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New anabolic steroid illegally used in cattle—structure elucidation of 19-norchlorotestosterone acetate metabolites in bovine urine

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Abstract

4-Chloro-estr-4-en-17-ol-3-one, trivially named 19-norclostebol acetate or 4-chloro-19-nortestosterone acetate (NClTA), has been identified on the European black market in the late 1990s for possible use in breeding animals. After oral and subcutaneous administration of NClTA to bovine, urine samples were collected over a period of three weeks, and chemical structure of main excreted urinary metabolites was determined. After oral administration, the most abundant metabolites were mainly reduced as 4-chloro-19-norandrostan-3ξ-ol-17-one and 4-chloro-19-norandrostan-3ξ,17ξ-diol. They were identified until 1 week after administration. Following subcutaneous injection, 4-chloro-19-norandrostan-3ξ-ol-17-one was again of major abundance, but so were 4-chloro-19-norandrost-4-ene-3ξ,17ξ-diol and 4-chloro-19-norandrost-4-en-3ξ-ol-17-one. They were detected at least 3 weeks after administration. Whatever the route of administration, metabolites were found mainly glucurono-conjugated; the only exception was metabolite 4-chloro-19-norandrostan-3ξ-ol-17-one which was identified both in the sulpho- and glucurono-fractions.

Keywords: Norclostebol; Bovine; Metabolite; Conjugate

1. Introduction

The use of anabolic steroids has been forbidden in the European Union since 1981 (directive 81/602/EEC) [1]. Chlorotestosterone acetate (ClTA) is a synthetic anabolic steroid which was illegally used in cattle as a growth promoting agent. In the beginning of the 1990s the analysis of injection sites revealed the illegal use of ClTA throughout Europe. However, ClTA could not be detected in urine samples due to the extensive metabolic breakdown of ClTA. Depending on the method of administration, different metabolites were found. Leyssens et al. [2] studied the metabolism of chlorotestosterone acetate in urine after intramuscular administration. The most abundant metabolites were 4-chloroandrost-4-en-17α-ol-3-one (α-ClT), 4-chloroandrost-4-ene-3α,17β-diol, 4-chloroandrost-4-ene-3,17-dione (ClAD) and 4-chloroandrost-4-ene-3-ol-17-one [2]. Later studies and experiments resulted in the same conclusions [3–6]. Le Bizec et al. [4] also examined the metabolism in urine after oral administration of ClTA. The presence of 4-chloroandrost-4-en-17α-ol-3-one (α-ClT), 4-chloroandrostan-3β-ol-17-one and especially 4-chloroandrost-4-ene-3α,17β-diol indicated the oral administration of ClTA. Less than 5% of the metabolites were excreted unconjugated. Therefore deconjugation is necessary to detect the metabolites of ClTA either with chemical or enzymatic approaches [4]. The differences between the conjugated forms recorded after oral and intramuscular administration were minor. The main metabolites, mentioned above, were easily identified 1.5 months after intramuscular administration and were presumably detectable long after that time.

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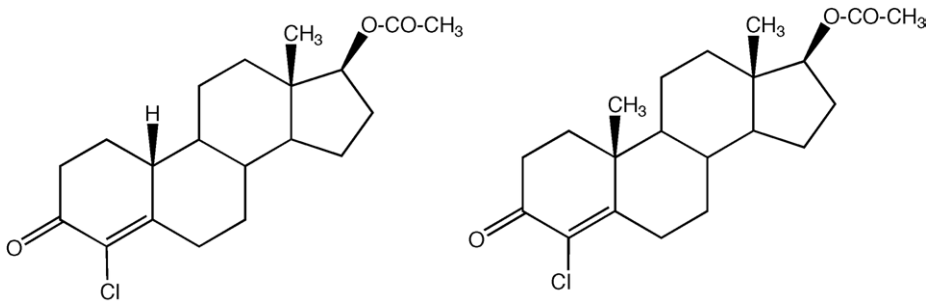


Fig. 1. Chemical structures of 4-chloro-19-nortestosterone acetate (left) and 4-chlorotestosterone acetate (right).

Recently, a new anabolic steroid, 4-chloro-19-nortestosterone acetate (norclostebol acetate, NClTA) was identified in black market preparations [7]. NClTA differs from ClTA in one methyl group on the C_{10}-position (Fig. 1). However, misuse (by means of biological matrices) has not been proven yet since there is no knowledge of norclostebol acetate metabolism.

Van Hoof et al. described the metabolism of NClTA after administration to the mysid shrimp *Neomysis integer*. *N. integer* has already been successively used as an alternative model to study steroid bio-transformation [8–10]. The enzymes of the cytochrome P450 system of *N. integer* are involved in the oxidation of steroids. Van Hoof et al. confirmed the expected resemblance of the metabolism of NClTA by *N. integer* with the metabolisation pathway of ClTA in vertebrates. Exposure of *N. integer* to NClTA indicated the production of two main metabolites, e.g. 19-nor-4-chlorotestosterone (NClT) and the oxidised form 19-nor-4-chloroandrost-4-ene-3,17-dione (NorClAD) (Fig. 2). Unambiguous identification was realised after synthesis of the two metabolites, saponification of NClTA (obtention of NClT) and oxidation of NClT (obtention of NorClAD) [11]. A better knowledge of the NClTA metabolites produced in vivo was necessary before any transposition into monitoring plans [10].

In this study, NClTA was administered both orally and subcutaneously to bovine. NClTA phase I metabolites were identified on the basis of their electron ionization mass spectra and conjugation was determined after specific separation of conjugates on anion exchange SPE cartridge [12].

2. Materials and methods

2.1. Reagents and chemicals

NClTA was obtained from Steraloids (Purity > 98%, Wilton, NY, USA). NClT was obtained by acid hydrolysis of NClTA (HCl (4 M) in acetone, 4 h reflux), and purified by semi-preparative HPLC according to previous published protocol [4]. The oxidised form of NClT, e.g. NorClAD, was in-house synthesised after oxidation of NClT with chromium trioxide/sulphuric acid/acetone (10/20/1000, m/v/v), 1 h at room temperature; a semi-preparative HPLC was performed for final purification [4].

Reagents and solvents were of analytical grade and provided by Solvents Documentation Synthesis (SDS, Peypin, France). Formic acid and triethylamine were from Sigma (St. Louis, MO, USA). The derivatization reagents were from Fluka (Buchs, Switzerland).

