Journal of Chromatography, 279 (1983) 533–542
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 134

PROCEDURE FOR THE GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC CONFIRMATION OF SOME EXOGENOUS GROWTH-PROMOTING COMPOUNDS IN THE URINE OF CATTLE

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SUMMARY

As gas chromatography–mass spectrometry is the most conclusive confirmation technique available today for the detection of ppb levels of anabolics in the urine of cattle, the following procedure was used. The urine is hydrolysed with *Helix pomatia* intestinal juice, extracted, and the extract cleaned by gel-permeation chromatography or with Extrelut. In one fraction are eluted diethylstilboestrol, dienoestrol, hexoestrol, methyltestosterone, ethinyloestradiol, zeranol and trenbolone. This fraction is injected into a two-dimensional high-performance liquid chromatography system. From the first column, the fraction containing the above-mentioned compounds is transferred to the second column, where a separation into two fractions is obtained. The first fraction contains zeranol and trenbolone, the second fraction the stilbenes, methyltestosterone and ethinyloestradiol. In general, the compounds are derivatized with an N,O-bis (trimethylsilyl)trifluoroacetamide–trimethylchlorosilane mixture to a trimethylsilyl derivative. With stilbene, confirmatory derivatization into heptafluorobutyryl derivatives is necessary. In combination with a Finnigan 4000/INCOS system, a CP-Sil-5 CB and a CP-Sil-19 CB capillary are used for final confirmation. Two capillaries with different polarities are necessary to overcome problems with possible interferences from compounds extracted from the urine. Recoveries at the ppb* level are better than 80%.

INTRODUCTION

In the autumn of 1980, the presence of diethylstilboestrol (DES) in calf meat was revealed in the Italian press. This was followed by similar press reports in several other European countries. Worldwide, natural and synthetic anabolics are used to increase meat production, about 5–15% due to improved feeding stuff conversion. In the Netherlands and the EEC the opinion prevailed that the use of hormonal anabolics should be prohibited, because of the possible hazards to the consumer. In The Netherlands, a survey programme based on screening of the urine of cattle for the determination of DES by radioimmunoassay (RIA)[1] was started. Urine is used, as the con-

* Throughout this article, the American billion (10^9) is meant.

0021-9673/83/$03.00

centration in urine is at least ten-fold higher than in meat. The RIA procedure is able to detect (with the same antibody) hexoestrol (HEX) and dienoestrol (DE), but with a lower sensitivity. Suspected samples must be confirmed using gas chromatography–mass spectrometry (GC–MS), as this method is the most conclusive confirmatory technique available today[2,3]. As it is expected that screening procedures will also be used for other anabolics, *e.g.*, zeranol (ZER) and trenbolone (TREN) we shall be confronted with problems with the confirmation of different anabolics in urine using GC–MS.

Many methods have been described for the determination of anabolics, often referring to a single or only a few hormones (stilbenes) in meat[4–9]. However, urine is not used often as a matrix[10,11], certainly not with GC–MS confirmation as the main aim. We have developed a method applicable to the extraction and clean-up of urine samples to obtain a multi-method for some important growth-promoting compounds used in the bio-industry.

APPARATUS

Gel-permeation chromatography (GPC)

The laboratory-built system consisted of a Waters M45 constant-flow pump, an automatic sampler (Waters Wisp 710 B) a UV detector (Waters 440) (wavelength 280 nm), a three-way valve (Chromatronix) controlled by a mechanical timer and an LKB Ultrarac fraction collector. The gel-permeation column (0.45 × 0.010 m I.D.) was thermostated at 40°C; packing material was Bio-Beads SX-3. The eluent was toluene–ethyl acetate (1:1) at a flow-rate of 1.0 ml/min.

