General and Comparative Endocrinology 126, 190–199 (2002)
doi:10.1006/gcen.2002.7793, available online at http://www.idealibrary.com on IDEAL®

Testosterone Metabolism in the Estuarine Mysid *Neomysis integer* (Crustacea; Mysidacea): Identification of Testosterone Metabolites and Endogenous Vertebrate-Type Steroids

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Accepted January 17, 2002

Testosterone metabolism by *Neomysis integer* (Crustacea; Mysidacea) was assessed to obtain initial data on its metabolic capacity. *N. integer* were exposed to both testosterone and [^{14}C]testosterone. Identification of testosterone metabolites and endogenous steroids was performed using thin-layer chromatography and liquid chromatography with multiple mass spectrometry. Endogenous production of testosterone in mysids was detected for the first time. *N. integer* were exposed to testosterone and metabolized administered testosterone extensively. At least 11 polar testosterone metabolites ($R_{f,\text{metabolite}} < R_{f,\text{testosterone}}$), androstenedione, dihydrotestosterone, and testosterone were produced *in vivo* by *N. integer*. A sex-specific testosterone metabolism was also observed, although this observation requires further confirmation. 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. These results reveal interesting similarities in enzyme systems in invertebrate and vertebrate species. Alterations in steroid hormone metabolism may be used as a new biomarker for the effects of endocrine disruptors in invertebrates. © 2002 Elsevier Science (USA)

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Key Words: Neomysis integer; testosterone metabolism; endocrine disruption; biomarker; vertebrate-type steroids.

INTRODUCTION

Changes of the hormone system due to environmental contaminants (endocrine disruptors) has recently become a widely investigated and politically charged issue (Colborn *et al.*, 1996; Krimsky, 2000). Invertebrates account for 95% of all animals (Barnes, 1980), yet surprisingly little effort has been made to understand their value in signaling potential environmental endocrine disruption (Alvarez and Ellis, 1990; Crisp *et al.*, 1997; Leblanc and Bain, 1997).

The use of hormones to regulate biological processes is a strategy common to both vertebrates and invertebrates. Endocrine control strategies and basic hormonal mechanisms to regulate biological processes have been widely conserved among animal phyla (DeFur *et al.*, 1999). Vertebrate-type steroids such as 17β-estradiol (DeLoof and DeClerk, 1986), testosterone (Burns *et al.*, 1984; Fairs *et al.*, 1989) and progesterone (Kanawaza and Teshima, 1971; Yano, 1985) have been detected in malacostracan crustaceans. Fragmented

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evidence even suggests a functional role for some of these compounds in crustaceans (Sarojini, 1963; Nagabhushanam and Kulkarni, 1981). There are, however, no data on the occurrence and metabolic pathways of vertebrate-type steroids in mysid shrimp. Very little information exists in literature on the endocrinology of mysids and crustaceans in general (DeFur *et al.*, 1999).

Recent studies have used steroid metabolism as a new biomarker for identifying endocrine-disrupting effects in invertebrates (Baldwin and Leblanc, 1994a,b; Baldwin *et al.*, 1997; Morcillo *et al.*, 1998; Oberdörster *et al.*, 1998a,b; Ronis and Mason, 1996). Alterations in steroid hormone metabolism often correlate with effects on steroid hormone-dependent processes such as growth and reproduction (Parks and LeBlanc, 1996).

Vertebrate-type steroids have been used as substrates to study the various P450 enzymes of the oxidative metabolism in invertebrates. These enzymes are also responsible for the biotransformation and elimination of lipophilic environmental pollutants (Hahn and Stegeman, 1994). Results from these studies provide information on the complexity of the P450 systems in invertebrates and its degree of similarity to P450s in vertebrates. It has been suggested that modulation of the P450 activity has potential as a biomarker of toxicant exposure in invertebrates (Baldwin and LeBlanc, 1994a,b).

The subtropical mysid *Americamysis* (=*Mysidopsis*) *bahia* is a standard organism used in laboratory toxicity testing (USEPA, 1987; ASTM, 1990). The available toxicity data suggest that mysids *sensu lato* are very sensitive to toxic chemicals (Roast *et al.*, 1998). Mysids have also been proposed as standard test organisms for evaluating the effects of endocrine disruptors (EDSTAC, 1998). There is, however, growing interest in using indigenous mysids in toxicity testing and environmental monitoring (Langdon *et al.*, 1996). Consequently, *Neomysis integer* has been identified as a possible European alternative to the standard American species (Emson and Crane, 1994; Wildgust and Jones, 1998; Roast *et al.*, 1999). *N. integer* has been reported to be present in all European coastal waters of the Atlantic Ocean (Tattersall and Tattersall, 1951), where it dominates the hyperbenthic fauna of the low-salinity regions of estuaries (Mees and Hamerlynck, 1992; Mees *et al.*, 1995).