2.2. Animal experiment

NClTA was administered by the oral and subcutaneous routes (500 mg each time) at two separated periods to a female cow (pie-noire, 543 kg). For the oral administration NClTA was suspended in propylene glycol; for subcutaneous

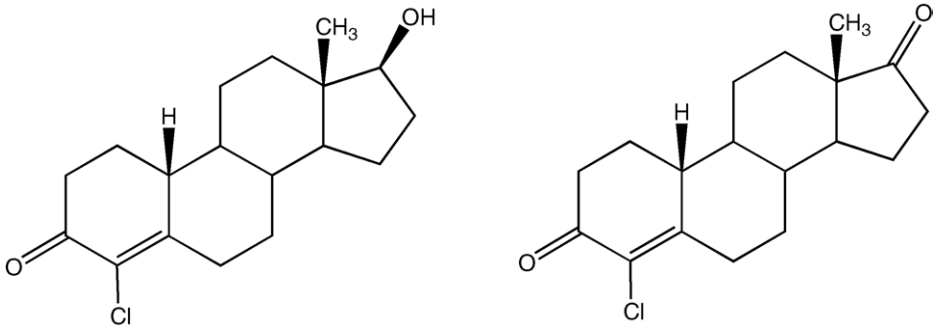


Fig. 2. Chemical structures of 4-chloro-19-nortestosterone (left) and 19-nor-4-chloroandrost-4-ene-3,17-dione (right).

injection, NClTA was suspended in sesame oil. In each experiment, urine was collected each day during 3 weeks.

2.3. Instrumentation

The gas chromatograph (HP-6890, Agilent®, Palo-Alto, CA, USA) was fitted with a capillary column, 30 m × 0.25-mm i.d., coated with a copolymeric stationary phase (MN-δ3, Macherey-Nagel, film thickness 0.25 μm). A glass insert, 4 mm i.d., loosely filled with silanized glass wool was used in the split/splitless GC injector (250 °C, purge splitless 1.5 min). Injection was performed at an oven temperature of 120 °C, increased at a programmed rate of 15 °C min^{-1} until 250 °C, and increased at 5 °C min^{-1} until 300 °C, and finally maintained 8 min. Helium (N55) was used as carrier gas at 1 mL min^{-1}. The electronic beam energy was set at 70 eV in the electron ionization (EI) mode. Low resolution (LR) full mass spectra were acquired on a quadrupole GC–MS system (HP-5973, Agilent®) at a scan rate of 0.5 s per decade in the mass range of *m*/*z* 50-600.

2.4. Analytical method

Ten millilitres bovine urine were adjusted to pH 5.2 with 2 mL of 2 M acetate buffer and then applied onto a SPE C_{18} cartridge (2 g stationary phase, SDS, Peypin, France), previously activated with 10 mL methanol and 10 mL water. After washing using 10 mL water, both free and conjugated forms steroids were eluted with 10 mL methanol.

The applied strategy to separate free, glucuronide and sulphate steroids, was already published [12]. Methanol extracts were applied onto a quaternary ammonium SPE cartridges (1 g stationary phase, SDS, Peypin, France) previously activated with 5 mL methanol, 5 mL water, 20 mL 0.5 M acetic acid in water, 20 mL water, and 5 mL methanol. As only the glucuronide and sulfate forms are retained on this stationary phase, the applied sample must be collected, using additional volume of methanol (total volume 12 mL) to elute the non-conjugated forms. In a second step, glucuronide forms are specifically eluted using a formic acid solution (0.75 M, 3.4%, v/v) in methanol (90–100% recovery for steroid glucuronide, determined by addition 17α-testosterone-d_3 glucuronide). For eluting sulfate forms, 20 mL triethylamine sulfate (TEAS) 1.4 M was used (80–90% recovery for steroid sulfates determined by addition of 17β-testosterone-d_3 sulfate). Phase II steroid hydrolysis was performed with *E. coli* glucuronidase (1 h, 50 °C) for glucurono-conjugates and solvolysis (ethylacetate/H_2SO_4, 1 h, 50 °C) for sulfo-conjugates. Hydrolyzed fractions were further purified onto a C18 SPE column as described above, and finally evaporated and derivatized with 20 μL of MSTFA/TMIS/DTE (1000:5:5, v/v/w) during 40 min at 60 °C.

3. Results

3.1. Phase I metabolites after oral administration

The detection of the metabolites was performed comparing peak to peak the total ion chromatograms of the extracted urine collected before and after administration of NClTA to the animal. Each appearing chromatographic peak different from those in blank urine, was considered as a potential

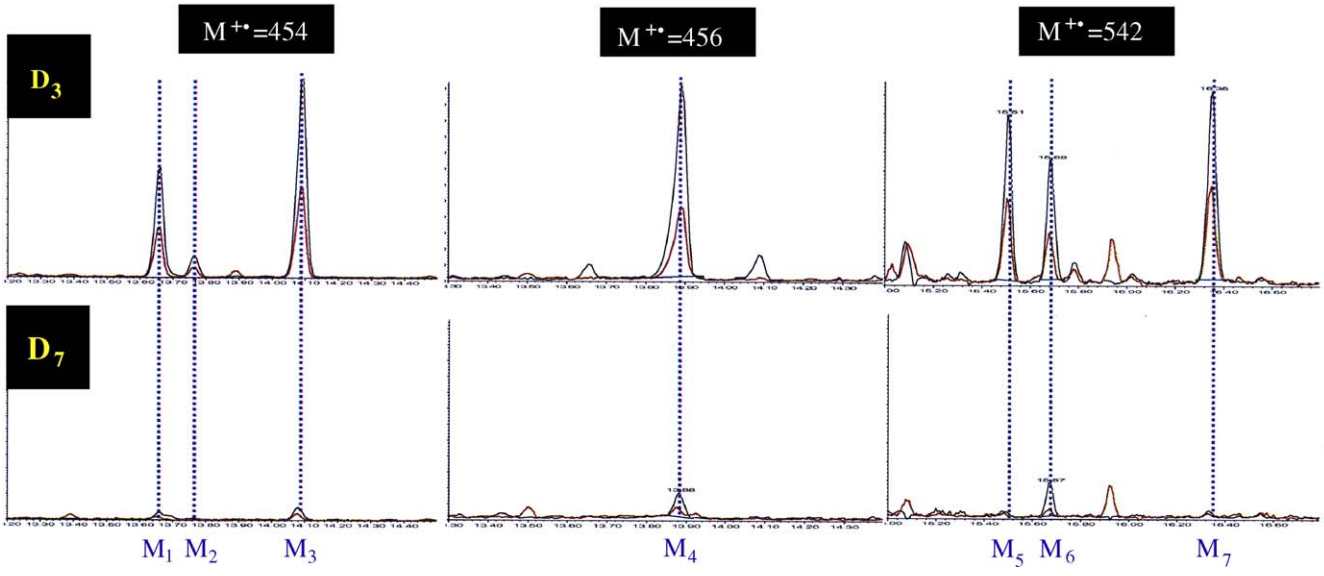


Fig. 3. Ion chromatograms showing the molecular ion signals of the seven main urinary metabolites (M1–M7) after oral administration of NClTA to bovine. *y*-axis: ion signal abundances; *x*-axis: retention time of metabolites. *m*/*z* 454, 456 and 542 (from the left to the right) and urine samples collected 3 days (D3, top) and 7 days (D7, bottom) after administration.

metabolite. The characteristic signal produced by the chlorine atom (^{37}Cl and ^{35}Cl in specific 3 to 1 abundance) in the mass spectrum confirmed strongly the metabolic link with NClTA.