High-performance liquid chromatography (HPLC)

The two-dimensional HPLC equipment was operated with one pump (Applied Chromatographic Systems), three Valco HPLC valves, which control the direction of the solvent flow (fore-flush, back-flush or heart-cut), and a Valco two-way valve for the solvent composition. A Siemens three-way valve, controlled by an air switch, was used for trapping different fractions. The system was remotely controlled by a Spectra-Physics 4000 data system. Fig. 1 shows the two-dimensional HPLC system.

Column I (10 μm LiChrosorb RP-18, 10 cm × 4.6 mm I.D.) was used for preconcentration of the sample with acetonitrile-water (5:95) as eluent A (Fig. 1B). Elution of column I was started by changing to eluent B (acetonitrile–water, 60:40). After about 10 min, a heart-cut of 1.5 min (Fig. 1C) transferred the anabolics to the second column (5 μm LiChrosorb RP-18, 15 cm × 4.6 mm I.D.). The first column was back-flushed for about 12 min with eluent B (Fig. 1D) and then elution of column II with eluent B was performed (Fig. 1C).

The anabolics in column II were thus separated into two fractions, which were collected for derivatization. The first fraction contained zeranol and trenbolone, the second fraction the stilbenes, methyltestosterone (METEST) (17α-methyl-Δ^4-androsten-17β-ol-3-one) and ethinyloestradiol (EEST) [1,3,5(10)oestratrien-17α-ethinyl-3,17β-diol].

Gas chromatography–mass spectrometry (GC–MS).

For confirmation purposes a Finnigan 4000/INCOS system was used. In the Fin-

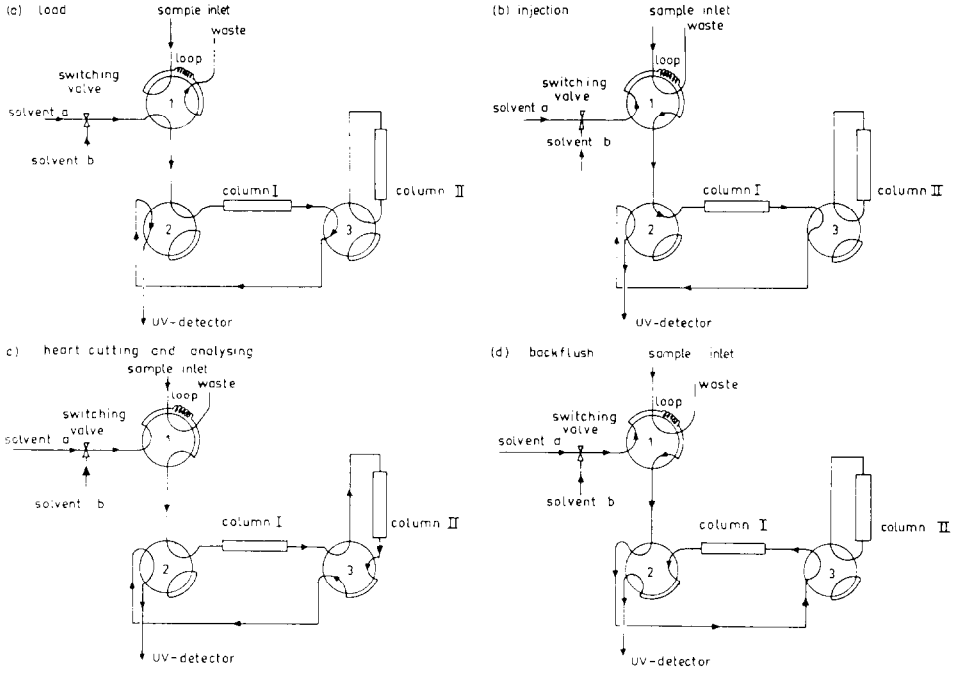

Fig. 1. Two-dimensional HPLC system. Valve I, injection loop = 500 μl; valves 2 and 3, switching flow directions. Eluents, acetonitrile–water: (A) 5:95; (B) 60:40.

