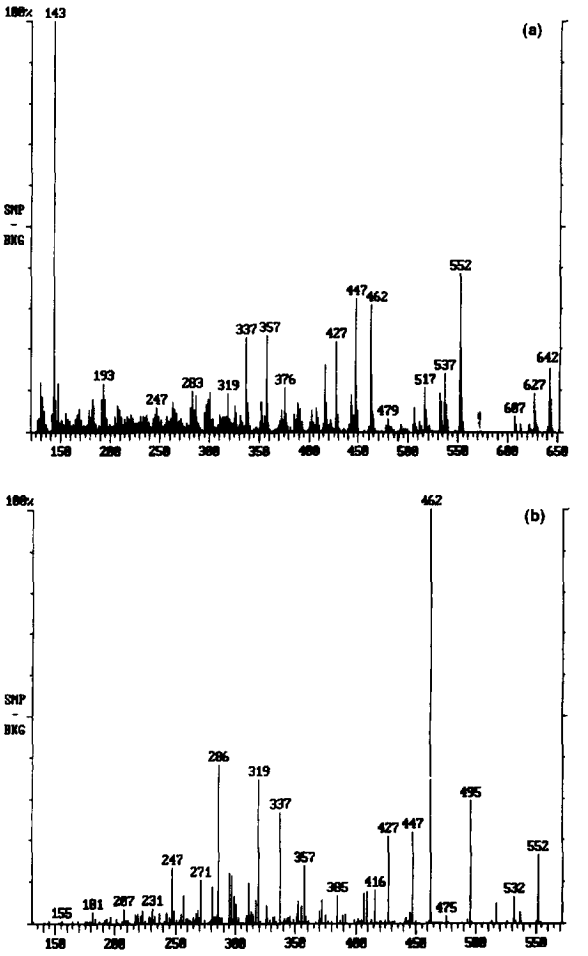

Fig. 3. MS (a) and MS–MS (b) spectra of the tetra-TMS derivative of androst-4-en-9α-fluoro-17α-methyl-3α,6β,11β,17β-tetrol. (Precursor, *m/z* 552; CID RF storage: 120 *m/z*; Non-resonant CID amplitude 76 V; Target 2000.)

olite androst-4-en-9α-fluoro-17α-methyl-3α,6β,11β, 17β-tetraol is shown in Fig. 3a. Main features are loss of methyl and TMS groups and HF from the derivative. The *m/z* 552 ion was chosen as the precursor ion due to its intensity, although there are a large number of ions at relatively high mass. A similar pattern is observed in the MS–MS spectrum (Fig. 3b). Using methanolic solutions and derivatization as reported above, 50 pg on-column could be detected with a signal-to-noise ratio of five-to-one. A urine sample found to contain fluoxymesterone metabolites was analyzed by GC–MS–MS (Fig. 4). The major peak corresponds to the tetra-TMS derivative of androst-4-en-9α-fluoro-17α-methyl-3α,6β,11β, 17β-tetraol. The minor peak at 14.12 min was found to contain all of the ions in the major metabolite. Since no standard material was available to verify its identity, we can only propose that it is an isomer of androst-4-en-9α-fluoro-17α-methyl-3α,6β,11β,17β-tetrol.

For most of the TMS-enol-TMS ether derivatives of steroids we have studied, structurally similar steroids usually fragment under similar CID conditions which allows detection of multiple metabolites with a single set of CID conditions. The isomeric steroids 4-hydroxystanozolol and 3′-hydroxystanozolol are a noteworthy exception. As shown in Fig. 5, 4-hydroxystanozolol readily undergoes CID fragmentation at a resonant CID energy of 0.4 V to yield a number of product ions. Under the same conditions, 3′-hydroxystanozolol produces only a single ion due to the loss of a methyl group (Fig. 6, top). The lack of fragmentation of 3′-hydroxystanozolol, even under rather extreme CID conditions, might be considered diagnostic for this isomer. Thus, rather than considering several ion ratios as identification criteria for a compound, the lack of fragmentation would be an identifying characteristic. Stanozolol and its metabolites also contain a pyrazol ring that should be amenable to chemical ionization. The uniqueness of chemical ionization in a steroid nucleus should prove to be quite selective. We are presently investigating the use of CI–MS–MS to confirm stanozolol. Furazabol and oxandrolone also contain oxygen functional groups that might be amenable to chemical ionization.