After oral administration of NClTA, seven main metabolites (M1 to M7), (Fig. 3) were detected by this way. The metabolites M1 till M3 showed a molecular ion at *m/z* 454, the metabolite M4 had a molecular ion at *m/z* 456 and M5 till M7 revealed a molecular ion at *m/z* 542 (Fig. 4). Since hydrolyzed NClTA gives a molecular ion at *m/z* 452 corresponding to 4-chloro-19-norandrostane-17-ol-3-one (NClT) (Fig. 5), the excreted metabolites are mostly reduced, hydroxylated (*m/z* 542) or not hydroxylated (*m/z* 454 and 456). The structure elucidation of the metabolites was based on the known fragmentation of androgens which was deeply studied in previous papers [4,13]. This knowledge is particularly wide for TMS-derivatized steroids and when EI is used as ionization mode. Starting from simple rules such as:

- 17-Keto steroids generate abundant *m/z* 169 ion ($C_9H_{17}OSi$) which might correspond to a C and D ring fragmentation (cleavage of 10–11, 13–14 and 15–16 bonds) with charge retention on the 17-OTMS part;
- androstane-3,17-diols release very easily their trimethylsilanol and methyl groups leading to intense ions corresponding to $(M–Me)^+$, $(M–TMSOH)^{\bullet+}$, $(M–TMSOH–Me)^+$ and $(M–2TMSOH–Me)^+$.
- Androst-4-en-3-one compounds lead to a 208 *m/z* fragment ($C_{12}H_{20}OSi$); according to an equivalent retro-Diels Alder mechanism; 4-chloro-19-norandrost-4-en-3-one compounds generate a 228 *m/z* ion ($C_{12}H_{19}ClOSi$);
- contrary to androst-4-en-3-one (which are mainly converted to androst-3,5-diene when TMS derivatized), androst-4-en-3-ol leads to a characteristic B ring fragmentation by cleavage of the 9–10 and 5–6 bonds with charge retention on the A ring. The resulting fragment for

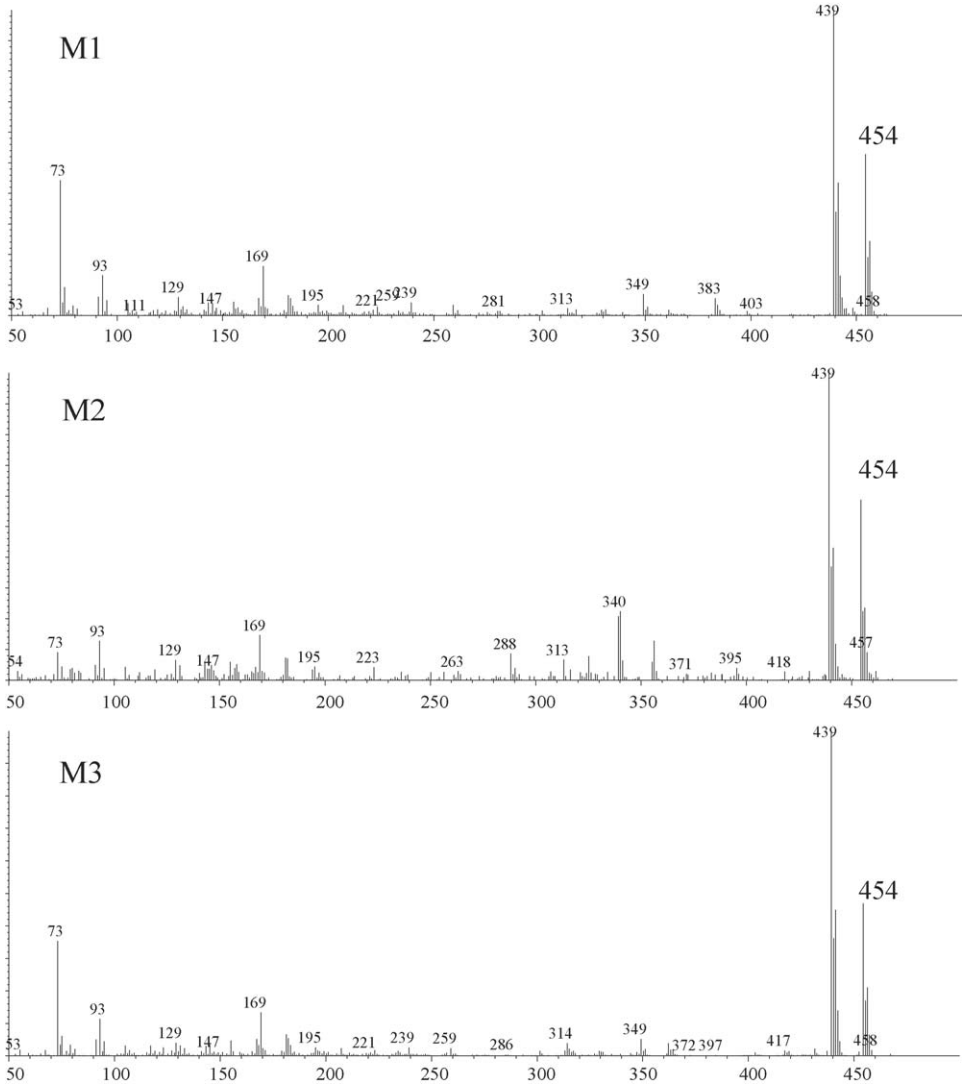


Fig. 4. Mass spectra of metabolites M1–M7 after oral administration.

