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Derivatization and gas chromatographic–mass spectrometric detection of anabolic steroid residues isolated from edible muscle tissues

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ABSTRACT

A method was developed for the detection of anabolic steroid residues in edible muscle tissues. After enzymic digestion of the tissue and purification on disposable C_{18} solid-phase extraction columns, the extract was injected onto a C_{18} reversed-phase high-performance liquid chromatographic column. Three fractions or windows were collected, each containing specific analytes. After evaporation to dryness, the residues were subjected to a derivatization procedure which yielded suitable derivatives. After gas chromatographic–mass spectrometric analysis, both gas chromatographic retention data and mass spectral data were used for the detection and identification of nortestosterone, testosterone, estradiol, ethinylestradiol, trenbolone, methyltestosterone, chlormadinone acetate, medroxyprogesterone acetate and megestrol acetate.

INTRODUCTION

Natural and synthetic hormones are used worldwide as growth-promoting agents. In Belgium, however, as in the other countries of the EEC, the use of these agents is forbidden. The control of the illegal use of anabolic steroids can be effective only if sensitive and specific tests are used for the detection and identification of these drugs and their metabolites. Radioimmunoassay (RIA) methods are sensitive enough but cross-reactivities are often observed so that the possibility of false-positive results remains. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), which are much more specific, are in most instances not sensitive enough to detect concentrations in the ppb range.

Diethylstilbestrol (DES), which was the first to be used on a broad scale, was also the first to be detected by gas chromatography–mass spectrometry (GC–MS) in several matrices [1–10]. Later, individual methods for trenbolone, zeranol, estrogens, nortestosterone and melengestrol acetate were developed [11–22]. This paper describes a method for the simultaneous isolation and identification of nortestosterone (NT), testosterone (T), estradiol (E2), ethinylestradiol (EE), methyltestosterone (MT), medroxyprogesterone acetate (MPA), chlormadinone

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acetate (CMA), megestrol acetate (MGA) and trenbolone (TBOH) in meat using GC–MS.

EXPERIMENTAL

Chemicals, glassware and solvents

Subtilisin A was purchased from Novo Industri (Copenhagen, Denmark), trishydroxymethylaminomethane (Tris) and benzene from Merck (Darmstadt, F.R.G.), diethyl ether was from Janssen Chimica (Geel, Belgium) and methanol and water of HPLC grade from Alltech (Deerfield, IL, U.S.A).

Heptafluorobutyric acid anhydride (HFBAA) from Machery-Nagel (Düren, F.R.G.), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) from Aldrich (Milwaukee, WI, U.S.A.), trimethylchlorosilane (TMCS) from Fluka (Buchs, Switzerland) and pyridine from Pierce (Rockford, IL, U.S.A.) were used as supplied.

C_{18} disposable extraction columns were obtained from Analytichem (Harbor City, CA, U.S.A.). The derivatization vials were silanized with a solution of dimethyldichlorosilane in toluene before use.

Extraction and clean-up

A tissue sample (1 g) was digested overnight (or for a minimum of 2 h) in an enzyme suspension containing 1 mg of subtilisin A at 60°C and pH 9 (0.1 *M* Tris solution) [10]. The digest was extracted with two 5-ml volumes of diethyl ether and the combined ether fraction was evaporated to dryness and dissolved in 0.1 ml of methanol and 4 ml of water were added. This solution was applied to a C_{18} disposable column, previously conditioned with 5 ml of methanol and 5 ml of water. After washing the column with 5 ml of water, the steroid residues were eluted with 2 ml of methanol. After evaporation to dryness, the residue was resuspended in 100 μl of methanol and a 50-μl portion was subjected to the fractionation step.

Fractionation

Fractionation was carried out on an HPLC system consisting of a Pye Unicam Model PU 4011 pump, a Gilson Model 231 automatic injector, a Gilson Model 203 fraction collector and a Pye Unicam Model PU 4020 UV detector operated at 254 nm.

The analytical column was a LiChrospher RP-18 (Merck, Darmstadt, F.R.G.) (12.5 cm × 4 mm I.D.), particle size 5 μm, and was protected by a 75-mm guard column (pellicular reversed phase, 30–50 μm). The mobile phase was methanol–water (65:35) at a flow-rate of 1 ml/min. The methanol–water fractions were evaporated to dryness under a stream of nitrogen.

The collection "windows" were determined by injecting 25 ng of each standard with the UV detector set at the wavelength of maximum absorption of the compound.

