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Alternative to vertebrate animal experiments in the study of metabolism of illegal growth promotors and veterinary drugs

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Abstract

The continuous production of new illegal veterinary drugs and related products requires residue laboratories to initiate research into developing fast and accurate extraction and detection methods for the identification (and/or quantification) of the major analyte or metabolites of these compounds. In practice, animal experiments are carried out in which vertebrate animals (bovine, porcine, . . .) are treated orally or intramuscularly with the illegal compound. Different matrices (urine, faeces, blood) are collected over 2 or 3 weeks until the animal is sacrificed. Edible matrices (meat, liver, kidney,. . .) are collected. Because of the complexity of the animal experiment and the method development, a lot of valuable time and money is consumed.

Recent studies have shown that some of these vertebrate experiments can be replaced by invertebrates metabolism studies. Vertebrate-type steroids such as testosterone have been used as substrates to study enzyme systems (cytP450) for the oxidative metabolism in invertebrates. Results from these studies provide information on the degree of similarity to the enzyme systems in vertebrates.

These findings are of great importance to the research of illegally used substances but also to the downscaling of vertebrate animal experiments and their considerable cost factors. The invertebrate *Neomysis integer* (Crustacea, Mysidacea) has been used as an alternative model for the partial replacement of vertebrate animals in metabolism studies with illegal growth promotors and veterinary drugs. The principle of this assay and some examples are described.

Keywords: Endocrine disruption; Animal experiment; Metabolisation; Growth promotor

1. Introduction

Because of the burning debate in science concerning the use of animal experiments to answer scientific questions, the controversy has driven scientists to develop alternative methods. In this case it was not only an animal welfare issue but also an issue with economic properties. Animal trials can be time-consuming and moreover have serious cost implications. Since time is money, alternative methods to time-consuming animal trials are requested.

Belgium has suffered from the latest residue problems: 'hormones', PCBs, tranquilizers, antibiotics and European and even world-wide agro-industries

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are affected. The use of illegal growth promoters has still not been eradicated. However, their widespread misuse has been reduced to a smaller few, misusers of legal and illegal products. Illegal products are still being seized at the farm and on other razzias. The list of misused anabolic steroids and other illegal products is long. A lot of scientific papers describe methods for the detection of these products. When browsing just one electronic database, 'Analytical Abstracts', for the keywords 'anabol* and GC or LC or MS' 131 papers could be found from 1990 to 2000. When the keyword 'metabolic' was added only 39 remained. Most metabolic studies were human-related. Metabolic studies of analytes used as growth promotors in cattle are even more complex. The analyte has to be ingested or injected. Different matrices, such as urine, faeces and plasma, have to be collected. Very often after a trial the animal is sacrificed.

Seized products are a topic for research. In general, animal trials are set up and an elimination profile is determined. Extraction and detection methods are developed and optimised. Knowledge of metabolism of an illegally used analyte is of great importance in the field of residue analysis. Very often the administered analyte is metabolised completely and only metabolites are excreted. This makes it even more difficult to detect misuse of growth promotors. Before an analyte can be identified in a matrix, a certified standard has to be available. It is not common that for every illegally used analyte, standards of metabolites are commercially available. It takes a long time to get an insight in the problems surrounding metabolic pathways of bovine species. The animal trial can take up to 3 weeks. The analyte and most important metabolites have to be extracted from complex matrices such as faeces and urine. Time and money can be saved with a simplified and faster procedure.

In this paper, the first preliminary results are given of a invertebrate model that can be used as a substitute for vertebrate animal experiments. An indication of the similarity between vertebrate, bovine, species and the invertebrate metabolism of *Neomysis integer* (Crustacea, Mysidacea) is given based on the metabolism of two exogenous illegally used anabolic steroids, stanozolol and norclostebol, and two endogenous anabolic steroids, testosterone and androstadienedione. In addition, the use of this model can open perspectives for the production of standards which are not commercially available or difficult to synthesize. Another perspective is the possibility to develop an alternative approach for validation by incorporating the metabolites produced by *N. integer*. In a 'quality controlled environment' certified analytical standards have to be available to confirm the presence of an analyte in a certain matrix. Because of the limited commercial availability of metabolites, a creative solution or alternative validation can be developed in which the extract of the exposure can function as reference material.