In this study testosterone metabolism by *N. integer* was assessed to obtain initial data on its metabolic capacity. Testosterone metabolites and endogenous vertebrate-type steroids were identified. Furthermore, results from this study provide information on the complexity of the P450 system in *N. integer* and its degree of similarity to P450s in vertebrates. Finally, these results are of value in future exposure studies to evaluate modulations of the testosterone metabolism profile by specific xenobiotics.

MATERIAL AND METHODS

Animal Collection and Maintenance

Initial *N. integer* populations were collected from the shore by handnet in the Galgenweel (a brackish water near Antwerp, Belgium). After a short acclimatization period, the organisms were transferred to 200-L glass aquaria. Culture medium was artificial seawater (Instant Ocean), diluted with aerated deionized tap water to a final salinity of 5. A 14-h light:10-h dark photoperiod was used during culturing, and water temperature was maintained at 20°C. Cultures were fed daily with 24- to 48-h-old *Artemia* nauplii *ad libidum*. Hatching of the *Artemia* cysts was performed in 1-L cylinder-conical vessels under vigorous aeration and continuous illumination at 25°C.

In Vivo Exposure

Adult male and female *N. integer* were taken from the 200-L aquaria and individually placed into 5-ml glass recipients containing 2 ml culture medium. Two micrograms of testosterone (Sigma-Aldrich, Bornem, Belgium) dissolved in 10 μl methanol was added to the recipients and a 16-h exposure period was applied. For the radioactive experiments 10 μl [4-^{14}C]testosterone (specific activity: 53.6 mCi/mmol; packaging: 0.04 mCi/ml ethanol) (NEN, Boston, MA) was added to the test medium and metabolized for 16 h. These test conditions were modified for *N. integer* from similar experiments with the Cladoceran *Daphnia magna* (Baldwin and LeBlanc, 1994a). Replication was used for these experiments and blanks were run to account

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TABLE 1
Retention of Authentic Steroid Standards Following TLC and LC-MSn

Standards	Chromatography		Identified	
	$R_{f,rel}$ TLC	$T_{r,rel}$ LC-MSn	Organism	Medium
Androstenedione[a]	1.18	0.90	•■	•■
Androstenolone[a]	1.17	1.05	—	—
Androstadienedione[a]	1.12	0.74	—	—
Dihydrotestosterone[a]	1.08	1.16	•■	•■
Androsterone[a]	1.07	1.26	—	—
17β-Estradiol[a]	1.02	0.96	—	—
Testosterone[b]	1	1	•■	•■
β-Boldenone[a]	0.89	0.87	•	•■
2α-OH[a]	0.82	0.76	•	•■
16β-OH[a]	0.77	0.72	•	•
6β-OH[a]	0.71	0.55	•	•
6α-OH[a]	0.63	0.41	•	•■
11β-OH[a]	0.53	0.79	•■	•■
7α-OH[a]	0.49	0.55	—	•■
16α-OH[a]	0.44	0.64	•	•■
11α-OH[a]	0.41	0.72	—	•
15α-OH[a]	0.36	0.53	—	•■

Note. Identification of testosterone metabolites and vertebrate-type steroids after *in vivo* exposure of *Neomysis integer* to 2 μg testosterone for 16 h. •, Identified by TLC; ■, identified by LC-MSn.
[a] Purchased from Steraloids Inc. (UK).
[b] Purchased from Sigma-Aldrich (Belgium).

for breakdown or microbial transformation of testosterone in the absence of mysids.

