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Simultaneous determination, in calf urine, of twelve anabolic agents as heptafluorobutyryl derivatives by capillary gas chromatography–mass spectrometry

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Abstract

A method to determine twelve anabolic hormones (diethylstilbestrol, hexestrol, dienestrol, 17β-estradiol, 19-nortestosterone, testosterone, 1-dehydrotestosterone, 17α-methyltestosterone, progesterone, estrone, 17α-ethynilestradiol, and trenbolone) is presented. Urine samples were extracted with octadecylsilica columns and clean-up was performed in two steps with basic alumina and silica solid-phase extraction cartridges. The extracts obtained were derivatized with heptafluorobutyric anhydride and analyzed by GC–MS. Stability of derivatives was good and compounds having keto groups produced enol derivatives that were stable also. SIM mode was applied to increase the sensitivity and, when possible, the higher m/z ions were selected to improve identification. Repeatability of the chromatographic analysis was evaluated on the basis of area repeatability, and the coefficient of variation obtained was lower than 13%. Absolute recoveries were in the range 35–60% (dehydrotestosterone and estrone <20%) with coefficients of variation between 14 and 37% for the whole procedure. [$^2H_3$]Testosterone and [$^2H_8$]diethylstilbestrol were evaluated to improve quantitative data. The recovery of [$^2H_3$]testosterone was found to be equal to or slightly higher than that of the other hormones, but the recovery of [$^2H_8$]diethylstilbestrol was lower than any other. [$^2H_3$]Testosterone was the most suitable for use as an internal standard, as its addition at the beginning of analytical procedure, corrected recovery results and greatly improved precision. Corrected recoveries from urine ranged from 72–110%, and coefficients of variation ranged from 6–15%, except for testosterone which yielded slightly higher values. The limit of detection was 0.5 ng/ml for all the compounds studied.

Keywords: Steroids; Diethylstilbesterol; Hexestrol; Dienestrol; 17β-Estradiol; 19-Nortestosterone; Testosterone; 1-Dehydrotestosterone; 17α-Methyltestosterone; Progesterone; Estrone; 17α-Ethynilestradiol; Trenbolone

1. Introduction

Hormonal anabolic compounds are illegally used in livestock production to promote growth rate and improve feed conversion efficiency. The EC has banned their use in the fattening of slaughter animals [1,2], because of their toxic or carcinogenic properties. To control illegal treatments it is essential to establish reliable analytical methods. The concentration of anabolic compounds in excreta, such as urine, is generally higher and remains high up to several weeks after administration [3]. Thus, the analysis of this kind of sample allows us to detect the use of these compounds. Moreover, it is the only sample available from living animals.

A wide variety of procedures have been described

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for the determination of anabolic residues in tissue, faeces, urine, feed, and plasma. Immunoassay techniques such as RIA and ELISA have been developed for several compounds [4–7]. Although these methods are quite sensitive and useful for screening purposes, they are limited to a single compound and can give false-positive results. Thus, another method is necessary to confirm positive samples. TLC and HPTLC were reported on by many authors [8–15]. These methods usually lack sufficient sensitivity and they involve several laborious purification steps. HPLC methods with different detection systems have been reported to determine illegal anabolics in urine. Trenbolone was analysed by normal-phase HPLC with on-line UV detection at 350 nm [16]; reversed-phase HPLC with detection at 240 nm was used to analyse diethylstilbesterol (DES) in urine after purification on a diol-column [17]; HPLC with fluorescence detector was used for trenbolone and testosterone [18]; voltametric detection made it possible to analyse the phenolic hormones [19]. Laganà and Marino [20] developed an HPLC multi-residue method with different detectors for the determination of steroids in tissue after separation of the neutral anabolics from the acidic ones.

GC–MS has been proved to be a sensitive and suitable technique to detect growth promoters after derivatization. Most reported multi-residue methods refer to muscle tissue and a few ones to urine. Anabolic estrogens in muscle are detected by negative ion chemical ionization GC–MS after formation of pentafluoropropionyl derivatives [21]. Electron-impact GC–MS was used to quantify seven anabolic drugs in meat after conversion to trimethylsilyl esters [22]. Tuinstra et al. [23] developed a multi-method for several growth-promoting compounds in urine. Purification was performed with two-dimensional HPLC system after size-exclusion chromatography or Extrelut column and GC–MS was used for final confirmation after conversion to TMS or HFB derivatives. Santarius and Rösel [24] used a GC–MS multi-detection method with a macroprogramme for analysis of hormone residues in calf urine, after forming silylderivatives. Some authors have used internal standards to quantify some hormones. A GC–MS method with a solid (moving-needle) injection system was developed to detect DES in meat after derivatization with HFBA. The reliability of the analytical procedure was improved by using [$^3H_6$]-DES as internal standard and the recovery was about 90% [25]. A recovery of 70±20% was obtained by Jansen et al. [26] with a method based on isotope dilution mass spectrometry for quantification of DES in bovine urine.

