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Analytica Chimica Acta 529 (2005) 311–316
ANALYTICA CHIMICA ACTA
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The study of some new anabolic drugs by metabolism experiments with *Neomysis integer*

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Received 8 May 2004; received in revised form 7 October 2004; accepted 7 October 2004
Available online 30 November 2004

Abstract

New illegal veterinary drugs are produced and distributed on the black market continuously. The anabolic drugs described here were found on the black market for body-builders, but are probably also available for growth promoting purposes in cattle.

The residue laboratories are obligated to develop extraction and detection methods to identify and/or quantify the metabolites of these continuously emerging new drugs. In practice, the formation of the metabolites is investigated with animal experiments in which vertebrate animals are treated with the illegal compound. Different matrices of the animal are collected and examined. Because of the complexity and duration of the animal experiment and the method development, a lot of time and money is consumed. Some of these vertebrate experiments can be replaced by invertebrate metabolism experiments [K. De Wasch, S. Poelmans, T. Verslycke, C. Janssen, N. Van Hoof, H. De Brabander, Anal. Chem. Acta 473 (2002) 59; T. Verslycke, K. De Wasch, H. De Brabander, C. Janssen, Gen. Comp. Endocr. 126 (2002) 190; S. Poelmans, K. De Wasch, Y. Martelé, R. Schilt, N. Van Hoof, H. Noppe, T. Verslycke, C.R. Janssen, D. Courtheyn, H.F. De Brabander, Proceedings of the Euro Food Chem XII Strategies for Safe Food, September 24–26, Brugge, Belgium, 2003, p. 74]. By using an invertebrate for the metabolism studies there is a reduction in time and money in comparison with vertebrate animal experiments.

In this study an invertebrate model, the mysid crustacean *Neomysis integer* (*Crustacea, Mysidacea*) was used as an alternative model for metabolism of some new anabolic drugs.

The investigated analytes were dehydroepiandrosterone (DHEA, (3β)-3-hydroxy-androst-5en-17one), maxterone (ADL: 5α-androstan-3β,17β-diol), 5-androstenedione (5AED, 5-androstene-3,17-dione), 5α-androstenedione (5αAED, 1-androstene-3,17-dione) and 1-testosterone (A1T, 1-(5α)T, 1-(5α)-androsten-17β-ol-3-one).

Keywords: Metabolism; *Neomysis integer*; Androgens; DHEA; ADL; 5AED; 5αAED; A1T

1. Introduction

The examined components, dehydroepiandrosterone (DHEA, (3β)-3-hydroxy-androst-5en-17one), maxterone (ADL, 5α-androstan-3β,17β-diol), 5-androstenedione (5AED, 5-androstene-3,17-dione), 5α-androstenedione (5α-AED, 1-androstene-3,17-dione) and 1-testosterone (A1T, 1-(5α)T, 1-(5α)-androsten-17β-ol-3-one), are all androgens. These products are illegally available via the internet and can be used for their positive influence in body-building and in meat production. The chemical structure of all examined compounds and of testosterone (βT, 17β-hydroxy-androst-4-en-3-one) is shown in Fig. 1. The choice

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doi:10.1016/j.aca.2004.10.026

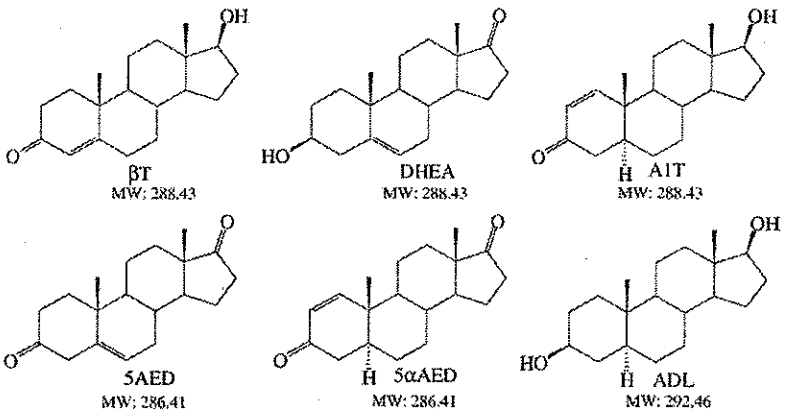

Fig. 1. Chemical structures and molecular weights of testosterone and the examined analytes.

of the tested drugs was based on which analytes are misused in human doping and which analytes can be linked to the boldenone-problem in cattle (boldenone origin; exogenous or endogenous) [4].