TABLE I

IMPORTANT IONS OF INVESTIGATED ANABOLICS

Anabolic	*Abbreviation*	*Derivative*	M^+	*m/z**
Diethylstilboestrol	DES	HFB	660	*660, 631, 447,* 417, *341,* 303
	DES	TMS	412	*412, 397, 383*
Dienoestrol	DE	HFB	658	*658,* 643, *629,* 461, *445,* 341
	DE	TMS	410	*410, 395, 381*
Hexoestrol	HEX	HFB	662	*331,* 332, *303,* 304
	HEX	TMS	414	*207, 192, 179*
17β-Oestradiol	ESTRA	HFB	664	664, 451, 409, 368, 356, 237
	ESTRA	TMS	416	416, 401, 326, 311, 298, 285
Oestriol	ESTRI	HFB	876	876, 663, 449, 368, 236
Oestrone	ESTRO	HFB	466	466, 448, 442, 409, 368, 356, 253
	ESTRO	TMS	342	342, 327, 357, 244, 234, 218
Ethinyloestradiol**	EEST	TMS	440	*440, 425, 300, 285*
	Underivatized		368	368, 353, 300, 285, 232
Methyltestosterone**	METEST	1TMS	374	374, 359, *317, 304, 284, 143*
	METEST	2TMS	446	*446,* 431, 412, *356, 314, 301*
	Underivatized		302	302, 284, 269, 245, 229
Zeranol	ZER	TMS	538	*538, 523, 433,* 335, *307*
Trenbolone	TREN	TMS	342	*342,* 327, *252, 237,* 224, *211*

* Values in italics are ions used for MID; underlined values are base peaks.

** See text.

nigan 9610 gas chromatograph, two capillaries are connected to the mass spectrometer. One column (25 m CP-Sil 19 CB fused silica) is connected to the standard Grob injector and the second column (25 m CP-Sil 5 CB fused silica) is connected to a modified injection port[12,13] also permitting injections according to Grob and Grob[14,15].

The first column is threaded through the stainless-steel transfer line union so that the exit of the column is up to the ion-source block entrance, making possible source removal without disconnecting the column. The seond column is connected to the 1/4-in. nut of the direct chemical ionization line. The linear velocity of helium in both columns is about 25 cm/sec at 240°C.

A volume of 5 μl is injected in the splitless mode with an injection port temperature of 230°C while the oven temperature is set at 80°C, after 4 min the oven temperature is raised to 240°C at 15°/min; 3 min after injection the splitter and the septum flush are reopened, with flow-rates of 20 and 3 ml/min, respectively. The temperature of the interface is set at 240°C and the temperature of the ion source at 250°C. Ionization is performed by electron impact at 70 eV and an emission current of 300 μA. The mass spectrometer is operated mainly in the multiple-ion detection mode (MID), with a total maximal scan time of 0.5 sec. At least ten scans per peak are acquired (see Table I).

METHOD

Hydrolysis

Add 12.5 ml of water to 25 ml urine and adjust the pH to 4.5–5 with acetic acid. Add 25 μl of β-glucuronidas–arylsulphatase solution *(Helix pomatia;* Merck) and incubate over-night at 37°C.

Extraction and clean-up

In our laboratory we use two alternative clean-up procedures with comparable results: GPC or extraction and clean-up with Extrelut.

GPC clean-up. Extract the hydrolysed urine twice with diethyl ether, shake the ether fraction with sodium carbonate–sodium hydrogen carbonate solution (dissolve 100 g of sodium carbonate in 1 l of water and adjust the pH to 10.3 with solid sodium hydrogen carbonate). Remove the aqueous phase, extract the ether phase with water and dry the ether over sodium sulphate. Evaporate the ether to dryness and dissolve the residue in 300 μl of ethyl acetate–toluene (1:1). Inject 240 μl of this solution into the GPC system, evaporate the fraction containing the anabolics to dryness and dissolve the residue in 300 μl of acetonitrile–water (1:1).