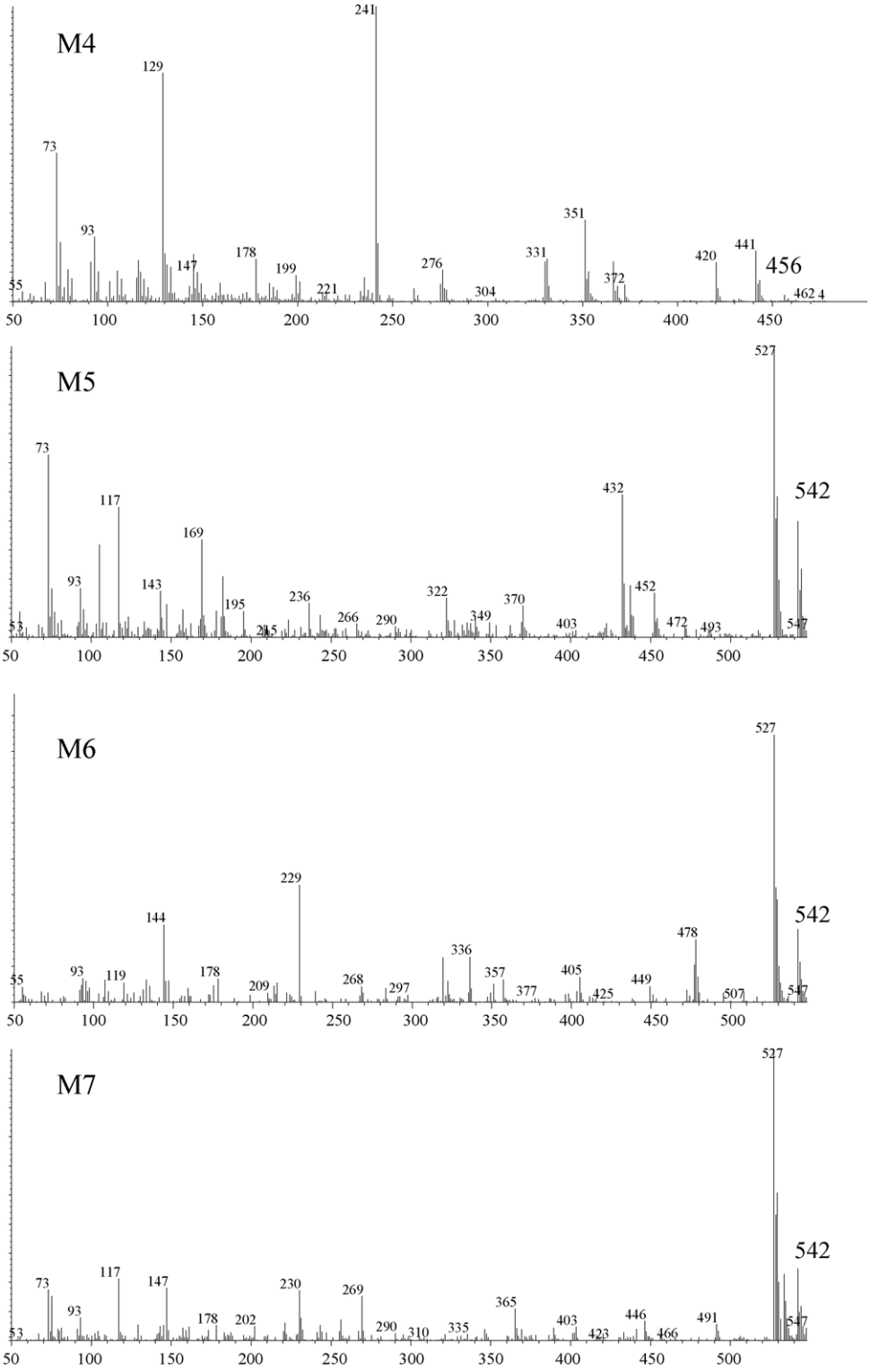


Fig. 4. (*Continued*).

4-chloro-19-nor-androst-4-en-3-ol steroids is a 201 *m*/*z* ion ($C_{10}H_{16}ClOSi$).

- The ion *m*/*z* 129 can be generated either by 17-hydroxysteroids (D ring cleavage with C13-17 and C14-15 ruptures and charge retention on the 17-OTMS fragment) or by 3-hydroxysteroid (A ring cleavage with C1–10 and C3–4 ruptures and charge retention on the 3-OTMS moiety).

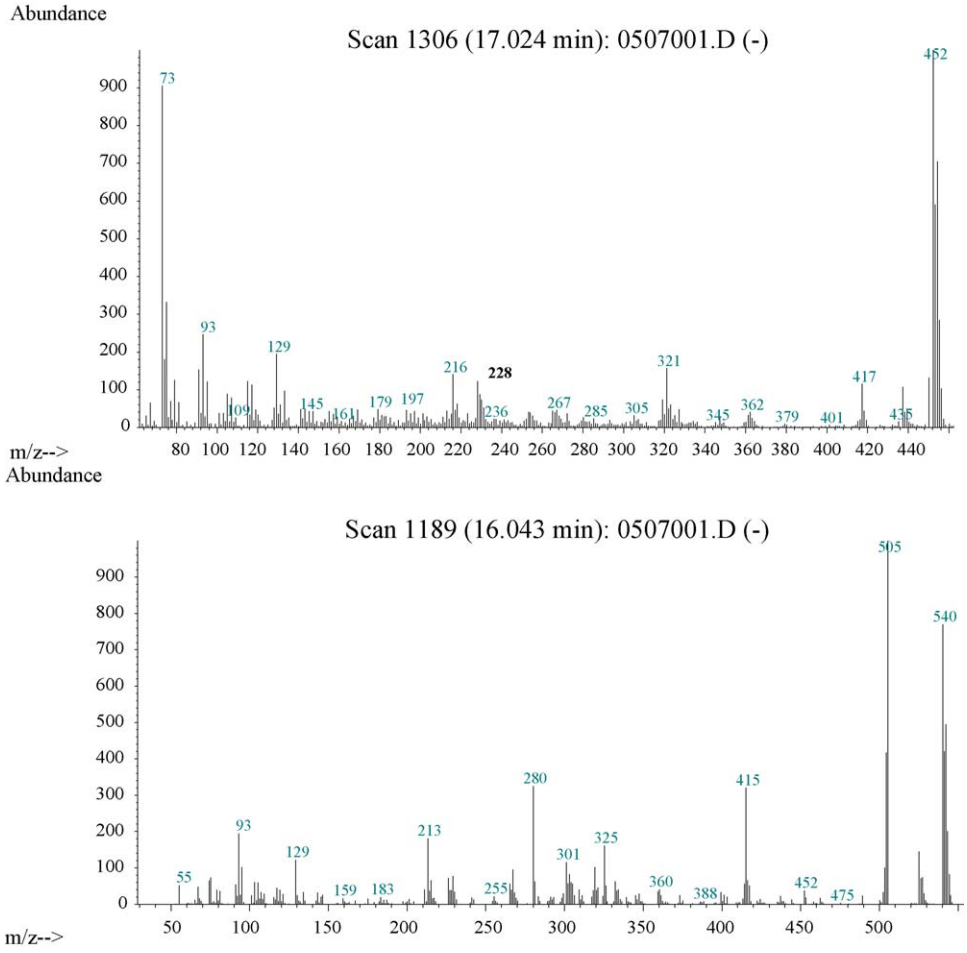


Fig. 5. Mass spectra of synthesised NClT and 6-hydroxy-NClT (EI ionization, TMS derivatization).