Here a capillary GC–MS multi-residue method to confirm the presence of eleven different hormones in calf urine that includes extraction and purification with SPE cartridges is presented. The use of internal standards to enhance the recoveries was evaluated.

2. Experimental

2.1. Solvents and reagents

All solvents were analytical grade from Merck (Darmstadt, Germany) and water was purified via Milli-Q (Millipore, Bedford, MA, USA). Deuterium-labeled diethylstilbestrol was supplied by CIL (Woburn, MA, USA) and the other standards were purchased from Sigma (St. Louis, MO, USA). β-Glucuronidase–arylsulfatase from *Helix pomatia* was obtained from Merck. Heptafluorobutyric anhydride (HFBA) was from Supelco (Bellefonte, PA, USA). Solid-phase extraction cartridges were Sep-Pak Plus from Waters (Milford, MA, USA).

2.2. Extraction and clean-up

A 20-ml aliquot of urine was adjusted to pH 5.0 with 0.1 *M* acetic acid and 25 μl of β-glucuronidase–arylsulfatase enzyme solution were added. The sample was incubated at 37°C overnight to allow hydrolysis of the hormone conjugate.

An octadecylsilica extraction column was activated with 5 ml methanol followed by 5 ml distilled water. After incubation the sample was slowly passed through the cartridge and it was allowed to dry under vacuum for 3 min. A basic alumina column was conditioned by rinsing with 5 ml methanol, then it was placed below the C_{18} column. The anabolic substances were eluted by passing 5 ml methanol through both cartridges. Thereafter, the C_{18} column was removed and another 3 ml methanol were applied to the alumina. The combined methanol eluates were taken to dryness in a rotary evaporator

at 45°C. The residue was dissolved with 1 ml hexane–diethyl ether (1:1, v/v) and passed through a silica column previously washed with 15 ml hexane. The cartridge was washed with 10 ml hexane and hormones were eluted with 10 ml dichloromethane–ethyl acetate (1:1, v/v). The solvent was partially evaporated and transferred quantitatively to a suitable screw-capped glass vial for derivatization.

2.3. Derivatization

After total evaporation, 200 μl acetonitrile and 50 μl HFBA reagent were added to the dry residue. The tube was closed with a PTFE coated cap and heated for 1 h at 80°C in a thermostatic block. After the tube was cooled, the solution was evaporated to dryness under a stream of nitrogen and the residue was redissolved with 100 μl iso-octane. A 1-μl volume of this solution was injected splitless onto the chromatographic system.

2.4. Chromatographic conditions

The analyses were performed on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5970A mass selective detector. A FSOT capillary column coated with 5% phenylmethylsilicone (20 m×0.18 mm I.D.) and 0.25 μm film thickness was used. The temperature program was: 1 min at 80°C and from 80°C to 280°C at a rate of 10°C/min; injection port and interface were set at 280°C; inlet pressure was 0.1 MPa and helium was used as a carrier gas.

Both identification and quantitation were carried out by selected ion monitoring (SIM) of characteristic ions of the heptafluorobutyril (HFB) derivatives. Individual SCAN of HFB derivatives was performed for each investigated anabolic standard to obtain the mass fragmentation pattern. The most suitable ions (high ion intensity, high mass and low background) were selected to perform the analysis with SIM mode. The reference compounds and ions chosen for the multi-residue analysis are listed in Table 1. However, decimal values had to be adjusted from time to time depending on the state of the mass selective detector.

2.5. Repeatability, recovery and sensitivity evaluation

Repeatability of the chromatographic analysis was determined by eight replicate 1-μl injections of a mixture of derivatized standards. Reproducibility for urine samples was evaluated by several injections of 1 μl of urine extracts obtained from fortified samples at 5 and 10 ppb levels. Coefficients of variation (R.S.D.s) were calculated.