1.1. Neomysis integer

N. integer is a mysid shrimp. Mysid shrimps are aquatic crustaceans with a well developed endocrine system. They are used in standardised tests by the US Environmental Protection Agency (EPA), the American Society for Testing and Materials (ASTM) and Organisation for Economic Cooperation and Development (OECD). Since the regulation of biological processes with hormones is universal in vertebrate and invertebrate organisms, vertebrate type steroids can be used to study the metabolism of invertebrates. Vertebrate-type steroids such as testosterone have been used as substrates to study enzyme systems (cytP450) for the oxidative metabolism in invertebrates. Results from these studies provide information on the degree of similarity to the enzyme systems in vertebrates [1–3]. These findings are of great importance in the research of illegally used substances and also to the downscaling of vertebrate animal experiments and their considerable cost factors. The invertebrate *N. integer* was used as an alternative model for the partial replacement of vertebrate animals in metabolic studies with illegal growth promotors and veterinary drugs.

1.2. Stanozolol

Stanozolol (Stan) is metabolized very quickly after administration. In sports doping, in man and in horseracing [4], Stan was the subject of metabolism research. The research group of the Deutsche Sporthochschule Köln (Germany) studied and synthetised urinary metabolites of Stan in man (Fig. 1) [5–7]. The major metabolite of stanozolol in bovine species has been identified as 16β-hydroxystanozolol (16OH-Stan) [8–11]. When studying the metabolism

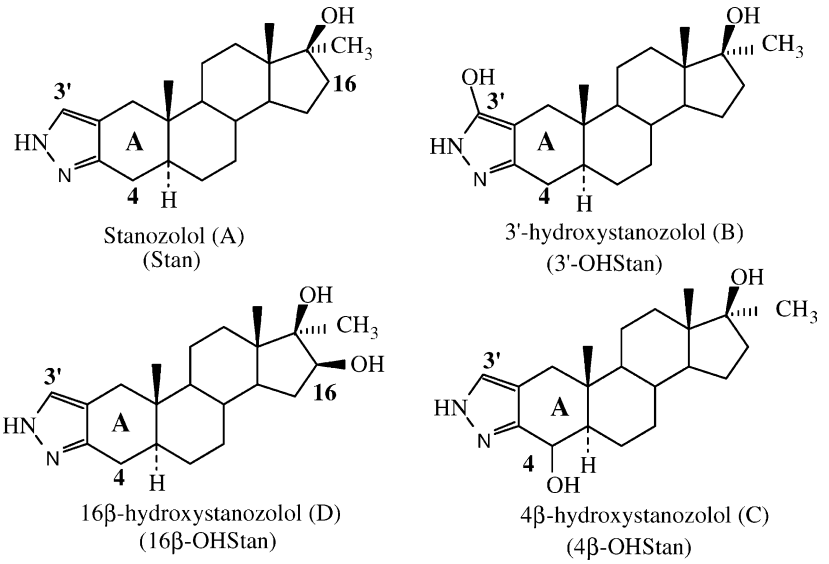

Fig. 1. The major metabolites of stanozolol in man.

one of the problems was to find a suitable internal standard for 16OH-Stan. Because of the 16β hydroxyl substitution, different properties were observed in comparison with Stan. A deuterated internal standard was available for stanozolol, stanozolol-D_3 (Stan-D_3). When using derivatisation with phenylboronic acid (PBA) [9] before detection, Stan and Stan-D_3 were not derivatised since PBA only reacts with two hydroxyl groups. In applications without derivatisation, Stan-D_3 was used as an internal standard for 16OH-Stan. Efforts have been made to synthetise 16OH-Stan-D_3 but the pathways for synthesis were difficult. *N. integer* was exposed to Stan and the metabolism was studied. In a second phase *N. integer* was also exposed to Stan-D_3 to develop an alternative synthesis pathway for 16OH-Stan-D_3.

1.3. Norclostebol

Norclostebol (Fig. 2) is an anabolic steroid that differs in one methyl group from chlorotestosterone. Chlorotestosterone has been misused as a growth promotor in the past. It was administered as chlorotestosterone acetate. Metabolic pathways have been studied intensively [12]. Metabolite identities can only be based on similarities with the metabolic pathway of chlorotestosterone acetate (ClTA). Recently, norclostebolacetate (NClTA) has been seized at a farm and has become a subject for research. Misuse has not yet been proved since there is no knowledge of the metabolic conversion. Instead of using an vertebrate animal trial, *N. integer* was exposed to norclostebolacetate.