The metabolites M1 till M3 had an intense ion fragment at *m*/*z* 169, indicating the presence of a keto-group in position 17 ($C_9H_{17}OSi$). These metabolites had a molecular ion at *m*/*z* 454. As a consequence, the 3-keto group and the C4–5 double bond were necessarily reduced. The α-/β-isomery in positions 3 (OH), 4 (Cl) and 5 still needs to be determined.

M4 showed a very classical fragmentation pathway with a sequence of ions at *m*/*z* 456, 441, 366, 351, 276, and 261 corresponding to an androstanediol. Confirmation is given by the series of ions at *m*/*z* 420, 330/331, 241 which match with $(M^{\bullet+}–Cl^{\bullet})^{+}$, $(M^{\bullet+}–Cl^{\bullet}–TMSOH)^{\bullet+}$, $(M^{\bullet+}–Cl^{\bullet}–2TMSOH)^{\bullet+}$ and a strong *m*/*z* 129. M4 is the completely reduced 4-chloro-19-norandrostane-3,17-diol, the isomery still being to be determined.

Metabolites M5 till M7 are obviously hydroxylated. Metabolite M5 had an intense fragment ion at *m*/*z* 169, indicating the presence of a keto-group at position 17 ($C_9H_{17}OSi$). As the mass spectrum indicated a molecular ion at *m*/*z* 542, M5 is by design reduced on the A ring and would be a 4-chloro-19-norandrostane-3,x-diol-17-one. M6 and M7 showed some similarities in their mass spectra with M5 especially regarding the ratio $M^{\bullet+}/(M^{\bullet+}–Me^{\bullet})^{+}$, i.e. 542/527, which was interpreted as a consequence of a 17-keto function. Due to a limited fragmentation, and to a noisy chromatographic region, the background subtraction did not permit to exploit more exhaustively the mass spectrum. For the same reason the position of the hydroxyl-group was difficult to determine for any of the three hydroxylated metabolites.

From a quantitative point of view, M1, M3, M4 and M6, were found as the most abundant metabolites and were still detectable 7 days after oral administration (Fig. 3).

3.2. Phase I metabolites after subcutaneous administration

Analysis of the ion chromatograms of the urines taken after subcutaneous administration of NClTA revealed five main metabolites (M3, M8, M9, M10, M11) (Fig. 6), four of them being different and generally more oxidised comparing to the oral route administration (Fig. 7).

The metabolite M8 had a molecular ion at *m*/*z* 450 obviously indicating a fully oxidised structure still keeping the

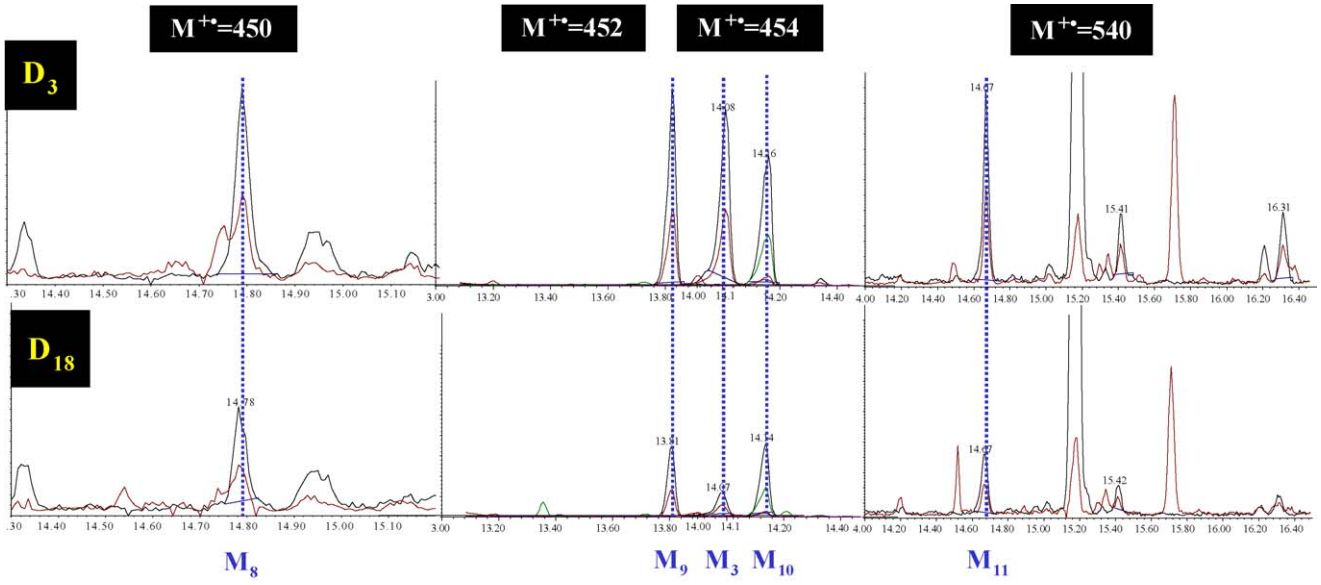


Fig. 6. Ion chromatograms showing the molecular ion signals of the five main urinary metabolites (M8, M9, M3, M10, M11) after subcutaneous administration of NClTA to bovine. *y*-axis: ion signal abundances; *x*-axis: retention time of metabolites. *m/z* 450, 452, 454 and 540 (from the left to the right) and urine samples collected 3 days (D3, top) and 18 days (D18, bottom) after injection.

C4–5 double bond. M8 was concluded as 4-norchloroandrost-4-ene-3,17-dione.