Analytical recovery of the method was evaluated for each anabolic compound. Five aliquots of the same urine sample were spiked with known amounts of hormones to obtain fortified urine samples at 10 ppb level. Extraction, purification and quantitative

Table 1
Ions of HFB derivatives of anabolics used for GC–MS

Abbreviation	Compound	M^a	M_d^b	m/z
DES	Diethylstilbestrol	268.4	660.4	303.2, 331.2, 341.2, 660.4
HEX	Hexestrol	270.4	662.4	275.2, 303.2, 331.2
DE	Dienestrol	266.3	658.3	317.2, 341.2, 445.3, 658.3
E	17β-Estradiol	272.4	664.4	356.2, 409.2, 451.3, 664.4
NT	19-Nortestosterone	274.4	666.4	306.1, 453.3, 666.3
T	17β-Testosterone	288.4	680.4	320.1, 355.1, 467.3, 680.4
DHT	1-Dehydrotestosterone	286.4	678.4	343.0, 369.1, 464.1, 678.2
MT	17α-Methyltestosterone	302.5	498.5	355.0, 369.1, 465.2, 480.2
PG	Progesterone	314.5	510.5	355.1, 425.1, 495.1, 510.2
ENA	Estrone	270.4	466.4	356.1, 409.2, 422.2, 466.3
EE	17α-Ethynylestradiol	296.4	492.4	353.0, 446.0, 459.1, 474.1
TREN	Trenbolone	270.4	664.4	356.1, 409.2, 451.2, 664.3

[a] Molecular mass.
[b] Molecular mass of HFB derivative.

analysis were performed using the described procedure. Mean recoveries, standard deviation (S.D.) and R.S.D. were calculated for each compound.

Urine aliquots spiked with increasing amounts of standards (0.5, 1, 2, 5 and 10 ppb) were analyzed to determine the detection limit of the method. A urine blank without added anabolics was analyzed at the same time in duplicate.

2.6. Use of internal standards

The use of deuterium-labeled testosterone ([$^2H_3$]T) and diethylstilbestrol ([$^2H_8$]DES) as internal standards (I.S.) were evaluated in order to compensate for any loss during sample preparation and to enhance the recoveries. Urine samples were fortified at 10 ppb level with six different anabolics (DES, HEX, DE, E, NT, and T) and [$^2H_3$]T and [$^2H_8$]DES were added as I.S. at the same time, before assay was performed.

3. Results and discussion

A total ion chromatogram of a standard mixture of HFB derivatives of the twelve substances studied is shown in Fig. 1. The two peaks seen for DES were due to *cis*- and *trans*-isomers of the derivatized compound. These isomers were well separated and the ion abundance of the *trans*-isomer was usually higher than the ion abundance of the *cis*-isomer. Since *cis*/*trans* ion intensity ratio was not constant, the sum of the peak areas of both isomers was used for quantitation. Although HEX and DE showed the same retention time, they could be distinguished by SIM, using specific ions (see Table 1). Also, it was possible to improve the separation of HEX and DE using a capillary column of I.D. 180 μm and 0.18 μm film thickness. E and TREN coeluted and yielded ions with the same values. Therefore, samples that present a peak at this retention time should be derivatized by an alternative reagent, such as BSTFA. Trimethylsilyl derivatives yielded different retention times and different molecular ions and fragments that make the identification and quantitation of E and TREN possible.

As can be seen in Table 1, HFBA reacted with

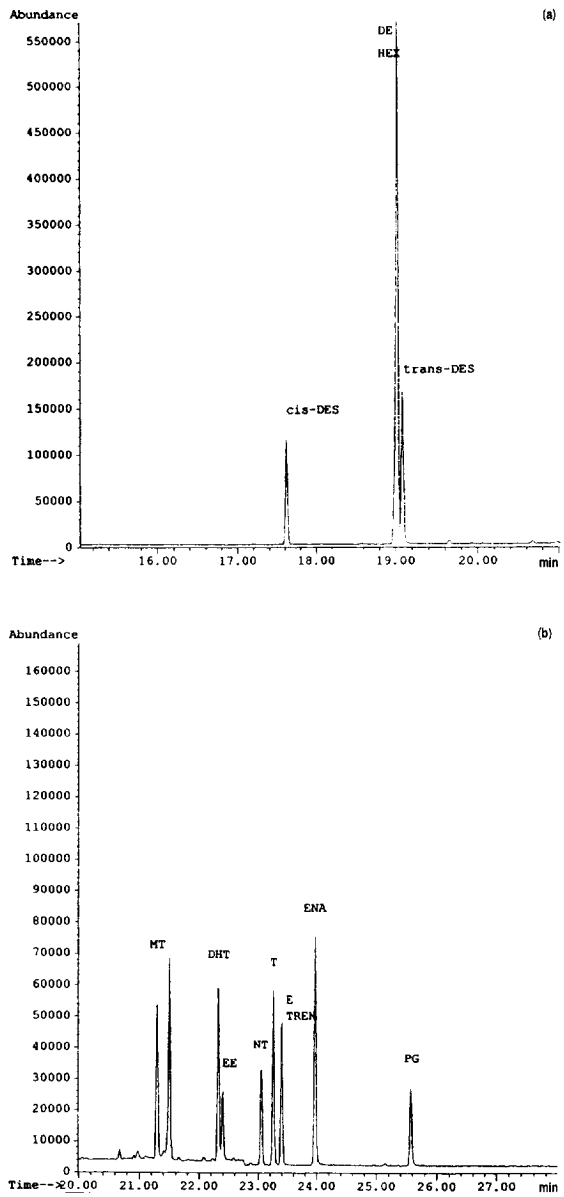

Fig. 1. GC–MS total ion chromatogram of a solution of HFB derivatives of anabolic standards. (a) DES, HEX, DE; (b) MT, DHT, EE, NT, T, TREN, E, ENA, PG. For conditions, see text.

