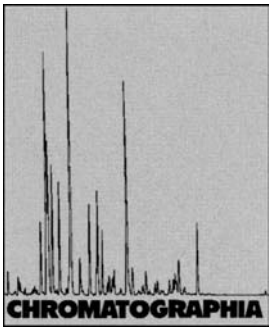
Norchlorotestosterone Acetate: An Alternative Metabolism Study and GC–MS^2 Analysis in Kidney Fat, Urine, and Faeces



2004, *59*, S85–S93

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In Honour of Professor Edward Houghton: A Celebration of 30 Years in Racing Chemistry

Received: 7 October 2003 / Accepted: 13 November 2003
Online publication: 9 March 2004

Abstract

Norchlorotestosterone acetate (NClTA) is an anabolic steroid which resembles chlorotestosterone acetate. It cannot yet be detected by routine methods used for anabolic steroids, because there is no knowledge of its metabolic pathway. The invertebrate *Neomysis integer* has been used as an alternative model to study the metabolism of NClTA. The experimental results indicated the presence of 4-norchloroandrost-4-ene-17-ol-3-one (NClT) and 4-norchloroandrost-4-ene-3,17-dione (NorClAD) as possible metabolites of NClTA. Subsequently NClTA and the synthesised metabolites NClT and NorClAD were incorporated into the routine multi-residue method for detection of anabolic steroids in kidney fat, urine, and faeces.

Keywords

Column liquid chromatography – mass spectrometry
Gas chromatography – mass spectrometry
Norchlorotestosterone acetate
Metabolism
Neomysis integer

Introduction

The use of anabolic steroids has been forbidden in the European Union since 1981 (directive 81/602/EEC) [1]. At the beginning of the 1990s many positive results were obtained indicating the presence of chlorotestosterone acetate (ClTA) at injection sites. Chlorotestosterone (clostebol, ClT) is an anabolic steroid which can be administered either orally or intramuscularly. It is used as a growth stimulant, even though it is strictly forbidden in the European Union. Chlorotestosterone acetate can be detected at injection sites. Negative results are, however, obtained when urine samples are screened for ClTA and ClT, because of extensive metabolic breakdown of the compounds. Leyssens et al. studied the metabolism of chlorotestosterone acetate by investigating unique metabolites in urine samples [2] and on the basis of results from this study a pathway for the metabolism of ClTA was proposed (Fig. 1). The most important metabolites in urine are 4-chloroandrost-4-ene-17 α-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]. Ninety-five percent of the metabolites are excreted in the sulfate form, except for 4-chloroandrost-4-ene-3-ol-17-one of which 25% is glucuronoconjugated. Less than 5% of the metabolites are excreted unconjugated. Deconjugation is therefore necessary to enable detection of the metabolites of ClTA [4].

A new anabolic steroid, norchlorotestosterone acetate (norclostebol acetate, NClTA) was recently discovered. NClTA differs from ClTA by one methyl group on the C_{10} position (Fig. 2). Although the anabolic steroid norchlorotestosterone (norclostebol) has been, or can be, misused as a growth promoter, misuse has not yet been proven because there is no knowledge of the metabolism of norclostebol acetate. It might be expected that the metabolic pathway of NClTA has some similarities to that of ClTA [7, 8].

Because most synthetic anabolic steroids are intensively metabolised, there is a need for animal experiments to study metabolic pathways. Because these experiments are complex and costly, valuable time and money are consumed. The invertebrate *Neomysis integer* was therefore used as an alternative model to study the metabolism of NClTA. The enzymes of

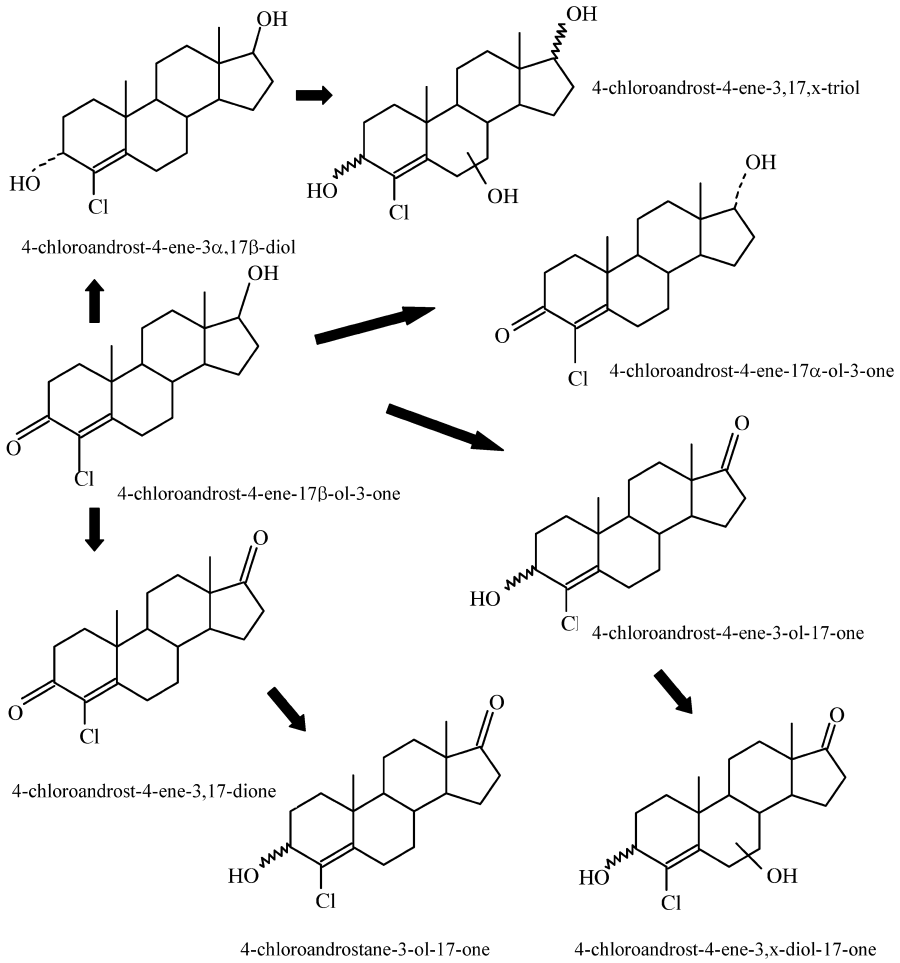

Fig. 1. Suggested metabolic scheme of CITA in cattle by Leyssens et al. (1994)