1.4. Testosterone

Testosterone is the major circulating androgen which is required for normal sexual differentiation in vertebrate species. Despite some doubts about their function, estrogens and androgens have been identified

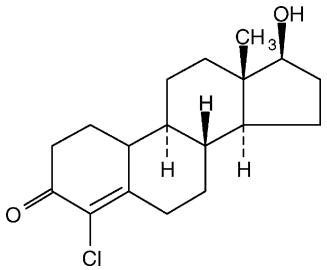

Fig. 2. Structural formula of norclostebol (19-nor-4-chloro-17-β-hydroxyestr-4-en-3-one).

in almost all investigated invertebrate species [3,13]. In some cases these steroid hormones seem to have an analogous function to that vertebrates [14]. Testosterone metabolism by *N. integer* was assessed by Verslycke et al. [3] to obtain initial data on its metabolic capacity. In this research, the anabolic steroid β-boldenone was also identified for the first time in invertebrates. The metabolic pathway leading to the formation of β-boldenone remains unknown, since the steroidal precursor androstadienedione could not be detected. The discussion around boldenone is a hot topic in laboratories that determine residues of illegally used anabolic steroids in cattle. The invertebrate model with *N. integer* can be used to try and determine the endogenous origin of boldenone or its formation after testosterone exposure.

1.5. Androstadienedione

Androstadienedione is a direct precursor of boldenone. Androstadienedione is commercially available through a website for body-building as a 'muscle builder' that has very mild side effects with little or no water retention. In the advertisement it is indicated that there is fast conversion to boldenone. Because it is a direct precursor, administration of androstadienedione could induce the formation of boldenone.

2. Material and methods

2.1. Animal experiments

N. integer (juvenile) was exposed to 2 μg (10 μl of 200 ng μl^{-1}) of the different analytes for 6 h in 2 ml of medium (5 ppt salinity water, diluted from 30 ppt artificial sea water Instant Ocean® with bidistilled water) in a temperature-controlled chamber (15 °C, Liebher®, Laborimpex, Brussels).

2.2. Reagents and chemicals

Standards were obtained from Sigma (St Louis, MO). NClTA was obtained from Steraloids (Wilton, NY). All chemicals used were of analytical grade from Merck (Darmstadt, Germany).

2.3. Extraction and clean-up procedure

The organism was transferred into an Eppendorf vial of 1.5 ml and shock-frozen in liquid nitrogen. The 150 μl of milliQ water was added to facilitate homogenisation with a PTFE potter. One milliliter of ethyl acetate was added. The mixture was vortexed and centrifuged (5 min, 14,000 rpm). The supernatant was transferred into a glass tube. The extraction was repeated once. The two extracts were combined and vacuum evaporated to dryness (Speedvac SC210A). The extract was reconstitued in 30 μl of methanol and 90 μl of 0.02 M formic acid. Sixty microliters were injected on column.

Also the medium was extracted twice with ethyl acetate, evaporated to dryness, reconstitued and injected on column.

2.4. LC-MS^2 detection

Chromatographic separation was achieved using a Symmetry C_{18} column (5 μm, 150 mm × 2.1 mm, Waters, Milford, MA). The flow rate was 0.3 ml min^{-1}. The detection of phenylboronic acid (PBA) derivatives and detection without derivatisation of stanozolol related species was published earlier [9]. NCBA was ionised using atmospheric pressure chemical ionisation (APCI) and chromatographed using an isocratic mixture of 60% methanol and 40% of 1% acetic acid. Testosterone and metabolites were also ionised using APCI and chromatographed using gradient elution. (from 60:40 0.02 M formic acid:methanol to 20:80 in 25 min, hold for 5 min). The liquid chromatography (LC) apparatus was composed of a TSP P4000 pump and a model AS3000 autosampler (TSP, San José, CA). The mass spectrometric (MS) detector was a Finnigan LCQdeca ion trap MS (ThermoFinnigan (San José, CA)) equipped with an electrospray interface in positive ion mode MS/MS full scan.

3. Results and discussion

3.1. Stanozolol

The aim of a preliminary trial was to test if *N. integer* produced 16OH-Stan, the major metabolite present in different bovine matrices, after exposure