The ability of the QIT to perform MS–MS–MS was explored to obtain a spectrum of 3′-hydroxystanozolol. The isolation and storage of a product ion from an initial CID experiment in the QIT are readily accomplished, and application of a second CID voltage waveform can result in fragmentation of the product ion (e.g., *m/z* 545) to yield a second generation spectrum. In the case of 3′-hydroxystanozolol, the MS–MS–MS spectrum (Fig. 6, bot-

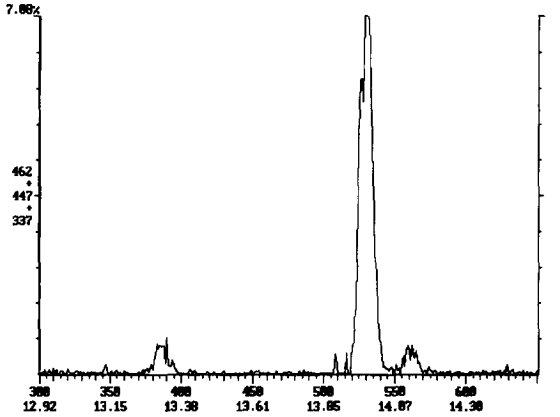

Fig. 4. GC–MS–MS analysis of a urine sample positive for fluoxymesterone. The major peak is the tetra-TMS derivative of androst-4-en-9α-fluoro-17α-methyl-3α,6β,11β,17β-tetrol. Note the presence of a peak at 14.12 min. MS–MS spectrum is consistent with ions generated by fluoxymesterone metabolite, although the structure is not known.

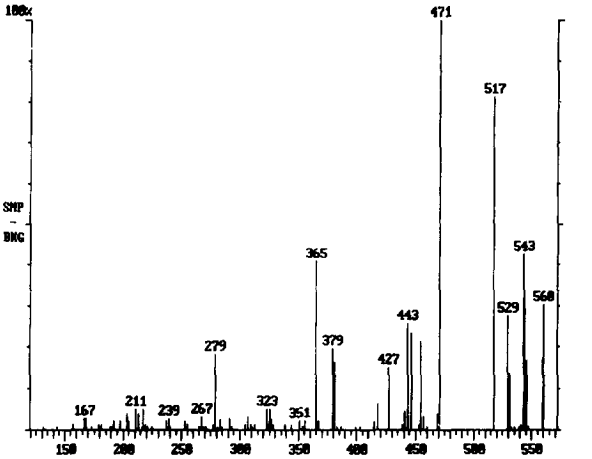

Fig. 5. MS–MS spectrum of 4-OH-stanozolol. (Precursor, *m/z* 560; CID RF storage: 200 *m/z*; Resonant CID amplitude 0.4 V; Target 5000.)

