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Metabolites of 4-chlorotestosterone acetate in cattle urine as diagnostic markers for its illegal use

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Abstract

Seven metabolites of 4-chlorotestosterone acetate were identified in urine of cattle that received a single injection of the drug. The steroids were isolated by means of a series of clean-up steps carried out before and after enzymatic hydrolysis. The obtained extract was fractionated by high-performance liquid chromatography and each fraction was examined both by high-performance thin-layer chromatography and by capillary gas chromatography–mass spectrometry of the *m*-ethoxime–trimethylsilyl derivatives. The metabolites were tentatively identified by studying the mass spectra of selected peaks not found in blank samples. The structures of two metabolites, *viz.* 4-chloroandrost-4-ene-3,17-dione and 4-chloroandrost-4-ene-3α,17β-diol were confirmed by chemical synthesis. The synthesized metabolites and 4-chloro-17α-testosterone, a third metabolite which was identified tentatively, were located on the thin-layer chromatograms obtained. This study led to the conclusion that the illegal use of 4-chlorotestosterone acetate can be detected by identifying one or more of its metabolites in urine.

1. Introduction

4-Chlorotestosterone acetate (4-chloroandrost-4-ene-17β-ol-3-one acetate, clostebolacetate, CLTA) is a synthetic anabolic steroid which can be illegally used in cattle as a growth promoting agent. Analysis of injection sites performed in our laboratory revealed an increasing number of cases of the illegal use of CLTA in Belgium. In 1990, 22.0% of the positive cases contained CLTA. In 1991 CLTA was present in 46.4% of the positive injection sites; this percentage increased to 58.3% in 1992 and 65.0% in the first half of 1993. Similar high percentages of CLTA abuse were also observed by others [1]. In contrast to these findings all urine samples screened for CLTA or for 4-chlorotestosterone (clostebol, CLT) were negative. This phenomenon could be due to extensive metabolic breakdown of CLTA and CLT, similar to what can be observed for other steroids. Indeed, particular metabolites of nortestosterone [2–6] and trenbolone [7,8] in cattle urine have been accepted as a valid marker for the illegal use of these compounds. CLTA metabolism in men has been studied extensively [9–11], but no information is available on the excretion and biotransformation of CLTA in cattle. The identification of typical

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and unique biotransformation products of CLTA could be of utmost importance when screening for illegal use of CLTA has to be performed on livestock cattle.

The main objective of the present study was to search for unique metabolites in urine samples collected from an 18 month old heifer and a 10 month old steer after one injection of CLTA.

2. Experimental

2.1. Experimental animals and samples

An 18-month old heifer and a 10-month old steer were treated with a solution of 500 mg CLTA in 20 ml of a 1:1 (v/v) mixture of pharmaceutical grade Miglyol and N-methyl-2-pyrrolidone. In a first experiment 350 mg CLTA was injected intramuscularly in a 18-month old heifer. Blank urine was collected before administration of the product and 12, 36, 84, 117 h and 7, 23 and 35 days after the injection. The same experiment was repeated by injecting 400 mg CLTA in a 10-month old steer. Blank urine was collected before administration and 2, 6, 18 and 24 h after the injection. Samples were kept frozen until analysis.

2.2. Chemicals and standards

All reagents and solvents used were of analytical grade. Purified water was obtained from the Milli RO water purification system (Millipore, Bedford, MA, USA). HPLC-grade methanol was obtained from Labscan (Dublin, Ireland). Tri-Sil-TBT (Pierce, Rockford, IL, USA), which consists of trimethylsilylimidazole–N,O-bis-(trimethylsilyl)acetamide–trimethylchlorosilane (3:3:2, v/v) was used for trimethylsilylation. Ethoxyamine hydrochloride was from Eastman Kodak (Rochester, NY, USA) and methoxyamine hydrochloride from Fluka (Buchs, Switzerland). Both reagents were used as a 2% solution in pyridine. *Succus Helix Pomatia* containing 10^5 Fishman units/ml β-glucuronidase and 10^6 Roy units/ml sulfatase was obtained from Sepracor (Villeneuve la Garenne, France). Bond-Elut C_{18} columns (3 ml) (catalog No. 1210-2028), BondElut silica columns (3 ml) (catalog No. 1210-2087) and BondElut amino columns (3 ml) (catalog No. 1210-2014) were from Varian (Harbor City, CA, USA). HPTLC nanoplates (10 × 10 cm) coated with Silica gel 60 without fluorescence indicator (Merck, Darmstadt, Germany; catalog No. 5631) were used for HPTLC analysis. CLTA was obtained from the National Reference Laboratory (Instituut voor Hygiëne en Epidemiologie, Brussels, Belgium). Other reference materials and internal standards were obtained from Sigma (St. Louis, MO, USA), Steraloids (Wilton, NH, USA) or Janssen Chimica (Beerse, Belgium).

2.3. Preparation of CLT

CLT was obtained after acid hydrolysis of 5 mg CLTA in a mixture of 2 mol/l hydrochloric acid and methanol (9:1, v/v) at 80°C for 4 h. After the reaction was completed, the solvent was evaporated under a stream of nitrogen at 60°C. The residue was dissolved in 2.5 ml of methanol.

