Pharmaceutical Research, Vol. 5, No. 1, 1988

Report

Structural Determination of Testosterone Esters in Oil Injectables by Thermospray Mass Spectrometry

Thomas Cairns,[1,2] Emil G. Siegmund,[1] and Thomas S. Savage[1]

Received June 16, 1987; accepted August 1, 1987

In the absence of analytical reference standards, the combination of thermospray and collision-activated dissociation mass spectrometry has been utilized to separate and identify the presence of several esters of testosterone in imported oil injectables. Structural identifications were based on generic daughter ions considered diagnostic of the steroidal ring structure of these anabolic steroids as well as daughter ions representative of the ester alkyl or aryl side chains.

KEY WORDS: testosterone esters; anabolic steroids; mass spectrometry; thermospray.

INTRODUCTION

The proliferation into the United States of imported injectable oil preparations of various anabolic steroids through various ports of entry has created subsequent illegal sales and distribution. There is now an urgent need to devise analytical protocols for rapid analysis, identification, and confirmation of these artificial steroids. While high-performance liquid chromatography (HPLC) has been the prime technique employed for the separation and detection of such steroids as testosterone propionate, progesterone, and estradiol benzoate (1), gas chromatography mass spectrometry (GC/MS) has also been used to determine testosterone propionate in human plasma (2) and horse urine (3). The analytical dilemma in dealing with oil-injectable mixtures of several anabolic steroids is threefold. First, the suspected thermal instability of the various esters related to testosterone (I) prohibits the singular use of GC/MS without prior derivatization. Second, the similar polarity of many steroidal molecules renders gas chromatographic separation difficult, with reverse-phase HPLC offering an alternate successful approach. Third, the lack of analytical standards for many of the imported testosterone esters requires a higher level of analytical sophistication to confirm the suspected structural moieties for regulatory purposes.

This paper now reports the application of thermospray (TSP) mass spectrometry in conjunction with collision-activated dissocation experiments in a rapid analytical approach to separate, identify, and confirm the presence of testosterone propionate (II), testosterone isocaproate (III), testosterone phenylpropionate (IV), and testosterone decanoate (V).

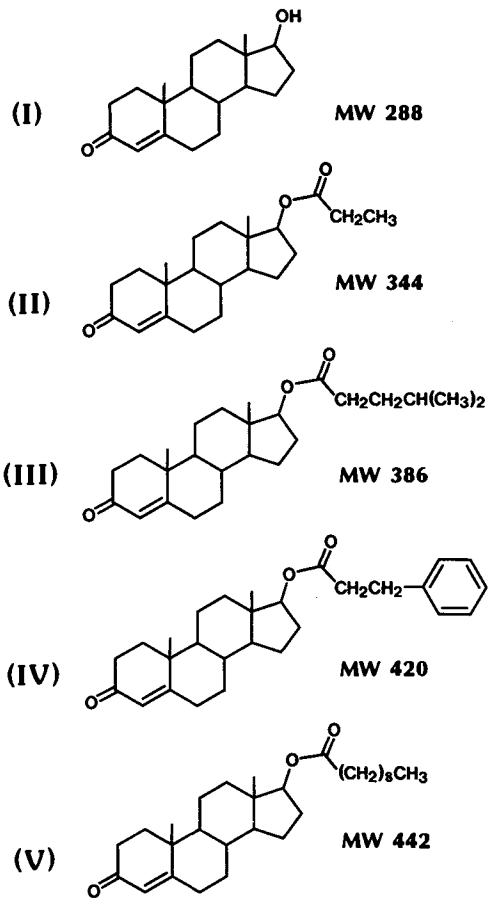
[1] Department of Health and Human Services, Food and Drug Administration, Office of Regulatory Affairs, Los Angeles District Laboratory, 1521 West Pico Boulevard, Los Angeles, California 90015.

[2] To whom correspondence should be addressed

MATERIALS AND METHODS

Apparatus

High-Performance Liquid Chromatography. Data were



Scheme I

acquired on a Spectra-Physics SP-8100 HPLC equipped with an SP-8440 ultraviolet-visible detector and a Hewlett–Packard HP-3385A integrator. Operating conditions were as follows: 254-nm monitoring wavelength, 5-μm ODS reverse-phase column (μ-Bondapak C-18, 30 cm × 3.9-mm id, Phenomenex, Palos Verdes Estates, Calif., and 10-μl sample loop. A gradient elution program using methanol and water as the solvent system was used: the methanol concentration was increased from 80 to 100% in a linear gradient over 20 min, held at 100% for 10 min, programmed linearly back to 80% in 2 min, and finally, held at 80% for 5 min using a flow rate of 1.0 ml.