3-oxo groups of T, NT, DHT and MT with formation of 3-heptafluorobutyryl enol esters, but some 17-hydroxyl groups of (MT, EE) were not converted. In brief, the procedure yielded mono-HFB derivatives for MT, PG, ENA and EE, whereas the rest of compounds yielded di-HFB derivatives. The stability

Table 2
R.S.D.(%) values of GC–MS analysis of standards

Compound	R.S.D. (%)
DES	5.8
HEX	5.3
DE	8.3
E	13.2
NT	7.3
T	7.0

Concentration: 1 ng/μl; injection volume: 1 μl; $n=8$

of these derivatives was good, but derivatized samples were not kept longer than three days.

Repeatability of the chromatographic analysis was evaluated for some standards (Table 2) and for spiked urine extracts at 5 and 10 ng/ml level (Table 3). The repeatability, stated as the coefficient of variation, was in the range 5–13%, R.S.D. values obtained from urine samples being slightly higher than those yielded for standards.

In the first phase of this work the analytical procedure was evaluated without using any I.S. Appropriate amounts of anabolic compound solution were added to urine aliquots to give fortified samples at the desired levels. Then, the whole procedure (extraction, clean-up, derivatization and chromatographic determination) was performed. The peak areas at one or two selected ions were obtained for each specific anabolic compound. Hormones were quantitated by comparing peak area from the spiked sample with that from the corresponding external standard and absolute recoveries were calculated. Derivatization of standards was performed for each set of samples. Recoveries were evaluated with urine samples spiked at 5 and 10 ng/ml level and data are reported in Table 4. The highest recoveries were

Table 3
R.S.D. (%) values of GC–MS analysis of urine samples

Compound	R.S.D. (%)	
	5 ppb ($n=5$)	10 ppb ($n=7$)
DES	10.6	7.3
HEX	9.5	6.9
DE	10.8	7.8
E	8.0	11.0
NT	6.6	5.6
T	8.7	6.8

Table 4
Recoveries of hormones from spiked urine samples ($n=5$)

Compound	Recovery (mean±S.D.) (%)	R.S.D. (%)
DES	45.0±7.6	17
HEX	47.4±6.7	14
DE	54.0±11.5	21
E	55.6±10.8	19
NT	59.8±11.1	19
T	54.6±13.5	25
MT	45.0±14.9	33
DHT	16.6±4.0	24
EE	44.6±7.2	16
ENA	19.8±7.2	36
PG	40.8±8.3	20
TREN	35.7±13.4	37

obtained for DE, E, NT, and T (>50%), whereas recoveries were lower for DHT and ENA. Table 5 shows recoveries obtained from urine spiked with increasing amounts of standards. As can be seen from these results, there is no effect of hormone concentration on the recovery of the substance at these levels. The limit of detection was 0.5 ng/ml for all the compound studied. At this concentration a defined chromatographic peak can be seen, as no important matrix interference appears at the retention time of the compounds. The matrix noise from the blank showed a signal lower than 0.2 ng/ml, but different urine samples can present different chromatographic profiles. As an example, Fig. 2 shows SIM chromatograms obtained from a urine sample fortified with several anabolic agents at 1 ppb level and those obtained from a urine sample free of anabolic residues and monitored at the same ions.

Since average recoveries obtained without adding internal standard were poor and R.S.D. were high, the use of an I.S. to improve results was evaluated.

Table 5
Recoveries from urine fortified with different amounts of anabolic

Compound	Recovery (%)				
	0.5 ppb	1 ppb	2 ppb	5 ppb	10 ppb
DES	37.4	36.2	43.3	53.8	35.2
HEX	39.4	38.4	47.5	54.5	39.3
DE	47.6	48.5	63.5	62.3	61.5
E	35.1	34.4	38.9	34.1	45.8
NT	47.7	46.8	46.8	37.8	46.3
T	72.5	53.6	56.8	41.8	49.8