DHEA is a major steroidal product secreted by the adrenal gland. DHEA has a similar effect to testosterone; it may stimulate the immune system and is used for improving the energy level and muscle formation. ADL is androgenic and does not convert into an estrogen. It will promote muscle hardening and help fat loss. 5AED and 5αAED both differ from androstenedione (AED, 4-androstene-3,17-dione), the precursor of testosterone, by the place of the double bond. AED has a 4–5 double bond, while 5AED and 5αAED have respectively a 5–6 and a 1–2 double bond. A1T closely resembles the natural hormone testosterone. The only difference is that the double bond from the 4–5 is now in the 1–2 position. Due to the absence of the double bond at the 4 position the androgen can no longer be aromatized to an estrogen. Therefore, the unwanted estrogenic side effects, such as water and fat retention are no longer present.

The formation of the metabolites after administration of those androgens was investigated by exposure of an invertebrate, the mysid crustacean *Neomysis integer* to these compounds. Mysids are already routinely used in laboratory toxicity tests and previous work with *N. integer* showed that it is useful to obtain a first indication of the formation of metabolites [1–3]. Afterwards the identified metabolites can be used for screening the abuse of steroids in human and in cattle. Previous studies have already confirmed similarities between the metabolite formation in invertebrates and in vertebrates [1–3]. The research on biotransformation processes, and thus the study of the formation of metabolites in food-producing animals is of great importance because of the relevance for veterinary pharmacotherapy and toxicology, but also for the health of man [5]. The detection method used for the identification of the metabolites was gas chromatography coupled to mass spectrometry (GC–MS).

2. Materials and methods

2.1. Standards and reagents

All chemicals used for extraction were of analytical grade from Merck (Merck-Eurolab, Overijse, Belgium). The derivatisation reagent used for GC–MS analysis, $MSTFA^{++}$, was prepared by adding 100 mg ammonium iodide (NH_4I) (Sigma, St. Louis, MO, USA) and 0.2 ml ethanethiol (Acros, Geel, Belgium) to 5 ml *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany), followed by dilution of 1.5 ml of this solution with 10 ml MSTFA. Analytes are derivatised for 60 min in 25 μl of derivatisation reagent at 60 °C.

Standards were obtained from Steraloids (Newport, Rhode Island, USA); (3β)-3-hydroxyandrost-5en-17one (DHEA, St A8500), 1-androstene-3,17-dione (1-(5α)AED, St A4400), 5-androstene-3,17-dione (5AED, St A8020), 1-testosterone (1,(5α)-androstene-17β-ol-3-one (A1T, St A4600), 5α-androstan-3β,17β-diol (ADL, St A1220). Testosterone (βT, T1500) and both the enzymes, sulfatase (S9626) and β-glucosidase (G0395) were obtained from Sigma (St. Louis, MO, USA).

2.2. Animal collection and maintenance

Initial *N. integer* populations were collected from the shore by hand net in the Braakman, brackish water near the Scheldt in Hoek (The Netherlands). The maintenance of the organisms in the laboratory was previously described by Verslycke et al. [2,5].