Derivatization

Heptafluorobutyrates (HFB). The residue obtained from the fractionation step was dissolved in 0.2 ml of benzene to which 0.05 ml of HFBAA was added. After vortex-mixing, the sample was heated at 60°C for 1 h and then concentrated to dryness at 40°C under a flow of nitrogen. The final residue was redissolved in 50 μl of hexane and a 2-μl portion was injected into the GC–MS instrument.

Trimethylsilyl (TMS) ethers. The residue obtained from the fractionation step was treated with 100 μl of pyridine–MSTFA–TMCS (10:3:1, v/v/v) and heated at 60°C for 1 h. After evaporation of the excess of reagent, the final residue was dissolved in 50 μl of hexane and a 2-μl portion was injected into the GC–MS instrument.

Gas chromatography–mass spectrometry

The analyses were carried out on an HP 5970 mass-selective detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) linked to an HP 5890 gas chromatograph equipped with an HP Ultra-2 (5% phenyl–methylsilicone) fused-silica capillary column (25 m × 0.2 mm I.D., film thickness 0.33 μm) and an all-glass moving-needle injection system. The ionization voltage of the ion source of the mass spectrometer was fixed at 70 eV.

The carrier gas was high-purity helium (L'Air Liquide, Liège, Belgium) at a flow-rate of 0.47 ml/min. The injection and interface temperatures were maintained at 290°C. The oven temperature was programmed from 200 to 280°C at 5°C/min, the final temperature being held for 10 min.

RESULTS AND DISCUSSION

Recoveries from the extraction and clean-up steps were measured by means of the radioactive homologues of the anabolic steroids, when available (T, MT, E2, EE and MPA). A known amount of radioactive material was added before the extraction and the recovery after the ultimate concentration was calculated from liquid scintillation counting data. The results are given in Table I.

The HPLC fractionation can be fully automated by the use of a programmable injection system and a programmable fraction collection unit. This means that this time-consuming step, which takes 45 min per run, can be completed overnight. The collection windows are determined by the injection of *ca.* 25 ng of each standard compound. This amount can be seen by the UV detector at the wavelength of maximum absorbance. It is recommended to check for possible memory effects by collecting in a consecutive run the windows of a blank injection. The shift of the windows as a function of time or ambient room temperature is minimal and needs to be controlled only once a month.

The retention windows (beginning and end of elution of a particular compound, in minutes after injection) are given in Table II.

The individual collection of each analyte, if possible, would make the analysis

TABLE I

RECOVERY AFTER EXTRACTION AND CLEAN-UP ON C_{18} DISPOSABLE EXTRACTION COLUMNS

Steroid	Recovery (mean ± S.D., $n=5$) (%)
T	97.0±4.0
E2	84.8±6.0
EE	99.3±1.0
MPA	97.5±2.0
MT	99.0±1.0
NT	97.0±4.0

cumbersome. Therefore, the steroids are collected in three distinct groups. The assignment is determined by, in addition to the retention time, the subsequent derivatization; all the compounds which are collected in one particular fraction can be converted into the same type of derivative, *i.e.*, heptafluorobutyrate (HFB) for fraction 1, which contains T, NT, E2, EE and TBOH, di-TMS for fraction 2, which contains only MT, and HFB for fraction 3, which contains MGA, MPA and CMA. It must be emphasized that as an additional proof of evidence, alternative but less appropriate derivatives may be formed, *i.e.*, TMS derivatives for fraction 1 and HFB derivatives for fraction 2. The conversion of MT into the corresponding diheptafluorobutyrate yields two peaks with similar mass spectra. For reasons of sensitivity, the conversion into a TMS derivative is to be preferred.

The advantage of the HFB derivatives is that they yield molecular ions with higher m/z values, so that theoretically interferences are less likely to be encountered.

Table III gives the retention data, expressed in methylene unit (M.U.) values of both derivatives, when available, together with the ions selected for individual monitoring. These selections were derived from the mass spectra. As an example, the electron impact (70 eV) mass spectra of nortestosterone-di-HFB and of nor-

TABLE II

RETENTION WINDOWS FOR THE ANABOLIC STEROIDS

Steroid	t_R (min)	Steroid	t_R (min)
TBOH	3.5 –4.5	MT	8.5–10.0
EE	5.17–6.17		
E2	5.5 –6.5	MGA	10.0–12.0
NT	5.5 –6.5	CMA	11.0–13.0
T	6.5–8.0	MPA	11.5–13.5

