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Determination of 19-nortestosterone, testosterone and trenbolone by gas chromatography–negative-ion mass spectrometry after formation of the pentafluorobenzylcarboxymethoxime–trimethylsilyl derivatives

RENZO BAGNATI* and ROBERTO FANELLI
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)
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ABSTRACT

The known reaction of 3-ketosteroids with carboxymethoxylamine (to form the corresponding carboxymethoxines), followed by esterification of the carboxyl group with pentafluorobenzyl bromide, has been used to obtain derivatives of 19-nortestosterone, testosterone and trenbolone suitable for high-sensitivity detection with gas chromatography–negative-ion chemical ionization mass spectrometry. These derivatives, after further silylation of the alcoholic groups of the steroids, showed excellent chromatographic and spectrometric characteristics and were detectable in the low picogram range. The derivatization gave rise to the formation of two isomers which were distinguishable by gas chromatography. The existence of the two isomers was also confirmed by high-performance liquid chromatography. Examples of the usefulness of this derivatization procedure are given for the analysis of 19-nortestosterone, testosterone and trenbolone in meat and urine samples. By the use of immunoaffinity extraction and addition of deuterated internal standards (synthesized by isotopic exchange), the new derivatization procedure allowed a correct identification and quantitation of the steroids and reached very low detection limits [0.02 ppb (10^9) for 19-nortestosterone and testosterone, 0.06 ppb for trenbolone].

INTRODUCTION

The determination of steroids in biological samples is an analytical problem of great importance. In fact, several immunological and instrumental methods [radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), gas chromatography (GC) and GC–mass spectrometry (MS)] have been developed for this purpose [1–8]. GC–MS is considered the most effective technique to obtain a selective and accurate measurement of these substances, but all the methods reported in the literature require derivatization of the molecules to achieve good chromatographic and spectrometric characteristics [4–8]. The most widely used derivatizing agents are methoxyamine (for the reaction with keto groups), silylating compounds (for alcoholic groups) and aliphatic fluorinated anhydrides (for both). Depending on the derivatization method, MS detection has been done with electron-impact (EI) ionization mode or negative-ion chemical ion-

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ization (NICI) mode, obtaining different sensitivities and specificities during the analysis of samples.

To extend the choice of derivatizing agents, we describe here the use of carboxymethoxylamine (a compound already used to introduce a carboxyl group into steroid molecules, for their subsequent conjugation to proteins [9]) as a derivatizing agent of the 3-keto groups of 19-nortestosterone, testosterone and trenbolone, followed by pentafluorobenzylation of the carboxyl group of the resulting carboxymethoximes. This attempt at introducing a pentafluorobenzyl ester moiety into the steroid molecules was made to exploit the high-sensitivity detection achievable for the compounds containing this group by GC–NICI-MS [10,11].

EXPERIMENTAL

Materials

19-Nortestosterone (19-norT) and testosterone (T) were from Sigma (St. Louis, MO, USA). Trenbolone (TBOH) was extracted and purified from pharmaceutical formulations. Carboxymethoxylamine hemihydrochloride (CMOA), pentafluorobenzyl bromide (PFBBr), deuterium oxide (^2H_2O), potassium deuteroxide (KO^2H, 40% solution in ^2H_2O), and monodeuteromethanol (CH_3O^2H) were from Aldrich (Milwaukee, WI, USA). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and methoxyamine hydrochloride, 2% solution in pyridine (MOX), were from Pierce (Oud Beijerland, Netherlands). Diisopropylethylamine (DIPEA) was from Fluka (Buchs, Switzerland). N,O-Bis(trimethylsilyl-$^2H_{18}$)acetamide (BSA-D_{18}) was from ICN Biomedicals (Cambridge, MA, USA). Immunoaffinity columns and extraction kits (Multi-Prep II) were from Genego (Gorizia, Italy).

Derivatization

Derivatization was done as follows. To dried standards or samples, in conical glass tubes, 100 μl of a solution of 1 mg/ml CMOA in acetonitrile–DIPEA (100:1, v/v) were added. The tubes were capped and heated at 60°C for 2 h. Then, 10 μl of a solution of PFBBr in acetonitrile (1:40, v/v) were added and the tubes were heated at 40°C for 10 min. Finally, after evaporating the solvents, 20 μl of BSTFA were added and the tubes heated at 60°C for 30 min.