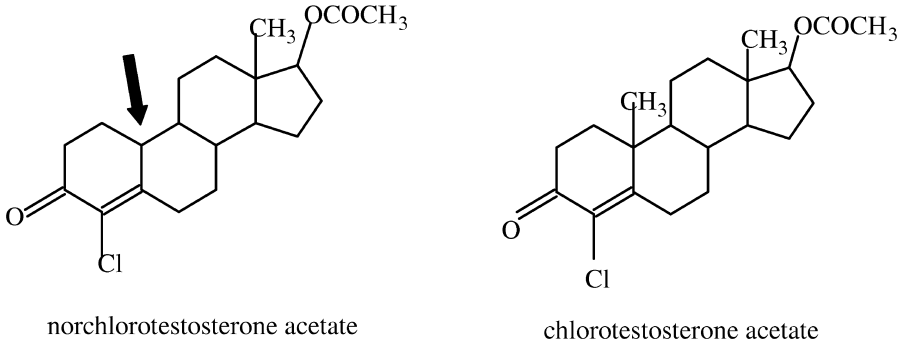

Fig. 2. Structural formulae of norchlorotestosterone acetate (left) and chlorotestosterone acetate (right)

the cytochrome P450 system of *N. integer* are involved in the oxidation of steroids. The mysid shrimp *N. integer* is a possible European alternative to the standard American species, *Americamysis bahia*, used by the US Environmental Protection Agency (EPA), the American Society for Testing and Materials (ASTM), and the Organisation for Economic Cooperation and Development (OECD) in standardised tests to study endocrine disruption. Verslycke et al. used the steroid testosterone as a substrate to study the extent of similarity of the P450 systems in *N. integer* and in vertebrates [9]. De Wasch et al. demonstrated the analogous metabolism of stanozolol in vertebrates (cattle) and invertebrates (*N. integer*). So exogenous and endogenous anabolic steroids have given an indication of the similarity of vertebrate and invertebrate metabolism [8].

In this study the invertebrate *N. integer* was used as an alternative model to study the metabolism of the anabolic steroid norclostebol acetate. The metabolites identified were subsequently synthesised and detected by means of a routine GC–MS^2 method for anabolic steroids in different matrices.

Experimental

Reagents and Chemicals

NClTA was obtained from Steraloids (Wilton, NY, USA). 4-Norchloroandrost-4-ene-17-ol-3-one (norchlorotestosterone, NClT) and 4-norchloroandrost-4-ene-3,17-dione (NorCLAD) were synthesised as described by Spruyt [10]. Other chemicals used were of analytical grade from Merck (Darmstadt, Germany). Stock standard solutions (1000 ng μL^{-1}) were prepared in ethanol. For preparation of working solutions methanol was used. All standard and working solutions were stored at 4 °C.

The derivatization reagent $MSTFA^{++}$ was prepared by dissolving 100 mg ammonium iodide (Sigma, St Louis, MO, USA) and 0.2 mL ethanethiol (Acros, Geel, Belgium) in 5 mL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA; Macherey–Nagel, Düren, Germany) then dilution of 1.5 mL of this solution with 10 mL MSTFA.

Animal Experiment and Extraction Procedure

N. integer was exposed to 4 μg NClTA for 16 h in 4 mL medium (5 ppt water, diluted with twice-distilled water from 30 ppt Instant Ocean artificial sea water) in a temperature-controlled chamber (15 °C, Liebher, Laborimpex, Brussels, Belgium). The organism was transferred to a 1.5-mL Eppendorf vial and shock-frozen in liquid nitrogen. MilliQ water (150 μL) was added to facilitate homogenisation with a Teflon plunger and 1 mL ethyl acetate was added. The mixture was vortex mixed and centrifuged (5 min, 14 000 rpm) and the supernatant was transferred to a glass tube. The extraction was repeated once. The two extracts were combined and evaporated to dryness under vacuum (Speedvac SVC 200, SC 210A Savant, Howe Gyrovap). The medium was also extracted twice with ethyl acetate and the combined extracts were evaporated to dryness [8]. The extracts from both the organism and the medium were examined

for traces of NClT, NorClAD, and the hydroxy metabolite of NClT.

Chemical Synthesis

Norchlorotestosterone (NClT) and 4-norchloroandrost-4-ene-3,17-dione (NorClAD) were synthesised as described by Spruyt [10].

Extraction and Clean-up

Several devices were used for extraction and clean-up [11]. The most important were solid-phase extraction coloums on Isolute Si-columns (3 mL, 500 mg) and Isolute NH_2-columns (1 mL, 100 mg) (IST International, Mid Glamorgan, UK) and preparative HPLC apparatus (HPLC Intelligent Pump; Merck, Darmstadt, Germany).

Extraction and Clean-up of Kidney Fat

Anabolic steroids were extracted from kidney fat by use of methanol. Clean-up was performed by liquid–liquid extraction with diethyl ether and solid phase extraction [11].