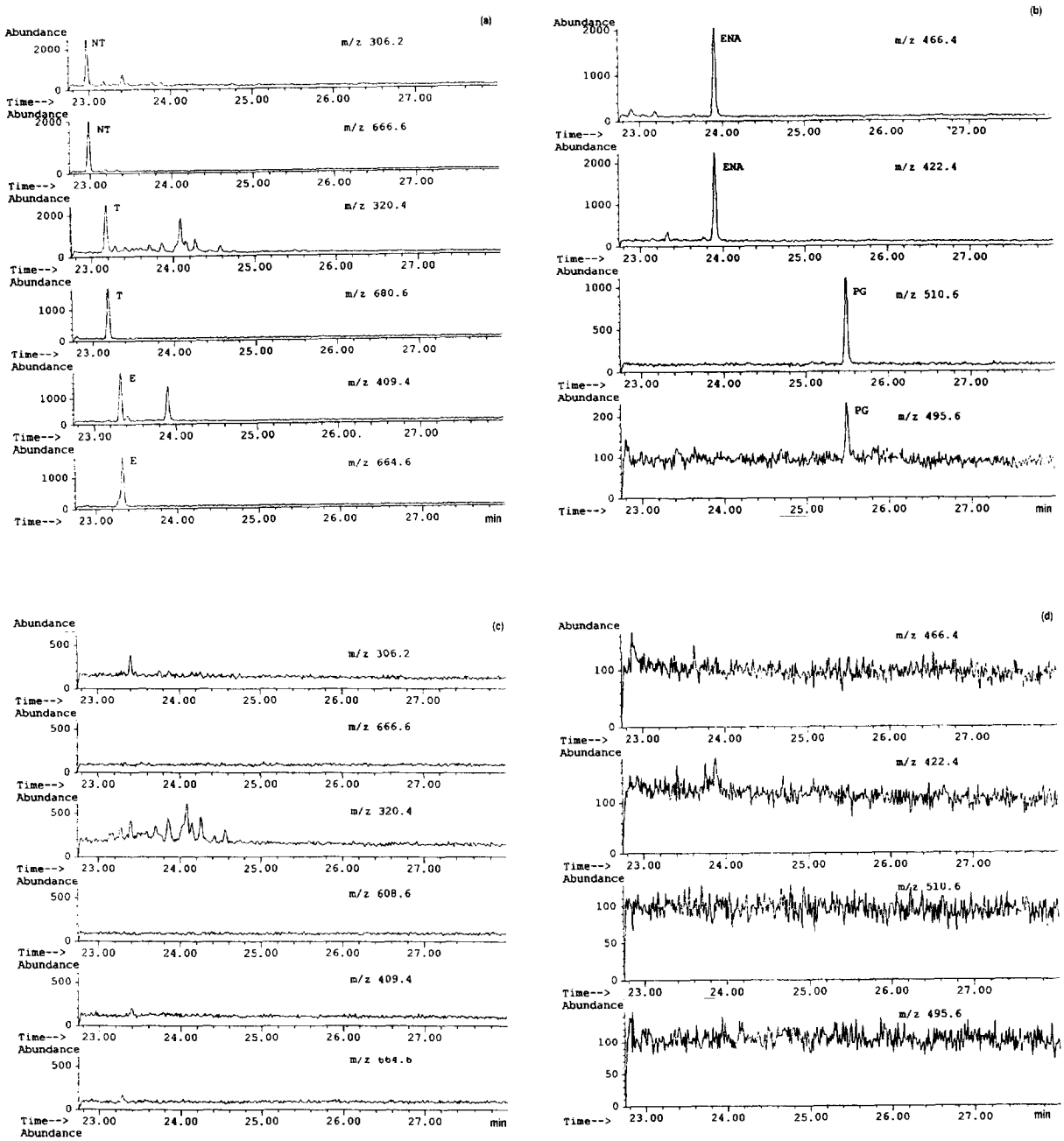

Fig. 2. GC–MS selected-ion monitoring chromatograms of urine spiked at 1 ppb level with NT, T, E, ENA, PG (a, b) and urine blank at the same selected ions (c,d). Two monitored ions are presented for each compounds: *m/z* 306.2, 666.6 (NT); *m/z* 320.4, 680.6 (T); *m/z* 409.4, 664.6 (E); *m/z* 466.4, 422.4 (ENA); *m/z* 510.6, 495.6 (PG). For conditions, see text.

The errors of the analytical procedure originate from extraction and purification steps, derivatization and detection. The use of an I.S. added at the beginning of sample preparation could compensate for the recovery losses during the assay. Deuterium-labeled testosterone and deuterium-labeled diethylstilbestrol

were evaluated for that purpose. Recovery experiments were performed with urine samples which were spiked by addition of known amounts of analytes and I.S. Then they were analyzed, and anabolic compounds were quantitated and the results were corrected by the ratio of peak area of each compound to peak area of the I.S. Selected ions to perform the analysis of deuterium-labeled I.S., obtained from the SCAN of HFB derivatives, were *m/z* 470.3 and 683.4 for [$^2H_3$]T and *m/z* 341.5 and 668.3 for [$^2H_8$]DES. Fig. 3 shows the chromatograms of calf urine spiked with MT and [$^2H_3$]T added as I.S.

