Journal of Chromatography B, 878 (2010) 1487–1492

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development, validation and comparison of LC–MS/MS and RIA methods for quantification of vertebrates-like sex-steroids in prosobranch molluscs

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ARTICLE INFO

Article history:
Received 30 September 2009
Accepted 25 March 2010
Available online 1 April 2010

Keywords:
RIA
LC–MS/MS
Comparative analysis
Steroids
Potamopyrgus antipodarum

ABSTRACT

The role of vertebrate-like sex-steroids (testosterone, T, progesterone, P, and 17β-estradiol, E2) in molluscs is still debated, but they could represent potential biomarkers of endocrine disruption. A radioimmunoassay (RIA) and a liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) methods have been developed and compared to measure their levels in a gastropod snail *Potamopyrgus antipodarum*. Both methods showed a good reproducibility despite the complex matrix and the very low levels of vertebrate-like sex-steroids. Only T and P were detected using the LC–MS/MS method, while the RIA method reached lower detection limits and enabled the detection of all three steroids. Results indicated that T and P were mainly present as unconjugated forms. Both methods were compared in the analysis of snails exposed to waste water treatment plant effluents and led to the same conclusions concerning the modulation of steroids levels. Moreover, they both were in agreement concerning T measurements. On the other hand, LC–MS/MS appeared to be more suitable when analyzing P levels due to a low sensitivity of the RIA method. As E2 was not measured using the LC–MS/MS method because of a higher detection limit compared to the other steroids, the results obtained with the RIA method should be interpreted with caution. LC–MS/MS remains the gold standard for sex-steroid determinations, however a relevant and alternative method based on RIA was developed, requiring fewer organisms. RIA seems a promising method as a screening tool for experimental use, allowing comparison of sex-steroid levels in the mudsnail both in laboratory and in field experiments.

1. Introduction

Vertebrate-like steroids (testosterone, T, progesterone, P, and 17β-estradiol, E2) have been found in several molluscs, bivalves or gastropods. Their origin (exogenous or endogenous) remains a question, although the main steps of their synthesis have been described [1–7]. Temporal variations of some of their levels have been linked to the reproductive cycle in bivalves [8–12] and gastropods [13]. Molluscs also seem able to accumulate high quantities of steroids in conjugating them with fatty acids [14]. These observations seem to point toward a physiological role of sex-steroids in mollusc reproduction.

Among the large number of chemicals released into the environment, endocrine disruptors (EDs) represent a structurally diverse group of compounds that may adversely affect the health of wildlife and fisheries by interaction with their endocrine system. The New Zealand mudsnail *Potamopyrgus antipodarum* (Mollusca, Hydrobiidea, Smith 1889) is a sensitive test organism, frequently used as an ecotoxicology biotest, recommended for use in the development of a reproduction test within the OECD guideline "*Ad hoc* Expert Group on Invertebrate Testing" [15,16]. As the main sex-steroids have been identified in *P. antipodarum* [17], their measurement in this organism could represent good biomarkers of endocrine disruption [18].

Quantification of the main vertebrate-like sex-steroids in such a complex matrix and with very low concentrations could be achieved by different techniques including radioimmunoassay (RIA) and gas or liquid chromatography coupled to mass spectrometry (GC–MS or LC–MS). Steroid measurements based on RIA methods are generally rapid and inexpensive, and usually exhibit satisfactory precision and accuracy while requiring little sample preparation. Nevertheless, GC–MS and LC–MS are considered the gold standard methods for sex-steroid identification and quantification. Especially LC–MS/MS methods have been shown to be suitable for investigating steroids with a relatively low quantity of sample but with time consuming sample preparation step [19].

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doi:10.1016/j.jchromb.2010.03.046

These methods are considered more specific and allow the simultaneous detection of several metabolite or compounds in a single assay while, in RIA-based methods, accuracy and specificity can be limited by cross-reactivity or subjected to interferences induced by the organic matrix in which steroids are included. The comparison of RIA with the reproducible and highly specific LC–MS/MS technique in the measurement of E2 in gonadal tissues of a mussel [20] has shown higher values with the RIA technique as compared to liquid chromatography analysis, due to the possible existence of a second estradiol immuno-reactive product [20] or interactions with the matrix.

The objectives of the present work were to develop and compare RIA and LC–MS/MS methods in regard of their advantages and drawbacks in the quantification of T, P and E2 in *P. antipodarum* in order to use these methods in large-scale experimental fields.