Extraction and Clean-up of Urine

Urine samples were first diluted and hydrolysed. Clean-up was performed by liquid–liquid extraction with diethyl ether. Levels of interfering matrix compounds in urine extracts were further reduced by preparative HPLC fractionation on a C_{18} reversed-phase column (unpublished work).

Extraction and Clean-up of Faeces

Anabolic steroids are extracted from faeces by use of diethyl ether. Defatting was performed with petroleumbenzin. Clean-up was performed by solid-phase extraction with an Si-column coupled to a NH_2-column. Levels of interfering matrix compounds in faeces extracts were further reduced by preparative HPLC fractionation on a C_{18} reversed-phase column [12].

Derivatisation

Steroids are high-molecular-weight polyfunctional substances which are difficult to analyse by GC–MS because of their polar and thermally labile character. Before GC–MS analysis they must, therefore, be derivatized to convert polar and active groups in the molecules to apolar and inert groups. This results in improved volatility during gas chromatography and improved thermal stability and peak symmetry.

Compounds in the final extracts were converted into enoltrimethylsilyl ether derivatives by reaction with $MSTFA^{++}$. $MSTFA^{++}$ (25 µL) was added to each vial, the mixture was incubated for 60 min at 60 ± 2 °C, then 1 µL was injected into the gas chromatograph.

LC–MS

HPLC of NClTA and its metabolites was performed with a TSP P4000 pump and model AS3000 autosampler (TSP, San Jose, USA). Chromatographic separation was achieved on a Symmetry C_{18} column (150 mm × 2.1 mm i.d., 5-µm particle; Waters, Milford, USA). The isocratic mobile phase was 60:40 (%, *v*/*v*) methanol–1% aqueous acetic acid at a flow rate of 0.3 mL min^{-1}. LC–MS–MS was performed in positive-ion mode with a Thermo Finnigan (San José, CA, USA) LCQ Deca ion-trap with an atmospheric-pressure chemical ionisation (APCI) interface. The extracts from the animal experiment were reconstituted in 150 µL of the same mixture of methanol and 1% aqueous acetic acid before injecting 30 µL on to the HPLC column.

GC–MS

Gas chromatography was performed with a ThermoQuest CE Trace GC gas chromatograph (Thermo Finnigan, Austin, TX, USA) with split/splitless injector. Compounds were separated on a non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 column (25 m × 0.22 mm i.d., 0.25-µm film; SGE, Austin, TX, USA). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. A Carlo Erba AS2000 autosampler (Thermo Finnigan) was used to inject the samples. Compounds were detected with a Polaris ion-trap mass spectrometer (Thermo Finnigan) with electron-impact ionization. The gas chromatographic and mass spectrometric conditions used for routine analysis of anabolic steroids are presented in Table 1.

Table 1. Gas chromatographic and mass spectrometric conditions used to perform GC–MS^n analysis

GC temperature program	
Initial temperature	100 °C
Segment 1	250 °C (17° min^{-1})
Segment 2	294 °C (2° min^{-1})
Segment 3	300 °C (30° min^{-1})
Isothermal segment	300 °C (hold for 1.30 min)
GC carrier gas	Helium
Column flow	1 mL min^{-1}
Injector (*splitless mode*)	
Temperature	250 °C
Split flow	60 mL min^{-1}
Polaris MS (*electron impact*)	
Ion-source temperature	200 °C
Transfer-line temperature	275 °C
Mass range	150–570 a.m.u.
MS–MS activating potential	0.70–1.30 V

Results and Discussion

Study of Metabolism

An experiment was set up with the invertebrate *Neomysis integer*. NClTA was administered to the shrimp to investigate the metabolic pathway. The results obtained gave only an indication, because there was no certainty that the cytochrome P450 enzymes of *N. integer* would oxidise NClTA in the same manner as those of vertebrates (cattle).

The calculated molecular masses of NClTA, NClT, NorClAD, and the hydroxy metabolite of NClT were 350, 308, 306, and 325, respectively, hence *m*/*z* 351, *m*/*z* 309, *m*/*z* 307 and *m*/*z* 326 were the pseudomolecular ions expected from LC–MS analysis in positive-ion mode. Analysis of the extract from the organism revealed the presence of ions of *m*/*z* 309 and *m*/*z* 351 in full-scan MS.