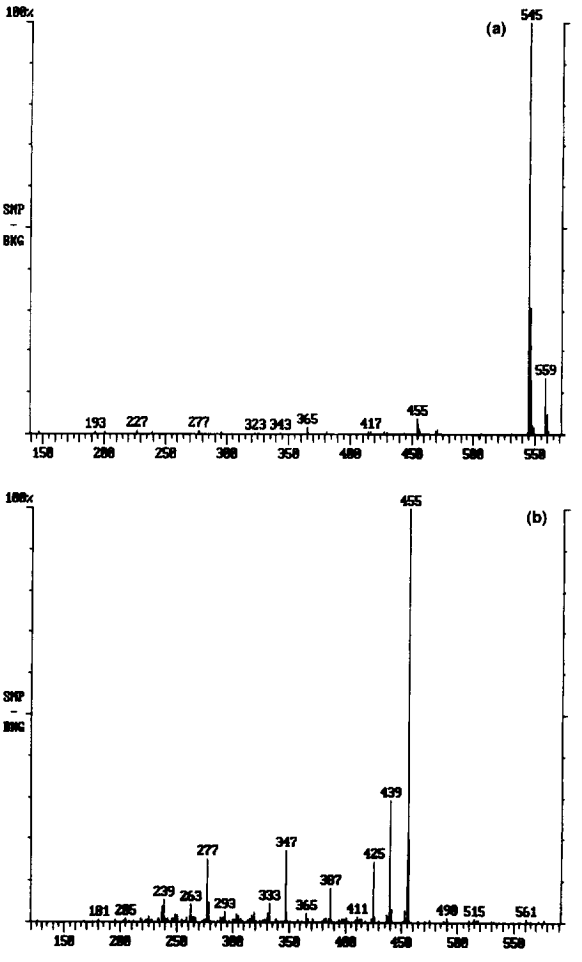

Fig. 6. MS–MS spectrum (a) and MS–MS–MS spectrum (b) of 3′-OH-stanozolol. (MS–MS conditions: Precursor, *m/z* 560; CID RF storage: 200 *m/z*; Resonant CID amplitude 0.4 V; Target 5000. MS–MS–MS conditions utilized the same initial step as MS–MS, but for the second isolation/CID step: Precursor, *m/z* 545; CID RF storage: 200 *m/z*; Resonant CID amplitude 2.0 V; Target 5000.)

tom) contains a variety of structurally-informative ions. For the purposes of forensic analysis, this is a more acceptable identification than the lack of fragmentation described above, although acceptable criteria for identification have not been clearly established for MS–MS–MS. We were able to achieve reproducible spectra at concentrations as low as 1 ng/ml in the urine matrix. The production of a single ion fragment from 3′-hydroxystanozolol in the MS–MS mode improves the MS–MS–MS detection limits significantly. As a result, identification of 3′-hydroxystanozolol is one of the first analytically useful applications of MS–MS–MS.

The tetrahydro metabolite of bolasterone presents the most difficult scenario for MS–MS detection. The predominant ion in the MS spectrum of the di-TMS derivative of tetrahydrobolasterone is *m/z* 143, arising from D-ring fragmentation. Unfortunately, this ion is present in all other 17α-methyl steroids and would not yield useful information from MS–MS for structural confirmation. Due to the low ion intensity of the $[M-15]^+$ ion (*m/z* 449), MS–MS analysis of the TMS-enol-TMS ether of tetrahydrobolasterone gave limits of detection of 1 ng on-column. One approach to improve detection limits for MS–MS would be the formation of a derivative that produces more intense high mass ions. Steffenrud et al. [16] prepared allyldimethylsilyl derivatives of steroids that readily fragmented by loss of the allyl moiety. Andersson and Sjövall [9] reported the formation of *tert.*-butyldimethylsilyl and mixed *tert.*-butyldimethylsilyl–trimethylsilyl derivatives of a variety of steroids. Although the allyl moiety may have an advantage due to the steric hindrance observed for the *t*-BDMS function at the 17β-hydroxyl group, the loss of the *tert.*-butyl function produces intense [M−57] ions which lend themselves to MS–MS analysis. The mono-*t*-BDMS derivative, containing a free 17β-hydroxyl group, could be efficiently chromatographed on the XLB column. The MS spectrum (Fig. 7b) is characterized by the loss of *t*-BDMS [M−57], loss of H_2O and apparent A–B ring fragments. The *t*-BDMS-enol-TMS ether derivative also gave good chromatographic peaks, a good precursor ion at *m/z* 449 and characteristic MS (Fig. 7c) and MS–MS spectra (Fig. 7d). Empirical studies showed that the mixed derivative could be detected at lower concentrations. The limit of detection was estimated at 20 pg on-column; a 50-fold improvement over the TMS-enol-TMS ether derivative.

4. Conclusions

Improved limits of detection continue to be a primary objective in anabolic steroid testing. Polysilphenylene–polydimethylsiloxane columns, al-