to 2 μg of stanozolol for 16 h. Vandewiele et al. described a detection method with and without derivatisation [9]. The MS^2-spectra of the derivatives produce more stable ion ratios (precursor ion with *m*/*z*: 431, product ions with *m*/*z*: 309 and 327) and less fragmentation than detection without derivatisation (precursor ion with *m*/*z*: 345, product ions with *m*/*z*: cluster ions in the range from 100 to 260 with most intense cluster ions 159 and 227; 309 and 327). Since stanozolol and stanozolol-D_3 are not derivatised with PBA, no internal standard is used. Retention time fluctuations can not be corrected by the retention time of an internal standard. Hence, to back-up the detection with derivatisation a detection without derivatisation was also performed. Ten juveniles of *N. integer* were exposed on different days. One batch was derivatised with PBA and the other batch was confirmed without derivatisation. In the extracts of medium and organism the presence of 16OH-Stan could be confirmed with both approaches. In Fig. 3 the presence of 16OH-Stan-PBA in an extract of an exposed juvenile organism is illustrated. No quantification was performed, only an estimation compared with a previously acquired standard injection. Quantification of the metabolisation rate will be examined further. In this stage the exposure conditions seemed good enough to produce 16OH-Stan for each exposed organism. The aim was only to give a qualitative confirmation of the presence of 16OH-Stan and to prove similar metabolism to in vertebrate species.

In a second trial the same experiments were repeated but with exposure to 2 μg of Stan-D_3. The major ions of 16OH-Stan-D_3 to be expected should differ by 3 amu from the non-deuterated 16OH-Stan. In the detection without derivatisation, fragmentation of the precursor ion with *m*/*z* 348 produced product ions with the following *m*/*z*: cluster ions in the range from 100 to 260 with most intense cluster ions 162 and 230; 312 and 330. For the PBA-derivatisation, fragmentation of the precursor ion with *m*/*z* 434 produced the major two product ions with *m*/*z* of 312 and 330. In Fig. 4 a chromatogram and three spectra are given of a underivatised extract of *N. integer* after exposure to Stan-D_3. The first peak corresponds to 16OH-Stan-D_3. The second peak is probably a 4β-hydroxy stanozolol-D_3 (based on retention time and analogy to the spectrum of 4β-hydroxy stanozolol). The third peak is as yet unidentified. Since the small amount of deuterated 16OH-Stan-D_3 was a generous gift from the RIVM, the spectra generated after exposure were compared and confirmed.

This experiment provides enormous perspectives for the production of deuterated internal standards.

3.2. Norclostebol

Norclostebolacetate is known to be misused as a growth promotor in cattle. A standard is commercially available. No standards are available of possible metabolites or of norclostebol that can be formed after hydrolysis of the acetate. After exposure to norclostebolacetate, the extract was examined for mass traces of norclostebol (*m*/*z*: 309), hydroxymetabolites (*m*/*z*: 325) and norClAD (*m*/*z*: 307)(4-chloronorandrost-4-ene-3,17-dione). A MS-full scan and MS^2-full scan of the MH^+ ions were acquired. A clear chromatographic peak for *m*/*z* 309 and a smaller intensity for *m*/*z* 307 was observed (Fig. 5). The identity of norclostebol cannot be confirmed yet since no certified standards are available. However, the presence of norClAD is to be expected from the study of Van Puymbroeck [12] in which he describes the formation of the metabolite ClAD after administration of clostebolacetate. Further experiments need to be performed but already a good estimation of identities of analytes after metabolisation is given. This knowledge can initiate research for further vertebrate animal experiments.

3.3. Testosterone

N. integer was exposed to testosterone in a study to assess testosterone metabolic in order to provide information on its metabolism capacity and thus susceptibility to contaminants in the environment. The extracts were tested for hydroxymetabolites, dihydrotestosterone, boldenone and androstenedione. Also extracts of *N. integer* after exposure to stanozolol, which is an exogenous anabolic steroid, were tested for the same analytes. Surprisingly, in both extracts small concentrations of β-boldenone were observed. An explanation can be given to the formation of boldenone after exposure to testosterone but it is more difficult to explain how after administration of stanozolol boldenone can be formed. Somehow the hormone metabolism is affected after administration