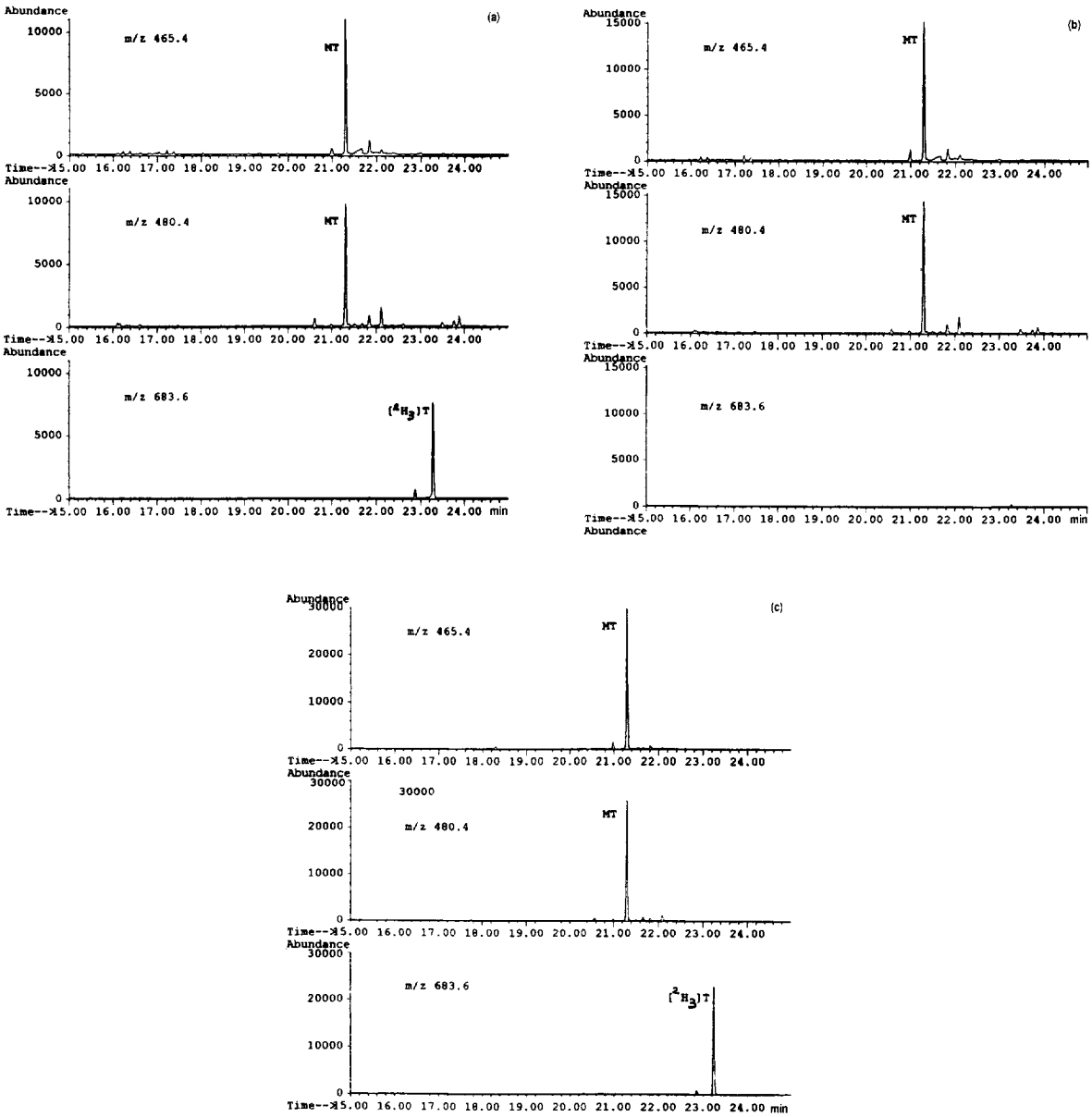

Fig. 3. GC–MS selected-ion monitoring chromatograms of a calf urine spiked with MT and [$^2H_3$]T as I.S. (a); the same urine spiked with MT but without the I.S. (b); mixture of standards (c). Ions used for specific detection of these two compounds were *m/z* 465.4, 480.4 (MT) and *m/z* 683.6 for [$^2H_3$]T. For conditions, see text.