The reagent solutions were prepared fresh every month.

Gas chromatography–mass spectrometry

A VG TS-250 mass spectrometer coupled to a HP 5890 gas chromatograph and equipped with an 11-250J data system was used for EI mode, and a Finnigan 4000 mass spectrometer coupled to a DANI 6500 gas chromatograph and equipped with a Teknivent 1050 data system (St. Louis, MO, USA) was used for NICI mode. Source conditions in NICI mode were: temperature, 250°C; emission current, 250 μA; electron energy, 100 eV. Ammonia was used as the reactant gas for electron-capture ionization at a source pressure of 0.2 Torr.

Samples were injected in 2-μl aliquots with the following GC conditions: column, CP Sil 5 CB, 25 m × 0.32 mm I.D., film thickness, 0.12 μm (Chrompack Italia, Cernusco sul Naviglio, Italy); oven temperature raised from 160°C (1 min) to 220°C at 25°C/min, then to 300°C (4 min hold) at 15°C/min; helium head pressure of the column, 0.4 bar.

The HP 5890 gas chromatograph was equipped with a split–splitless injector (operated in splitless mode at 240°C), and with an 'on-column' injector; the DANI gas chromatograph was equipped with a programmable temperature vaporizer (PTV) injector (Dani, Monza, Italy) which was initially kept at 60°C and then heated quickly to 275°C immediately after injection. The split–splitless injector gave sensitivity problems when injecting the pentafluorobenzylcarboxymethoxime–trimethylsilyl (PFBCMO–TMS) derivatives, probably because of thermal instability during the flash heating. The PTV injector and the on-column injector did not suffer such problems and sensitivity was always good.

High-performance liquid chromatography

HPLC injections were done on a Beckman System Gold chromatograph, equipped with a 20-μl loop injector and with a UV detector (set at 266 nm). The column was a Chromspher C_{18}, 100 × 3.0 mm (Chrompack Italia) operated at a flow-rate of 0.5 ml/min of methanol–water (60:40).

Deuterium exchange of 19-nortestosterone, testosterone and trenbolone

Deuterated analogues of 19-norT, T and TBOH, to be used as internal standards in GC–MS quantitation, were obtained by isotopic exchange in deuterated solvents under alkaline conditions. Briefly, 1 mg of 19-norT and T and 0.1 mg of TBOH were incubated, at room temperature, with a mixture of 10 μl of 10% KO^2H in ^2H_2O and 200 μl of CH_3O^2H. The isotopic exchange of the weakly acidic protons adjacent to the 3-keto group and to the double bond in the 4–5 position was monitored by GC–MS. After 10 days for 19-norT and TBOH and 20 days for T the incubation was stopped. The mixtures were diluted with 2 ml of ^2H_2O and the steroids were extracted with three 2-ml volumes of ethyl acetate. T and TBOH were found by GC–MS to have exchanged five protons, giving [$^2H_5$]testosterone (T-D_5) and [$^2H_5$]trenbolone (TBOH-D_5), while 19-norT exchanged six protons, giving [$^2H_6$]19-nortestosterone (19-norT-D_6). The isotopic purity of these compounds was about 80%, owing, mainly, to the presence of pentadeuterated 19-norT and tetradeuterated T and TBOH. However, the amount of completely non-deuterated molecules was negligible (<0.1%), as measured by GC–MS. The deuteration mixtures also contained other minor unidentified substances which were separated by GC and did not interfere in the quantitation procedure of the non-deuterated compounds.

Immunoaffinity extraction

The immunoaffinity purification of urine samples was performed using Multi-Prep II columns and buffers and following the manufacturer's instructions. Briefly, the procedure was as follows: the samples were diluted to 8 ml with extraction buffer, loaded on the columns and gently mixed with the gel for 1 min; the columns were then left to elute by gravity and washed with washing buffer (2 × 10 ml) and distilled water (2 ml); finally, immunoabsorbed substances were recovered by elution with acetone–water (95:5 v/v; 2 × 1.5 ml). The eluates were dried with a stream of air at 60°C and derivatized as described.