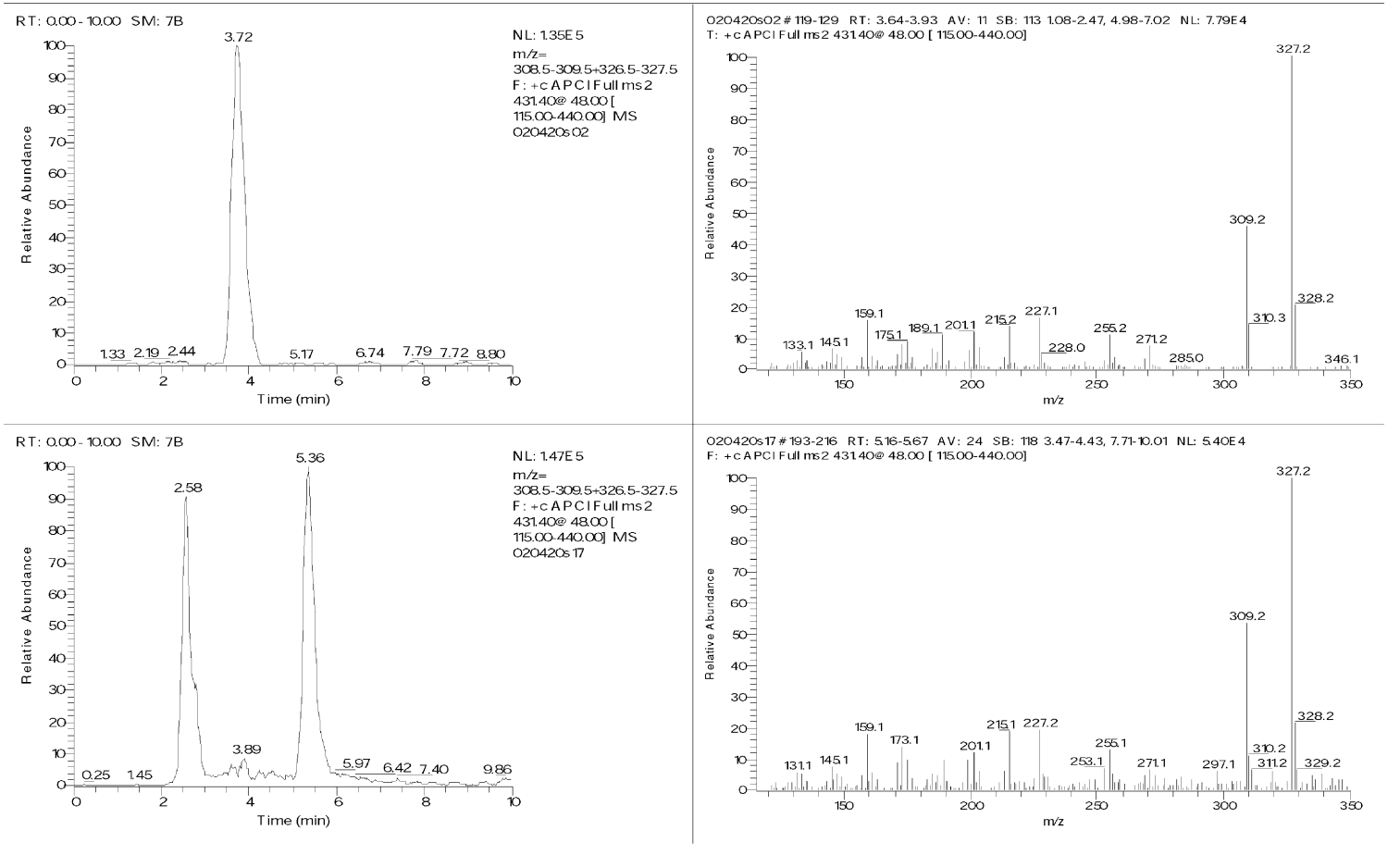

Fig. 3. Standard injection of 16OH-Stan-PBA (upper trace) and extract of exposed *N. integer* indicating the presence of 16OH-Stan-PBA (lower trace).