2. Experimental

2.1. Chemicals and reagents

All solvents (HPLC grade) and chemicals were purchased from Sigma–Aldrich, except the deuterated testosterone-1,2-d2 and progesterone-2,2,4,6,6,17α,21,21,21-d9 from CDN Isotopes. The degree of purity was at least 98% for all the standards; they were used without any further purification. Pure water was obtained from a Milli-Q device from Millipore.

For the LC–MS/MS experiments, individual stock solutions of 200 mg/L were prepared in methanol and stored at −23 °C. Composite working solutions were prepared by diluting suitable aliquots of each individual solution in Milli-Q water and were used within a week.

2.2. Experimental animals

P. antipodarum used for the validation of the RIA analysis were obtained from long-term cultures established in our laboratory (Cemagref, Lyon, France). These cultures were initiated with snails that originated from natural populations collected in clean sediment in a canal close to the Rhône river. The snails were kept at 16 ± 1 °C in 20 L stainless aquaria filled with reconstituted water (mixture of natural spring and deionised waters) and fed with Tetramin® fish food (Tetrawerke, Melle, Germany). The snails used for the validation of the LC–MS/MS analysis were directly collected in the Rhône river. To compare RIA and LC–MS/MS techniques, an *in situ* exposure of caged *P. antipodarum* was conducted upstream and downstream of a waste water treatment plant (WWTP) effluent discharge of Bourgoin-Jailleu, on the Bourbre river, France. As soon as collected, organisms were frozen at −23 °C until extraction.

2.3. Sample preparation

2.3.1. Preparation for LC–MS/MS analyses

Pools of 20 whole organisms were used. Each pool was first homogenized in 1 mL of water with UltraTurrax for 1 min. In order to prepare a range of standard additions, 4 pools were spiked with 50 μL of standard solutions of respectively 0, 3, 6 and 9 μg/L. To enhance the dissolution of steroids from binding proteins, 250 μL of 0.1N HCl was added and the fractions were submitted to 10 min of sonication (125 W). To neutralize homogenates, 0.5 mL of 0.28 M Na_2HPO_4 was added. Methylene chloride (3× 3 mL) was used to extract the steroids. After centrifugation at 10,000 × *g* for 10 min, organic phase was removed, pooled and dried under nitrogen gas at 40 °C. Fractions were dried and reconstituted in 5 mL heptane–ethyl acetate (60/40, v/v). Each fraction was applied to aminopropyl SPE columns (200 mg/3 mL) from JT Baker, previously conditioned with 4 mL heptane and 4 mL heptane–ethyl acetate (60/40, v/v). In these conditions, the steroids were not retained by the stationary phase and elution was completed with the addition of 6 mL volume of heptane–ethyl acetate (60/40, v/v). The purified extract was dried and reconstituted in 250 μL methanol.

As it has been shown that molluscs conjugate steroids with fatty acids [14], the determination of total steroids (free + conjugated) was performed: methanol containing 2% KOH was added after neutralization with Na_2HPO_4 and the pools were kept at 45 °C for 3 h. After the saponification step, 3 mL of Milli-Q water was added, and the samples were extracted with methylene chloride (3× 3 mL).

2.3.2. Preparation for RIA analyses

To remove the interfering substances, a simple extraction procedure was performed, adapted from Gooding and LeBlanc [21], to measure the levels of total T, P and E2 (free and conjugated). Pools of 2 whole frozen *P. antipodarum* were manually homogenized with Teflon pester in 1% (v/v) trifluoroacetic acid (TFA) for 3 min. Homogenates were extracted twice with methylene chloride (2× 2 mL) and centrifuged at 1000 × *g* for 10 min. The organic extract was evaporated and then underwent a saponification step (1 mL methanol containing 2% KOH for 3 h at 45 °C). Following saponification, 4 mL of reverse-osmosed water was added to each tube. Steroids were extracted twice with methylene chloride (2× 2 mL) and resuspended in an adequate volume of steroid-free bovine serum albumin 1% (BSA).

2.4. LC–MS/MS

Chromatography was performed using a Waters 2695 HPLC system including a vacuum degasser, binary pump and well-plate. The separation was performed on Zorbax Eclipse XDB C18 column (100 mm × 2.1 mm, 3.5 μm) with the corresponding pre-column. Two gradient elution programs were applied, both with a 0.2 mL/min flow-rate. For the analyses of P and T, the mobile phase was (A) pure water and (B) acetonitrile, with a linear gradient from 35 to 75% (B) in 15 min. For E2 analysis, the mobile phase was (A) pure water and (C) methanol, with the following gradient: from 30 to 100% (C) in 10 min.