2.4. Preparation of 4-chloroandrost-4-ene-3,17β-diol

4-Chloroandrost-4-ene-3,17β-diol was prepared from CLT by reduction with sodium borohydride as described previously [12]. GC–MS analysis of a crude extract revealed two peaks with nearly identical mass spectra, representing two isomers carrying a differently oriented 3-hydroxyl group. The more polar isomer (methylene unit value 28.21) was present in a 4-fold excess as compared with the less polar isomer. By analogy with similar molecules it was estimated that the more polar isomer corresponds to the 3β-hydroxy-isomer while the less polar isomer was designated as the 3α-hydroxy-isomer (methylene unit value 27.54).

2.5. Preparation of 4-chloroandrost-4-ene-3,17-dione

4-Chloroandrost-4-ene-3,17-dione was prepared from CLT by oxidation with chromium trioxide as described previously [12]. GC–MS

analysis of the residue of the crude extract revealed the presence of a single peak (methylene unit value 30.81).

2.6. Preparation of reference solutions, internal standard solutions and quality control samples

Stock solutions (1.0 mg/ml) of reference standards and of internal standards 1 (equilenin, I.S.1) and 3 (androsterone, I.S.3) were prepared in methanol. Of I.S.1 and I.S.3, 5 ng/μl working solutions were prepared by dilution of the stock solutions with methanol. A 150 ng/μl working solution of internal standard 2 (naphthalene, I.S.2) was prepared by dissolving 15 mg in 100 ml methanol–water (65:35, v/v). For GC–MS analysis, stock solutions of all reference standards were mixed and diluted with methanol to a single 2 ng/μl working solution. For the daily control of the HPLC fractionation system, a calibration solution was prepared by evaporating 200 μl of the same 2 ng/μl working solution of reference standards and dissolving the residue into 170 μl of the I.S.2 working solution. For HPTLC analysis, several 50 ng/μl working solutions containing appropriate combinations of reference standards were used. Stock solutions were stored at −20°C. Working solutions were stored at 4°C. Spiked quality control samples were freshly prepared by adding 50 μl of the 2 ng/μl working solution (100 ng of each compound) to 50 ml blank urine.

2.7. Preparation of urine samples: isolation of steroid conjugates and enzymatic hydrolysis

All urine samples, collected from the animals treated, were analyzed several times on different days. The sample clean-up procedure was a modification [13] of a method previously developed in our laboratory [14]. To 50 ml of urine, adjusted to pH 7, 100 μl of working solution I.S.1 (= 500 ng, corr. to 10 μg/l) and 1 ml of methanol was added. The pretreated urine was centrifuged at 1500 *g* for 10 min and then transferred to a BondElut C_{18} column, conditioned by subsequently passing methanol and water. After washing the column with water and drying under negative pressure elution was carried out with methanol. The eluate was evaporated at 60°C under a stream of nitrogen, dissolved in 100 μl of methanol and further diluted with 10 ml of phosphate buffer 0.1 mol/l (pH 7.0). Finally, 250 μl of *Succus Helix Pomatia* was added and the mixture was incubated overnight at 37°C.

2.8. Preparation of urine samples: isolation of hydrolyzed steroids and clean up

The hydrolyzed extract was loaded on a preconditioned BondElut C_{18} column. The column was subsequently washed with water and methanol–water (30:70, v/v). Traces of water were displaced by passing through 3 ml of hexane. Elution was carried out using 6 ml of ethyl acetate. After evaporation of the eluate at 60°C under a stream of nitrogen, the dry residue was dissolved in 500 μl of chloroform. The solution obtained was diluted with 5 ml *n*-hexane and placed on top of a BondElut silica column. The column was washed with hexane. Finally, the column was placed on top of a BondElut amino column and eluted with 5 ml chloroform–acetone (4:1, v/v). The eluate was evaporated at 60°C under a stream of nitrogen.

2.9. HPLC of extracts

The instrumentation consisted of a modular HPLC system equipped with a high-pressure pump (Spectra Physics, San Jose, CA, USA; Model SP8700 XR/SP8750), an automatic injector (Gilson, Middleton, WI, USA; Model 231 with Rheodyne 7010 injection valve), a variable wavelength monitor (LKB, Bromma, Sweden; Model 2151 operated at 244 nm), a fraction collector (Gilson Model 202) and a recorder (Shimadzu, Kyoto, Japan; Chromatopack CR-1B). The HPLC column was a 250 × 4.6 mm I.D. stainless-steel tube packed with 5-μm silica (Chromspher C_{18}, Chrompack, Middelburg, Netherlands). The mobile phase for isocratic elution was methanol–water (67:33, v/v) at a flow-rate of 1.2 ml/min. The residue obtained from the clean-up step was dissolved in 170 μl of working solution I.S.2 and transferred to an autosampler cup. The sample controller was

programmed for full-loop injections (loop volume: 140 μl). Fractionation of the column eluate was done as described by Smets *et al.* [15,16]. The collector was operated in time mode. The time windows were controlled and adjusted at regular intervals by injecting the calibration solution (330 ng of each standard on-column). Routinely, four fractions were collected with relative retention-time windows ranging from 0.45 to 0.63 (fraction I), from 0.63 to 0.78 (fraction II), from 0.78 to 0.97 (fraction III) and from 0.97 to 1.30 (fraction IV). In unknown samples both retention time and peak height of I.S.2 were evaluated. To avoid contamination a blank sample was injected after each calibration. Fractions I to IV were evaporated separately to dryness under a stream of nitrogen at 60°C and dissolved in 50 μl methanol. Aliquots (25 μl) of each fraction (equivalent to 25 ml urine) were pooled in a 2-ml screw-cap vial and processed for GC–MS analysis. From the remaining of each fraction, 8 μl was taken for HPTLC analysis.