Thermospray Mass Spectrometry. Mass spectra were recorded on a Finnigan Model 45A triple-stage quadrupole mass spectrometer equipped with a chemical ionization source, Incos data system, and Finnigan TSP device. Typical instrument parameters used were as follows: extractor, −7 V; lens, −150 V; electron energy, 100 V; quad entrance, −27 V; source pressure, 300 mtorr; electron multiplier, −1050 V; and source, 170°C. TSP operating conditions were as follows: jet stream, optimized via visible characteristics before installation (stream to be no less than 15 cm at a flow rate of 2 ml/min); solvent system, 90% methanol/water with 0.1 *M* ammonium acetate as buffer; column, 5-μm bonded cyano (Altex—Ultrasphere cyano, 15 cm × 4.6-mm id); flow rate, 1.0 ml/min; and jet temperature, set to 185°C for mass spectral optimization of TSP reagent spectrum—*m/z* 50 (100%), *m/z* 77 (50%), *m/z* 36 (24%), and *m/z* 18 (12%). Collision-activated dissociation studies were carried out using argon as the collision gas at a pressure of 1 mtorr with the collision energy set at 29 eV.

Sample Preparation

Oil-injectable preparations were obtained as samples for regulatory analysis. Samples for analysis were diluted with chloroform to correspond to a concentration of ca. 0.3 mg of testosterone propionate/ml based on the label declaration of content. Amounts injected on the column were 3 μg and 500 ng for analysis by HPLC and TSP, respectively.

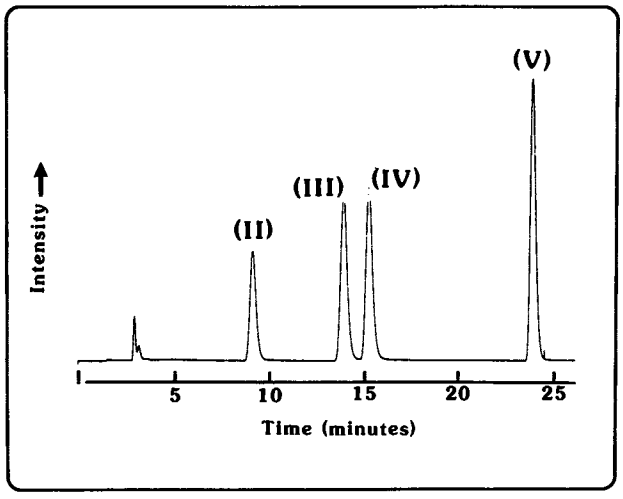


Fig. 1. Liquid chromatographic separation of major components of an oil-injectable preparation of testosterone esters: (II) testosterone propionate, (III) testosterone isocaproate, (IV) testosterone phenylpropionate, and (V) testosterone decanoate.

RESULTS AND DISCUSSION

Liquid Chromatography

Preliminary separation of the various esters of testosterone was accomplished using gradient elution and a reverse-phase ODS column (Fig. 1). While the identification of testosterone propionate (II) was accomplished via the availability of a standard reference material, the structural identity of the other esters could be inferred only tentatively based on increased retention times (i.e., dependent on lipophilicity) and label declarations since no reference standards existed for testosterone isocaproate (III), testosterone phenylpropionate (IV), and testosterone decanoate (V). Because of this inability to identify these three esters by direct comparison with reference standards, the application of TSP mass spectrometry was attempted to resolve the issue via molecular weight determination and fragmentation.

Thermospray Mass Spectrometry

Since this method of ionization normally requires the employment of ammonium acetate as buffer to favor the production of protonated molecular ions (4) from the esters under investigation, an alternate solvent system (90% methanol/water with 0.1 *M* ammonium acetate) to the gradient elution system had to be developed for on-line TSP use. While this transition in developing another solvent system may be viewed as cumbersome, it is fundamental to the successful operation of this technique. Besides, the importance of obtaining molecular weight information via TSP for these anabolic steroids is a cornerstone to the general philosophy that protonated molecular ions are excellent candidates for subsequent collision-activated dissociation (CAD) in providing unambiguous structural identity, especially since no significant fragment ions were initially observed.