Characterization and quantification were performed with a mass spectrometer Waters Quattro Micro in the positive (analyses of T and P) or negative (analysis of E2) electrospray ionisation mode, using multiple reaction monitoring (MRM). Precursor and product ion masses as well as the individual cone and collision energy voltages are presented in Table 1.

2.5. RIA

Sex-steroid measurements were performed using different commercial kits for E2 (Orion Diagnostica, Espoo, Finland or DiaSorin, Saluggia, Italy), P and T (Diagnostic Systems Laboratories, TX, USA). Samples were read on a RIA Star Packard® gamma counter. For P and T, measurements were made according to the protocols provided with the RIA kits. For E2 measurements, modifications were performed, including 40 μL 1% steroid-free BSA incubation overnight at room temperature before performing competition with the tracer for 1 h at 37 °C. The E2 assay was performed in a non-equilibrium state assay in order to significantly increase the sensitivity of the test, as it has been described by Paulson et al. [22].

3. Results and discussion

Because of the complexity of the matrix and the low detection limits required to establish the presence of traces of steroids in tissue samples, very sensitive analytical procedure should be developed, including extraction and purification steps, with acceptable recovery and reproducibility. Since steroids have been reported

Table 1
Precursor and product ions, cone voltage (V_{cone}), collision energy (E_{col}), limits of detection (LOD) of the LC–MS/MS method, and intra-assay precision (CV) for T and P.

	Precursor ion (m/z)	Product ions (m/z)	V_{cone}–E_{col}	LOD (μg/L)	CV (%)
T	289	109	25 V–20 eV	0.50	Lab.: 2.4
		97	25 V–20 eV		Upstream: 6.7 Downstream: 13.7
P	315	109	25 V–20 eV	0.40	Lab.: 0.4
		97	25 V–25 eV		Upstream: 5.2 Downstream:8.4
E2	271	183	40 V––40 eV	nd	nd
		145	45 V–45 eV		

nd: not determined.

to be present mainly as fatty acid-derived conjugates in molluscs, we tried to determine their respective levels with and without a saponification step, by both LC–MS/MS and RIA methods. Since the saponification step induces complete hydrolysis of fatty acid-derived steroids, the use of both preparation steps should allow on one hand the quantification of total steroids after total hydrolysis step and, on the other hand, only of the free fraction of steroids when this step was omitted. However, the LC–MS/MS method only allowed the identification of free forms of T and P while the RIA method was reliable only after the saponification step for P and E2, thus measuring the total levels of T, P and E2.

3.1. LC–MS/MS and RIA method developments

3.1.1. LC–MS/MS optimization

For the LC–MS/MS method, in order to optimize the MRM, full scan and product ion spectra, acquisition in positive and negative ion modes were performed by the infusion of standard solutions at 10 mg/L of each analyte. The base peak selected for quantification of E2 (m/z = 271) corresponded to the deprotonated molecule $[M-H]^-$. The major product ions corresponded to the A–B ring moiety (m/z = 145) and to the opening of C ring together with the loss of D ring (m/z = 183). On the other hand, T and P were detected as protonated molecules $[M+H]^+$ respectively m/z = 289 and m/z = 315. In MS/MS spectra of T and P, main product ions were m/z = 109 and m/z = 97, deriving respectively from the cleavage of the B- and C-ring as observed by several authors [23–25].

3.1.2. Sample preparation

3.1.2.1. Extraction of samples. Given the small size of organisms, in order to ensure a representative sample, tests with LC–MS/MS method were carried out from pools of 20 individuals. Various strategies such as freeze-drying, acidification of the sample, or the need for ultrasonication have been tested from samples spiked with sex-steroids (50 μL of a 100 μg/L spiked solution). The freeze-drying increased the amount of extracted steroids, but this step exhibited a poor reproducibility and was finally not adopted. Addition of chlorhydric acid permitted to enhance the dissociation of steroids from binding proteins, and extraction was increased by a few minutes under ultrasounds

For the preparation of samples for RIA analysis, several solutions were tested (SDS 1%, Tris–HCl 1%, Nonidet NP40 1%, Triton X-100 1%, HCl 1N, PBS 1X, TFA 1%) to homogenize the snails. Only TFA 1% did not induce a background noise. Moreover this acid enhanced the dissociation of sex-steroids from binding proteins.