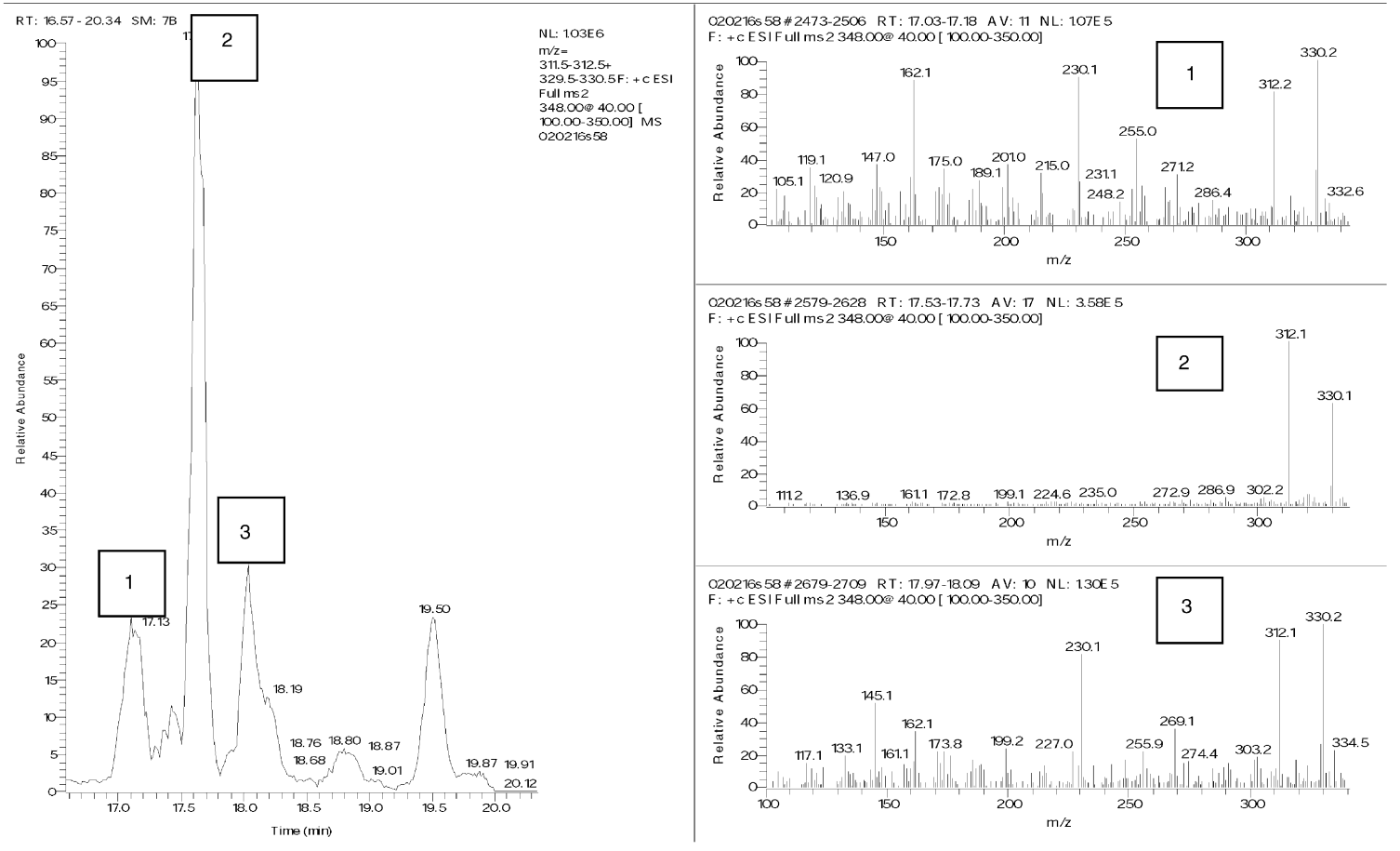

Fig. 4. A chromatogram and three spectra of a underivatised extract of *N. integer* after exposure to Stan-D_3. The first peak (1) corresponds to 16OH-Stan-D_3. The second peak (2) is probably a 4β-hydroxy stanozolol and the third peak (3) is as yet unidentified.