Extrelut clean-up. Apply 20 ml of hydrolysed urine to the Extrelut column, wait 15 min, then elute with 80 ml diethyl ether and shake the eluate with 25 ml of sodium carbonate–sodium hydrogen carbonate solution (see above). Remove the aqueous phase, shake the ether extract with 25 ml of water, separate the layers and remove the aqueous phase. Dry the ether phase over sodium sulphate, evaporate to dryness and dissolve the residue in 300 μl of acetonitrile–water (1:1).

Final clean-up and isolation of the anabolics (HPLC)

Inject the cleaned extract obtained by GPC or Extrelut clean-up into the HPLC system and subject it to the two-dimensional analysis. Collect two fractions, one con-

TABLE II

RELATIVE RETENTION TIMES (*trans*-DES = 1.000) OF THE TMS AND HFB DERIVATIVES OF THE ANABOLICS ON TWO CAPILLARIES

Anabolic	*HFB derivatives**			TMS derivatives*	
	CP-Sil-5 CB	CP-Sil-19 CB		CP-Sil-5 CB	CP-Sil-19 CB
DES	1.000	1.000		1.000	1.000
DE	0.992	0.990		0.997	1.002
HEX	0.997	1.007		0.992	0.996
ESTRA	1.205	1.236		N.D.	1.117
ESTRI	1.214	1.277		N.D.	N.D.
ESTRO	1.232	1.342		N.D.	1.182
EEST**	—	—		1.434	1.233
EEST (Underivatized)	—	—		N.D.	1.260
METEST**	—	—	1TMS	1.415	1.334
METEST	—	—	2TMS	1.402	1.196
METEST (underivatized)	—	—		N.D.	1.433
ZER	—	—		1.507	1.294
TREN	—	—		1.317	1.298

* N.D. = not determined; — = no HFB derivative.

** For TMS derivatization of EEST and METEST, see text.

taining zeranol and trenbolone and the other the stilbenes, ethinylestradiol and methyltestosterone. Evaporate the two fractions to dryness.

Derivatization

With no indications from the screening analysis (RIA), the trimethylsilyl (TMS) derivative is the first choice (Table I). In confirmation for DES, DE and HEX, the hepta fluorobutyryl (HFB) derivative is preferred.

TMS derivatives. All the anabolics in Table I are derivatized with a freshly prepared mixture of trimethylchlorosilane (TMCS) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (1:99). Dissolve the fractions in 50 μl of TMCS–BSTFA, and allow them to react for 1 h at 60°C in a closed reaction vial[16]. Add 200 μl of acetonitrile, evaporate to dryness at 40°C under a gentle stream of nitrogen and dissolve the residue in 25 μl of isooctane with the aid of a vortex mixer.

HFB derivatives. Dissolve the stilbene-containing fraction in 250 μl of HFBA (Merck)–acetone (1:4). Allow the mixture to react for 1 h at 60°C in a closed reaction vial. Evaporate to dryness and dissolve the residue in 25 μl of isooctane with the aid of a Vortex mixer[16].

DISCUSSION AND CONCLUSION

The growth-promoting compounds are excreted in urine mainly as conjugates of glucuronic or sulphuric acid or, to a minor extent, as free hormones[17]. Hydrolysis of the conjugates is carried out with *Helix pomatia* intestinal juice[10,18,19] at 37°C. As only DES glucuronide (DES-G; Aldrich) is commercially available, no real recovery experiments with the conjugates could be carried out for the other anabolics. On the