To correlate the HPLC retention times, R_F values and GC retention times of the CLTA metabolites, urinary extracts from CLTA-treated animals were subfractionated into HPLC fractions of 30 s and kept for further evaluation.

2.10. High-performance thin-layer chromatography

HPTLC was carried out in the "4 × 4" mode as described by De Brabander *et al.* [17]. Aliquots (8 μl, the equivalent of 8 ml urine) of the remaining 25 μl of each HPLC fraction were applied to a 10 × 10 cm silica gel 60 plate using an automatic micro spotter (Camag, Muttenz, Switzerland; Linomat IV) set at a velocity of 1 μl/min. Chromatography was carried out in two dimensions using chloroform–acetone (27:3, v/v) as solvent I and cyclohexane–ethyl acetate–ethanol (18:12:0.75, v/v/v) as solvent II. Staining and detection of the steroids were carried out using 5% sulfuric acid in acetic anhydride according to Verbeke [18].

2.11. Derivatization for gas chromatography–mass spectrometry

A 25-μl volume of working solution I.S.3 (= 125 ng androsterone) was added to a vial containing the evaporated HPLC fractions. The solvent was eliminated under a stream of nitrogen at 60°C. Ethoxime-trimethylsilyl (EO-TMS)- and methoxime-trimethylsilyl (MO-TMS)-derivatives were prepared by adding 100 μl ethoxyamine (EO-TMS)- or methoxyamine (MO-TMS)-solution and allowing the mixture to react for 1 h at 80°C. Subsequently, the excess of reagent was removed by evaporation at 60°C under a stream of nitrogen. A 25-μl volume of silylating mixture was added to the dry residue and the mixture was allowed to stand overnight at 80°C. A volume of 1 μl (corresponding to 10 ng I.S.1 at 100% recovery and to 5 ng I.S.3) was injected into the GC–MS system. For the preparation of enol-TMS-derivatives the silylating mixture was added directly after addition of I.S.3.

2.12. Gas chromatography–mass spectrometry

All GC–MS analyses were performed on a Varian Saturn I system (Walnut Creek, CA, USA), which included a Model 8100 autosampler, a Model 3400 capillary gas chromatograph and an ion-trap mass spectrometer. The analyzer was controlled by a Compaq 386/20e personal computer. The GC column used was a DB-5 MS, 30 m × 0.32 mm I.D. fused-silica column with 0.25 μm film thickness (J&W, Folsom, CA, USA). Temperature settings were as follows: injector: 260°C; transfer line: 280°C; oven program: initial temperature: 50°C for 0.7 min, 50 to 190°C at 33°C/min, 190° to 320°C at 4.2°C, 320°C for 1 min. The carrier gas was helium at a flow-rate of 1.0 ml/min. A 1-μl aliquot was injected on a split–splitless injector in the split mode. In routine conditions, the instrument was operated in the electron-impact ionization (EI) mode. A run was completed within 35 min. Chemical ionization (CI) spectra were recorded using both ammonia and isobutane as reagent gasses.

2.13. Calibration and direct quality control of GC–MS performance

Mass spectra, relative retention times and response factors for quantitation were obtained

by the injection of a reference mixture prepared by evaporation and direct derivatization of 125 μl of the 2 ng/μl working solution. To calibrate the GC–MS instrument, a 10 ng/μl solution (high control) was used. Similarly, a 0.8 ng/μl solution (low control) was prepared by treatment of 10 μl of the 2 ng/μl working solution. Comparison of the results obtained after an injection of both high and low control samples allowed a daily check of the linearity of the GC–MS response, the detection limits, and the chromatographic performance.

2.14. GC–MS-data handling

For routine analysis, a reversed library search method was set up for approx. 40 target compounds by using the Autoquan software. For positive identification using the automatic procedure the following criteria had to be fulfilled: (1) The compound eluted within the preset relative retention time window ±20 s. (2) The mass spectrum matched the library spectrum containing the 8 to 10 most diagnostic fragment ions of an analyte. The fit-threshold was set at 900. For each positively identified compound a semi-quantitative result was obtained by measuring the peak-area ratio of a selected fragment-ion to the area of ion m/z 360 of I.S.3 (methylene unit value 26.01). A two-point calibration was carried out by injection of direct control samples. Each analyte was quantified in triplicate using three different fragment ions. After termination of the automated procedure, false positive results were eliminated by exact matching of the retention time (±1 s), by visual comparison of the sample spectrum with the detailed reference spectrum and by visual inspection of background signals. Furthermore the presence of unknown compounds was evaluated by studying the total ion chromatogram. In contrast to the well known target analytes, an estimation of the concentration levels of newly detected metabolites was based on total ion responses.

2.15. Quality control of the overall method

The overall GC–MS and HPTLC methods and extraction recoveries were checked for each batch of samples prepared by processing a blank sample spiked at a level of 2 μg/l. Therefore, 50 μl of the 2 ng/μl working solution was added to 50 ml urine. An internal control for real unknown samples was provided by the addition of I.S.1 (methylene unit value 28.29) at an easily detectable level of 10 μg/l. The mean recovery of I.S.1 was approximately 40%.