RESULTS AND DISCUSSION

The efficiency of the derivatization procedure described was tested by full-scan GC–MS injections of pure steroid standards. The chromatograms did not show any peak corresponding to underivatized or partially derivatized steroids, indicating that the derivatization reactions were complete.

The derivatization of 19-norT, T, and TBOH with CMOA can give rise to the formation of two isomers for each substance, because of the possible *cis–trans* conformation relative to the nitrogen atom of the oxime group. The same kind of isomerization has been described in the literature after the derivatization of 3-ketosteroids with methoxyamine [12]. Indeed, the injection of the PFBCMO–TMS derivatives of either 19-norT, T or TBOH, by GC–MS, showed the presence of two peaks (Fig. 1) with identical mass spectra in both EI and NICI modes (Fig. 3). To confirm the existence of the two isomers and to exclude the possibility of GC artifacts (*e.g.* false double peaks [13]), the derivatized steroids were also analyzed by HPLC. Each steroid (50 μg) was derivatized by the method described but omitting the silylation reaction (because of incompatibility of silyl ethers with HPLC eluents), dissolved in methanol (0.5 ml) and injected (5 μl) into a liquid chromatograph under the conditions described in the Experimental section. The results confirmed the presence of two isomers for all of the three steroids considered. The isomers eluted in reverse order with respect to GC (the minor isomers eluted after the major isomers) and had retention times distinguisable from those of the underivatized compounds (Fig. 2).

The ammonia NICI spectra of the PFBCMO–TMS derivatives of steroids (Fig. 3) are characterized by three major peaks: one is the usual fragment of PFB esters, corresponding to the loss of the PFB group (M − 181, carboxylate anion [11,14,15]), and the other two (common to all derivatives) probably correspond to the fragments $CHOCOO^-$ (*m/z* 73) and $HOCH_2COO^-$ (*m/z* 75), resulting from further cleavage of

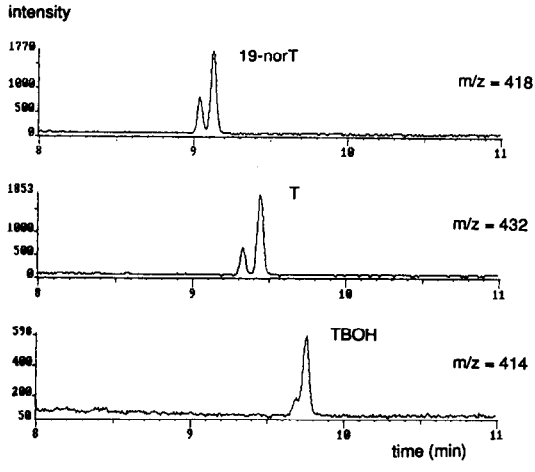

Fig. 1. GC–NICI–SIM tracings of 16 pg of each of the PFBCMO–TMS derivatives of 19-norT, T and TBOH, showing total or partial separation of the *cis–trans* isomers relative to the nitrogen atom of the oxime group. Conditions were as described in the Experimental section.

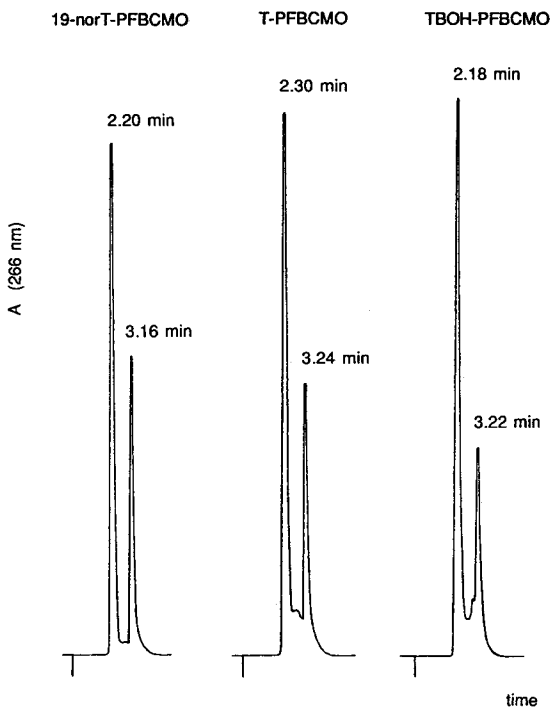

Fig. 2. HPLC profiles of the PFBCMO derivatives of 19-norT, T and TBOH, with retention times of the two isomers of each compound. The retention times of underivatized 19-norT, T and TBOH, under the same conditions (see Experimental section), were 2.90, 2.96 and 2.92 min, respectively. See also Fig. 1 and text for other explanations.