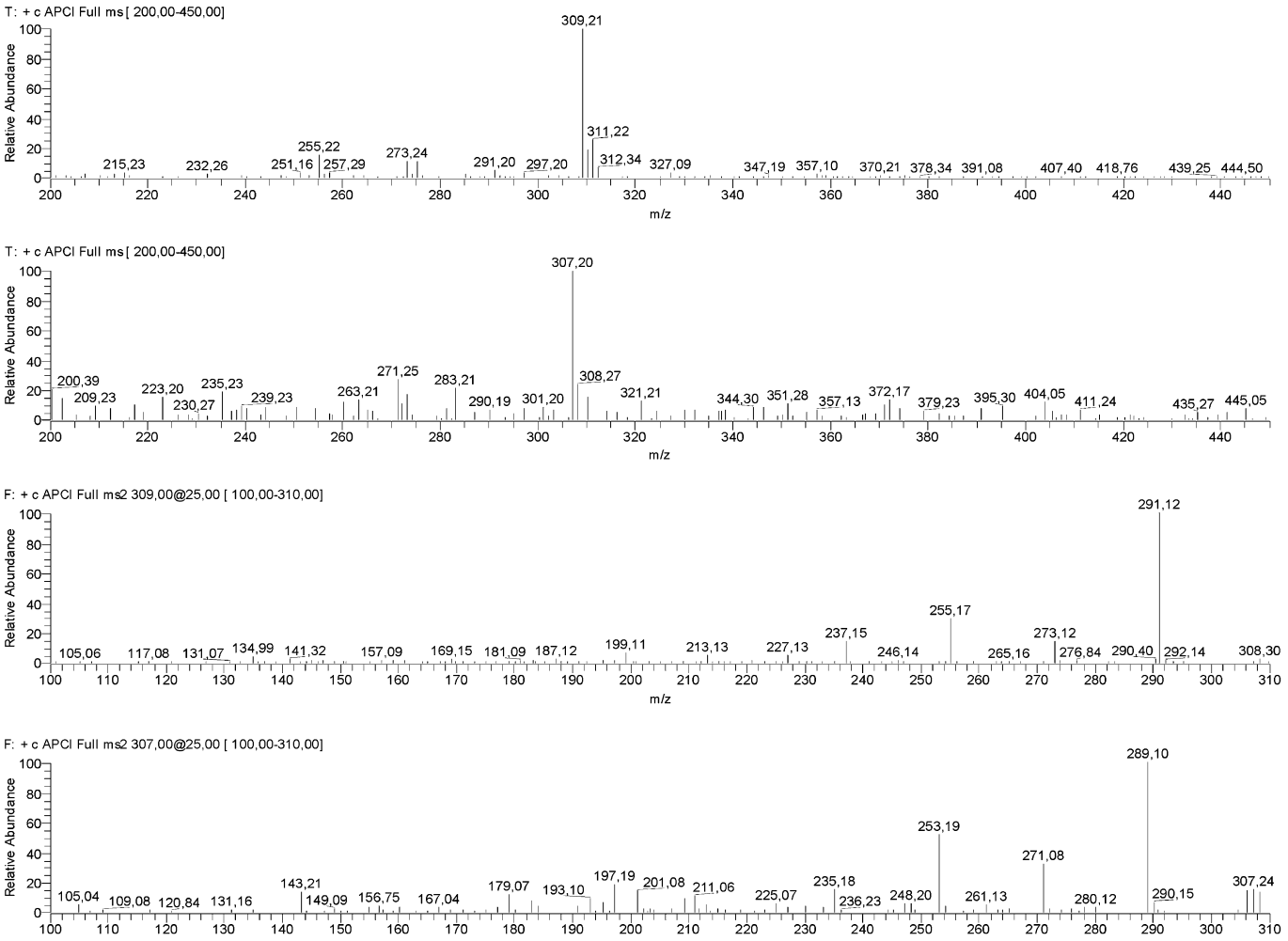

Fig. 3. Analysis of the extract of the medium with LC–MS and LC–MS^2

These ions gave an indication of the presence of the metabolite NClT in the organism. Analysis of the extract obtained from the medium revealed the presence of ions of m/z 307, m/z 309, and m/z 351 in full-scan MS. Full-scan MS^2 was also performed on these ions (Fig. 3). These ions gave an indication of the presence of metabolites NClT and NorClAD in the medium.

To obtain complementary information the extracts were also analysed by GC–MS. The molecular masses calculated for the enoltrimethylsilyl ether derivatives of NClTA, NClT, NorClAD, and the hydroxy metabolite of NClT were 422, 452, 450, and 541, respectively. Analysis of the extract from the organism revealed ions of m/z 422, m/z 452, and a trace of m/z 450 in full-scan MS. These ions gave an indication of the presence of the metabolites NClT and NorClAD in the organism. Analysis of the extract from the medium revealed the ions of m/z 422, m/z 452, and m/z 450 in full-scan MS. Full-scan MS^2 was also performed on these ions (Fig. 4). The ions m/z 450 and m/z 452 were more clearly present in the extract from the medium than in that from the organism. Differences between medium and organism will not be discussed here, however.

The expected resemblance of the metabolic pathway of ClTA in vertebrates and in *N. integer* was confirmed by the experiment. The mass spectrum of the metabolite NorClAD obtained by full-scan MS^2 resembled that obtained for ClAD by GC–MS^2 analysis. There was an expected mass difference of 14 between the most important product ions of NorClAD and ClAD—ions of m/z 309, 325, 345, 399, 415, and 435 of NorClAD (Fig. 4) were 14 mass units lower than ions of m/z 323, 339, 359, 413, 429, and 449 in the mass spectrum of ClAD. Because no ClT standard was available, no comparison could be made between NClT and ClT. By comparing NClT with NorClAD it could be concluded there was a mass difference of 2 between the product ions after GC–MS^2 analysis, because of the presence of a hydroxyl group in NClT instead of a keto group in NorClAD. Further experiments must be performed but a good indication of the metabolites of NClTA has already been obtained.

Identification of the Synthesised Products NClT and NorClAD

The synthesised compounds NClT and NorClAD were identified by infrared spectrometry and mass spectrometry [10].