2.3. Animal experiments

Juvenile *N. integer* were isolated from their aquarium and individually exposed to 2 or 10 μg (10 μl of 200 or 1000 ng μl^{-1}, respectively) of the different analytes for 16 h in 2 ml of culture medium in a 5 ml glass tube in

a temperature-controlled chamber (15 °C, Liebher®, Laborimpex, Brussels). After exposure, the juvenile was gently taken out of the culture medium, shock-frozen in liquid nitrogen, and homogenized on ice in 100 μl deionized water using a motor-driven Teflon® pestle. The analyte and the formed phase I metabolites were extracted from the homogenized organisms using 2 ml ethyl acetate (2× 1 ml) and phase-separated using centrifugation (5 min at 14 000 g). The two ethyl acetate fractions were pooled for analysis. Metabolites were extracted from the medium in the same way using 4 ml ethyl acetate (2× 2 ml) [6]. Following ethyl acetate extraction, the remaining polar phase II metabolites were hydrolyzed according to Baldwin and LeBlanc [7]. In short, the assay medium was separated in two equal parts and evaporated. Subsequently the conjugates were hydrolyzed for sulfate- and β-glucose-conjugated metabolites. Extraction was performed with 4 ml ethyl acetate (2× 2 ml). The ethyl acetate was evaporated to dryness, reconstituted with 300 μl ethanol and passed into a GC–MS vial. After drying, 25 μl of derivatisation reagent was added to perform derivatisation for 1 h at 60 °C.

2.4. GC–MS analysis

A trace GC 2000 (ThermoFinnigan, Austin, USA) coupled to an ion trap mass spectrometer Polaris (ThermoFinnigan, Austin, USA) was used for GC–MS analysis. A Carlo Erba AS2000 (ThermoFinnigan, Austin, USA) autosampler was used to inject. Analyses were performed using a non-polar 5% phenyl-polysilphenylenesiloxane SGE BPX-5 GC-column (25 m × 0.22 mm i.d., 0.25 μm) (SGE Inc., Austin, TX, USA). Glass injector liners (10.5 cm × 3 mm) were supplied by SGE Inc. (Austin, TX, USA). The injector, ion source and transferline temperature were respectively 250, 200 and 275 °C. The temperature gradient started at 100 °C and increased with 17 °C min^{-1} until 250 °C. Further the temperature increased to 273 °C in steps of 2 °C min^{-1} and finally ramped at 30 °C min^{-1} to 300 °C. Helium was used as carrier gas at a flow of 1 ml min^{-1}. About 1 of 25 μl of sample was injected with a split–splitless injector (split flow 20 ml min^{-1}, splitless time 1 min). Electron impact (EI) mode was used to ionise the analytes which were acquired in positive ion mode MS full scan.

3. Results and discussion

Standard solutions were injected on column individually for acquiring information of the retention time and the spectra of the administered analytes. When looking for "unknowns", MS full scan will acquire necessary information in a certain mass range.

After exposure of *N. integer* to the analytes, the organism and the culture medium were investigated for the presence of metabolites. But first the presence of the analyte itself was investigated in the extracts. On the one side to test the extraction procedure of the analytes from the medium and the organism and on the other side to verify the uptake of the analyte by the organism. The added analyte was continually present in the media, which proved the extraction method was successful. While, only 5αAED and ADL and a trace of A1T were found in the organisms. This could mean that the organism did not take up the analyte, or in concentrations below the limit of detection (LOD) or that there was a fast conversion to metabolites. It is unlikely that no analyte was taken up because the excretion of metabolites by the organism in the media was clear for all administered analytes.

To detect potential metabolites in the chromatograms possible *m/z*-values were calculated from the parent ion in comparison with the metabolism process of testosterone (βT). Testosterone is structurally similar to all tested analytes and its metabolism process has already been investigated thoroughly [2]. βT is transformed to different hydroxy testosterone metabolites and other metabolites. The hydroxy testosterone metabolites all have a different retention time in the chromatogram but show the same fragment ions in the spectra. The possible formation of hydroxy metabolites caught our attention for further research on the unknown metabolites of the examined administered analytes. Hydroxylation of the administered compound is an important way of metabolism.

Because of the same molecular weight of βT, DHEA and A1T, the same ions were expected in MS full scan for the hydroxylated metabolites. Similar to the hydroxy testosterone spectra, the ions with $m/z = 520$ and 505 were present in the spectra of the possible metabolites of DHEA and A1T. As can be seen in Fig. 2 the metabolites of the different analytes with a molecular weight of 288 do show the same ions in the spectra but in a different ratio. The occurrence of the same ions for the metabolites in the spectra complicates finding out the origin of the analyte by the presence of its metabolites. The same difficulty was presented between the different androstenediones. AED, 5AED and 5αAED have the same molecular weight (286) and as a consequence the same ions (518 and 503) occurred in the spectra of possible metabolites.

