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ORIGINAL PAPER

Quantification of steroids and endocrine disrupting chemicals in rat ovaries by LC-MS/MS for reproductive toxicology assessment

Nadia Quignot · Mikaël Tournier · Charlène Pouech · Cécile Cren-Olivé · Robert Barouki · Emmanuel Lemazurier

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Abstract Reproductive function is controlled by a finely tuned balance of androgens and estrogens. Environmental toxicants, notably endocrine disrupting chemicals (EDCs), appear to be involved in the disruption of hormonal balance in several studies. To further describe the effects of selected EDCs on steroid secretion in female rats, we aim to simultaneously investigate the EDC concentration and the sex hormone balance in the ovaries. Therefore, an effective method has been developed for the quantification of the sex steroid hormones (testosterone, androstenedione, estradiol, and estrone) and four endocrine disrupting chemicals (bisphenol A, atrazine, and the active metabolites of methoxychlor and vinclozolin) in rat ovaries. The sample preparation procedure is based on the so-called "quick, easy, cheap, effective, rugged, and safe" approach, and an analytical method was developed to quantify these compounds with low detection limits by liquid chromatography coupled with a tandem mass spectrometer. This analytical method, applied to rat ovary samples following subacute EDC exposure, revealed some new findings for toxicological evaluation. In particular, we showed that EDCs with the same described in vitro mechanisms of action have different effects on the gonadal steroid balance. These results highlight the need to develop an integrative evaluation with the simultaneous measurement of EDCs and numerous steroids for good risk assessment.

Keywords Endocrine disrupting chemicals · Hormonal balance · LC-MS/MS · Steroids · Toxicity

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N. Quignot (✉) · E. Lemazurier
Experimental Toxicology Unit, INERIS,
Parc Technologique ALATA, BP-2,
60550 Verneuil-en-Halatte, France
e-mail: nadia.quignot@gmail.com
e-mail: nadia.quignot@ineris.fr

M. Tournier · C. Pouech · C. Cren-Olivé
Département Service Central d'Analyse, UMR CNRS 5280,
Institut des Sciences Analytiques,
Chemin du Canal,
69360 Solaize, France

R. Barouki
INSERM UMR-S 747, Université Paris Descartes,
45 rue des Saints-Pères,
75006 Paris, France

Introduction

Humans and animals are exposed to numerous chemicals that can modulate or disrupt the endocrine system [1]. One potential target for environmentally and toxicologically relevant chemicals, such as endocrine disrupting chemicals (EDCs), is steroidogenesis [2], which allows a finely tuned sex hormone balance. Of high concern is the impact of EDCs on the reproductive system [3]. Among them, atrazine (ATZ), bisphenol A (BPA), methoxychlor (MXC), and vinclozolin (VCZ) have long been studied and have been described as estrogeno-mimetic (ATZ, BPA, and MXC) or anti-androgenic (VCZ) in vitro [4–7]. Despite the high importance of the androgen-to-estrogen balance in evaluating reproductive effects [8], the great majority of in vivo studies have dealt with the determination of estrogens in females

and androgens in males [9]. Moreover, the biological processes that occur between administration of the dose and tissue response are often poorly investigated and thus poorly understood. However, they might provide a strong mechanistic basis for findings in whole-animal systems. For example, a better understanding of both target tissue dosimetry and hormonal status would permit better evaluation of the reproductive effects.

To better understand the impact of EDCs on the hormonal system, it is necessary to develop highly sensitive analytical methods able to simultaneously detect and quantify the endogenous hormones and EDCs [10, 11]. The quantification of steroid hormones in urine [12], serum [13], water [14], milk [15], and mammalian tissues is fairly common but only rarely is it performed on mammalian gonads [10]. This is probably explained by the fact that the gonad, as an extremely complex biological matrix with a high level of lipids, demands intensive pre-treatment. Two major methods are reported in the literature for the extraction of hormones from animal tissues. The first is a solid/liquid extraction followed by solid-phase purification [16]; the second is based on the more recent so-called "quick, easy, cheap, effective, rugged, and safe" (QuEChERS) approach [17]. The latter is already a reference method for the extraction and purification of multi-class and multi-residue analysis of pesticides in food matrices [18]. The original "QuEChERS" method has been continuously adapted with regard to sample comminution and the extraction of pH-dependent compounds. Therefore, it can now be successfully applied to animal tissues [19]. However, while the "QuEChERS" sample preparation has been extensively developed for chemical residue analysis, it was rarely used for the extraction of hormones [20].