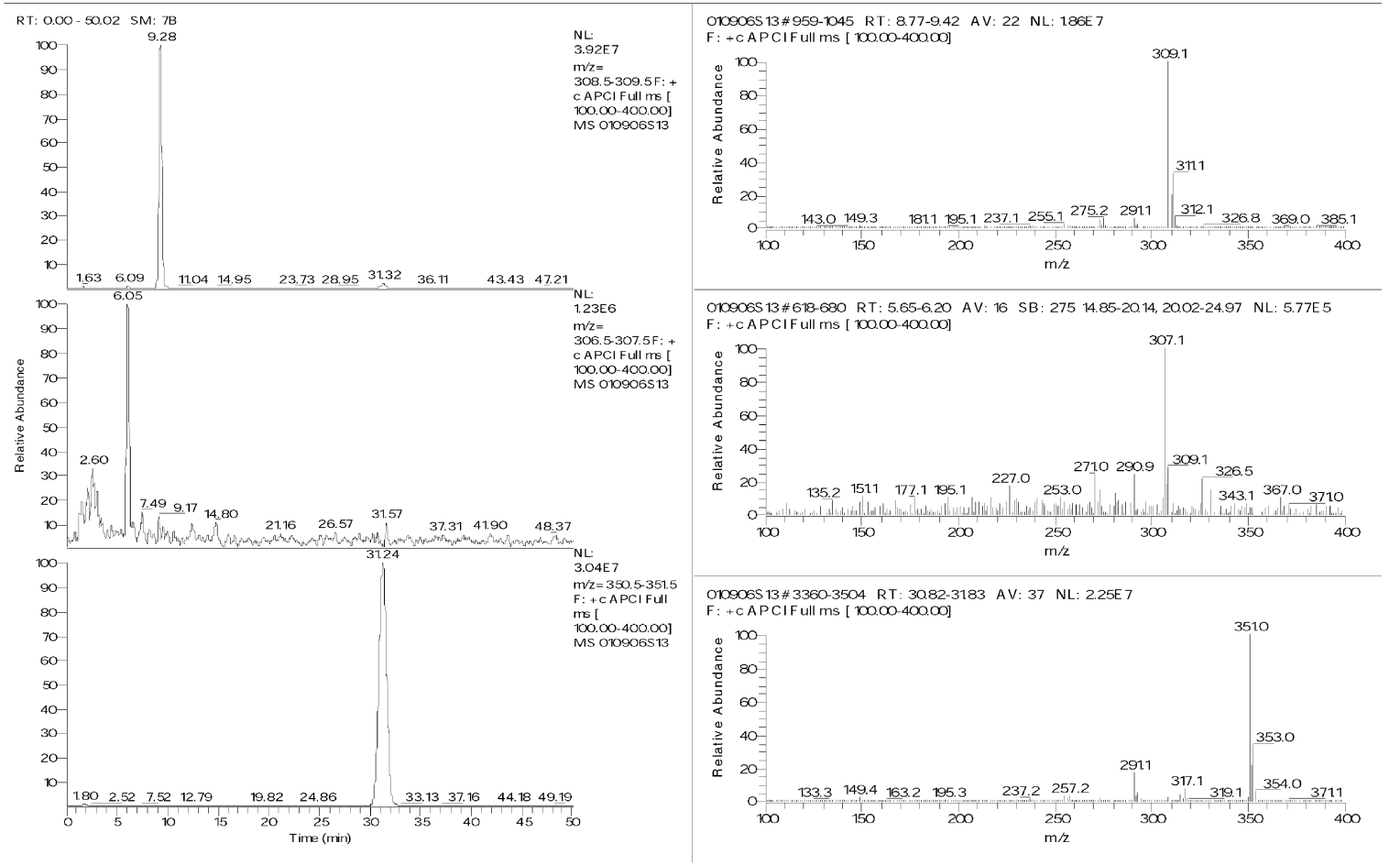

Fig. 5. Chromatogram and three spectra of an extract of *N. integer* after exposure to NCBA. The first mass trace corresponds to norclostebol. The second mass trace corresponds with norClAD and the third peak is NCBA.