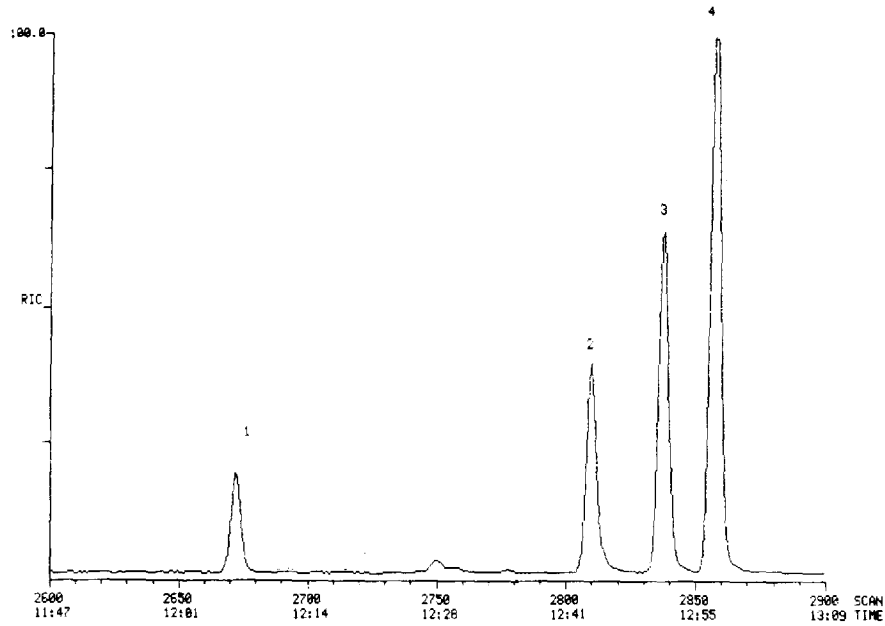

Fig. 2. Separation of DES-, HEX- and DE-HFB derivatives on a CP-Sil 19 CB column. Peaks: 1 = *cis*-DES-HFB; 2 = DE-HFB; 3 = *trans*-DES-HFB; 4 = HEX-HFB.

other hand, the fact that in urine, using this hydrolysis procedure with RIA and GC–MS techniques, several anabolics could be detected at the ppb level indicates its practical utility[4,6,11]. Recoveries by use of the GPC[18] and Extrelut columns are nearly 100%. With the procedure described naturally occurring hormones (*e.g.*, oestradiol, oestriol and oestrone) are also extracted. These hormones can also be collected with the GPC clean-up. The advantage of GPC is that it can be used with automated sample injection

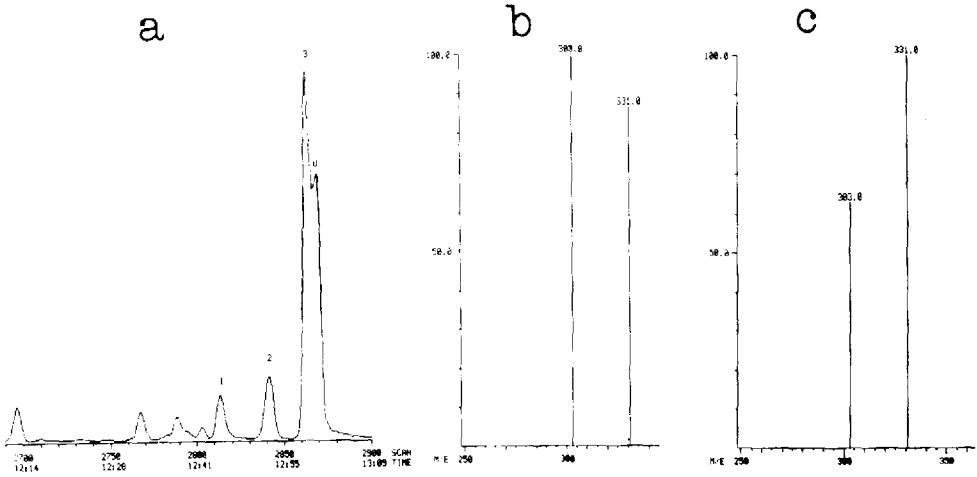

Fig. 3. Analysis on a CP-Sil 19 CB column. Urine spiked with DES, DE and HEX. (a) Total ion current for HFB derivatives in urine, peaks: 1 = DE-HFB, 2 = DES-HFB, 3 = HEX-HFB, u = unknown; (b) HEX-HFB in urine, MID; (c) HEX-HFB standard, MID.