3. Results and discussion

3.1. GC–MS identification of selected compounds

Knowledge of the composition of a blank urinary profile simplifies the search for unknown metabolites of exogenous origin. The full scan capabilities of ion-trap GC–MS at residue levels has allowed us to locate a number of endogenous background substances present in blank urine extracts of cattle [19]. Based on these data a mass spectral library was constructed which was found to be suitable for the detection of exogenous illegal hormones in the presence of a background of endogenous steroid metabolites. This feature was extremely useful to locate new xenobiotic substances.

Analysis of urine extracts obtained from the two animals treated with CLTA and subsequent examination of the data indicated the presence of at least seven metabolites. Neither CLTA nor CLT were ever found in the urine samples investigated. Higher mass fragments of the unknown spectra all showed an isotope ratio diagnostic for the presence of a chlorine atom. None of the substances were detected in the urine samples collected before administration of CLTA. Mass spectra of the EO-TMS derivatives of the unknown compounds and CLT are shown in Fig. 1. Typical chromatographic profiles of the unknowns are shown in Fig. 2. Exact retention data expressed as methylene unit values, molecular masses of derivatives and proposed formulas are listed in Table 1. The compounds were labeled alphabetically according to their GC-elution order.

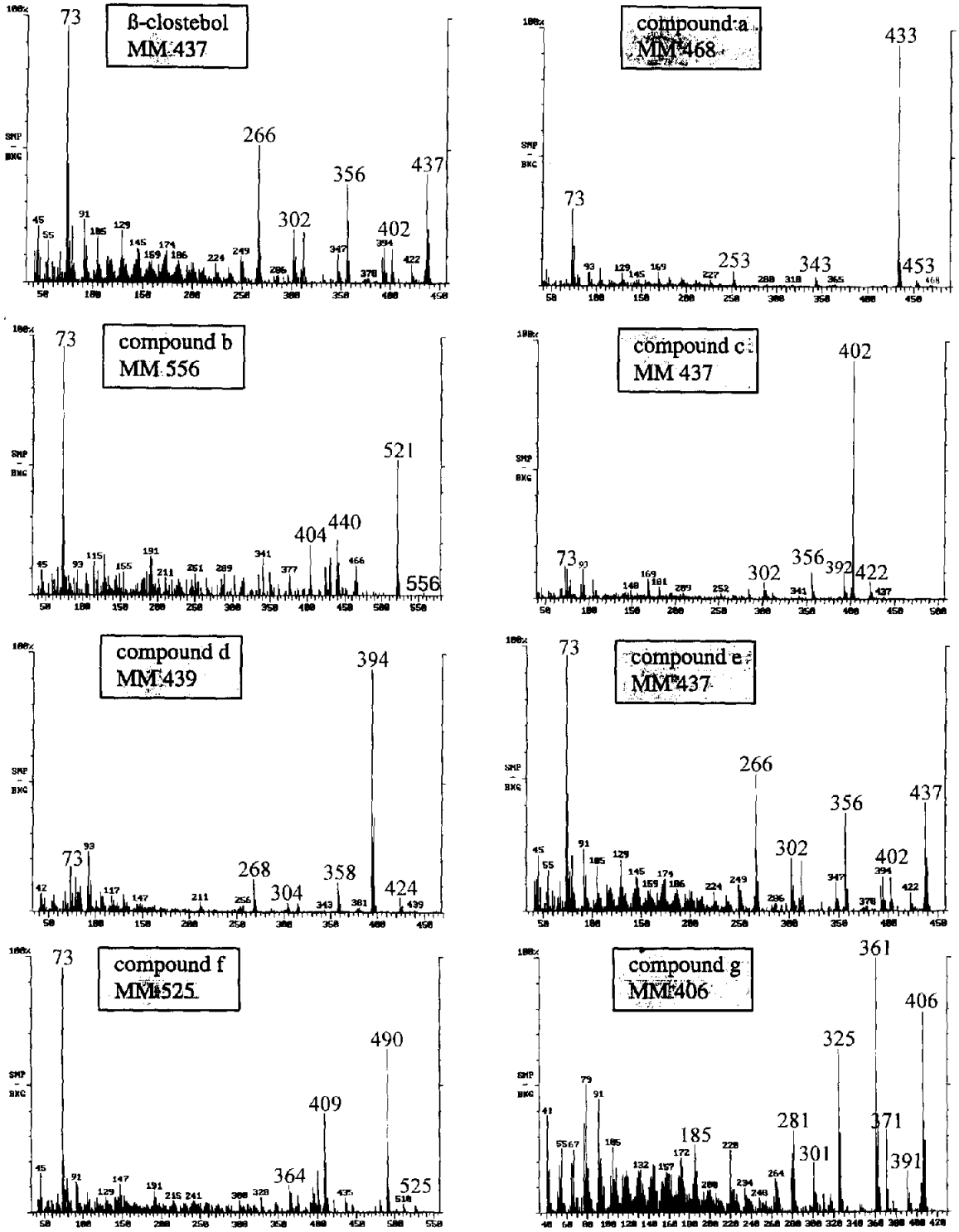

Fig. 1. Mass spectra of EO-TMS derivatives of CLT and seven metabolites found in the urine of cattle injected with CLTA.

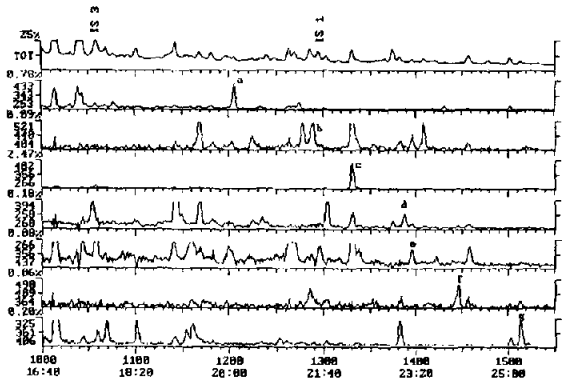

Fig. 2. Typical gas chromatographic profile of CLTA metabolites in cattle urine, showing the total ion current (top) and the selected ion traces obtained by summing up the three typical ions.