the oxygen–nitrogen bond of the carboxymethoxime group, with rearrangements involving a hydrogen atom. The possibility that these last two peaks could originate from the silyl groups was excluded by taking NICI spectra of PFBCMO derivatives in which the hydroxyl groups were reacted with deuterated BSA (BSA-D_{18}) to form the nonadeutero-trimethylsilyl (TMS-D_9) derivatives. These derivatives had the same peaks at m/z 73 and m/z 75, indicating that the silyl groups were not involved in the fragmentation.

The NICI mass spectra of the deuterated analogues of the steroids (19-norT-D_6, T-D_5 and TBOH-D_5) showed the corresponding carboxylate anions (with complete conservation of the isotopic abundances) and the same m/z 73 and m/z 75 ions as the non-deuterated compounds.

Selected-ion monitoring (SIM) of the carboxylate anions of the PFBCMO–TMS derivatives (m/z 418 for 19-norT, m/z 424 for 19-norT-D_6, m/z 432 for T, m/z 437 for T-D_5, m/z 414 for TBOH and m/z 419 for TBOH-D_5), during GC injections, gave highly specific and sensitive detection. The injection of pure standards of derivatized steroids produced chromatograms free of interferences for all the compounds tested, and the detection limit was about 1 pg injected into the gas chromatograph.