Thin-Layer Chromatography (TLC) Analysis of Testosterone and [^{14}C]Testosterone Metabolites

Extraction and thin-layer chromatography of testosterone metabolites was adopted from Baldwin and LeBlanc (1994a,b) with minor modifications. *N. integer* were isolated from the exposure medium, shock-frozen in liquid nitrogen, and homogenized on ice in 100 μl deionized water using a motor-driven teflon pestle. Testosterone metabolites were extracted using 4 ml ethyl acetate (2 × 2 ml) and phase-separated using centrifugation. The ethyl acetate fractions were pooled and evaporated under a stream of nitrogen. The residue was redissolved in 40 μl ethyl acetate and spotted onto an aluminum-backed silica gel TLC plate (Merck; 20 cm × 20 cm × 0.25 mm). The metabolites were separated with a double-solvent system consisting of methylene chloride:acetone (4:1, v/v) and chloroform: ethyl acetate:ethanol (4:1:0.7, v/v/v). All of these solvents were of HPLC grade (Sigma-Aldrich, Belgium). The [^{14}C]testosterone metabolites were visualized using autoradiography film (Kodak X-OMAT AR). For visualization of the nonradioactive testosterone metabolites and authentic standards, the plates were sprayed with a H_2SO_4/acetone (1:1, v/v) mixture and charred in the oven at 160° for 15 min. The different metabolites were identified on the basis of their cochromatography with the authentic standards (Table 1).

Liquid Chromatography with Multiple Mass Spectrometry (LC-MSn) Analysis of Testosterone Metabolites and Endogenous Steroids

Exposure and extraction procedures for LC-MSn analysis of the metabolites were identical to those for the TLC analyses. The HPLC apparatus was composed of an Agilent 1100 series pump, autosampler, and vacuum degasser (Agilent, Palo Alto, CA). Separation was performed on a Symmetry C18 column (5 μm, 150 × 2.1 mm; Waters, Milford, MA). Analysis

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was carried out using an LCQ^{DECA} Ion Trap Mass Analyzer (ThermoQuest, San Jose, CA), with an APCI interface and XCalibur 1.2. software. The metabolites were detected in MS-MS-full scan positive ion mode. The solvents for preparation of the mobile phase were HPLC grade obtained from Merck (Darmstadt, Germany). To separate the different compounds, a linear gradient consisting of a mixture of 60% 0.02 M formic acid and 40% methanol was used. The methanol percentage increased from 40 to 80% in 25 min. The column was allowed to equilibrate under initial conditions for 8 min. The different testosterone metabolites were identified on the basis of their relative retention times (calculated as a ratio of the retention time of testosterone) as compared with the retention times of authentic standards (Table 1).

RESULTS

TLC Analysis of Testosterone Metabolites

Female and male *N. integer* produced at least 11 polar metabolites ($R_{f,\text{metabolite}} < R_{f,\text{testosterone}}$) and a number of nonpolar metabolites ($R_{f,\text{metabolite}} > R_{f,\text{testosterone}}$) during *in vivo* exposure to [^{14}C]testosterone (Fig. 1). The metabolites were identified on the basis of their comigration with authentic standards, using the solvent system described under Material and Methods (Table 1).

The nonpolar metabolites were poorly resolved from one another in the TLC solvent system used, and only androstenedione and dihydrotestosterone could be identified and confirmed by LC-MSn.

Of the 11 polar metabolites, β-boldenone, 9 monohydroxy metabolites (2α-, 16β-, 6β-, 6α-, 11β-, 7α-, 16α-, 15α-, and 11α-hydroxytestosterone), and an unknown metabolite were identified by their relative retention in the solvent system used. Of these polar metabolites, β-boldenone and 6 monohydroxy metabolites (2α-, 6α-, 11β-, 7α-, 16α-, and 15α-hydroxytestosterone) could be confirmed with LC-MSn.

From the autoradiogram depicted in Fig. 1, it can be concluded that the nonpolar lipophilic metabolites were preferentially retained by *N. integer*, while polar hydrophilic metabolites were most abundant in the test medium.