The mass spectrum of M9 revealed a molecular ion at *m/z* 454, as M1, M2 and M3. However, the absence of fragment ion at *m/z* 169 and the high abundance of the ion *m/z* 129 was interpreted as a androstanediol structure. Moreover the classical sequences $M^{\bullet+}$, $(M^{+}–TMSOH)^{\bullet+}$, $(M^{\bullet+}–2TMSOH)^{\bullet+}$ occurring at *m/z* 454, 364, 274, and $M^{\bullet+}$, $(M^{\bullet+}–Cl^{\bullet})^{+}$, $(M^{\bullet+}–Cl^{\bullet}–TMSOH)^{\bullet+}$, $(M^{\bullet+}–Cl^{\bullet}–2TMSOH)^{\bullet+}$ at *m/z* 454, 419, 329, 239 and $M^{\bullet+}$, $(M^{\bullet+}–Me^{\bullet})^{+}$, $(M^{\bullet+}–Me^{\bullet}–TMSOH)^{\bullet+}$, $(M^{\bullet+}–Me^{\bullet}–2TMSOH)^{\bullet+}$ at *m/z* 454, 439, 349, 259 confirmed the first hypothesis. By difference, the double bond is still in-between C4 and C5. We concluded M9 as 4-chloro-19-norandrost-4-ene-3,17-diol, the isomery of the hydroxyl group in positions 3 and 17 still to be determined.

The reading of M10 mass spectrum highlighted the mass value of the molecular ion (*m/z* 452). Signals at *m/z* 437 and 417 indicated the removal of a methyl group and a chlorine atom, respectively. These two fragments led to the *m/z* 347 and 327 ions, corresponding both to one trimethylsilanol elimination. A second 90 U loss on these ions was not observed indicating the presence of only one hydroxyl group in the molecule. The presence of *m/z* 169 and the presence of a weak *m/z* 129 are in favour of a 17-keto function in position 17. By deduction, as the molecular ion was known to be at *m/z* 452, the 3-keto function was evidently reduced and the C4–5 double bond remained unchanged. We came to the conclusion that M10 was the 4-chloro-19-norandrost-4-en-3ξ-ol-17-one. The isomery of the 3-hydroxyl group is still to be determined.

The molecular ion *m/z* 540 of metabolite M11 suggested an hydroxylation of the original structure. The mass spectrum is disturbed by some co-eluting compounds making the interpretation of weak abundant signals difficult. The comparison of M11 mass spectrum with synthesised 4-chloro-19-norandrost-4-en-6,17β-diol-3-one (6-hydroxyNCT) (Fig. 5) clearly shows that the hydroxylation occurred in another position than C6. The absence of the *m/z* 129 and 169 would indicate that the D-ring has the hydroxyl group, and *m/z* 205 would suggest a C16 position of this hydroxyl group. We hypothesize that M11 was the 4-chloro-19-norandrost-4-ene-6,17-diol-3-one. M3, M9 and M10 were the most abundant metabolites. They were still identifiable 18 days after subcutaneous administration. Whatever the administration route, M3 can be considered a specific marker of norclostebol acetate misuse in cattle (Fig. 6). The metabolism pathway proposed in Fig. 8 summarises most of the information developed in this paragraph, highlighting overlapping between oral and subcutaneous NClTA metabolism.

3.3. Phase II metabolites

The strategy used to determine the profile of phase II metabolites relied upon the specific separation of free, glucuronide and sulfate forms of 19-norchlorotestosterone acetate metabolites into three distinct fractions, and then the specific hydrolysis of both categories of conjugates. After TMS derivatization of hydrolyzed metabolites, GC–MS analysis was performed. We observed that the majority of the metabolites were excreted in the glucuronide fraction, apart from metabolite M3 which was found mainly in the sulfate fraction particularly after subcutaneous administration. Almost no free metabolites were detected (Fig. 9).

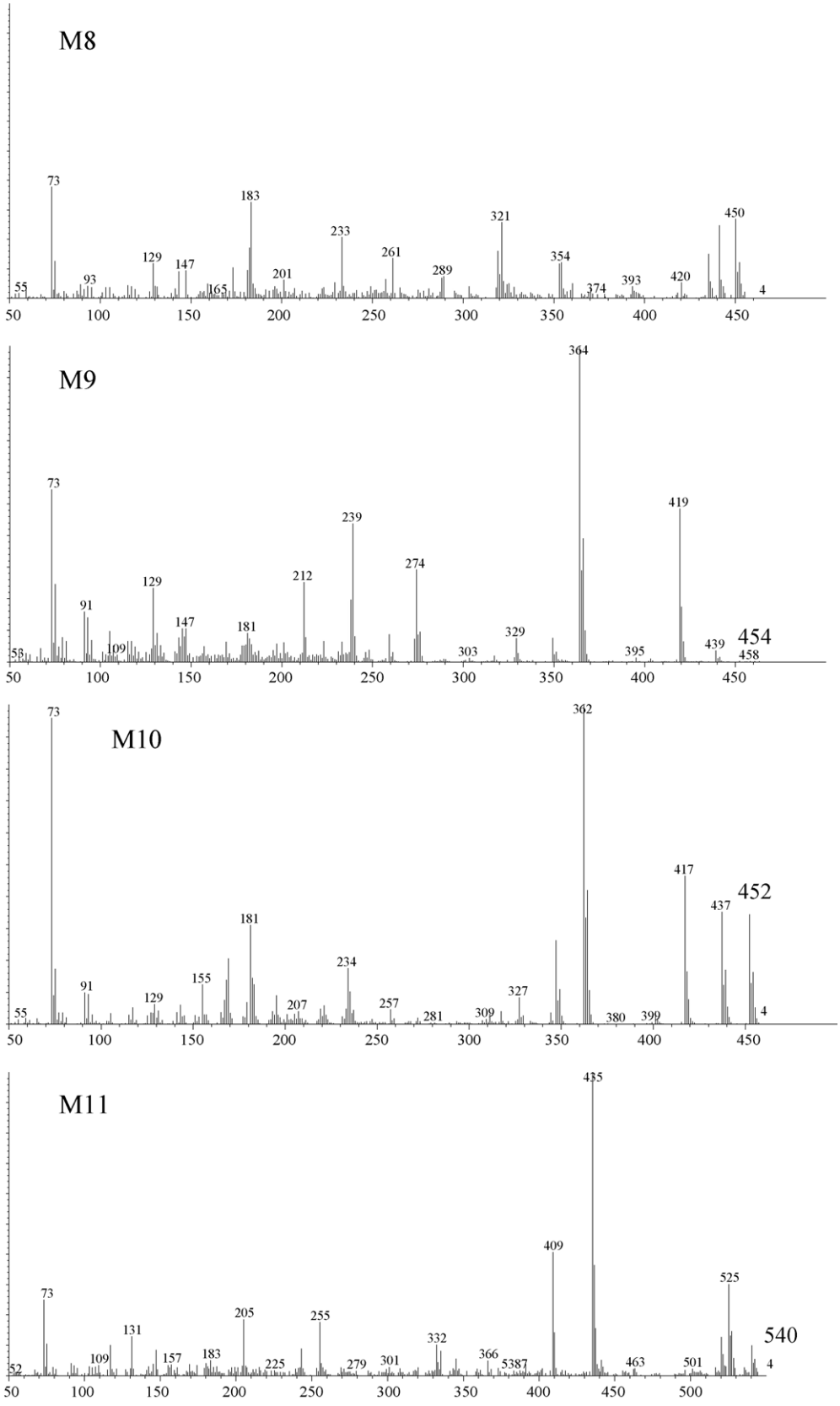


Fig. 7. Mass spectra of metabolites M8–M11 detected after subcutaneous administration.