Metabolites were more present in the extracts of the medium in comparison to the extracts of the organism. By the formation of metabolites it is the intention of the organism to excrete exogenously administered analytes as more hydrophilic compounds in the culture medium. The comparison of the invertebrate metabolism experiments with the metabolism of vertebrate animals can be made by investigating and comparing the metabolites excreted in the medium during the invertebrate experiment with the metabolites excreted in the urine of a treated vertebrate animal. Phase I biotransformations of steroid hormones can be oxidative (e.g. hydroxylation) or reductive (e.g. reduction of a double bond). In addition many xenobiotics are conjugated with endogenous ligands that render a molecule more polar and thus are more easily excreted. These conjugations are commonly referred to as phase II reactions. Major conjugation reactions include glucuronidation, glucosidation and sulfation [7].

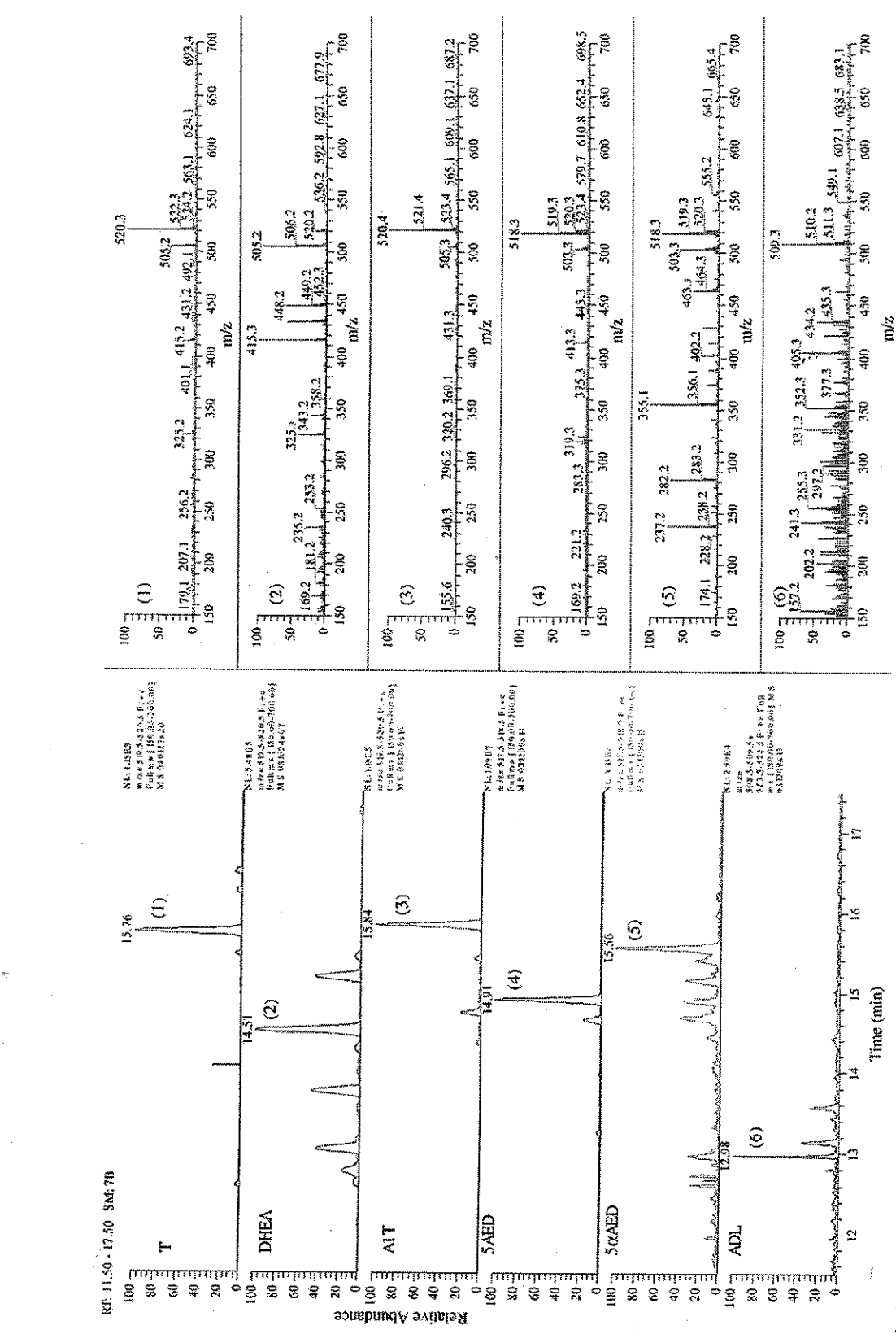

Fig. 2. Chromatograms and spectra, of the most intense peak in the chromatogram, of possible metabolites after administration of 10 μg of T (1), DHEA (2), A1T (3), 5AED (4), 5αAED (5) and ADL (6).

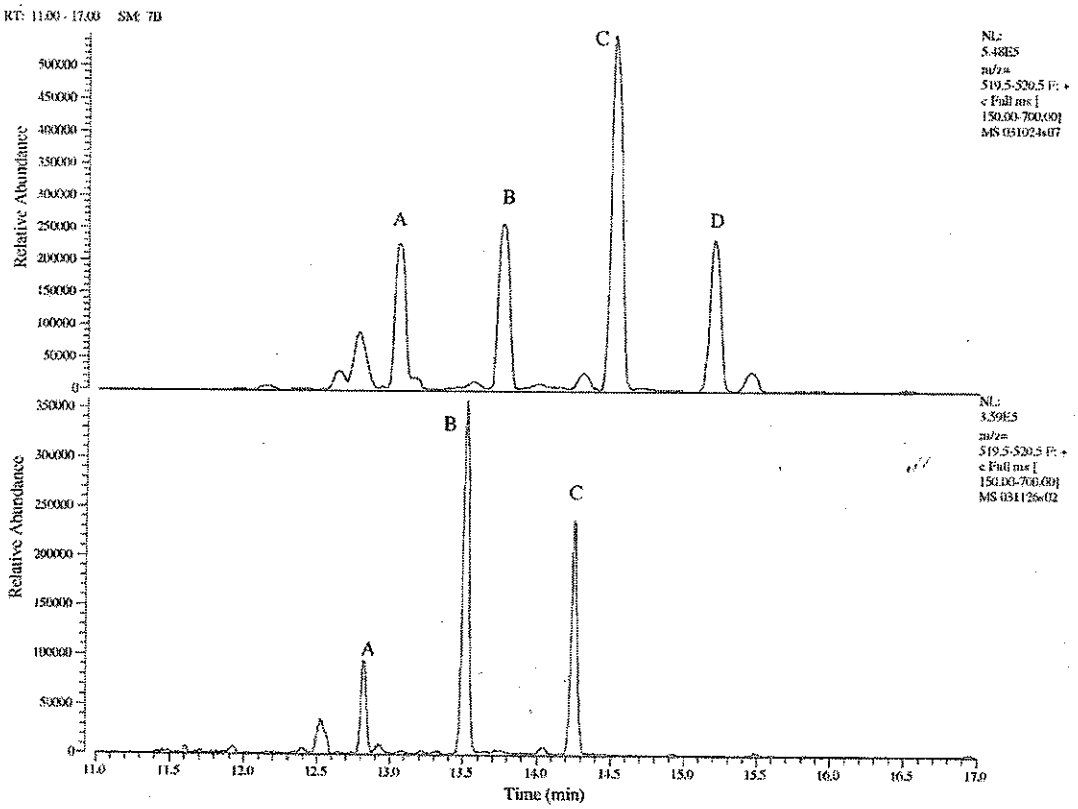

Fig. 3. Metabolites formed after administration of different concentrations of DHEA (lower chromatogram represents the administration of 2 μg and the upper of 10 μg of DHEA). Equal letters represent equal relative retention times.