3.2. Compound a

From the CI spectrum (isobutane), the molecular mass of compound a was estimated at 468. The EI spectrum (Fig. 1) showed a base peak at m/z 433 ($[M-35]^+$) revealing the loss of the chlorine atom from the weak molecular ion at m/z 468. The absence of oxo groups was evidenced by the fact that neither spectra nor retention time were influenced by oximation. Moreover, the poor detectability of compound a when using ammonia as the CI reagent gas in contrast to isobutane was in agreement with the presumption that no nitrogen atom was present in the molecule. The presence of 2 TMS groups was indicated by the presence of $[M-15]^+$ (m/z 453), $[M-90]^+$ (m/z 390), $[M-105]^+$ (m/z 363), $[M-35-90]^+$ (m/z 343), $[M-35-90-90]^+$ (m/z 253) and m/z 73 fragments. The isobutane CI spectrum typically showed the consecutive loss of two HOTMS moieties from the $[M+1]^+$ ion. Presuming the presence of two TMS-hydroxyl groups the molecular mass of the underivatized compound was estimated at 324. This corresponds to an analog of CLT in which the 3-oxo group is transformed into a 3-hydroxyl group. Both, the 3α- and the 3β-isomer of the postulated compound were obtained by chemical reduction of CLT using sodium borohydride. It was shown by GC–MS and by HPTLC that

Table 1
Analytical data of metabolites of CLTA

Substance	GC–MS data					Molecular formula	HPLC data		HPTLC data		
	MU EOTMS	MU MOTMS	MM EOTMS	MM MOTMS	MM		Fraction	Approximate t_R (min)	R_F (I)	R_F (II)	Appearance (366 nm)
βCLTA	30.68	30.52	407	393	364	$C_{19}H_{29}ClO_3$	>IV	–	0.85 ($n=2$)	0.85 ($n=2$)	grey-blue
βCLT	30.10	29.64	437	423	322	$C_{19}H_{27}ClO_2$	III	11.1	0.56 ($n=2$)	0.53 ($n=2$)	light-blue
a	27.54	27.54	468	468	324	$C_{19}H_{29}ClO_2$	IV	12.5	0.55 ± 0.02 ($n=10$)	0.62 ± 0.05 ($n=12$)	yellow-green
b	28.43	28.43	556	556	340	$C_{19}H_{29}ClO_3$	II	8.3	–	–	–
c	28.85	28.29	437	423	322	$C_{19}H_{27}ClO_2$	III	9.5	–	–	–
d	29.44	28.88	439	425	324	$C_{19}H_{29}ClO_2$	IV	12.0	–	–	–
e	29.51	29.04	437	423	322	$C_{19}H_{27}ClO_2$	IV	14.1	0.57 ± 0.03 ($n=20$)	0.62 ± 0.02 ($n=20$)	orange-red
f	30.00	29.54	525	511	338	$C_{19}H_{27}ClO_3$	I	5.8	–	–	–
g	30.81	29.69	406	378	320	$C_{19}H_{23}ClO_2$	II	8.3	0.77 ± 0.03 ($n=12$)	0.71 ± 0.03 ($n=12$)	green-yellow

compound a was identical to 4-chloroandrost-4-ene-3α,17β-diol.

3.3. Compound b

The EI spectrum of compound b (Fig. 1) showed a base peak at *m/z* 73 revealing the presence of several TMS groups. The appearance of the spectrum was not influenced by oximation excluding the presence of oxo groups. The compound was undetectable by CI using ammonia but revealed a molecular mass of 556 using isobutane as the reagent gas. The isobutane CI spectrum typically showed the consecutive loss of three HOTMS moieties from the $[M+1]^+$ ion. Presuming the presence of three TMS-hydroxyl groups the molecular mass of the underivatized compound was calculated to be 340. This corresponds to a 4-chloroandrost-4-ene-3,17,*x*-triol which is a hydroxylated form of compound a. No further attempts were made to verify the postulated structure.

3.4. Compounds c and e

The mass spectra of compounds c and e (Fig. 1) showed the same fragments as the mass spectrum of 17β-CLT. The molecular mass of the EOX-TMS derivative of both compounds was shown by CI (ammonia, isobutane) to be 437. The typical retention time shift of the corresponding MOX-TMS derivative of 0.5 methylene unit downwards and the difference in molecular mass of 14 revealed the presence of 1 oxo group. Furthermore, the presence of *m/z* 73 and the loss of HOTMS (very weak for compound e, very strong for compound c) in the CI spectrum was evidence for the presence of a TMS-hydroxyl group. On the basis of these findings, the molecular mass of the underivatized compounds were calculated to be 322 as for CLT.