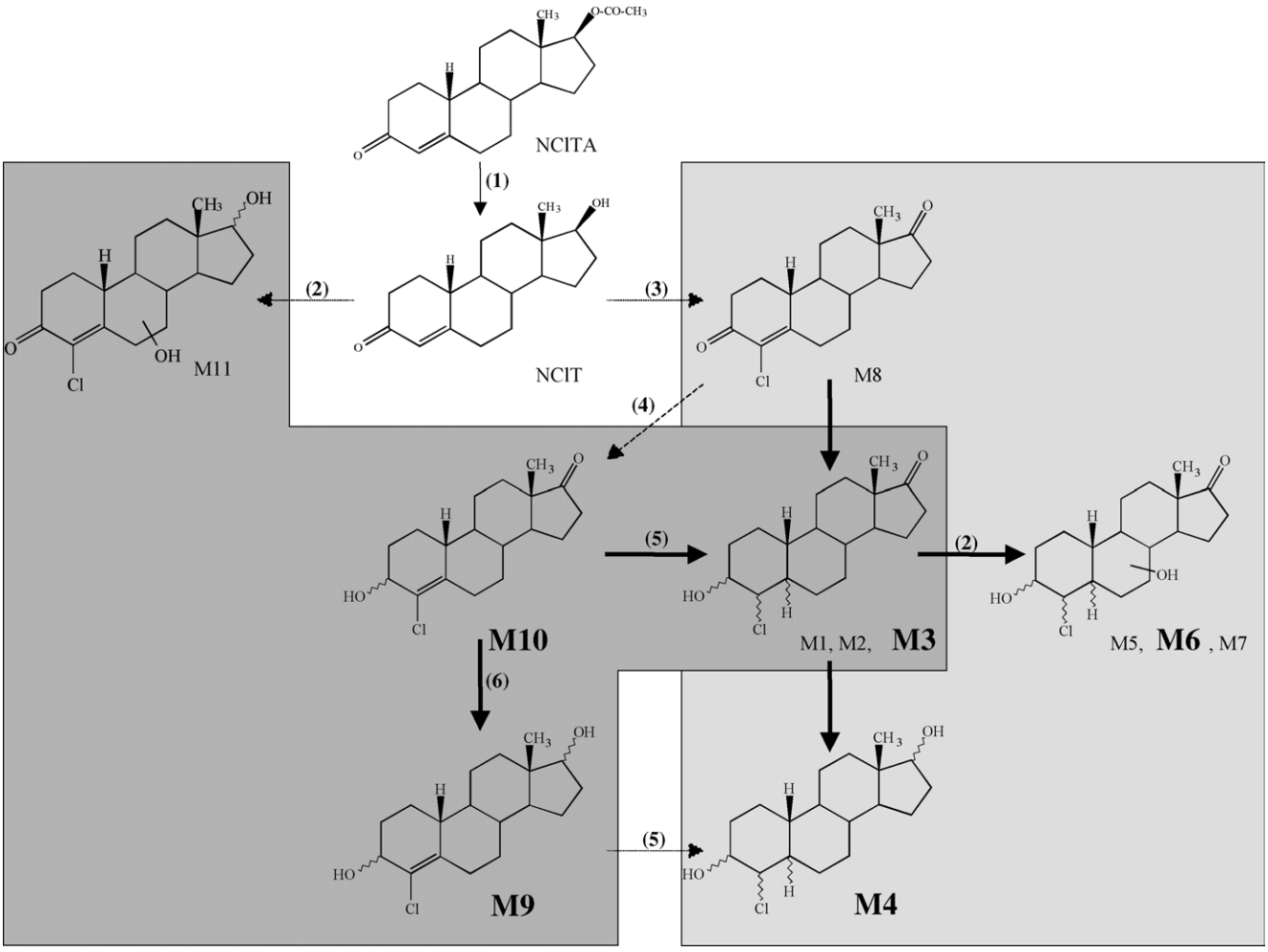


Fig. 8. Proposed metabolism pathway (phase I) of NClTA in bovine. In dark grey, metabolites identified after subcutaneous injection; in light grey, those detected after oral administration: (1) hydrolase; (2) steroid hydroxylase; (3) 17-hydroxysteroid dehydrogenase; (4) 3-ketosteroid-reductase; (5): 5-steroid-reductase; (6): 17-ketosteroid-reductase.

		M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9	M_{10}	M_{11}
ORAL	F	0	0	0	0	0	0	0	0	-	-	-
	G	+++	+	++	+++	+	+++	+	+	-	-	-
	S	0	0	+++	0	0	0	0	0	-	-	-
		>7 days		>7 days	>7 days		>7 days					

		M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9	M_{10}	M_{11}
SUB-CUT	F	-	-	0	-	-	-	-	-	0	0	0
	G	-	-	0	-	-	-	-	-	+++	+++	+
	S	-	-	+++	-	-	-	-	-	0	0	0
				>18 days						>18 days	>18 days	

Fig. 9. Summary of NClTA metabolites (M1–M11) after oral and subcutaneous administration (F fraction: free metabolite; G fraction: glucurono-conjugate metabolite; S fraction: sulpho-conjugate metabolite). (0) non detected; (+) weak abundance; (++) moderate abundance; (+++) high abundance.

4. Discussion

After oral administration of NClTA only reduced metabolites were detected. The most abundant metabolites were 4-chloro-19-norandrostan-3-ol-17-one (M1 and M3), 4-chloro-19-norandrostane-3,17-diol (M4) and 4-chloro-19-norandrostan-3,x-diol-17-one (M6) (Table 1). In a previous 4-chlorotestosterone acetate metabolism study, Le Bizec et al.