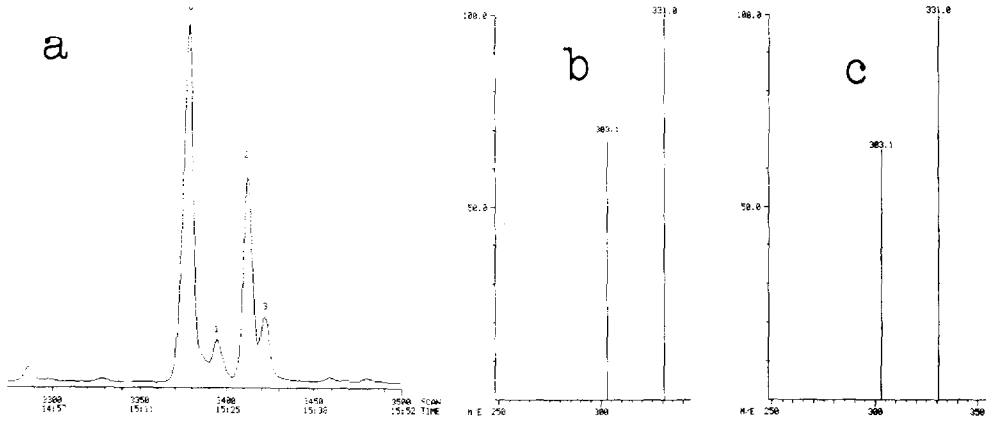

Fig. 4. Analysis of same sample as in Fig. 3 on a CP-Sil 5 CB column. (a) Total ion current for HFB derivatives, peaks: 1 = DE-HFB, 2 = HEX-HFB, 3 = DES-HFB, u = unknown; (b) HEX-HFB in urine, MID; (c) HEX-HFB standard, MID.

and fraction collection. A drawback is the laborious extraction of the hydrolysed urine. If only a few samples are to be analysed Extrelut is preferable. Both clean-up procedures in combination with HPLC give good recoveries and clean extracts. The clean-up with the sodium carbonate–sodium hydrogen carbonate solution is very important in both the GPC and Extrelut procedures. Of special importance is the pH of 10.3. Higher pH values result in considerable losses, and lower values in poorer clean-up[20]. The drawback of the complexity of the described HPLC procedure is off-set by the advantages that diluted samples can be introduced and concentrated on the first column, whereas the heart-cutting technique not only improves the separation but also provides for only a small part of the urine matrix being carried over to the analytical column. As only acetonitrile–water (60:40) flows through the analytical column, a long lifetime of the analytical column and excellent reproducibility of the retention time are ensured. Although the first column is cleaned by back-flushing, the column must be replaced after about 250 samples.

For nanogram amounts of DES, HEX and DE good conversion is obtained into TMS or HFB derivatives[16,21]. In Table II the relative retention times (RRT) are given for the different anabolics on both columns for HFB and TMS derivatives. The resolution of the stilbene-TMS is insufficient on the CP-Sil 5 CB column. If no baseline separation between DES-TMS (M = 412) and DE-TMS (M = 410) is obtained, as is often the case with nonpolar stationary phases[4,6], the presence of DE-TMS results in an interference with the base peak (412) of DES-TMS. With the HFB derivative the separation is slightly improved (see also Fig. 4) but on a CP-Sil 19-CB column (Fig. 2) a baseline separation is obtained. The other reason for choosing the HFB derivative is the lower specificity of the TMS derivatives (for DES and DE). With the probability based matching (PBM) system of McLafferty and co-workers[22–24] it is possible to establish the uniquenes of a spectrum. A combination of low masses, low abundances and few fragments scores a much lower uniqueness than a combination of high masses, high abundances and many fragments. The base peak for DES-TMS (412) under our conditions fragments only into two weak, structure-dependent fragments (397 and 389),