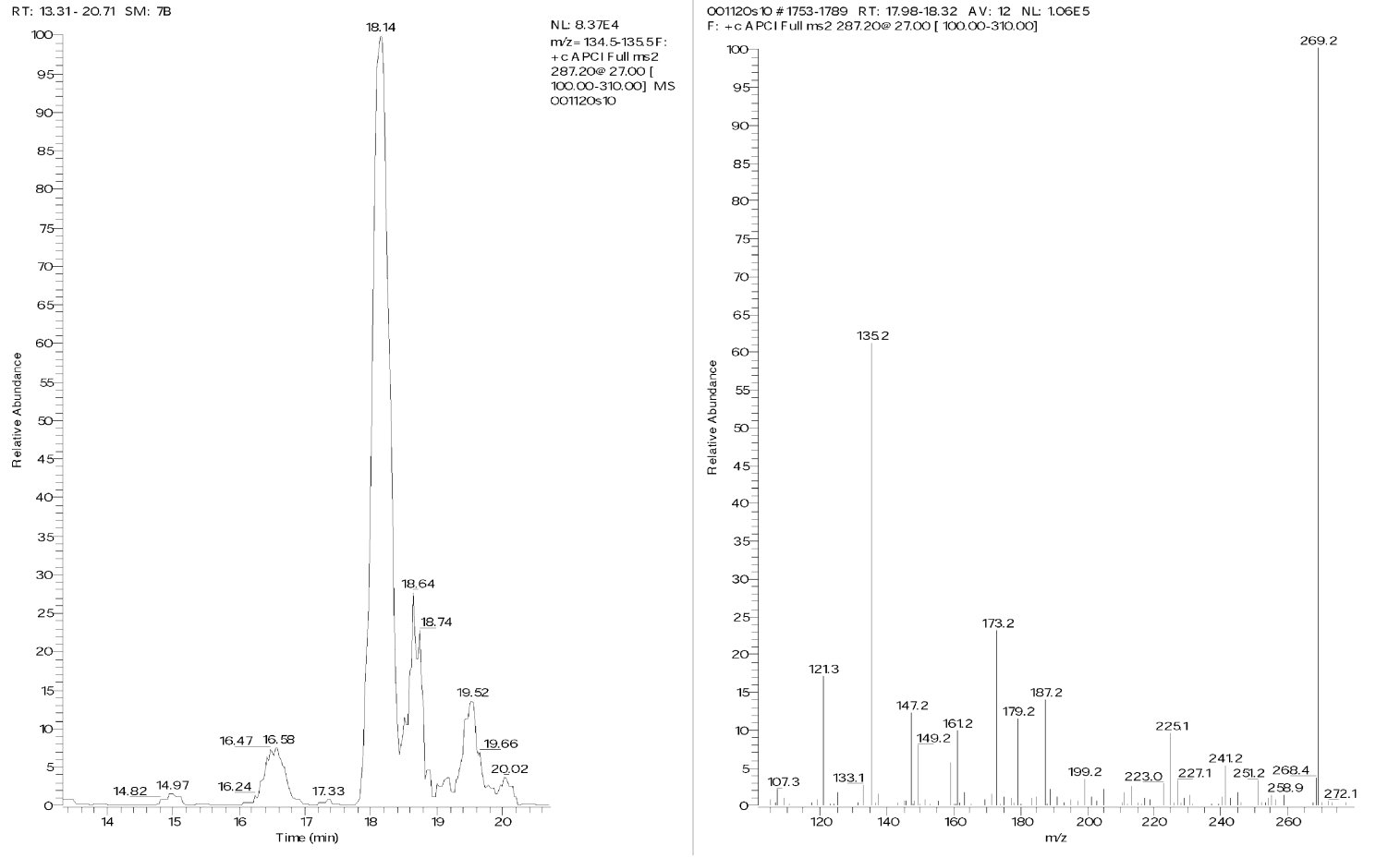

Fig. 6. Chromatogram and spectrum of β-boldenone present in a *N. integer* extract after exposure to testosterone or stanozolol.

of an endogenous or exogenous anabolic steroid. A theory for the formation of boldenone after exposure to testosterone lies in the biosynthesis of testosterone. Androstenedione (AED) is a precursor of testosterone but can also be formed from androstadienedione after dehydrogenation in positions 1,2. Transformation of the keto-group into an alcohol explains the formation of boldenone. Or maybe it can be synthesised directly from testosterone by dehydrogenation in position 1,2. Boldenone could not be measured in blank (not exposed) samples. Only after exposure to a high concentration of an anabolic steroid (testosterone and also stanozolol) was boldenone formed and detected in the medium (highest response) and organism (lowest response). A hypothesis could be that the equilibrium of the formation of testosterone from AED has been shifted towards AED with formation of boldenone as a next step. Still this is only a hypothesis. In Fig. 6 a chromatogram and tandem mass spectrum is illustrated of β-boldenone present in a *N. integer* extract. Important to mention is that the reproducibility of the experiments in the case of boldenone was a problem. During a certain period all exposed samples were clearly positive for β-boldenone. Later we had only a few positive responses and sometimes none showed a response for boldenone. The cause of this phenomenon is as yet unknown. The discussion of boldenone being endogenous or present in vertebrate extracts after administration is still ongoing. These experiments can help in solving the problem.

3.4. Androstadienedione

When exposing *N. integer* to androstadienedione the formation of androstenedione and β-boldenone was observed (data not shown). This knowledge can also serve in the discussion about the origin of boldenone.

4. Conclusions

The use of *N. integer* as an invertebrate model for metabolism studies has shown interesting perspectives. An analogy with vertebrates has been demonstrated with the production of 16OH-Stan after exposure to Stan. Exposure to Stan-D_3 gave rise to the formation of 16OH-Stan-D_3.

Metabolism of norclostebol acetate showed information of similar analytes as in a vertebrate animal trial after administration of ClTA.

Exposure of *N. integer* to testosterone and androstadienedione opened interesting perspectives in solving problems about the origin of the formation of boldenone.

Because of the ‘clean’ nature of the extracts, they can be considered for further clean-up and finally for production of standards for use as reference material. Next to the production of standards an alternative validation procedure can be discussed in which the extracts ‘in se’ can function as a kind of internal standard or reference material.

Because of the simplicity of the invertebrate animal experiments comparised to vertebrate animal experiments a lot of valuable time and money can be saved and in the end lesser vertebrate animals have to be sacrified.

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