A variety of organic solvents (methylene chloride, ethyl acetate, ether/ethanol 4/1, diethylether) were tested for their ability to extract T, P and E2. Methylene chloride was proved as the best universal solvent for extracting these steroids from lipid-rich tissues, as previously shown in other invertebrate tissues [26]. This solvent extraction was consequently used for both sample preparations in LC–MS/MS and RIA analyses.

3.1.2.2. Purification for LC–MS/MS analyses. In order to purify the extracted samples and reduce interferences during the subsequent analyses by LC–MS/MS, SPE has been chosen being a technique of choice for sample clean-up. Indeed it is easy to use, adapted to small sample volume and the wide variety of sorbents commercially available offers many interaction possibilities. To find an efficient method of clean-up limiting the loss of steroids, different protocols have been tested from standard solutions of T and P (2 μg/L) and E2 (200 μg/L), based on the principle that unwanted interferences remain on the solid phase and the compounds of interest are eluted. Thus the silica gel SiOH, Florisil, and the bonded phase aminopropyl cartridges were tested with different elution solvents alone or in combination (ethyl acetate, heptane, methylene chloride, acetone). Aminopropyl cartridges used with the mixture heptane–ethyl acetate (60/40, v/v) allowed best recovery rate while minimizing interferences. All recoveries were above 80%.

3.1.2.3. Purification for RIA analyses. Snail extracts of snails induced strong matrix interference, leading to lower precision and accuracy when measuring sex-steroids in RIA. To circumvent this problem, we tried to further isolate steroids using SPE C18 cartridge (Waters) and acetonitrile as elution solvent. However this process did not strongly diminish matrix interferences and SPE methods were discarded for RIA analysis. To control these interferences we added a saponification step, which actually resulted in a significant reduction and allowed a complete hydrolysis of complex lipids and conjugated steroids. Initially, RIA methods for E2 measurements were based on DiaSorin kits. However, this kit proved unsuitable to measure this steroid after the saponification step. Consequently, another highly sensitive kit (Orion Diagnostic coated kit) was used for determining E2 levels in *P. antipodarum*.

3.1.3. Quantification of the LC–MS/MS method

Deuterated analogues of the analytes represent ideal internal standards, as their identical chemical properties make them behave exactly as the analytes during the preparation steps, but the mass difference makes them easily identifiable with MS/MS detection.

At first, these standards were used to quantify steroids in snail extracts. However, we observed problems during the ionisation of deuterated in samples having undergone a saponification step. Indeed, the molecular ions $[M+H]^+$ = 291 and $[M+H]^+$ = 324 corresponding respectively to deuterated T-d2 and P-d9, present in the majority in the mass spectrum of the non-saponified samples (Fig. 1a), decreased by a factor 10 in saponified samples (Fig. 1b). It is worth noting that the peak $[M+H]^+$ = 324 disappeared completely in favour of masses centered on m/z = 318. It is likely that hydrogen–deuterium exchange takes place in the electrospray source, but so far we can make no assumptions about the nature of the mechanisms involved in this transformation. Therefore, no quantification of T and P could reliably be performed after saponification step using LC–MS/MS.