As far as analysis techniques are concerned, due to cross-reactivity, some immunoassay-based steroid hormone assays have problems quantifying steroids. The two analytical methods that are mainly used for the analysis of hormones and EDCs are gas chromatography (GC) coupled with a tandem mass spectrometer [21] and liquid chromatography coupled with a tandem mass spectrometer (LC-MS/MS) [13, 14, 22]. Most of the investigations using GC require a derivatization or a hydrolysis step; LC-MS/MS does not and gives higher sensitivity and specificity.

The aim of this work was to develop a method for the simultaneous and direct quantification of four free hormones (testosterone, androstenedione, estrone, and estradiol) and four EDCs (ATZ, BPA, VCZ metabolite M2 (M2), and the MXC metabolite 2,2-*bis*-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)) with low detection limits in rat ovaries. Even if we dosed animals with the parent chemicals MXC and VCZ, we aim to quantify their toxicologically active metabolites M2 and HPTE [23, 24]. Furthermore, MXC is rapidly metabolized by the liver and VCZ is very unstable. The method presented herein consists of two steps: the extraction of analytes based on the "QuEChERS" method and the analysis of the extracts by LC-MS/MS without derivatization or hydrolysis, thus avoiding complex and time-consuming sample preparation procedures. Another goal was to apply this method to a subacute (14 days) toxicity study in rats, to better characterize EDC reproductive toxicity in females.

This paper describes the optimization of the method, the validation strategy, and the findings from the subacute study.

Materials and methods

The in vivo subacute study

Test chemicals

Atrazine (CAS number 1912-24-9; purity, 97.1 %) was provided by TCI Europe (Zwijndrecht, Belgium). Vinclozolin (CAS number 50471-44-8; purity, 99.5 %) was from Greyhound Chromatography (Birkenhead, UK). Methoxychlor (CAS number 72-43-5; purity, >95 %) and bisphenol A (CAS number 80-05-7; purity, 99 %) were purchased from Sigma-Aldrich Chemical Co. (Saint-Quentin-Fallavier, France).

Animals

Female Sprague–Dawley rats were obtained from Janvier, Le Genest-Saint-Isle, France. The rats were housed with a 12-h light and 12-h dark cycle and received food and water ad libitum. All of the procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the INERIS.

Study design

The animals were approximately 8 weeks old at the time of treatment initiation and were divided into 6 groups. Each group consisted of 20 females. Each animal was administered orally with atrazine (200 mg kg^{-1}, dissolved in 0.5 % methylcellulose (Sigma-Aldrich Chemical Co.)), vinclozolin (100 mg kg^{-1} in corn oil (Sigma-Aldrich Chemical Co.)), methoxychlor (200 mg kg^{-1} in corn oil), bisphenol A (200 mg kg^{-1} in corn oil) or vehicle alone every day for 14 days. The dose volume was set at 5 ml kg^{-1} of body weight. The dosage and treatment period were chosen based on previous studies showing adverse effects on the reproductive system [25–28]. For estrous cycle staging, vaginal smears were collected once or twice a day and classified microscopically as diestrus, proestrus, estrus, or metestrus [29]. On the first diestrus stage of the estrous cycle following the 2-week treatment, ten animals per group were

sacrificed by a lethal intraperitoneal pentobarbital injection. The ten females remaining of each group were housed without treatment for an additional period of 14 days (encompassing three estrous cycles). These remaining females were sacrificed on the first diestrus stage after the recovery period. During the treatment and recovery period, all animals were observed carefully for mortality, body weight, and gross behavioral changes.

Sample collection

The ovaries and uterus were harvested and weighed. For each animal, one ovary was fixed in Bouin's solution, and the other was homogenized in phosphate-buffered water for tissue analysis. The uterus of each animal was fixed in paraformaldehyde.

Histological analysis

The organs were processed and embedded into paraffin using an automated processor. Five micrometer tissue sections were cut and mounted onto glass slides. The organ sections were colored with hematoxylin–eosin.