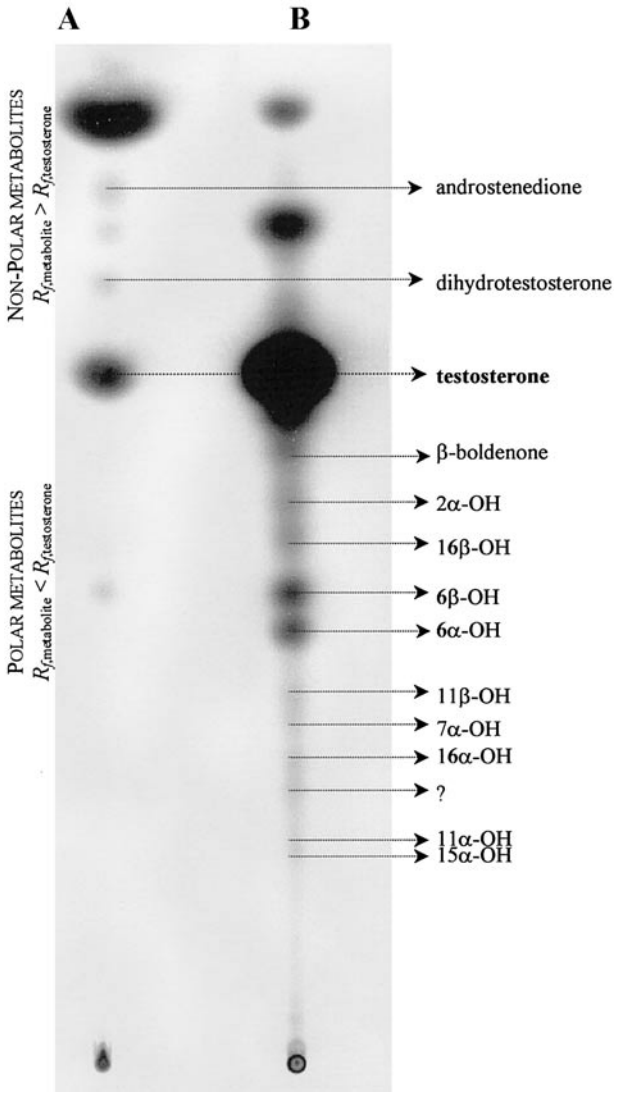

FIG. 1. Autoradiography (96 h) of [^{14}C]testosterone metabolites in homogenates (A) and in the test medium (B) of exposed *N. integer* following TLC. The different metabolites are identified on the basis of their cochromatography with authentic standards.

LC-MSn Analysis of Testosterone Metabolites

The metabolites in *N. integer* and the test medium after *in vivo* exposure to testosterone were identified using LC-MSn (Fig. 2). A protocol was adopted to separate nine different monohydroxy standards and vertebrate-type steroids in one run. The relative retention times (calculated as a ratio of the retention time of testosterone) of the different steroids on the LC-MSn are shown in Table 1 compared with the relative retentions in the TLC solvent system. Six monohydroxy

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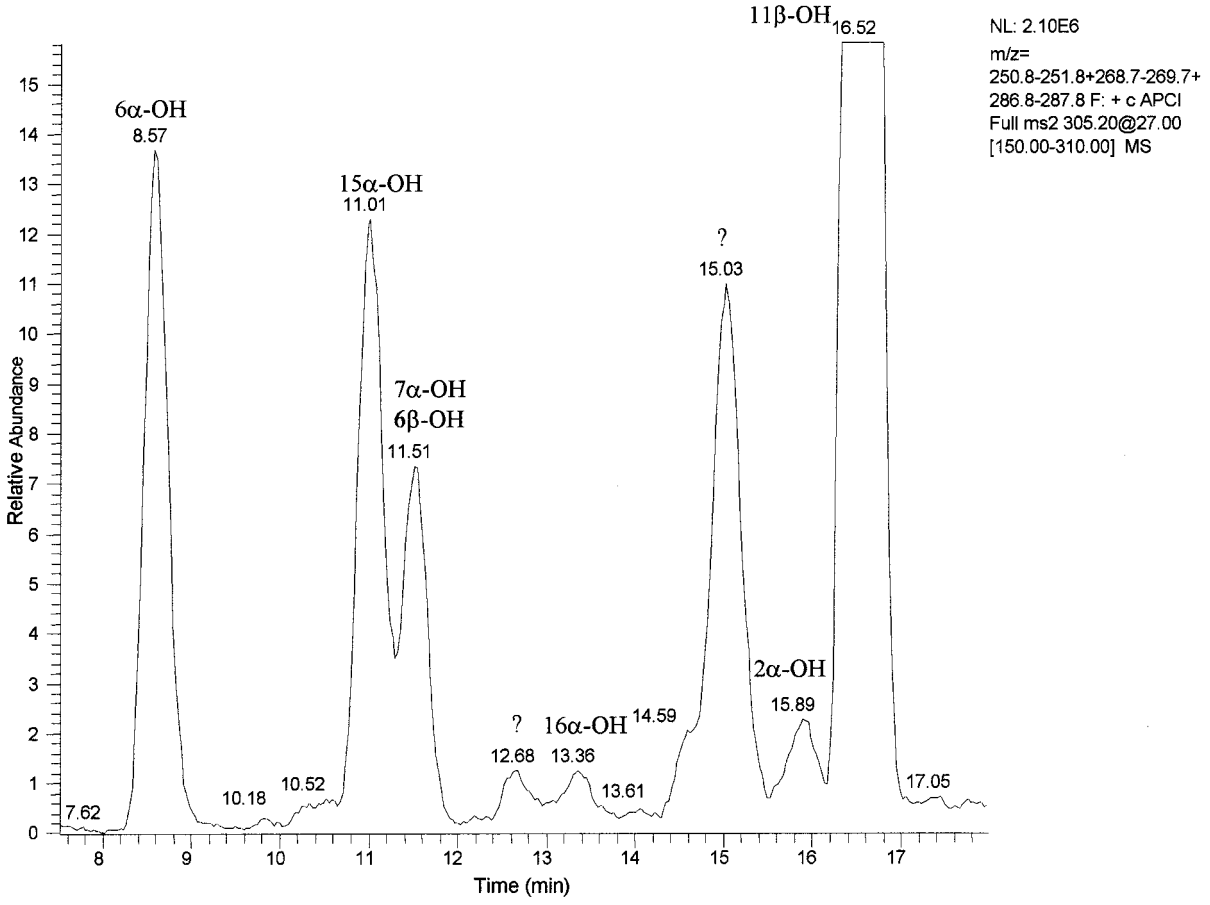