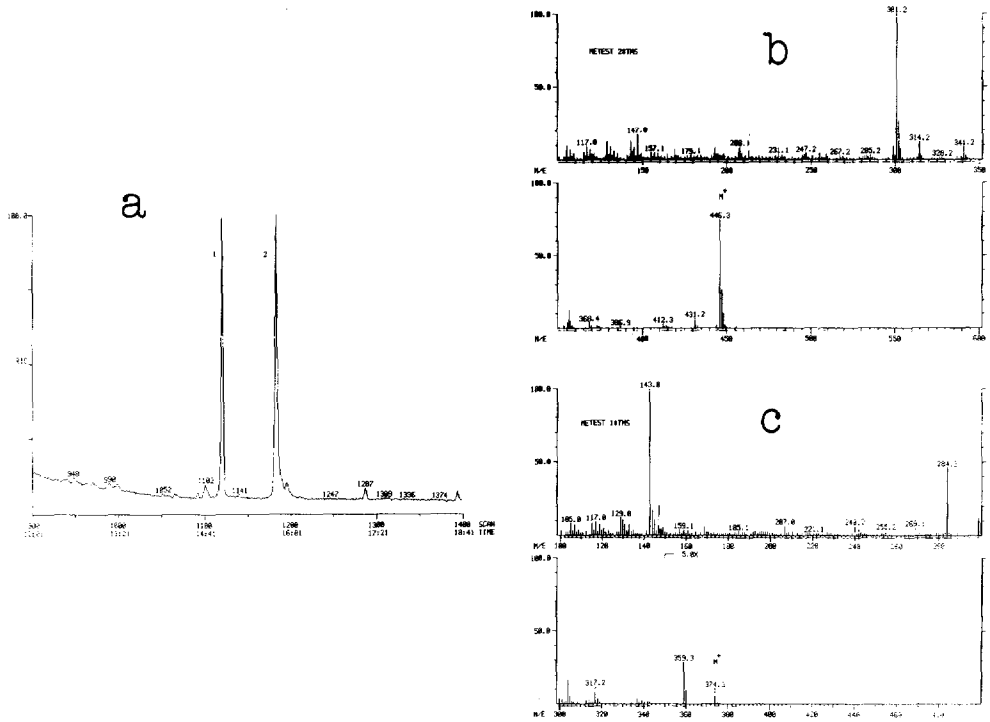

Fig. 5. Analysis of METEST on CP-Sil 19 CB. (a) Total ion current, peaks: 1 = METEST 2TMS, 2 = METEST 1TMS; (b) spectrum of METEST 2TMS; (c) spectrum of METEST 1TMS.

whereas DES-HFB (M^+ = 660) fragments into more ions with higher abundances (341, 447, 631).

For DE-HFB, a spectrum comparable to that of DES-HFB is obtained. Unfortunately, the spectrum obtained for HEX-HFB consists of only two structure-dependent fragments.

For confirmation of HEX, a full spectrum must be obtained, proving the presence of the two fragments of HEX-HFB as well as the isotope masses and the absence of other fragments. On the CP-Sil 19 column, interferences with compounds from the urine can be encountered (Fig. 3), whereas on the CP-Sil 5 column no compounds are eluted from the urine that interfere with HEX-HFB (Fig. 4).