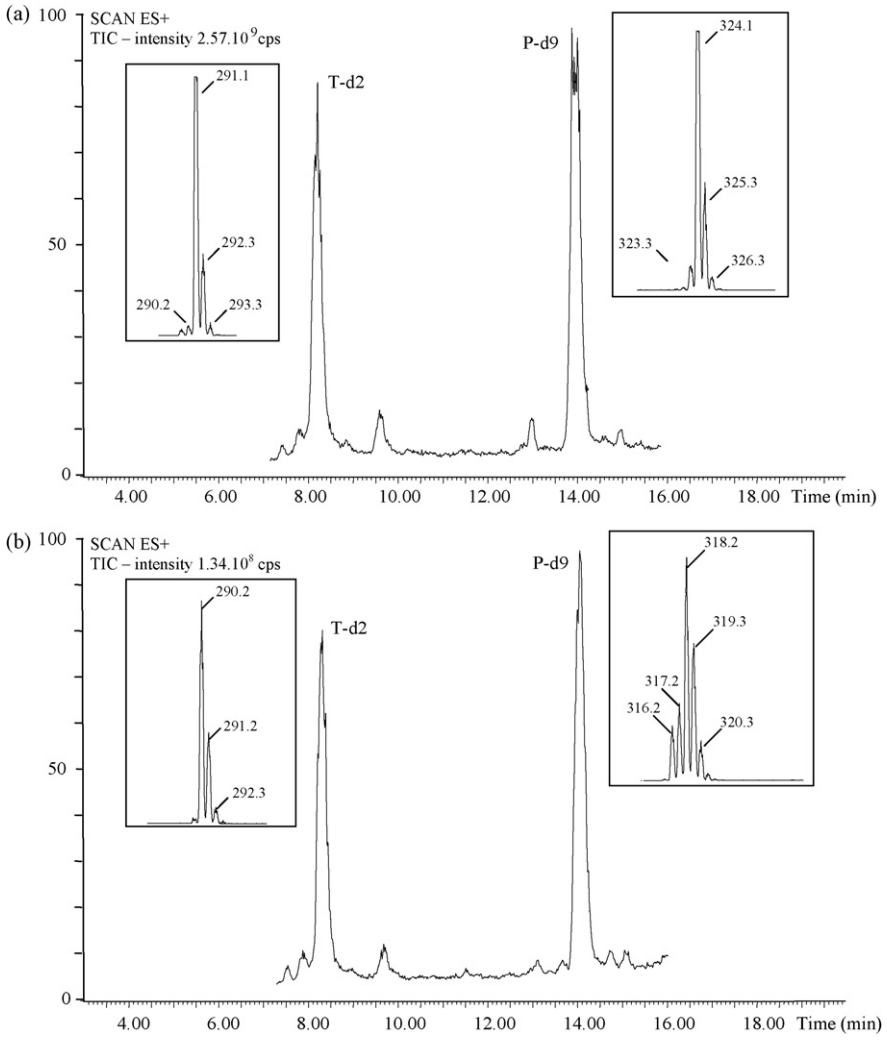

Fig. 1. Chromatograms of standard mixtures of T-d2 and P-d9 and their corresponding mass spectra without saponification (a) and after the saponification step (b).

3.1.4. Methods performances

3.1.4.1. LC–MS/MS. The linearity of the instrumental method was studied by injecting 6 standard concentrations of the target compounds within the range 1–50 μg/L for T and P corresponding to expected levels in organisms [27]. A good linearity was observed over the specified range with correlation coefficients 0.9989 and 0.9993 for T and P, respectively. Linearity of the whole method (preparation and analysis) was evaluated by linear regression of standard additions, which varied between 0.8220 and 0.9997 for T and between 0.7502 and 0.9925 for P. Repeatability of the method was obtained by the determination of T-d2 and P-d9 added in 8 replicates with 20 organisms in each pool (spiking at 40 ng/mL). The intra-assay precision (Table 1) was dependent of the origin of the organisms (from laboratory, upstream or downstream of WWTP) (Fig. 2).

3.1.4.2. RIA. Linearity was evaluated by measuring steroids in 2, 4, 6 and 8 snails. Linearity was very limited in the absence of the saponification step and was not respected beyond 4 snails (data not shown). However, the linearity of the method was confirmed for the range from 2 to 8 snails when this additional saponification step was performed. In this case, correlation coefficients were greater than 0.986. However, the slope of P measurements was lower indicating a very low sensitivity of the assay for this compound (Fig. 3). Concerning T measurements, linearity was achieved with and without saponification step, suggesting that this step could be avoided for this steroid only. For practical reasons, all assays were performed after saponification.

Recoveries were obtained by analyzing 10 replicates of spiked snails homogenates (2.88 ng/mL for T, 109 pg/mL for E2 and 20.44 ng/mL for P). Percent recoveries were calculated by dividing

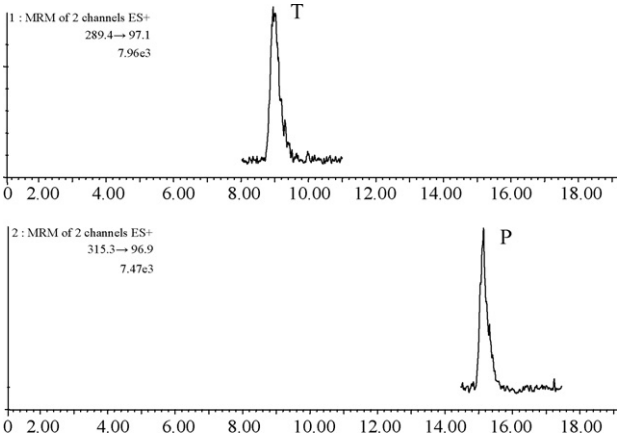

Fig. 2. MRM chromatograms of spiked (0.14 ng T and 0.17 ng P) samples from extraction of 20 snails upstream of WWTP.