Analytical procedures for steroid hormones and EDC quantification

Materials and reagents

Testosterone (CAS number 58-22-0; purity, >99 %), androstenedione (CAS number 63-05-8; purity, >98 %), estradiol (CAS number 50-28-2; purity, >98 %), estrone (CAS number 53-16-7; purity, >99 %), atrazine (CAS number 1912-24-9; purity, >98 %), bisphenol A (CAS number 80-05-7; purity, 99 %), and HPTE (CAS number 2971-36-0; purity, 97 %) were obtained from Sigma-Aldrich Chemical Co. Vinclozolin M2 (CAS number 83792-61-4; purity, >98 %) was purchased from Cayman Chemical (Ann Arbor, USA).

All deuterated standards used as internal standards were of 98 % purity and were obtained from CDN Isotopes (Sainte Foy La Grande, France), 17β-estradiol-2,4-d_2 (CAS number 81586-94-9), estrone-2,4-d_2 (CAS number 350820-16-5), testosterone-1,2-d_2 (CAS number 204244-83-7), 4-androsten-3,17-dione-2,2,4,6,6,16,16-d_7 (CAS number 67034-85-9), bisphenol A-2,2′,6,6′-d_4 (CAS number 102438-62-0), and atrazine-d_5(ethyl-d_5) (CAS number 163165-75-1).

Phenacetin-ethoxy-1-^{13}C (CAS number 72156-72-0), used as an injection control, was purchased from Isotec (Miamisburg, USA). Methanol (MeOH) and hexane were of HPLC grade, and acetonitrile (MeCN) was of LC-MS grade. UltraPure water was obtained from a MilliQ device (Millipore, Molsheim, France). Standard solutions were prepared in MeCN at 1 mg ml^{-1} and stored at −18 °C; working solutions were prepared by mixing standard solutions with homogenized ovaries.

QuEChERS citrate buffers (Agilent SampliQ QuEChERS Kits) were purchased from Agilent Technologies (Massy, France). For the first step, where the citrate-buffered version was chosen, a mixture containing 4 g anhydrous magnesium sulfate ($MgSO_4$), 1 g sodium chloride, 1 g trisodium citrate dehydrate, and 0.5 g disodium hydrogen citrate sesquihydrate was employed. For the second step, the clean-up one, two dispersive solid-phase extraction (dSPE) were tested: (1) primary and secondary amine exchange (PSA) phase with 150 mg $MgSO_4$ and 25 mg PSA; (2) PSA/C18 phase with 150 mg MgSO4, 25 mg PSA, and 25 mg C18.

Sample comminution

The rat ovaries were homogenized with a Precellys® homogenizer (Cayman Chemical) in phosphate-buffered water (5 μL/mg of organ) and were then submitted to a 10-min sonication step. The homogenates were stored at −80 °C until extraction and analysis.

Sample extraction

An extraction procedure was developed for rat ovaries weighing between 30 and 80 mg. In summary, the homogenized ovary was spiked at a concentration of 30 ng ml^{-1} with the internal standard solution. A volume of MeCN was added in a ratio of V_{MeCN}/V_{water} of 2.6, then a volume of hexane was added in a ratio of V_{MeCN}/V_{hexane} of 2. Approximately 7 mg of Agilent SampliQ QuEChERS EN 15 662 extraction salt per mg of ovary was added; after shaking and vortexing, the sample was centrifuged at 5,000 rpm for 5 min. Finally, a 350-μl aliquot of the centrifuged and cleaned MeCN phase was transferred into an autosampler vial and evaporated under a stream of nitrogen at 40 °C. The sample was reconstituted in an 80/20 solution of water/MeCN spiked with 5 ng ml^{-1} of phenacetin C13.

LC-MS/MS analysis

An Applied Biosystems API-5500 triple quadrupole MS/MS with electrospray ionization (ESI) coupled to an Agilent 1290 LC (binary pump) was used. The analytical column was an Agilent ZORBAX Eclipse Plus C18, 50×2.1 mm, 1.8-μm pore size. A pre-filter of 0.2-μm pore size frit with 2.1-mm diameter was added. The injection volume was 5 μl, and the flow rate was 0.3 ml min^{-1}. Mobile phase A was 0.1-mM ammonium acetate in water, and mobile phase B was MeCN. In negative mode, the gradient conditions started at 90 % A and were ramped linearly to 33.5 % A