Prior to on-line separation through the cyano LC column, however, the sample extract was first injected into the solvent system stream to obtain a preliminary nonchromatographic survey of potential molecular weights present in the extract (Fig. 2). As can be observed, the suspected testosterone esters are clearly evident as $[MH]^+$ ions at *m/z* 345 (propionate), *m/z* 387 (isocaproate), *m/z* 421 (phenylpropionate), and *m/z* 443 (decanoate). This survey method of using the TSP as a probe device rather than a separatory technique has merit in that such preliminary information can often indicate that sufficient sensitivity of detection is available to proceed with the full-scan LCMS experiment. Admittedly, the presence of additional ions derived from the oil base in this probe sample experiment could confuse the issue without prior knowledge of which molecular ions are expected to be observed. It could be argued at this juncture that, through the use of CAD experiments on these four ions of interest, structural identity could be inferred. While that approach could result in a successful conclusion to this case, the possibility of interferences from other compounds giving rise to ions with the same *m/z* values might have precluded unambiguous structural identifications.

Figure 3 illustrates the mass spectral data obtained

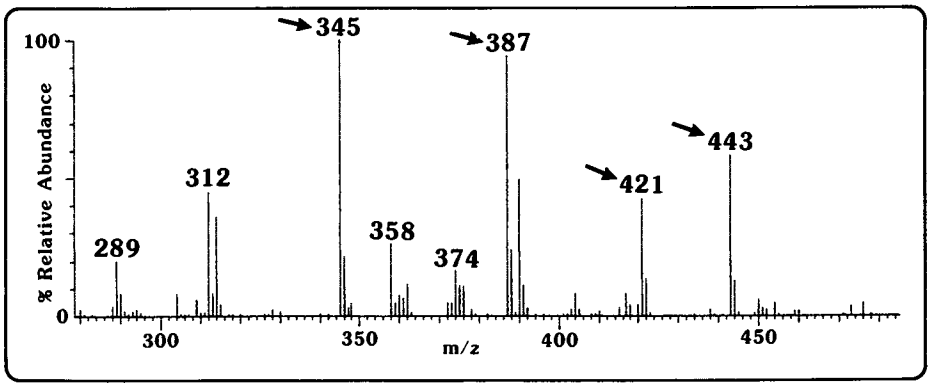


Fig. 2. Thermospray mass spectrometry of sample obtained by injecting into solvent system stream to observe anabolic constituents via appearance of their protonated molecular ions as indicated by arrows.

when the sample was separated and ionized via the TSP approach. Resultant ion profiles for the various testosterone esters constructed from the data base clearly indicate the order of elution—a different elution order was observed because of the change in the mobile phase. The long retention time for the decanoate (V; *m/z* 443) and the peak width have seriously reduced the sensitivity of the technique for this anabolic steroid. The appearance of an ion at *m/z* 443 at the retention time expected for testosterone phenylpropionate (IV) is probably an adduct ion (i.e., $M + 23$) resulting from trace levels of sodium in the sample.

The final step in this experimental approach for the rapid analysis of oil injectables was structural identification of the anabolic steroids involved. With the availability of CAD as an additional tool for structural analysis, the protonated molecular ions for the various esters were then sequentially permitted to collide with argon gas in the second quadrupole, and the resultant daughter spectra recorded (Fig. 4). In the case of the parent steroid, testosterone (I) (Fig. 4A), only two major daughter ions were observed at *m/z* 97 and 109. The exact origin of these ions was investigated using the 4-chloro derivative of testosterone, which revealed that both daughter ions were derived from ring A. Empirically these daughter ions represented the intact ring A enone system with or without the angular methyl group. What is significant about these ions is that they were also