FIG. 2. Detection of monohydroxy testosterone metabolites with LC-MSn after *in vivo* testosterone biotransformation in *Neomysis integer*.

testosterone metabolites and two unknown polar testosterone metabolites were detected in the test medium by their chromatographic retention and mass spectrum (MS_2). Only 11β-hydroxytestosterone could be identified in extracts of *N. integer* exposed to testosterone.

Detection of Vertebrate-Type Steroids by LC-MSn in Testosterone-Exposed and Unexposed N. integer

Testosterone-exposed and unexposed *N. integer* were tested for different vertebrate-type steroids (Table 1). In both male and female unexposed *N. integer*, endogenous testosterone was identified (Fig. 3). Preliminary quantification results indicate significantly higher (about five times higher in male; data not shown) endogenous testosterone levels in male mysid than in females. Androstenedione was endogenously produced by male mysids, but could not be detected in female organisms. Further confirmation is needed to investigate the possibility of a sex-specific testosterone metabolism in *N. integer*.

β-Boldenone, an anabolic steroid, was detected in the test medium of exposed male and female organisms, but the steroid precursor androstadienedione could not be detected. Dihydrotestosterone and androstenedione were also found in the test medium of *N. integer* exposed to testosterone (Fig. 4).

DISCUSSION

N. integer produced all the monohydroxy testosterone metabolites (2α-, 16β-, 6β-, 6α-, 7α-, 15α-) found in a similar study with the crustacean *D. magna* (Baldwin