over the course of 5 min, then ramped to 100 % B from 5 to 6 min and held there until 11 min. In positive mode, the gradient profile started at 75 % of eluent A, decreased linearly to 33.5 % A over 4 min, then ramped to 100 % of eluent B over 1 min and held there for 5 min. The re-equilibration time before every run was 5 min, giving total run times of 16 min in negative mode and 15 min in positive mode. The MS/MS conditions were optimized using direct infusion into the ESI source in both modes to provide the highest detection and sensitivity for the quantification of each analyte. A second MS/MS transition was selected as the qualitative confirmation. The source temperatures were 400 °C and 600 °C in negative and positive mode, respectively; the ion spray potentials were set to −4,500 and 5,500 V, respectively. The operating settings, i.e., the nitrogen flow for the nebulizer gas (GS1) and for the turbo gas (GS2), were 276 and 345 kPa, respectively, for negative mode, and 345 and 414 kPa, respectively, for positive mode. AB Sciex Analyst 1.5.2 software was used for data collection and instrument control.

Method validation

The method combining the sample preparation protocol and the LC-MS/MS analysis has been validated according to international conference harmonization (ICH) directives [30]. Thus, the limits of detection and quantification, linearity, extraction recovery, matrix effects, repeatability, reproducibility, and accuracy were determined.

The limits of detection (LOD) and quantification (LOQ) were defined as the analyte concentrations that produced peak signals of three and ten times the background noise of the chromatogram, respectively.

Whole method linearity was checked with extracted matrices spiked with molecules of interest at five concentrations: 1, 3, 10, 25, and 50 times the LOQ.

The recovery, the matrix effect, the repeatability and the reproducibility were evaluated by triplicate analyses of samples spiked at three internal standard concentrations, specifically 3, 10, and 50 times the LOQ. Extraction recovery was calculated by comparing the peak areas of the analytes spiked in matrices before or after extraction. Matrix effects were evaluated by comparing the mean peak areas of the spiked samples after extraction (A_{extract}) with those obtained from standards at the same concentration prepared in the mobile phase (A_{solvent}). The percentage matrix effect was then calculated according to the following equation: matrix effect $(\%) = \left(\frac{\text{Aextract}}{\text{A}_{\text{solvent}}} - 1\right) \times 100$. To meet the ICH standard, more than nine evaluations of intra- and inter-day precision (3 concentrations/3 replicates/3 days) were performed. The intra-day (repeatability) and inter-day (reproducibility) precisions were expressed as the relative standard deviation (RSD) of a series of measurements [31]. Internal calibration, considered to be an effective method to eliminate matrix effects on the accuracy and reproducibility of data [32], was performed for each molecule in our study.

Measure accuracy, which refers to the degree of closeness between the value obtained and the value considered as the true value [30], was determined for each concentration level.

Statistical analysis

GraphPad software (Prism 5.02) was used for the statistical analyses. The results were analyzed with a Mann–Whitney non-parametric test. All of the data are expressed as the mean±error of the mean (SEM). Differences with a p of less than 0.05 were considered to be statistically significant.

Results

The in vivo study

The estrous cycle

Several animals treated with EDCs showed estrous cycle disruption (Table 1). For the period of the 2-week atrazine exposure, females presented a lengthened estrous cycle, which persisted during the 2-week recovery period. This lengthening was characterized by a blockage in the diestrus stage of the estrous cycle. The mean estrous cycle length also increased in MXC-treated females, and returned to normal after MXC withdrawal. Some females treated with VCZ showed continuous estrus with cycles of more than 5 days.

Histological analysis

Ovaries Fine analysis of follicle numbers and type was summarized in Table 1. There were no significant histopathological alterations in BPA- and VCZ-treated animals. Females treated with ATZ or MXC showed little ovaries. In ATZ-treated females, a decrease in the number of previously formed corpora lutea (corpus albicans) was noted, and currently formed corpora lutea (corpus hemorrhagicum) were rarely detected in those ovaries (Table 1). In MXC-treated females, an increased number of degenerating oocytes was found in the primary follicles (Fig. 1).

Uterus Uterine atrophy, with low stromal density, was observed in some ATZ-treated female rats (Table 1 and Fig. 2).