The IR spectrum of NClTA was characterised by intense absorption at 1740 cm^{-1} and 1680 cm^{-1} by the C=O bonds of, respectively, the acetate group and the carbon–oxygen double bond at

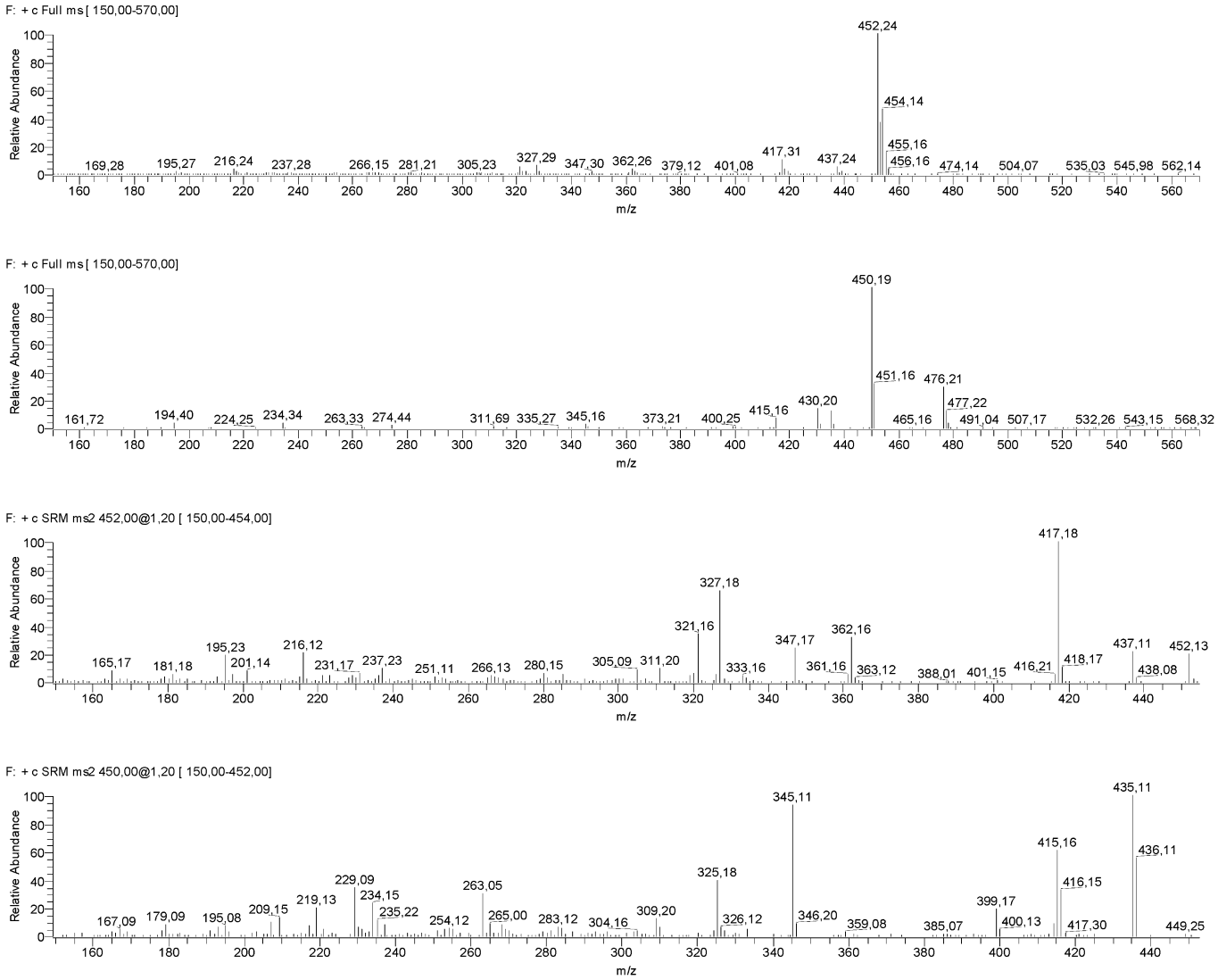

Fig. 4. Analysis of the extract of the medium with GC–MS and GC–MS^2

the 3-position. The IR spectrum of NClT contained a broad peak at approximately 3500 cm^{-1}, indicating the presence of a hydroxyl function in the structure. The absorption of the acetate group had disappeared. The IR spectrum of NorClAD contained an intense peak at 1737 cm^{-1}, indicative of oxidation of NClT.

When 50 ng NClT and 50 ng NorClAD were injected on to the HPLC column two chromatographic peaks were observed. The first peak furnished the pseudomolecular ion m/z 307 in full-scan MS indicating the compound was NorClAD. Full-scan MS^2 of the pseudomolecular ion furnished a mass spectrum resembling that of NorClAD obtained during the experiment with *N. integer* (Fig. 5). Ions of m/z 289, 253, 271, and 235 were present in both spectra and their relative intensities were similar. The second chromatographic peak furnished the pseudomolecular ion m/z 309 and its Cl isotope m/z 311 in full-scan MS, indicating the presence of the metabolite NClT. Full-scan MS^2 of the pseudomolecular ion furnished a mass spectrum which resembled that of NClT obtained during the experiment with *N. integer* (Fig. 6). Ions of m/z 291, 255, 273, and 237 were present in both spectra and their relative intensities were similar. When 10 ng NClT and NorClAD was injected on to the GC column the first chromatographic peak furnished an ion of m/z 450 in full-scan MS, indicative of NorClAD, and the second peak furnished an ion of m/z 452, indicative of NClT.

Complimentary information from IR spectroscopy and mass spectrometry indicated the identities of the synthesised products to be NClT and NorClAD. MS data from the synthesised products were the same as those obtained from the invertebrate experiment. Hence the synthesis was correct but incomplete. Because traces of NClTA and NClT were still observed after LC–MS and GC–MS analysis, hydrolysis of NClTA and oxidation of NClT were both incomplete. To obtain pure compounds further purification was necessary. Another problem was lack of knowledge about the stereospecificity of NClT (i.e. whether it was α- or β-NClT). By analogy with ClTA the α form can be expected in urine samples [2].