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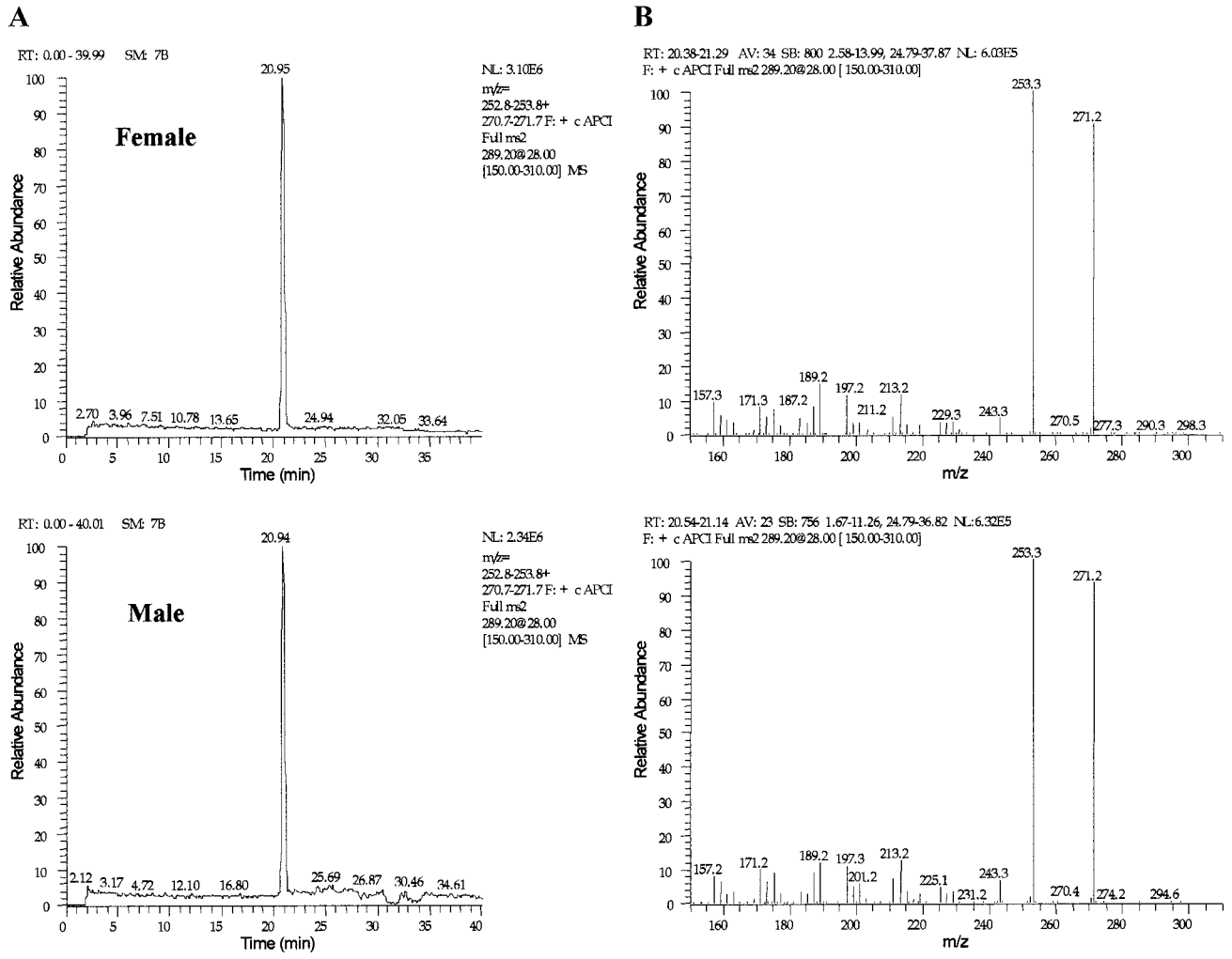

FIG. 3. Homogenates of *Neomysis integer* were extracted and analyzed on LC-MSn. The chromatogram shows evidence of endogenous testosterone production in male and female *N. integer*. (A) Retention time for testosterone; (B) mass spectrum for testosterone.

and Leblanc, 1994b), but also three new monohydroxy metabolites (11β-, 16α-, 11α-) which were not identified in the daphnid study. This could be a result of the greater identification capability of the combined chromatography methods (TLC and LC-MSn) used in the present study.

Of the nonpolar metabolites, only androstenedione and dihydrotestosterone could be identified. Since the nonpolar metabolites were very poorly resolved by TLC, a more detailed analysis for vertebrate-type steroids was carried out on extracts of exposed and unexposed *N. integer* with LC-MSn. This led to the identification of β-boldenone (1,4-androstadiene-17β-ol-3-one), a close derivative of testosterone (4-androstene-17β-ol-3-one) differing only by the addition of a second double bond in the A ring of the structure. The direct precursor of boldenone is androstadienedione, which differs from testosterone's direct precursor only by the same alteration (Fig. 5). To our knowledge, boldenone has never been identified in invertebrates. As it was not detected in unexposed organisms, it can be hypothesized that it is a biotransformation product of testosterone. The metabolism of this compound is not clearly understood in vertebrates, where it is mostly used as a performance enhancer in the form of boldenone-17-undecylenate. Androstenedione was produced endogenously by male mysid and was found in male and female *N. integer* exposed to testos-