Table 1 The estrous cycle duration and pathological findings in female rats treated with atrazine, vinclozolin, methoxychlor, or bisphenol A daily for 2 weeks

Treatment	Control 1	Atrazine	Control 2	Vinclozolin	Methoxychlor	Bisphenol A
Estrous cycle						
No. of animals examined	20 (10)	20 (10)	20 (10)	20 (10)	20 (10)	20 (10)
Mean estrous cycle length (days)	3.76±0.25 (3.48±0.18)	8.53±2.34 ***p<0.0001; (5.8±2.12) **p=0.0043	3.96±0.21 (3.91±0.52)	4.15±0.71 (4.33±0.52); *p=0.0355	6.28±1.91 ***p<0.0001; (3.58±0.69)	4.01±0.40 (4.00±0.40)
No. of animals showing irregular estrous cycle (>5 days)	0 (0)	20 (7)	3 (1)	6 (3)	14 (2)	2 (2)
Gross pathology						
No. of animals examined	10 (10)	10 (10)	10 (10)	10 (10)	10 (10)	10 (10)
No. of animals with small[a] ovaries	0 (0)	6 (0)	0 (0)	(0)	4 (0)	0 (0)
No. of animals with a small[a] uterus	0 (0)	8 (2)	0 (0)	(0)	0 (0)	0 (0)
Histopathology						
No. of animals examined	5 (5)	5 (5)	5 (5)	5 (5)	5 (5)	5 (5)
Ovary: loss of currently formed corpora lutea (corpus hemorrhagicum)	0 (0)	4 (1)	0 (0)	0 (0)	3 (0)	0 (0)
Ovary: decrease in numbers of previously formed corpora lutea (corpus albicans; <4/ovary)	0 (0)	0 (0)	0 (0)	0 (0)	4 (0)	0 (0)

Control 1 was used for atrazine treatment, and control 2 was used for vinclozolin, methoxychlor, and bisphenol A treatment. Values are mean±SEM of 20 or 10 animals/group. Values inside parentheses represent findings after recovery period

**p<0.01; ***p<0.001 versus corresponding control

[a] Organ weight from treated animal is <40 % compared with mean organ weight from control animals

Method development

LC-ESI-MS/MS optimization

First, the optimum detection settings for each analyte were determined. Table 2 shows the characteristics of the analytes and the internal standards.

Optimization of the LC conditions was performed by injecting a mixture of all analytes. The nature of the mobile phases, the flow and the gradient were determined: the compounds were separated within 4 min in the positive mode and within 5 min in the negative mode. Several mobile phases were tested. MeOH and MeCN were first compared as solvent B, and MeCN was the solvent that provided the best separation, especially on the couples estrone-estradiol and testosterone-androstenedione. For solvent A, we choose to compare the commonly used solutions formic acid (0.1 %) and ammonium acetate aqueous

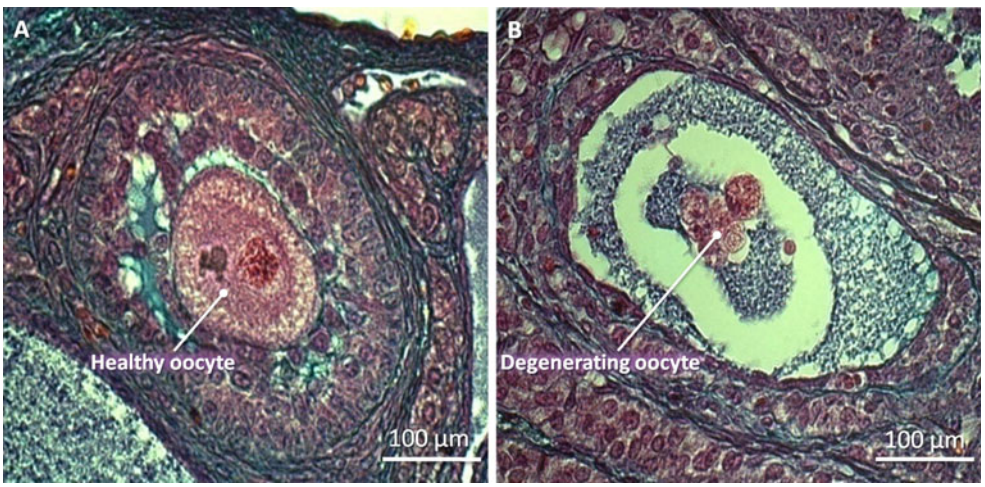

Fig. 1 Exemple of oocyte degeneration in methoxychlor-treated females (**B**) compared with healthy oocyte in control animals (**A**). We reported an elevated number of degenerating oocytes in methoxychlor-treated female ovaries (example in B) compared with the control group (example in (**A**)). Hematoxylin–eosin staining was used