The spectrum of compound e was nearly identical to the spectrum of β-CLT which was suggestive for the epimer 17α-CLT. Additional evidence for this assumption was found in the observation that the shift in methylene units (0.59) of compound e relative to β-CLT was in agreement with the values found for other 17α-/17β-epimers (17α-nortestosterone/17β-nortestosterone: 0.46, 17α-testosterone/17β-testosterone: 0.47, 17α-trenbolone/17β-trenbolone: 0.58). The structure of compound e was further confirmed by the evaluation of the retention-time ratio of the enol-TMS derivatives of compound e with respect to CLT. The obtained value of 0.963 was in agreement with the data ($t_{R_{rel}}$ 0.959) provided by Le Bizec *et al.* [20].

Compound c, on the other hand, showed less abundant lower mass fragments while the abundance of the M − 90 fragment in the CI spectrum was very high. These findings argued for a 17-oxosteroid. This was suggestive for a switch from the 3-oxo-17-hydroxyl configuration to a 17-oxo-3-hydroxyl configuration. This compound was found to be the major metabolite in men [10]. The structure of 4-chloroandrost-4-ene-3α-ol-17-one was confirmed by studying the mass spectrum of the enol-TMS derivative, which was in agreement with the data obtained by others [21].

3.5. Compound d

The mass spectrum of the EOX-TMS of compound d (Fig. 1) showed a base peak at *m/z* 394 revealing the loss of an ethoxime moiety from the molecular ion. In contrast to the other compounds found, the loss of chlorine was not a major fragmentation route, which argued for the absence of a 4,5-double bond. In a similar way as described above, CI- and EI-mass spectral data of both the EO-TMS- and MO-TMS-derivatives confirmed that compound d contained an oxo- and a hydroxyl group and that the molecular mass was 439. On the basis of these findings, the molecular mass of the underivatized compound d was calculated to be 324. Taking into account the low relative abundances of the lower mass fragments in the EI spectrum and the abundant $[M-90]^+$ fragment in the CI spectrum, compound d was tentatively designated to one of the isomers of 4-chloroandrostane-3-ol-17-one.

3.6. Compound f

The mass spectrum of the EOX-TMS of compound f (Fig. 1) showed a base peak at m/z 73 revealing the presence of more than one hydroxyl group. The fragment at m/z 490 represented the loss of a chlorine atom from the molecular ion at m/z 525. In a similar way as described above, CI- and EI-mass spectral data of both the EO-TMS- and MO-TMS-derivatives confirmed that compound f contained an oxo- and two hydroxyl groups and that the molecular mass was 525. On the basis of these findings, the molecular mass of the underivatized compound f was calculated to be 338 which corresponds to a hydroxylated derivative of CLT. Taking into account the low relative abundances of the lower mass fragments in the EI spectrum and the abundant $[M-90]^+$ (loss of TMSOH moiety) and $[M-180]^+$ (loss of 2 TMSOH moieties) fragments in the CI spectrum, compound f was tentatively identified as one of the isomers of 4-chloroandrost-4-ene-3,x-diol-17-one.

3.7. Compound g

The molecular mass of the EOX-TMS derivative of compound g (Fig. 1) was unequivocally shown by CI (ammonia, isobutane) to be 406. The ease of detection when using ammonia as the reagent gas strongly argued for the presence of nitrogen atoms. Since an even molecular mass was found, at least two nitrogen atoms should be present. The retention time shift of the corresponding MOX-TMS derivative of 1 methylene unit downwards and the difference in molecular mass of 28 indicated the presence of 2 oxo groups. The absence of m/z 73 in the EI spectrum and of fragments representing the loss of HOTMS groups in the CI spectrum, was not in agreement with the presence of hydroxyl groups. The molecular mass of the underivatized compound g was calculated to be 320 corresponding to an analog of CLT in which the 17-hydroxy group was transformed into a 17-oxo group. This compound was synthesized by chemical oxidation of CLT. It was unequivocally shown by