It is therefore often necessary to inject a sample also into the CP-Sil 5 column. Up to now results of HFBA derivatization have been unsatisfactory for ZER, TREN, METEST and EEST. Therefore, the TMS derivatives are used. To avoid contamination of the ion source caused by precipitation of silica on the lenses, the silylation reagent is removed after the reaction by diluting the mixture with acetonitrile, evaporating to dryness and then dissolving the residue in isooctane. In our hands, METEST shows irreproducible behaviour during derivatization. This is probably caused by the use of a commercial TMCS–BSTFA mixture. In Fig. 5 two peaks are shown. The second peak (M^+ = 374) on the CP-Sil 19 column is the one expected. The first peak (M^+ = 446) contains an extra TMS group, probably the enol TMS ether more or less analogous to the behaviour of testosterone[16]. Sometimes, even the underivatized com-

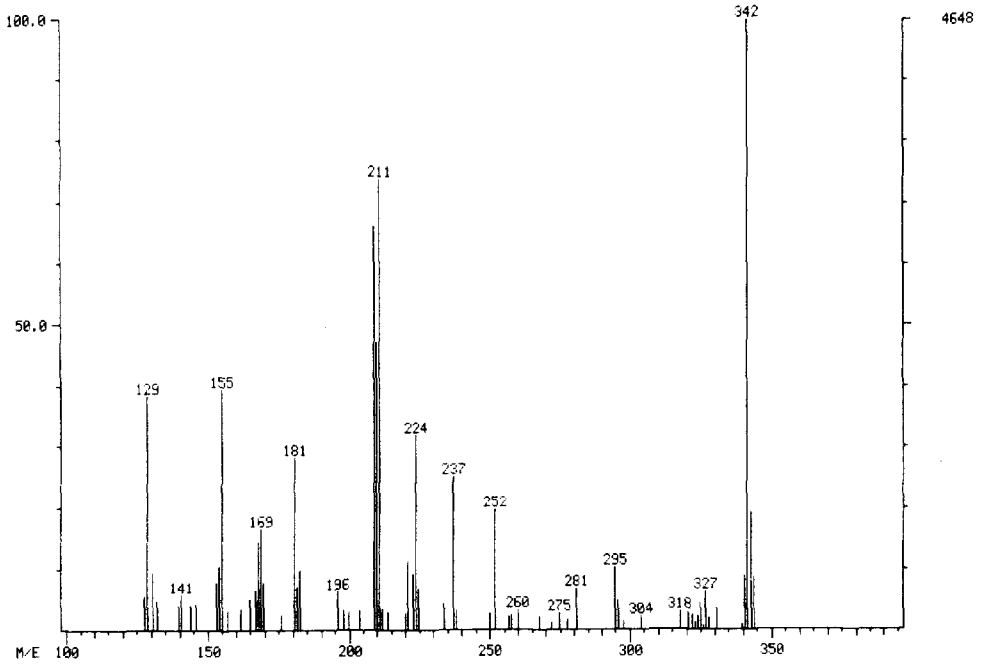

Fig. 6. Spectrum of trenbolone TMS derivative.

pound appears and this can also occur when EEST is derivatized. When pyridine is added as a catalyst, these problems seem to be lessened.

ZER always gives good results. TREN at 5 ppb causes no problems (Fig. 6).

For positive confirmation of anabolics in urine we choose the following criteria: (a) the retention time should be in agreement with the standard derivative and should not deviate more than ± 5 sec; (b) the abundances of the MID fragments (≥ 4), expressed as a percentage of the base peak, should not deviate more than 10% from the ratio of the analogous fragments in the spectrum of the standard derivative. (With HEX-HFB, a full spectrum is needed, analysed on non-polar column.)

Most experience has been obtained with the stilbenes, as these compounds have top priority. Up to now RIA screening was only used for the stilbenes. RIA-suspected samples (from a check-sample programme) have been analysed by GC–MS. The presence of DES, HEX and DE, alone and in combination, has been proved at ppb levels.

For the other anabolics, results with spiked samples have shown that recoveries and confirmation at the ppb level will present no problems, so that when rapid screening methods are developed, GC–MS confirmation can be carried out.

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