Table 2
Performances of the RIA technique.

	Mean recovery	Intra-assay CV	Intra-assay ER	Inter-assay CV	Inter-assay ER	LOQ
E2	93 ± 19%	16%	17%	14%	15%	0.14 pg/mL
T	101 ± 12%	11%	1%	12%	1%	0.03 ng/mL
P	84 ± 8%	10%	18%	12%	11%	0.03 ng/mL

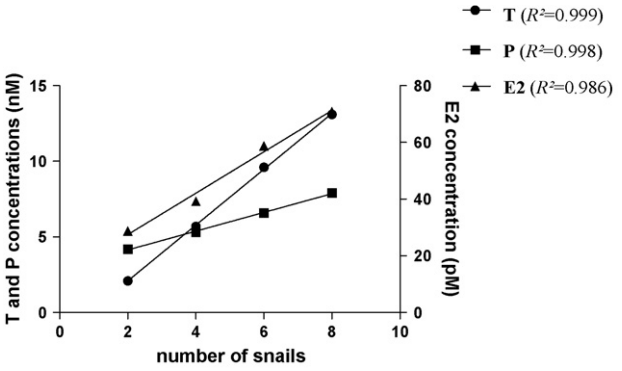

Fig. 3. Linearity of the RIA method in 2, 4, 6 and 8 snails for T (slope = 1.845), P (slope = 0.62) and E2 (slope = 7.281).

Table 3
Levels of T, P, and E2 using LC–MS/MS (organisms collected in a river) and RIA technique (laboratory animals).

Steroid levels	LC–MS/MS (pg/ind)	RIA (pg/ind)
T	3.3 ± 0.1	15.27 ± 6.27
P	2.3 ± 1.2	1.98 ± 1.83
E2	nd	1.58 ± 0.46

nd: not detected.

the measured concentration by the theoretical spiked concentration. Mean recovery values (Table 2) ranged from 84 to 101%. These high recoveries further suggest a good selectivity of the assay, limiting the existence of other endogenous interfering components which could be a potential source for variable results between subjects.

We tried to develop a measurement method which would be reproducible and would generate reliable data suitable for variability interpretation. Intra-assay precision and accuracy were evaluated by determining steroid concentrations in 10 snails equally spiked. Controls (0.66 ng/mL of T, 80 pg/mL of E2 and 1 ng/mL of P) were added in each measurement in order to ascertain inter-assay precision and accuracy. The greater the variability of the assay, the less discriminative the measurement method is. These data (Table 2) suggest that performance of the RIA method was acceptable across the range of measurement based on validation criteria (±20% Coefficient of Variation, CV, and Relative Error, ER). No notable effects of the origin of the snails (laboratory, caging experiments) were observed on these parameters.

3.2. Application of RIA and LC–MS/MS methods

3.2.1. Applications of the LC–MS/MS method

The LC–MS/MS method was successfully applied to the analysis of T and P in organisms collected in the river (Table 3), but did not allow the determination of E2 due to a higher detection limit of the method for this steroid.

3.2.2. Applications of the RIA method

We performed T measurements with or without saponification step of the extract prior RIA analysis successfully. Measured levels were similar (15.3 and 12.3 pg/snail, respectively) suggesting that, in laboratory *P. antipodarum*, conjugated T is not the major form. T is preferentially present as conjugated to fatty acids and this storage form appears to be a mechanism for regulating free levels (considered to be the active fraction) of the steroid in molluscs [28,29]. However the higher level of total T measured downstream of WWTP effluents (Table 4) suggested that *P. antipodarum* is able to bioaccumulate T.

3.2.3. Comparison of the performances of LC–MS/MS and RIA methods

In order to compare RIA and LC–MS/MS methods, an *in situ* exposure of caging *P. antipodarum* was conducted upstream and downstream WWTP effluent discharge for 21 days. Indeed it is now well established that endocrine disruptor compounds can enter the environment mainly through WWTP [30]. Analyses of P, T and E2 were performed simultaneously on 5 or 6 pools of 2 snails with RIA and (4× 20) snails with LC–MS/MS methods.