However, the advantages of the use of these derivatives are more clearly shown

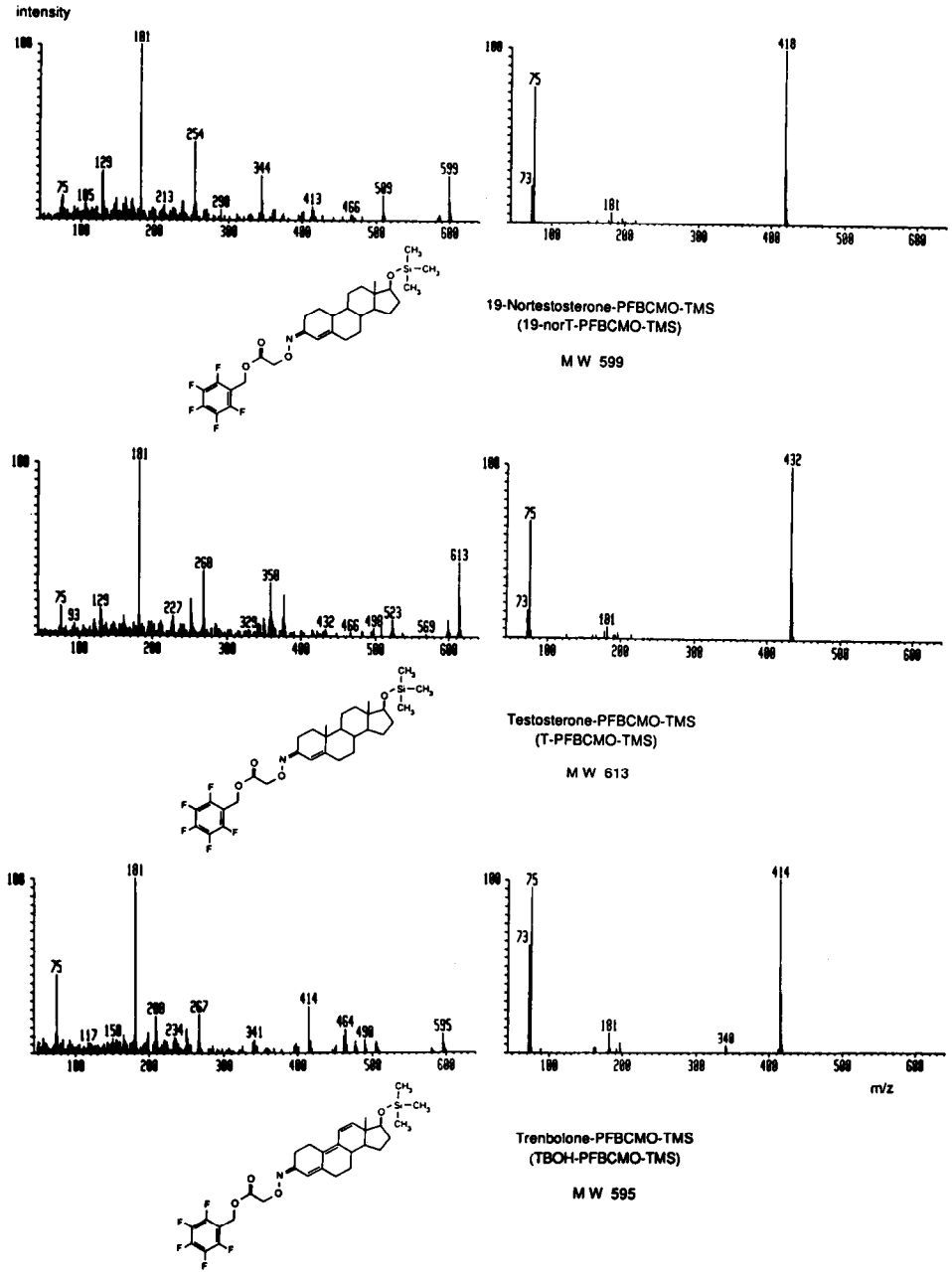

Fig. 3. EI (left) and NICI (right) mass spectra of the PFBCMO–TMS derivatives of the steroids indicated. MS conditions were as described in the Experimental section. MW = Molecular weights.

by the analysis of biological samples. Two extraction procedures of such samples, one using conventional methods (liquid–liquid and solid–phase extractions, HPLC) and one using an immunoaffinity method, were used to evaluate the detection improvements which could be obtained.

As a first case, a meat sample from a commercial food, suspected of being contaminated by 19-norT and which was extracted and purified by solid-phase extraction and HPLC, was divided into two aliquots to be examined by different GC–