Detection of NClTA and its Metabolites in Different Matrices

Evidence of abuse of anabolic steroids in cattle can be detected in several matrices,

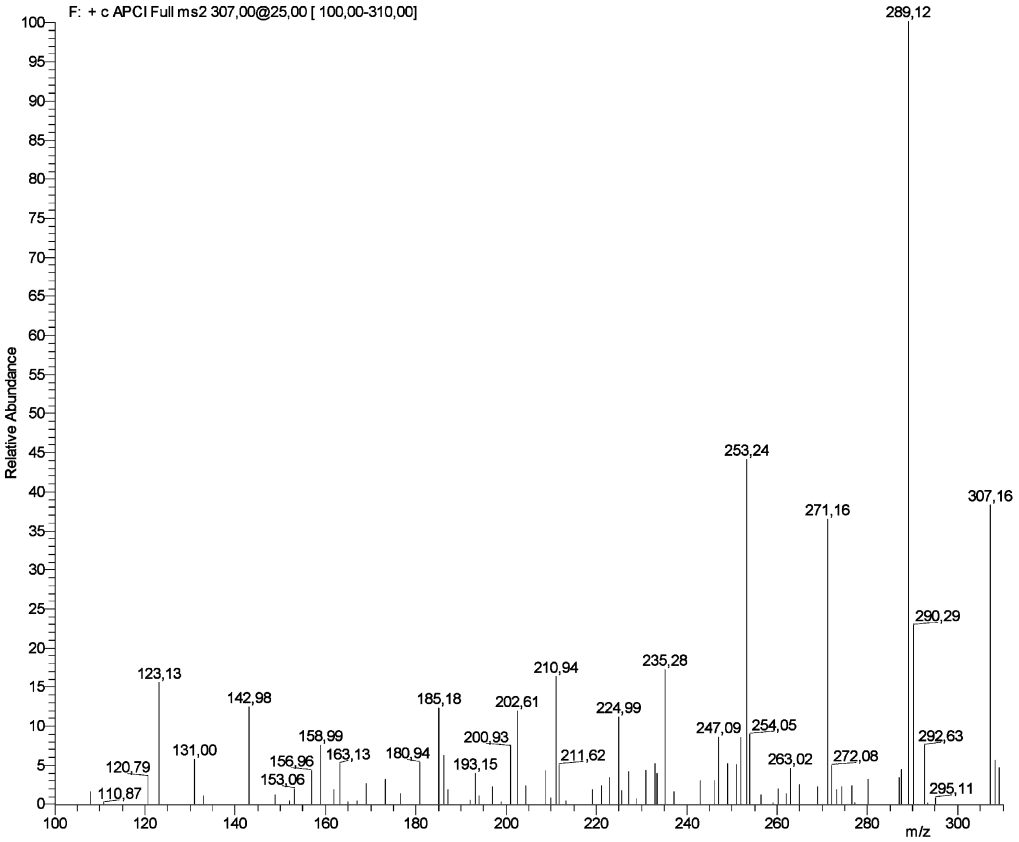

Fig. 5. The mass spectrum of the synthesised NorClAD analysed with LC–MS^2

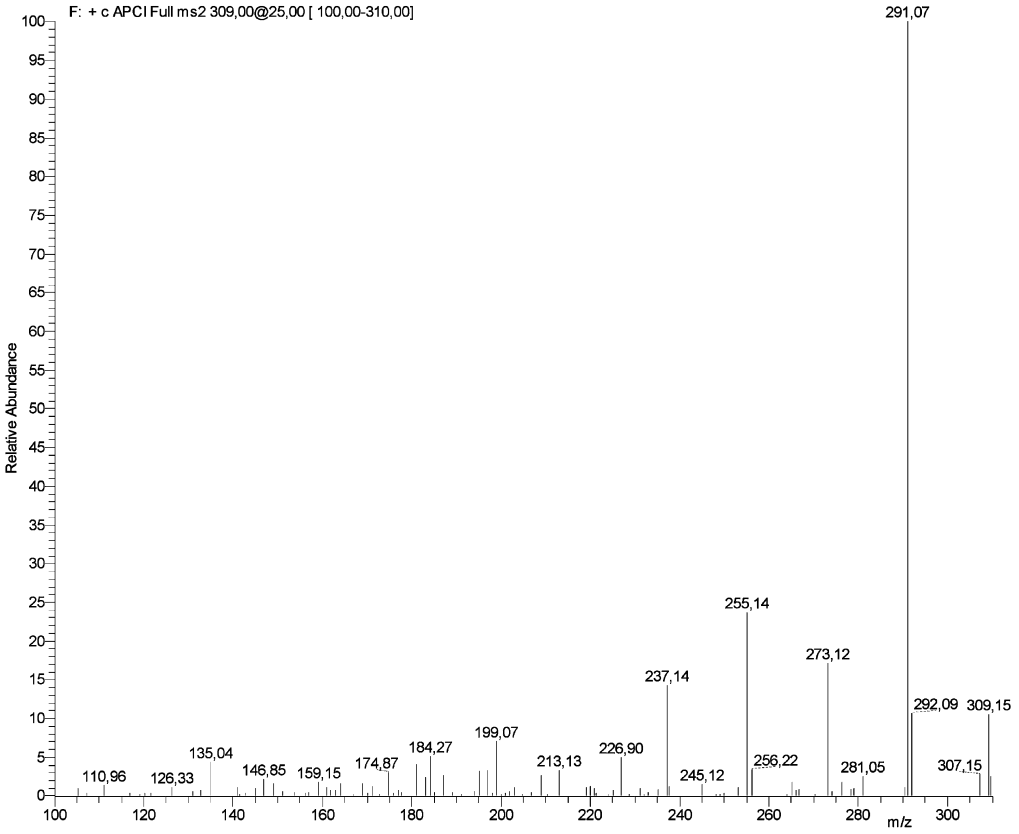

Fig. 6. The mass spectrum of the synthesised NClT analysed with LC–MS^2

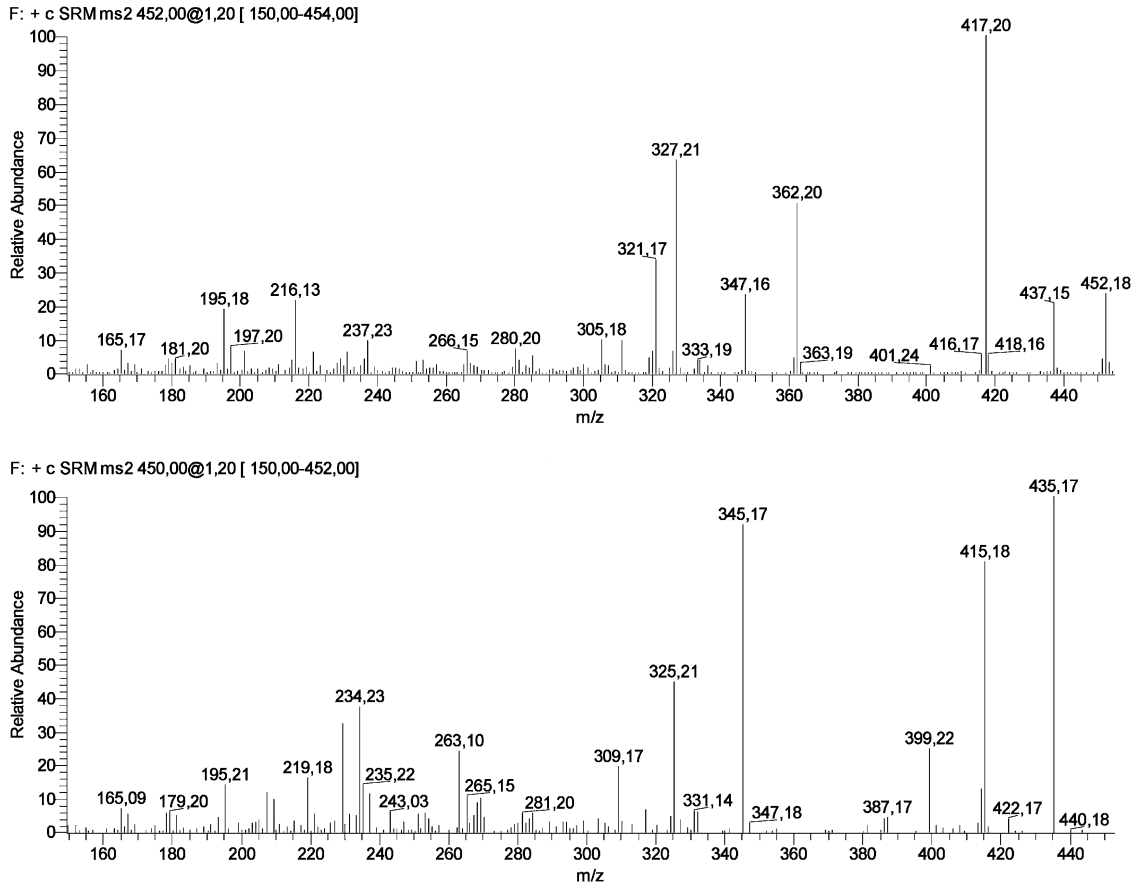

Fig. 7. Fractions 5 and 6 (upper mass spectrum) and fractions 3 and 4 (bottom mass spectrum) from urine sample spiked with NClT (upper mass spectrum) and with NorClAD (bottom mass spectrum)

for example meat (e.g. injection sites), kidney fat, urine, and faeces. In regulatory control analysis to detect steroid abuse the identity of the anabolic steroid is unknown; it is, therefore, important to screen samples for a wide range of anabolic steroids in one analytical run. A multi-residue procedure must be employed. The aim of this study was to incorporate NClTA and its synthesised metabolites in routine multi-residue methods for detection of anabolic steroids in kidney fat, urine, and faeces. Although a national MRPL level has not yet been imposed by the Belgian authorities, monitoring of NClTA should be included in routine residue analysis because it might be (mis)used for cattle fattening.

Impens et al. have already included NClTA in the routine method for analysis of anabolic steroids in kidney fat. The decision limit, $CC\alpha$, is the limit at and above which it can be concluded with an error probability α that a sample is non-compliant. The $CC\alpha$ for NClTA was 3 μg kg^{-1} [13].

To check whether the routine method for anabolic steroids in urine and faeces could also be used to detect the metabolites of NClTA, i.e. NClT and NorClAD, blank urine samples were spiked with the synthesised compounds NClT and NorClAD. As already mentioned NClT and NorClAD were not pure products, so the concentration needed to spike the blank urine samples was adapted. Comparison was made with standard 4-chloroandrost-4-ene-3,17-dione (ClAD), which could be detected at a level of 2 ng by $GC–MS^2$. To obtain chromatographic peaks for NClT and NorClAD of intensity similar to that for ClAD 10 ng was necessary, probably because of the impurity of the compounds. The national MRPL for ClAD in urine and faeces is 5 μg kg^{-1}. Because it was possible to detect ClAD at this concentration with the routine method for anabolic steroids in urine and faeces, the concentration of NClT and NorClAD added to the blank urine samples was based on this ratio and the national MRPL for ClAD.

After clean-up of the spiked urine samples the extracts were fractionated. Both NClT and NorClAD were observed in the spiked urine samples, even though the intensity for NorClAD was rather low (Fig. 7). The metabolites NClT and NorClAD could therefore be detected by use of the routine method for anabolic steroids in urine. The concentration at which the metabolites were detectable was comparable with the national MRPL for ClAD.