Fig. 4 shows the chromatograms of urine fortified with DE and [$^2H_8$]DES as I.S. [$^2H_8$]DES shows two peaks corresponding to its *cis*- and *trans*-isomers. As can be seen, no interference from matrix was found for the selected ions at the retention time of deuterium-labeled compounds.

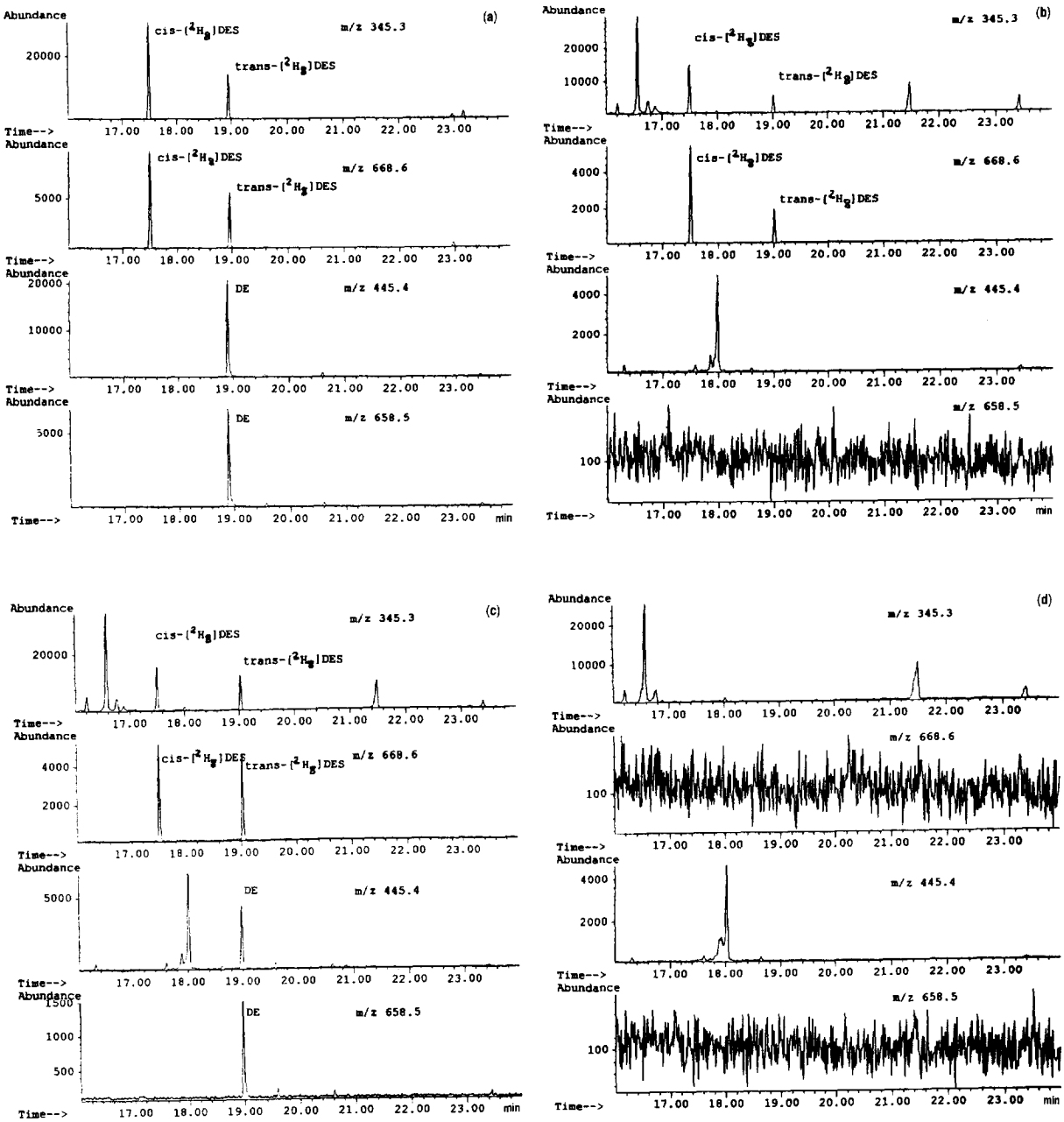

Fig. 4. GC–MS selected-ion monitoring chromatograms of a mixture of DE and [$^2H_8$]DES as I.S. (a); urine spiked only with the I.S. (b); the same urine spiked with DE and [$^2H_8$]DES (c); control unspiked urine (d). Ions selected for monitoring were *m/z* 445.4, 658.5 (DE) and *m/z* 345.3, 668.6 for [$^2H_8$]DES. For conditions, see text.