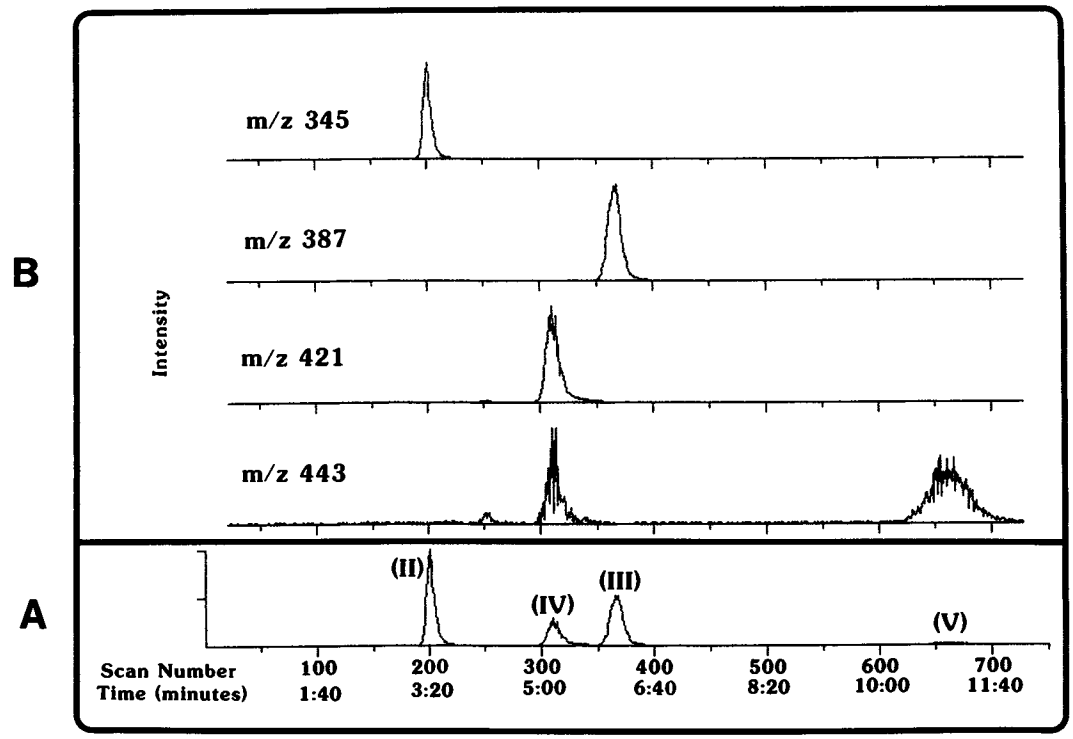


Fig. 3. Thermospray mass spectrometry of sample obtained by separation on column prior to ionization: (A) reconstructed ion current; (B) mass chromatograms corresponding to the protonated molecular ions for the suspected testosterone esters present (II through V).