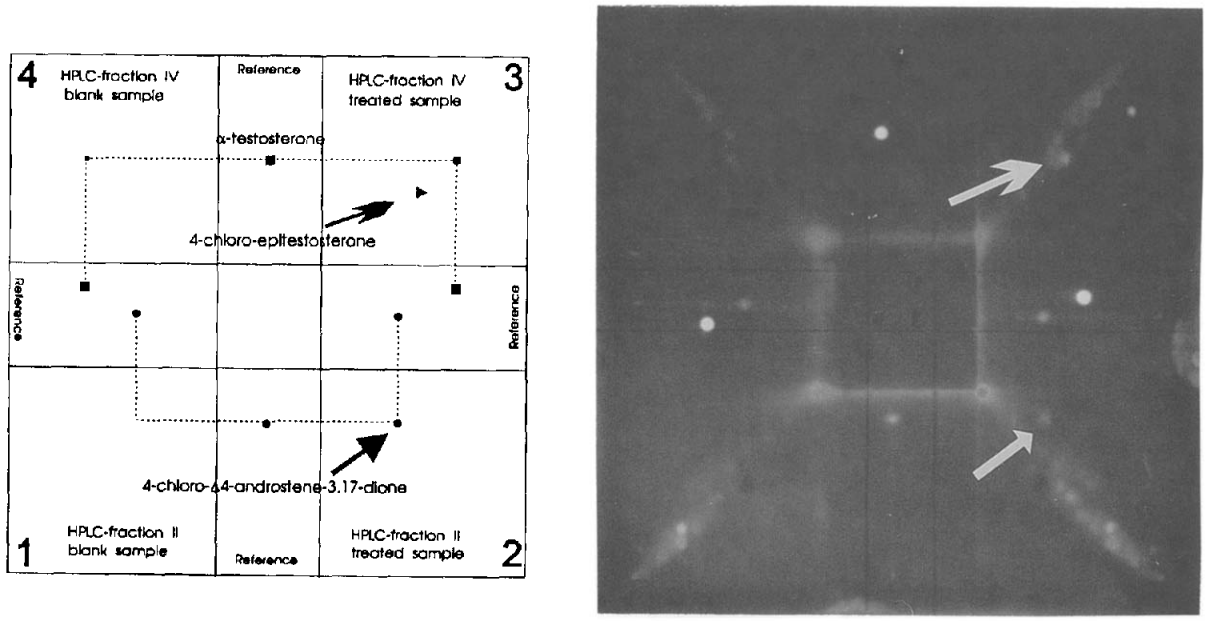

Fig. 3. HPTLC chromatogram of a urine sample obtained from an eighteen-month-old heifer before and after injection of CLTA: 1. HPLC-fraction II, blank sample; 2. HPLC-fraction II, treated sample: presence of 4-chloroandrost-4-ene-3,17-dione (top arrow); 3. HPLC-fraction IV, treated sample: presence of 17α-CLT (bottom arrow); 4. HPLC-fraction IV, blank sample.

GC–MS and by HPTLC that compound g was identical to the synthetically prepared reference product. Therefore it was concluded that the structure of compound g corresponded with 4-chloroandrost-4-ene-3,17-dione.

3.8. HPLC and HPTLC results

Subfractionation of urinary extracts by HPLC into fractions of approx. 30 s, followed by HPTLC- and GC–MS analysis of each subfraction, allowed to locate the major metabolites on HPTLC chromatograms and to estimate their HPLC retention time. The results of these experiments are listed in Table 1.

A typical HPTLC chromatogram obtained from a CLTA positive urine sample is shown in Fig. 3. 4-chloroandrost-4-ene-3,17-dione appeared in fraction II as a yellow-green spot at 366 nm with $R_F = 0.77$ (solvent I) and $R_F = 0.71$ (solvent II). Furthermore, 17α-CLT was identified as an orange-red spot in fraction IV at $R_F = 0.57$ (solvent I) and $R_F = 0.62$ (solvent II). 4-Chloroandrost-4-ene-3α,17β-diol appeared as a yellow-green spot in fraction IV at $R_F = 0.55$ (solvent I) and $R_F = 0.62$ (solvent II). Since 4-chloroandrost-4-ene-3α,17β-diol and αCLT appeared at nearly identical R_F values a clear distinction of both metabolites was not possible by the normal HPLC fractionation procedure. This problem was solved by subfractionation of fraction IV into two fractions. The spots discussed were not found in control urine.

3.9. Relative excretion of CLTA metabolites

Based on the results of this study, a pathway for the metabolism of CLTA in cattle was proposed (Fig. 4). Due to the lack of purified reference materials, it was not feasible to draw accurate excretion curves for each metabolite from the results obtained from the treated animals. However, attempts were made to estimate their relative excretion by using the ratio of the total ion current area to the total ion current area of I.S.3, of which an equal mass was added to all fractions injected. Long term data were obtained for the 18-month old heifer only. These

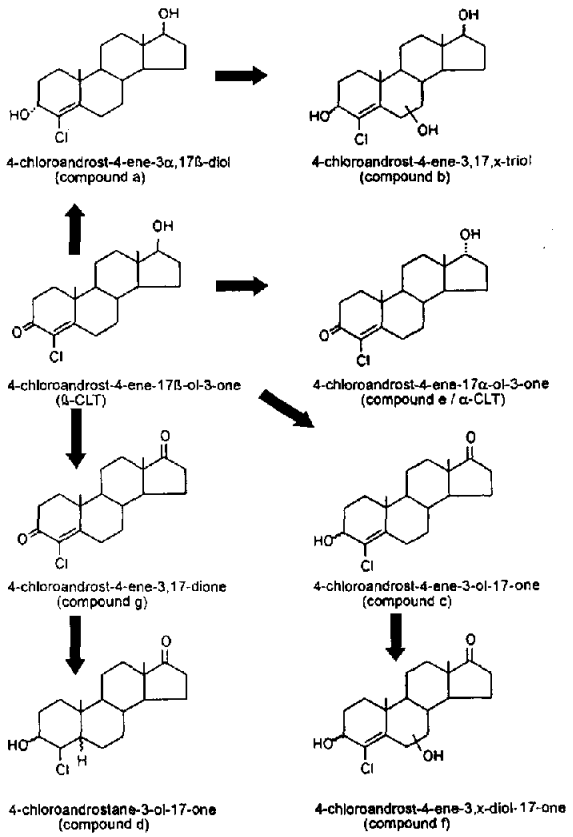

Fig. 4. Suggested metabolic scheme of CLTA in cattle.

are summarized in Fig. 5. The GC–MS results were in agreement with the conclusions drawn from the visual inspection of the typical spots found by HPTLC.