Table 6
Recoveries of deuterium-labeled standards ($n=5$)

Compound	m/z	Recovery (mean±S.D.) (%)	R.S.D.(%)
$[^2H_3]$T	683.4	53.2±10.0	19
	470.3	58.7±10.9	18
$[^2H_8]$DES	668.3	39.2±6.2	16
	345.1	43.4±6.8	16

The absolute recovery of $[^2H_3]$T was found to be equal to or higher than that of the other substances, but the recovery of $[^2H_8]$DES was lower than that of any other (Table 6). Thus, using $[^2H_8]$DES as I.S. resulted in recoveries higher than 100% for the compound studied. In addition, slight differences were observed with respect to the ion selected for peak integration. Table 7 shows recoveries calculated using $[^2H_3]$T as I.S. and for both selected ions (m/z 683.4 and 470.3). Table 8 gives recoveries obtained from the same urine sample using $[^2H_8]$DES as I.S. and for m/z 668.3 and m/z 345.1. An increase in the mean recoveries and a decrease in the R.S.D. values were observed using $[^2H_3]$T to correct the results. This compound seems suitable for use as I.S. As can be seen from Table 8, the use of $[^2H_8]$DES yielded recoveries far from the expected values and high R.S.D. values, except for DES and HEX when m/z 345.1 was used. From these results, it can be concluded that the most suitable I.S. is $[^2H_3]$T.

As a wide variation in the recovery of the deuterium-labeled substances was observed, the use of I.S. added just before derivatization and chromatographic analysis was evaluated. Four aliquots of the same urine were fortified with DES, HEX, DE, NT, T, MT, EE, and PG at 10 ppb level and extraction and clean-up were performed as described in Section 2.2. After drying the extracts and just before derivatization, 200 ng of $[^2H_3]$T were added. Table 9 compares the mean recovery and the R.S.D. obtained for each substance with correction by means of I.S. and without any correction. A decrease in the R.S.D. value of recoveries was observed, especially for E, T, MT and PG, by using $[^2H_3]$T as I.S., but the recoveries of some hormones remain low (E, NT, T, PG). This means that adding I.S. before derivatization clearly enhances precision, but recoveries are not affected equally. These results suggest that extraction and clean-up steps have a strong influence on the recovery of these compounds that have similar molecular structures but different functional groups, so that, adding I.S. at the beginning of analytical

Table 7
Recoveries of hormones from fortified urine using $[^2H_3]$testosterone as I.S.

Compound	m/z 683.4		m/z 470.3	
	Mean±S.D. (%)	R.S.D. (%)	Mean±S.D. (%)	R.S.D. (%)
DES	81.2±12.6	15	72.2±11.4	16
HEX	86.1±10.0	12	76.3±7.1	9
DE	94.9±5.5	6	84.5±8.3	10
E	105.9±10.0	9	93.8±4.3	5
NT	109.6±8.2	7	97.3±5.5	6
T	112.4±24.4	22	99.3±17.6	18

Table 8
Recoveries of hormones from fortified urine using $[^2H_8]$DES as I.S.

Compound	m/z 668.3		m/z 345.1	
	Mean±S.D. (%)	R.S.D. (%)	Mean±S.D. (%)	R.S.D. (%)
DES	116.6±23.4	20	104.2±10.0	10
HEX	122.3±19.2	16	109.6±4.5	4
DE	121.5±28.6	23	123.4±12.6	10
E	147.1±31.0	21	131.8±18.9	14
NT	155.2±36.6	23	138.2±18.7	13
T	140.4±34.1	24	128.7±36.6	28

Table 9
Recoveries with and without I.S. added before derivatization

Compound	I.S.: [$^2H_3$]T		Without I.S.	
	Mean±S.D. (%)	R.S.D. (%)	Mean±S.D. (%)	R.S.D. (%)
DES	121.9±16.6	14	77.4±10.5	14
HEX	69.3±10.0	14	44.5±9.4	21
DE	118.3±14.8	12	75.1±9.5	13
E	46.8±5.7	12	30.1±6.4	21
NT	66.8±5.1	8	42.6±5.5	13
T	59.2±7.3	12	38.2±9.2	24
MT	114.7±4.5	4	31.3±8.2	26
EE	95.0±17.8	19	25.6±7.1	27
PG	56.2±3.0	5	15.3±3.9	25

procedure is the best choice to quantify these anabolic compounds by the method proposed.

4. Conclusion

The multi-residue method described is useful for screening and confirmatory purposes. A combination of three SPE columns provided urine extracts without interference from matrix, suitable for GC–SIM-MS analysis, after derivatization with HFBA. TMS derivatives have to be used for samples containing estradiol and/or trenbolone. The use of [$^2H_3$]testosterone as I.S., added at the beginning of analytical procedure, greatly improved precision and corrected recovery results.

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