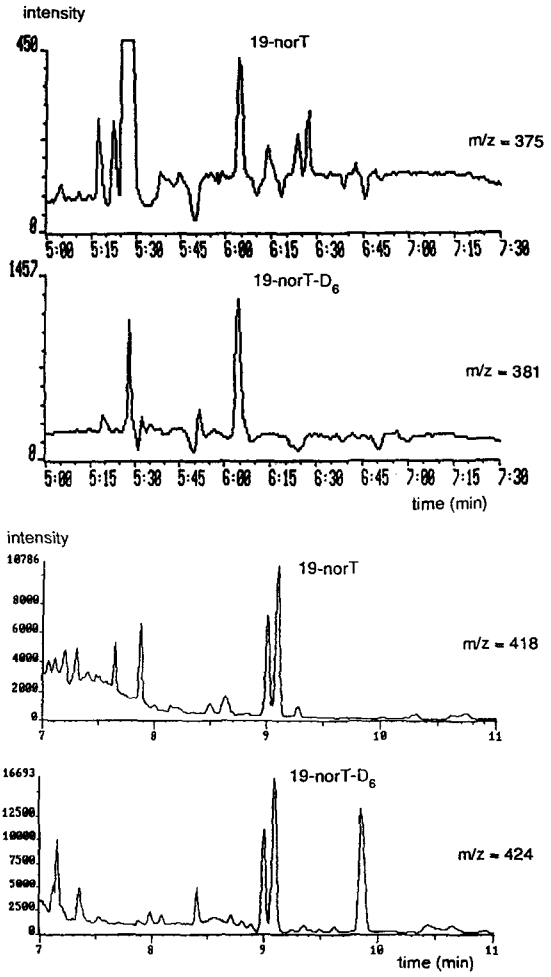

Fig. 4. GC–SIM tracings of an extracted meat sample, contaminated by 19-norT, after two different derivatization and ionization techniques. (A) Tracings obtained after conversion of steroids to the MO–TMS derivatives and after detection with EI mode; 2.5 ng of 19-norT-D_6 were added as external standard. Upper trace: molecular ion of 19-norT–MO–TMS. Lower trace: molecular ion of 19-norT-D_6–MO–TMS. (B) Tracings obtained after conversion of steroids to the PFBCMO–TMS derivatives and after detection with NICI mode; 1 ng of 19-norT-D_6 was added as external standard. Upper trace: carboxylate ion of 19-norT–PFBCMO–TMS. Lower trace: carboxylate ion of 19-norT-D_6–PBFCMO–TMS. The chromatograms of the PFBCMO–TMS derivatives show the separation of the two derivatization isomers of the steroids. See Fig. 1 and text for other explanations.

MS detection procedures. An aliquot was derivatized with MOX and BSTFA to give the methoxime–trimethylsilyl (MO–TMS) derivatives [12], and the other was derivatized by the method described. To both samples was added, before derivatization, 19-norT-D_6 as external standard (2.5 ng for the aliquot derivatized with MOX and 1 ng for the aliquot derivatized with CMOA). The MO–TMS derivatives were detected by GC–SIM using EI mode and monitoring the molecular ions (*m/z* 375 for 19-norT and *m/z* 381 for 19-norT-D_6), while the PFBCMO–TMS derivatives were detected, as described, using NICI mode. The same GC conditions (see Experimental section) were used for both samples. The use of the latter derivatization and ionization technique considerably increased the specificity and sensitivity of detection (Fig. 4). The improved sensitivity can be attributed to the increase in the absolute signal given by the analytes and to the reduced interferences in the chromatograms. In fact the PFBCMO–TMS derivatives of 19-norT and 19-norT-D_6 had longer retention times than MO–TMS derivatives and eluted into clean zones of the chromatograms. (The MO–TMS derivatives, unlike the PFBCMO–TMS derivatives, did not show the separation of two isomers with the GC conditions which were used.) For the meat sample analysed, a more certain identification and a more accurate quantitation of 19-norT could be obtained from the chromatograms of the PFBCMO–TMS derivatives. The quantitation of these samples, based on calibration curves made with standards containing known amounts of non-deuterated and deuterated analytes

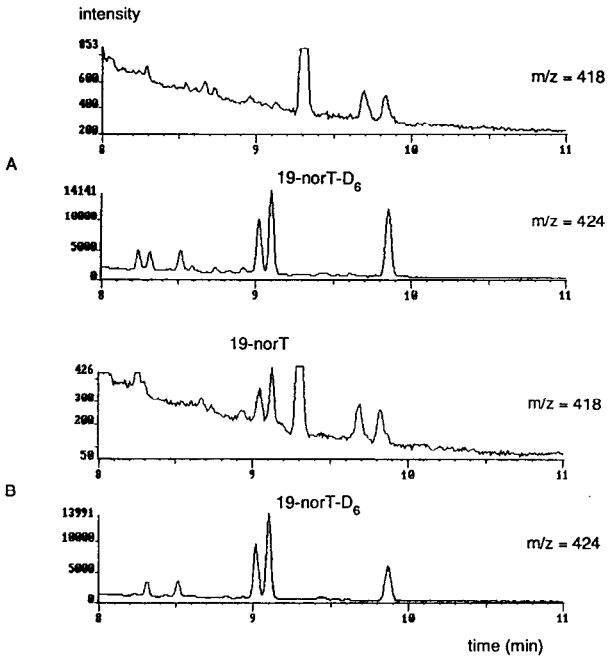

Fig. 5. GC–NICI–SIM tracings of two blank urine samples (1 ml) spiked with 19-norT and 19-norT-D_6, extracted with immunoaffinity columns and derivatized to form the PFBCMO–TMS derivatives of the two compounds. (A) Sample spiked with 1 ng of 19-norT-D_6 (lower trace) and not spiked with 19-norT (upper trace). (B) Sample spiked with 1 ng of 19-norT-D_6 (lower trace) and 20 pg of 19-norT (upper trace). The chromatograms show the separation of the two derivatization isomers of the steroids. See Fig. 1 and text for other explanations.