In a manner similar to the experiments with urine, blank faeces samples were spiked with the synthesised NClT and NorClAD. Again the metabolites NorClAD and NClT were detected. Both NClT and NorClAD were observed in the spiked faeces samples, even though the intensity of the NorClAD signal was rather low (Fig. 8).

Conclusion

The metabolism experiment with the invertebrate *Neomysis integer* indicated

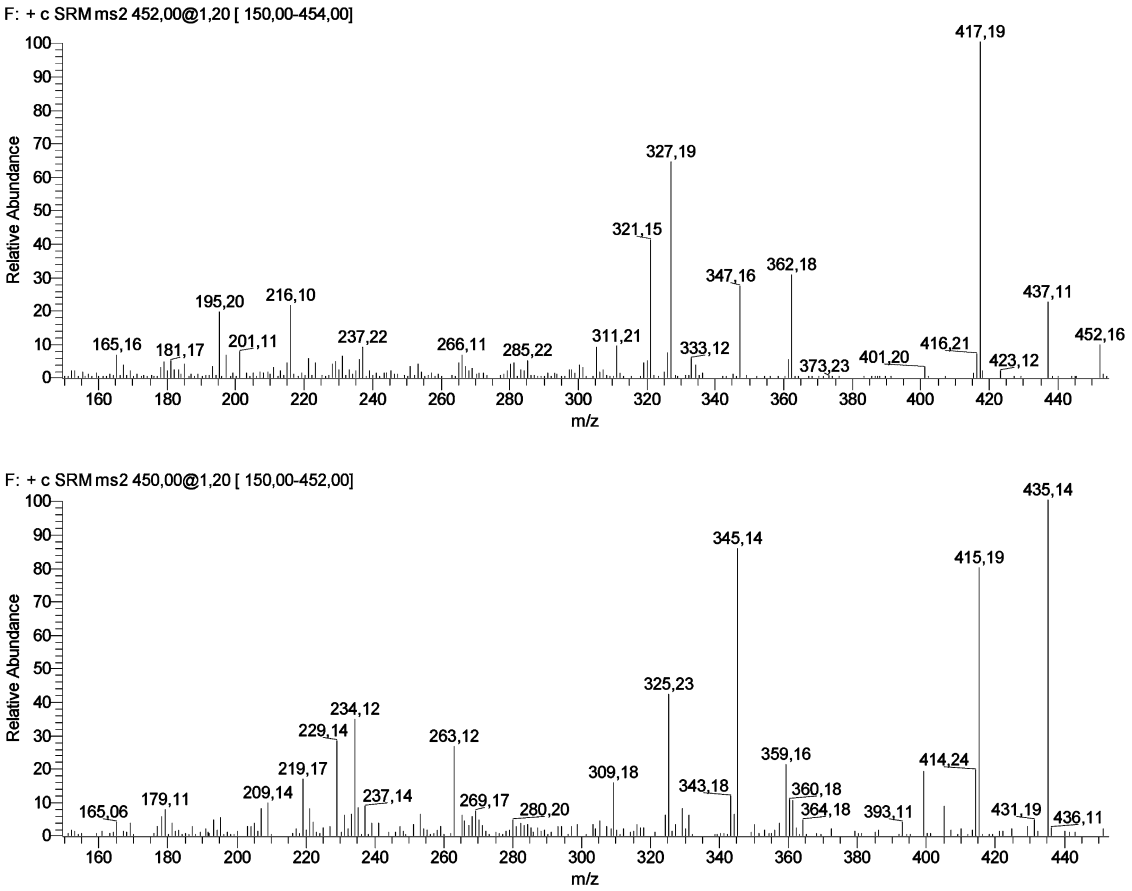

Fig. 8. Fractions 5 and 6 (upper mass spectrum) and fraction 3 and 4 (bottom mass spectrum) from faeces spiked with NClT (upper mass spectrum) and with NorClAD (bottom mass spectrum)

NClT and NorClAD were present as possible metabolites of NClTA. These results confirmed the expected resemblance with the metabolic pathway of ClTA. Because there is no certainty about the similarity of vertebrate and invertebrate metabolism, study of the metabolism of NClTA in bovine species is still recommended.

The compounds NClT and NorClAD were subsequently synthesised and identified by use of infrared and mass spectrometry [10]. The mass spectra obtained from full-scan MS^2 of NClT and NorClAD were identical with those obtained during the experiment with *N. integer*. This confirmed that synthesis was successful. It was, however, incomplete because traces of NClTA and NClT were still observed after MS analysis. NClT and NorClAD are, therefore, impure products and further purification is necessary.

Finally, NClTA and its synthesised metabolites were incorporated into routine multi-residue methods for detection of anabolic steroids in kidney fat, urine, and faeces. Impens et al. had already included NClTA in the routine method for anabolic steroids in kidney fat. The $CC\alpha$ for NClTA was 3 μg kg^{-1} [13]. The metabolites NClT and NorClAD were included in the routine method for anabolic steroids in urine and faeces, and detection and identification of these metabolites was possible. The concentration at which the synthesised compounds were detectable was comparable with the national MRPL for ClAD (5 μg kg^{-1}). Because the synthesised metabolites still contained many undesired interferences the exact concentrations at which NClT and NorClAD were detectable was not known and so quantification of the metabolites was not yet possible.

Acknowledgements

The authors wish to thank the Belgian Federal Ministry of Public Health and the Federal agency for the safety of the food chain for the grant financing this research (S6044/S3). The authors are grateful to Dirk Stockx and Ann Houvenaghel for assistance with experimental work and skilful operation of the $GC–MS^n$ equipment.

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