3.10. CLTA metabolites as diagnostic markers for the illegal use of CLTA

Eighteen urine samples obtained from slaughterhouses which were found to be negative by the classical screening method clearly showed (Fig. 6) that the illegal use of CLTA is widespread. This is in agreement with the frequent finding of CLTA in injection sites. Additional evidence for the diagnostic value of the analysis of the metabolites of CLT was obtained by analysis of a urine sample obtained from an animal which was found to have a positive injection site. The compounds investigated were

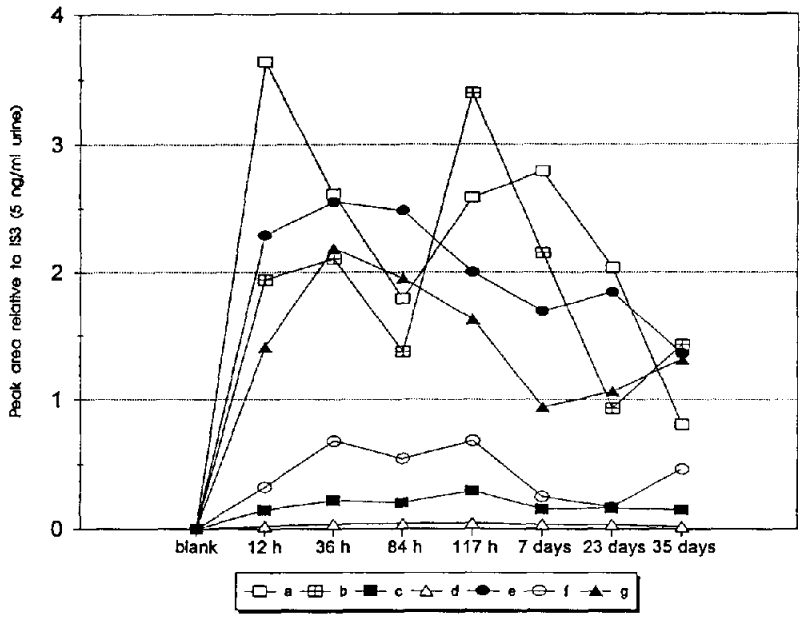

Fig. 5. Estimate of the relative excretion of seven metabolites of CLTA as determined by GC–MS in an eighteen month-old heifer treated with 350 mg CLTA. The symbols used in the legend refer to the respective metabolites a to g.

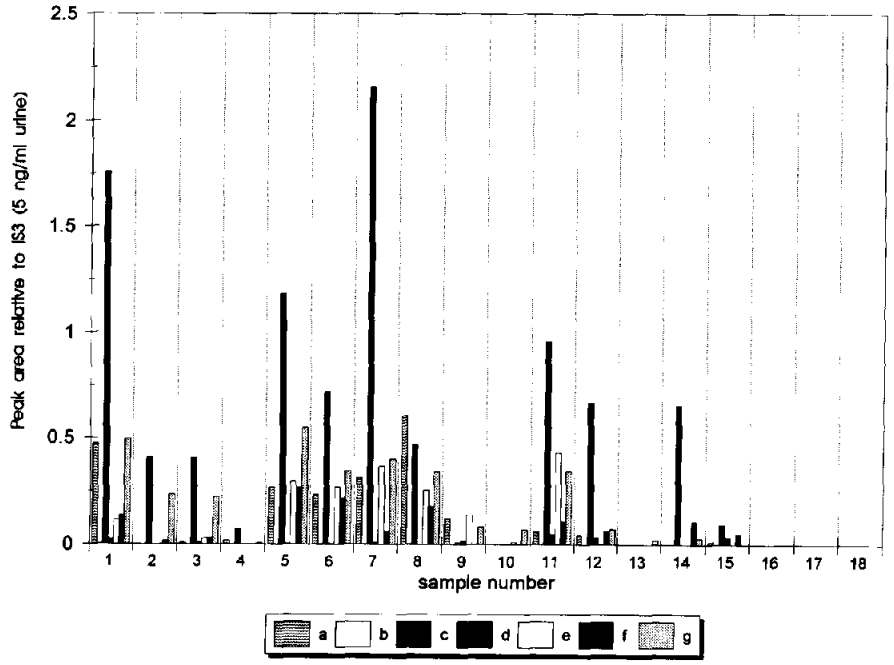

Fig. 6. Relative abundancy of the CLTA metabolites in 18 samples of cattle urine from slaughterhouses as determined by GC–MS. The symbols used in the legend refer to the respective metabolites a to g.

unequivocally identified by HPTLC and GC–MS.

In view of their HPTLC detectability, αCLT (metabolite e, fraction IV) and 4-chloroandrost-4-ene-3,17-dione (metabolite g, fraction II) can be used as diagnostic markers for illegal CLTA use when analyzing cattle urine by means of HPLC–HPTLC. Preferably, 4-chloroandrost-4-ene-3α,17β-diol (metabolite a, fraction IV) should be detected as well. However, distinction between 4-chloroandrost-4-ene-3α,17β-diol and αCLT is difficult when using the normal HPLC fractionation procedure. Confirmation by GC–MS should always be performed even if all typical spots are present. As can be derived from the results shown in Fig. 6, confirmation by GC–MS generally revealed the presence of 4-chloroandrost-4-ene-3,17-dione, 4-chloroandrost-4-ene-3-ol-17-one, 4-chloroandrost-4-ene-3α,17β-diol and α-CLT. From a quantitative viewpoint 4-chloroandrost-4-ene-3-ol-17-one was considered to be of major importance.

4. Conclusion

In conclusion, using GC–MS, 7 metabolites were found in urine of animals treated with CLTA. The structures of 4-chloroandrost-4-ene-3α,17β-diol and 4-chloroandrost-4-ene-3,17-dione were confirmed by chemical synthesis. The identification of at least one of these metabolites or αCLT in cattle urine unequivocally demonstrates the use of CLTA. The metabolites can be detected by means of the normal HPLC–HPTLC procedure. Additional evidence can be obtained by GC–MS.

5. References

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