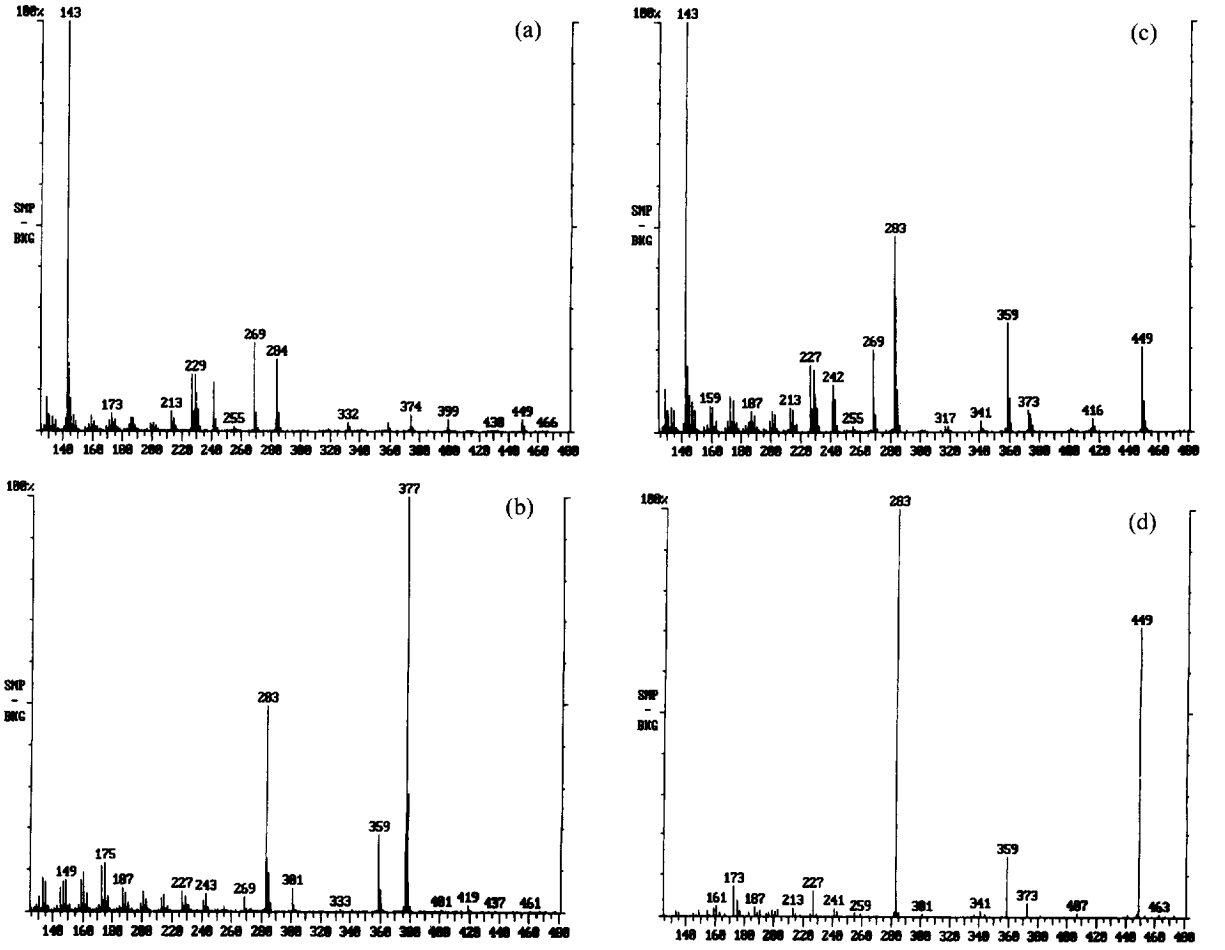

Fig. 7. MS and MS–MS spectra of several derivatives of tetrahydrobolasterone (5α-androst-7α,17α-dimethyl-3β,17β-diol). (a) MS spectrum of the TMS-enol-TMS ether derivative; (b) MS spectrum of the *t*-BDMS-enol derivative; (c) MS spectrum of the *t*-BDMS-enol-TMS ether derivative; (d) MS–MS spectrum of the *t*-BDMS-enol-TMS ether. (Precursor, *m/z* 449 ($[M-57]^+$); CID RF storage: 100 *m/z*; Non-resonant CID amplitude 40 V; Target 5000.)

though reducing column bleed substantially, do not provide improved resolution for steroid isomers. No single column was able to resolve all of the steroids under the conditions tested. It appears that the preparation of the column and phase has a significant role in determining column selectivity for TMS-derivatives of steroids. The application of QIT MS–MS to detection of steroids resulted in significant decreases in the amount of steroid that could be detected. The improvement in detection limits for norandrosterone using the same derivatization was 100-fold over selected ion monitoring on a quadrupole mass filter. The decrease for a metabolite of fluoxymesterone was 50-fold. The generation of structurally useful ions in the MS–MS spectrum of steroids can be difficult to achieve. In the case of bolasterone metabolite, a change in derivatization scheme allowed a significant improvement in detection limits. For 3′-hydroxystanozolol, the use of MS–MS–MS gave informative spectra and good detection limits. Clearly ion trap MS–MS and MS–MS–MS can play a significant role in detecting anabolic steroids for longer periods of time after administration.

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