Table 1
Summary of the main metabolite chemical structures

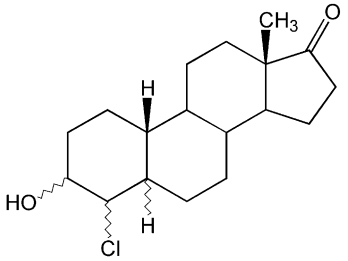
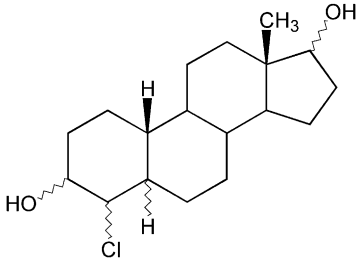
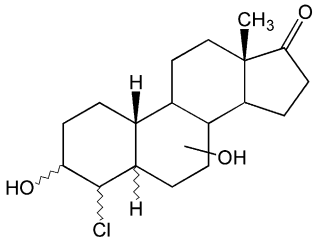
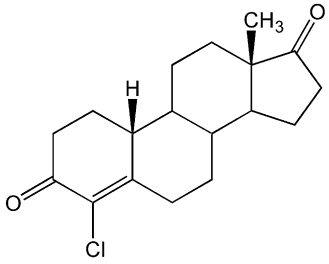
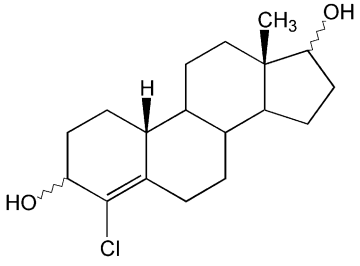
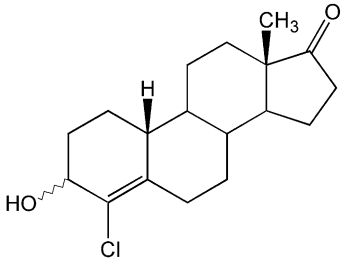
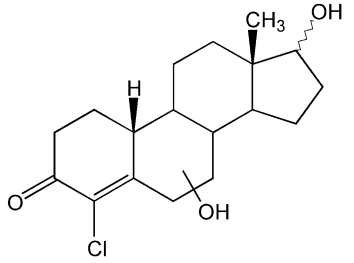
	Metabolite name	Chemical structure
M1–M3	4-chloro-19-norandrostan-3ξ-ol-17-one	
M4	4-chloro-19-norandrostane-3ξ,17ξ-diol	
M5–M7	4-chloro-19-norandrostane-3ξ,x-diol-17-one	
M8	4-chloro-19-norandrost-4-ene-3,17-dione	
M9	4-chloro-19-norandrost-4-ene-3ξ,17ξ-diol	
M10	4-chloro-19-norandrost-4-en-3ξ-ol-17-one	

Table 1 (*Continued*)

Metabolite name		Chemical structure
M11	4-chloro-19-norandrost-4-ene-3-one-x,17ξ-diol	

[4] reported three main residues, e.g. 4-chloroepitestosterone, 4-chloroandrostan-3β-ol-17-one, and 4-chloroandrost-4-ene-3α,17β-diol. Walshe et al. [5] described clearly 4-chloroandrostan-3β-ol-17-one as main indicator of ClTA administration. There is obviously a parallel which can be made in-between our study dedicated to NClTA and the two papers previously mentioned on ClTA, especially regarding the high abundance of a metabolite (4-chloro(19-nor)androstan-3β-ol-17-one metabolite) reduced in position 3 (3β-ol), $C_{4–5}$ (androstane or estrane), and oxidised in position 17 (17-keto).

After subcutaneous administration, the metabolite profile was significantly different compared to the oral route. The most important metabolites were 4-chloro-19-norandrostan-3-ol-17-one (M3), 4-chloro-19-norandrost-4-ene-3,17-diol (M9) and 4-chloro-19-norandrost-4-ene-3-ol-17-one (M10) (Table 1). After intramuscular administration of ClTA, Le Bizec et al. [4] described mainly oxidised metabolites and 4-chloroepitestosterone, 4-chloroandrostan-3β-ol-17-one (equivalent to M3) and 4-chloroandrost-4-ene-3,17-dione as main markers.

In summary, a parallel in-between previously published work on 4-chlorotestosterone and the observations performed in this study onto 4-chloro-19-nortestosterone, is difficult to establish. The 17α-epimer of 4-chlorotestosterone was identified as one of the main residues regardless the administration route [4]. However, this steroid was not detected in the metabolism study NClTA. This difference is difficult to explain especially when referring to the compared metabolism of testosterone and 19-nortestosterone (nandrolone) where the 17α-epimers (epitestosterone and epinandrolone, respectively) of both steroids are by far the leading metabolites, and used in all control laboratories as indicators of illegal administration. For NClTA, the metabolite called M3 (4-chloro-19-norandrostan-3-ol-17-one) appeared as the main strategic one to monitor the illegal use of this new androgenic anabolic steroid. The organic synthesis is now necessary to prove the definitive steroid stereochemistry and to be able to include this NClTA metabolite in the official monitoring plan.

5. Conclusion

The metabolism study of 4-chloro-19-nortestosterone acetate led to the identification of 7 and five metabolites after oral and subcutaneous administration, respectively (Table 1). In both cases M3 (4-chloro-19-norandrostan-3ξ-ol-17-one) was found to be amongst the most pertinent one to prove the illegal administration of NClTA. For confirmatory purposes and to establish the administration route, M1 (one of the isomers of M3), M4 (fully reduced structure) and M6 (hydroxylated 17-keto metabolite), would indicate a per os treatment, whereas two 4-ene-3-ol metabolites, e.g. 4-chloro-19-norandrost-4-ene-3,17-diol (M9) and 4-chloro-19-norandrost-4-ene-3-ol-17-one (M10) would designate a subcutaneous injection. The stereochemistry of most compounds still needs to be determined. All detected metabolites were still detectable 7 and 18 days after oral and subcutaneous administration, respectively. The phase II metabolism study revealed that most metabolites were unsurprisingly glucurono-conjugated. M3 was significantly different from the other ones, as it has been proved to be mainly sulfoconjugated, and to some extent linked to a glucuronic acid (only per os experiment) (Fig. 8). According to our observations, the use of β-glucuronidase alone (extracted and purified from *E. coli* or bovine liver) would not be sufficient to reveal the presence of M3 especially in case of subcutaneous administration. In analytical methods dedicated to NClTA control, the combined action of β-glucuronidase and sulfatase (or better a solvolysis step) would be necessary for an efficient monitoring of M3.

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