Detectable levels of vertebrate-like sex-steroids are confirmed in *P. antipodarum* but absolute levels were quite different regarding the method used (Table 4). Nevertheless, steroid levels were in the same order of magnitude when measured by RIA and LC–MS/MS methods. The measured steroid levels with both methods were consistent with those previously described in other molluscs species [27].

The sensitivity of the RIA method was an order of magnitude higher than the one of the LC–MS/MS method (Tables 1 and 2), which enabled the measurement of the steroids in fewer snails. This makes our RIA method more appropriate in experimental survey. The lesser sensitivity of the LC–MS/MS technique did not allow the detection of E2, hence results obtained with the RIA method must be used with caution.

Our RIA method underestimated P levels compared to LC–MS/MS, which could be explained by a residual matrix-dependence or more certainly, by the low sensitivity of the method

Table 4
Steroids levels (in ng/g) in snails exposed to a WWTP effluent discharge using RIA and LC–MS/MS techniques.

	T		P		E2
	RIA	LC–MS/MS	RIA	LC–MS/MS	RIA
Day 0 (n=5)	0.47 ± 0.39	0.60 ± 0.08	0.31 ± 0.17	1.02 ± 0.63	0.18 ± 0.10
Day 21 (n=6)					
Bion upstream	1.92 ± 0.32	0.84 ± 0.11	0.15 ± 0.08	0.92 ± 0.07	0.35 ± 0.15
Bourbre upstream	3.24 ± 1.28	1.25 ± 0.09	1.17 ± 0.52	0.50 ± 0.26	0.53 ± 0.26
Confluence	3.27 ± 0.84	1.78 ± 0.14	0.70 ± 0.49	0.61 ± 0.02	0.63 ± 0.26
Bourbre downstream	3.20 ± 0.92	1.65 ± 0.40	1.35 ± 0.96	1.18 ± 0.06	0.45 ± 0.10

used, probably dependent of a low antibodies avidity. These hypotheses would also explain the lower recoveries observed for this compound with the RIA method compared to E2 and T. Moreover in the field survey presented here (Table 4), P levels measured by both methods showed no correlation (Spearman test $P > 0.05$), but comparison could only be made on five measurements performed on different snails issued from the same experimental pool. The very poor dynamic range of the RIA method for P (Fig. 3) seems to indicate that it is not a suitable technique to assess variations of its levels in the mudsnail. The LC–MS/MS analysis should be preferred for P measurements. Our RIA method also generally produces higher values of T levels than the LC–MS/MS method as it was observed in human serum [31].

Spearman correlation coefficient between RIA and the T levels measured by LC–MS/MS, reach 0.9 ($P < 0.05$).

The effluent discharge seemed to modulate some steroids levels. Regardless of the measurement technique, no effects on P levels were observed after the exposure to the effluent discharge. The T and E2 levels were lower at the beginning of the exposure. It is very possible that the higher levels observed after exposure could be attributed to bioaccumulation of compounds discharges by the WWTP effluent and present in the river. Interestingly both methods show similar results in our survey.

4. Conclusions

Both RIA and LC–MS/MS methods enabled to detect sex-steroids in the gastropod snail *P. antipodarum* despite low amounts and complex matrix. They both are in agreement concerning T measurements, but LC–MS/MS should be preferred when analyzing P levels. As E2 was not measured using LC–MS/MS method, levels determined by RIA method must consequently be used with caution. Both methods showed sufficient sensitivity and reproducibility to be used as potential measurement method of endocrine disruptive compounds exposure in the mudsnail *P. antipodarum*. As RIA method requires far fewer snails (2) compared to LC–MS/MS method (4× 20) and is better adapted for numerous samples, it is more useful for screening purposes in courant ecotoxicology testing. RIA method developed in this work is reliable to assess variations of T and E2 levels in the mudsnail. Vertebrate-like sex-steroid measurement is a very promising biomarker to better understand the mode of action of EDs impairing mollusc reproduction.

Acknowledgements

The present work was funded by ONEMA. The authors wish to thank the Endocrinology Laboratory of the National Veterinary School of Lyon for the financial grant of the RIA analysis. Particular acknowledgements are addressed to the reviewer whose helpful comments greatly improved this manuscript.

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