TABLE III

GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC IDENTIFICATION DATA

Steroid	M.U. value		Ions monitored *(m/z)*	
	HFB	TMS	HFB	TMS
Fraction 1				
NT	24.40	26.84	666,453,306	418,194,182
T	24.63	27.28	680,467,320	432
E2	24.66	27.28	664,451,409	416,285
EE	25.87	28.42	492,409,356	440,425,285
TBOH	24.71	30.03	662,448	413
Fraction 2				
MT	22.72	28.26	480,456,369	446,301
Fraction 3				
MPA	29.47		582,479.439,383	
MGA	29.09		580,477	
CMA	30.55		497	

testosterone-di-TMS are shown in Fig. 1. A particular steroid is considered to be identified if it appears at the right retention time in the gas chromatogram, indicated in Table III, and if all the selected ions in Table III simultaneously show up. Although it has been indicated that the relative abundances of all selected ions monitored from the analyte should match those of the standard analyte [23], it has been found from practical experience that this is a purely theoretical criterion. Indeed, samples which were shown to contain a residue of a particular steroid by means of, *e.g.*, RIA, or to which the analyte was added by spiking prior to extraction, should have been declared negative if those rules were to be applied too stringently.

The method described has already been succesfully applied to muscle tissue samples obtained from animals that had been anabolized under experimental conditions with a view to the production of reference materials. This means that the method is sensitive enough to permit the detection of parenterally administered doses of anabolic steroids. Thirty-four samples of minced beef, purchased at random from the retail trade, were analysed in the same way for fractions 1 and 2, except trenbolone. There were eight positive samples (24%), all of which contained NT. The other results are summarized in Table IV.

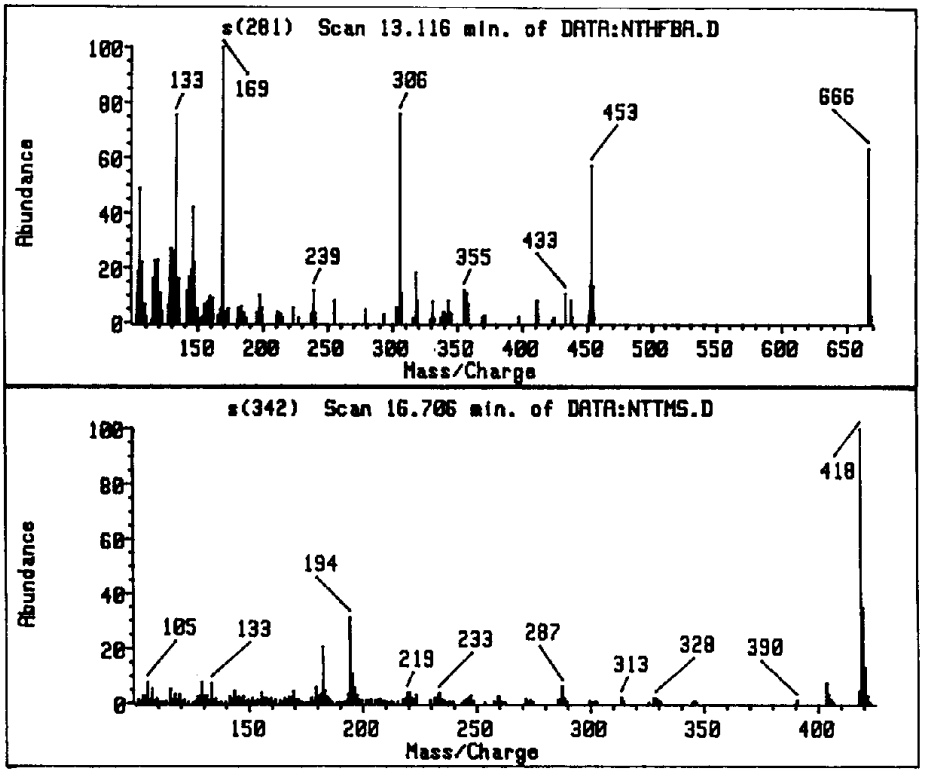

Fig. 1. Electron impact (70 eV) mass spectrum of NT-di-HFB and NT-di-TMS.

TABLE IV

RESULTS OF THE ANALYSIS OF 34 MINCED BEEF SAMPLES FROM THE RETAIL TRADE

Sample No.	NT	T	MT	E2	EE
1	+	−	−	−	−
2	+	−		−	−
6	+	+	+	−	−
7	+	−		−	−
11	+	−	−	−	−
21	+	−	−	−	−
25	+	+	−	−	−
31	+	−	−		−

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