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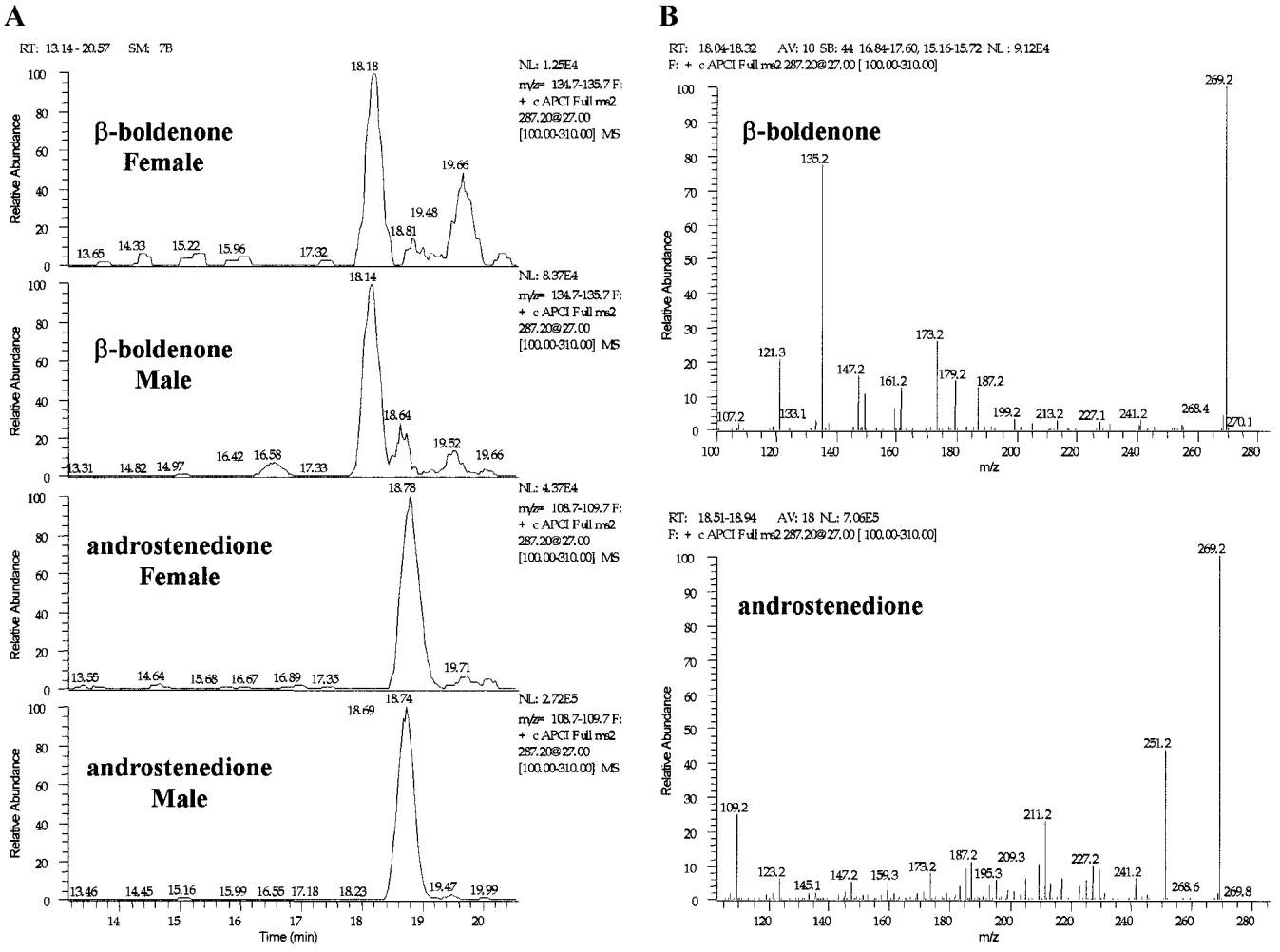

FIG. 4. Detection of the anabolic steroid β-boldenone and the vertebrate-type steroid androstenedione by LC-MSn in male and female *Neomysis integer* exposed to 2 μg testosterone, (A) Retention time; (B) mass spectrum.

terone, which illustrates the presence of the widely distributed body enzyme 17β-hydroxysteroid dehydrogenase (which interconverts testosterone–androstenedione and boldenone–androstadienedione between inactive 17-keto and active 17-beta hydroxy form) in *N. integer*. Unexpectedly, androstadienedione could not be detected. Further research is required to elucidate the possible biotransformation pathways of boldenone and its metabolites.