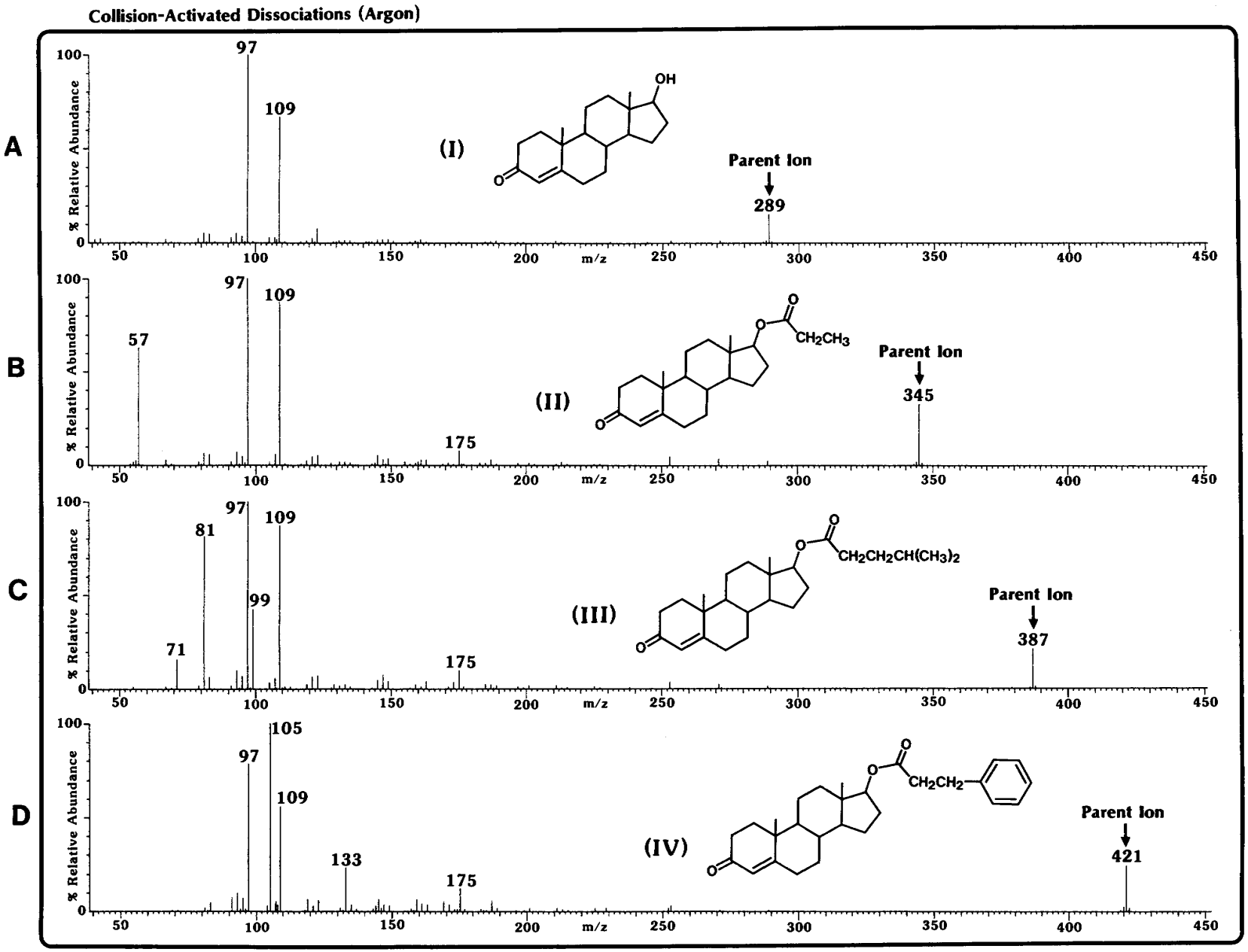


Fig. 4. Daughter spectra obtained via collision activated dissociation using argon of protonated molecular ions for (A) testosterone, (B) testosterone propionate, (C) testosterone isocaproate, and (D) testosterone phenylpropionate.

observed in the ester CAD spectra. It would appear, therefore, that *m/z* 97 and 109 are generic diagnostic daughter ions for the testosterone skeletal structure. In the case of testosterone propionate (II) (Fig. 4B), the ion at *m/z* 57 is indicative of the formation of COC_2H_5. Similar behavior was observed for testosterone isocaproate (III) (Fig. 4C), where the formation of the COR ion gave rise to *m/z* 99 and the formation of the R ion to *m/z* 71. Finally, in the case of testosterone phenylpropionate (IV), the formation of the COR ion gave rise to *m/z* 133, while the ion at *m/z* 105 corresponded to the R grouping itself. The low sensitivity of *m/z* 443 for the decanoate ester (V) did not permit the recording of its daughter spectrum under on line conditions. Under probe conditions, however, the CAD daughter spectrum was observed to be in concert with the results observed for its other ester relatives.

The CAD spectral characteristics of these testosterone esters have indicated an improved analytical technique for detection and confirmation based on daughter ions resulting from both the substituent alkyl or aryl groups, cleavage between the carbonyl and the ether oxygen atom, and the appearance of generic diagnostic daughter ions which strongly indicate the presence of the testosterone steroidal nucleus.

Two useful techniques have evolved from this study. First, for wide-scale surveillance analysis of oil injectables, a probe TSP sample can be quickly analyzed to determine the potential steroidal constituents via observance of protonated molecular ions. Additionally, collision experiments on those protonated molecular ions can confirm the suspected identity through daughter ion spectral profiles. Second, the utility of the two generic daughter ions representing the testosterone ring structure could be employed as a useful tool to screen for the presence of such molecules by strictly requesting the identification of those eluting compounds that have parent ions resulting in those particular daughter ions.

REFERENCES

1. L. S. Friedersdorff, R. W. Schaller, and A. C. Barnes *Anal. Lett.* **19**:229–237 (1986).
2. S. Baba, M. Fujioka, Y. Shinohara, and T. Furuta *J. Chromatogr.* 337:205–212 (1885).
3. M. C. Dumasia, E. Houghton, and S. Sinkins *J. Chromatogr.* 377:23–33 (1986).
4. R. D. Voyksner, J. T. Bursey, and E. D. Pellizzari *Anal. Chem.* 56:1507–1514 (1984).