The phase I metabolites found in the extracts of the medium are discussed first. After exposure to DHEA more distinct metabolites were formed than after exposure to the other androgens. Fig. 2 shows more chromatographic peaks for DHEA than for the other administered analytes in full scan MS. A hypothesis for this is that DHEA is more easily metabolised than the other analytes by the invertebrate *N. integer*.

Exposure to the different analytes was performed with two different concentrations to examine the influence of the concentration on the amount of metabolism. Respectively 2 and 10 μg of the analyte was added to the medium. The differences, between both concentrations of administered DHEA, which are discussed, can be seen in Fig. 3. It was obvious that for the highest concentration (10 μg) more metabolites were present in the extracts than for the lowest concentration (2 μg). This became clear by the number of peaks present in the chromatograms, for the highest concentration additional peaks were present besides the peaks that were also present in the lower concentration. A second difference between both concentrations was the ratios of the different peaks in the chromatograms. A third remark was that, without quantifying, it seemed that the organism was able to excrete higher concentrations of metabolites at the highest concentration of administered analyte. The relative abundance of the metabolite peaks in the chromatogram was higher for 10 μg exposure, except for peak B, which has a higher relative abundance in the 2 μg exposure. No quantification was performed by means of an internal standard. Quantification was not required for achieving the aim of our study; elucidating the metabolite formation after administration of androgens by interpreting its metabolite pattern. But, the simplicity of the extraction method (only a liquid–liquid extraction with ethyl acetate) and the comparison of the samples with standards make it possible to compare the absolute areas. The differences in absolute areas between the metabolite formation after administration of 10 and 2 μg are due to the differences in administered concentration and are most probably not due to deviations of the extraction method or the detection equipment. The latter are very small. The variation of injection and extraction does not play an important role for the interpretation of these data.

Phase II metabolites were examined with the same GC–MS method, but a deconjugation step was performed before extraction as described earlier. We performed only hydrolysis with β-glucosidase and sulfatase, because glucuronidation is rare in invertebrates [8] in comparison to vertebrates, and deconjugation with α-glucosidase was found to be negligible in mysids [6]. The chromatograms of the phase II metabolites showed fewer peaks in comparison

with the chromatograms of the phase I metabolites. Phase II metabolism seems to be less important than phase I metabolism.

The chromatograms of the extracts of all analytes showed a certain pattern of metabolite peaks dependent on the administered concentration. Such a pattern was also seen by the hydroxylated testosterone metabolites. Together with the spectra, these metabolite peak patterns could be used as a tool to identify the exogenous administration of a certain analyte. The spectra of the metabolites indicated specific ion ratios, comparable with the spectra of the testosterone metabolites.

We cannot be sure what the identity of the metabolites was. This can only be confirmed by comparison with standards of the metabolites if these were commercially available. The identical molecular weight of the analytes makes it very difficult to recognize and to distinguish the origin of the metabolites present in an extract. But the combination of the patterns in the chromatograms, the peak ratios and the retention times, and in the spectra, the ion ratios, could make it possible to get an idea of the origin of the metabolites and thus of the exogenously administered analyte.

The phase II metabolism of *N. integer* plays a less important role for extrapolation to vertebrates, because less chromatographic peaks were detected and because different conjugations occur in invertebrates and vertebrates.

4. Conclusions

Metabolite identification of "unknown" exogenously administered analytes in any kind of extracts will always be difficult. The use of GC–MS makes it possible to recognize metabolites formed by an organism after administration of an analyte. The combination of the information obtained by the patterns present in the chromatograms and in the spectra is very promising for the identification of metabolites. The metabolite formation is very easily, cheaply and quickly performed by the use of the invertebrate *N. integer* in comparison with vertebrate animal testing. The results of the invertebrate tests can be used as a first indication for what kinds of metabolites are formed by vertebrates and to inform about the chromatographic pattern that can be of great importance for elucidating the abuse of, in this case, anabolic compounds.

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