One step in identifying testosterone and its metabolites with LC-MSn was to look for endogenous testosterone production in *N. integer*. Baldwin and Leblanc (1994a) found no indication of testosterone production by daphnids. The results of our study clearly show the presence of endogenous testosterone in unexposed male and female *N. integer*. To our knowledge, evidence for testosterone production has only been described for some decapod crustaceans (Burns *et al.*, 1984; Fairs *et al.*, 1989), but never for mysid shrimp. The limited available data on vertebrate-type steroids in crustaceans are insufficient to conclude that these steroids function as hormones (DeFur *et al.*, 1999). Future studies will address the possible functional role for testosterone or its metabolites in *N. integer*.

The main vertebrate estrogen 17β-estradiol could not be detected in *N. integer*. Although previous studies have identified this compound in malacostracan

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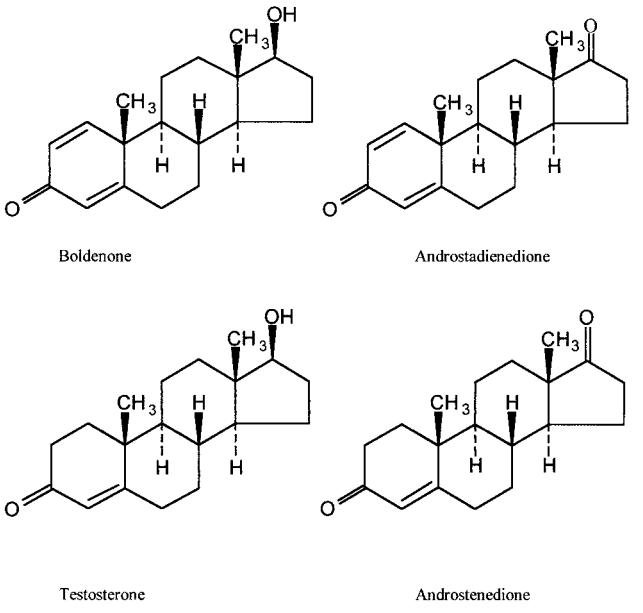

FIG. 5. Structure of testosterone, the anabolic steroid boldenone, and their respective precursors androstenedione and androstadienedione.

crustaceans (DeLoof and DeClerk, 1986), the functional role of estrogens has not been established in arthropods. Androsterone, a 17-keto steroid derived from testosterone in humans, and androstenolone (dehydroepiandrosterone), a precursor of androstenedione, also were not detected in our study.

The results from the *in vivo* biotransformation experiments with *N. integer* demonstrate the presence of a complex steroid hydroxylase system consisting of different P450 isozymes. The remarkable diversity of testosterone hydroxylation exhibited should stimulate further studies on the induction, stereospecificity, and regulation of the enzyme systems of *N. integer*. These results are also in line with previous observations that have indicated that testosterone is a good steroid substrate for the study of enzyme systems in crustaceans (James, 1989).

Steroid hydroxylation reactions are catalyzed by most P450 isozymes found in mammalian liver. Theoretically, testosterone can be hydroxylated at 20 different sites by different isozymes. Since each P450 isozyme has a distinct hydroxylation pattern, this pattern can be used to determine the relative concentration of these enzymes (Correia, 1995). These hydroxylation patterns can be affected by exposure to xenobiotics through a number of different mechanisms (e.g., transcriptional inhibition or induction of P450 enzymes, competition of inducible or constitutive enzymes for limited cellular heme pools, perturbation of the hormonal regulation of P450 enzyme expression, etc.) (Waxman, 1988; Yeowell *et al.*, 1987). Endocrine-disrupting compounds also have the potency to affect hydroxylation patterns in invertebrates (Baldwin *et al.*, 1995). As a result, modulation of the P450 activity in *N. integer* can potentially be used as a predictive biomarker for exposure to endocrine disruptors.

The LC-MSn method described here has the advantage over the TLC method that results are obtained rapidly and without the use of radioactive substrates. Difficulties due to interference with endogenous steroid concentrations, which are not a problem when working with labeled compounds, may be encountered. Another advantage of working with labeled compounds is the possibility of detecting very small quantities of a metabolite by increasing the autoradiography exposure period. An autoradiography period of 96 h was applied for the identification of the different metabolites in the homogenates of exposed *N. integer*. These metabolites were below the detection limit of the LC-MSn method. The LC-MSn method is, however, convenient for use in routine biomonitoring, and additional studies are underway to further identify and quantify metabolites by LC-MSn and validate these results as a biomarker for endocrine disruption in *N. integer*.

ACKNOWLEDGMENT

This research was supported by a research grant of the Flemish Institute for the Promotion of Scientific and Technological Research in Industry (I.W.T.).

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