[15], gave values of 0.70 and 0.64 ppb (10^9) of 19-norT for the MO–TMS and PFBCMO–TMS derivatives respectively.

A second application of the derivatization procedure was performed to evaluate exactly the accuracy and the sensitivity achievable in the determination of 19-norT, T and TBOH. Several samples of female calf urine, spiked with known amounts of these steroids, were analysed after purification with immunoaffinity columns (IACs). The IACs (Multi-Prep II) contained anti-19-norT antibodies immobilized to a Sepharose gel and could also recover other similar steroids as T and TBOH. (Recovery efficiencies claimed by the manufacturer are 87% for 19-norT, 85% for T and 35% for TBOH.) The samples (1 ml urine, in triplicate) were spiked with 0, 20, 100 and 1000 pg of 19-norT, T and TBOH and with a constant amount (1000 pg) of 19-norT-D_6, T-D_5 and TBOH-D_5 and then extracted using the procedure described in the Experi-

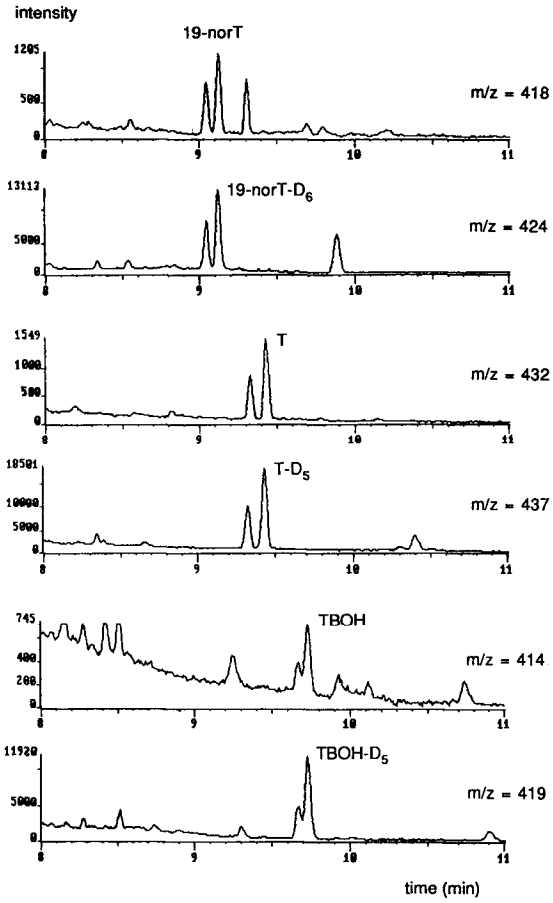

Fig. 6. GC–NICI–SIM tracings of a sample of female calf urine (1 ml) spiked with 100 pg of 19-norT, T and TBOH and with 1 ng of 19-norT-D_6, T-D_5 and TBOH-D_5. The sample was extracted with an immunoaffinity column containing antibodies raised against 19-norT and derivatized to obtain the PFBCMO–TMS derivatives of the steroids indicated. The chromatograms show the separation of the two derivatization isomers of every compound. See Fig. 1 and text for other explanations.

mental section. Injections by GC–NICI–SIM showed that urines spiked with deuterated standards only did not show any peak corresponding to unlabelled 19-norT and TBOH. The lowest level of 19-norT which could be detected corresponded to a concentration of 0.02 ppb (Fig. 5). The detection limit of TBOH, instead, was estimated to be about 0.06 ppb, because of the lower recovery from the IACs. For T the detection limit was not measurable in that way, because of its natural presence in urine at these low levels; however a detection limit of 0.02 ppb was obtained by extracting, in the same way, pure buffer samples spiked with T and T-D_5. The coefficients of variation for these determinations, obtained by extracting three urine samples spiked at the 0.1 ppb level, were 5.6% for 19-norT, 8.7% for TBOH and 6.3% for T. The complete chromatograms of one of these samples are shown in Fig. 6.

CONCLUSIONS

We have introduced a new derivatization procedure which expands the possibilities of choice for GC–MS detection of some 3-ketosteroids. This procedure was useful for the detection of 19-norT, T and TBOH at very low levels in biological samples. The method is suitable for extension to other similar steroids.

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