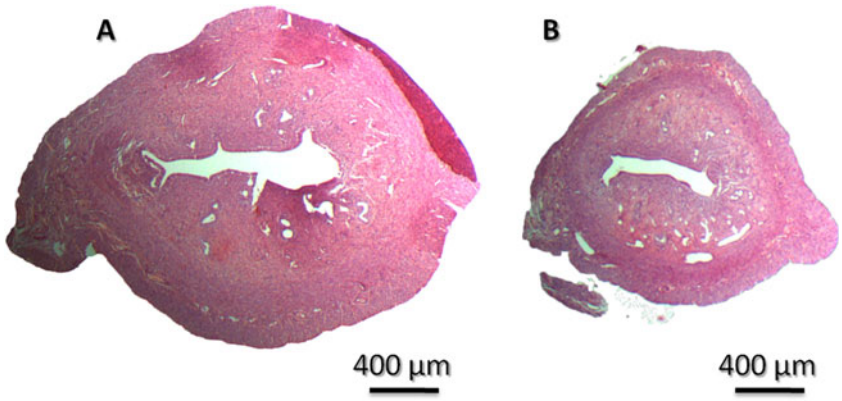

Fig. 2 Details of uterus measurements from control (**A**) and atrazine-treated females (**B**). Atrazine-treated females (**B**) presented an atrophic uterus compared with the control animals (**A**). Hematoxylin–eosin staining was used

solution (0.1 mM). Formic acid showed suppressed ionization in negative mode, whereas ammonium acetate did not affect the three analytes (ATZ, testosterone and androstenedione) in positive mode and improved the signals in negative mode. Hence, ammonium acetate solution and MeCN were chosen as solvents A and B, respectively.

Extraction

The so-called "QuEChERS" method consists of two major steps: a liquid/liquid buffered extraction followed by a dSPE clean-up. In this study, three settings were optimized: (1) the MeCN/water volume ratio (V_{MeCN}/V_{water}) for the extraction step, (2) the dSPE-based purification step, and (3) the MeCN/hexane volume ratio (V_{MeCN}/V_{hexane}) for the final clean-up step. For each optimization step, the ovaries were spiked at the same concentration (500 µg l^{-1}).

The V_{MeCN}/V_{water} ratio for the extraction step V_{MeCN}/V_{water} ratios of 2.3, 2.6, 3, and 3.5 were tested. The best ratio for most analytes was 2.6 (Fig. 3).

The purification step Two different types of dSPE sorbents were tested: PSA and PSA/C18; one sample was also extracted without the clean-up step. Compared with PSA clean-up and with the extraction without dSPE, the use of PSA/C18 decreased the recovery responses by a factor two (Fig. 4). Our results revealed that neither the PSA/C18 nor the PSA sorbents were suitable for the dSPE clean-up step proposed in the original QuEChERS method [33].

The V_{MeCN}/V_{hexane} ratio for the clean-up step To carry out a cleaning step on the biological matrix, a method was tested using hexane. It has been shown that this solvent allows the elimination of co-extracted compounds such as non-polar and fatty compounds [34]. Several V_{MeCN}/V_{hexane} ratios ranging from 0 to 4 were tested. Equivalent responses were observed for the ratios ranging from 2 to 4 (Fig. 5), but the ratio of 2 allowed easier phase partitioning and easier handling of the cleaned extract.

Method validation and method performance

Quantitative analysis Table 3 shows the limits of detection and quantification for all the analytes. For the hormones, all the LOQs were inferior to 3 ng g^{-1}. For the EDCs, M2 and ATZ gave LOQs inferior to 1 ng g^{-1}. Due to the weak ESI, the LOQ of HPTE was 15 ng g^{-1}. The BPA LOQ was high at 25 ng g^{-1}.

Linearity The calibration curve for each analyte, based on five concentration points, was linear over the entire concentration range and all correlation coefficients were superior to 0.99.

Recovery=extraction yield Recoveries and RSDs, calculated from three replicates, are given in Table 2. Recoveries were superior to 70 %, except for HPTE.

Matrix effects Ion suppression was observed, with a maximum value of 16, 10, and 8 % for BPA, E1, and E2, respectively (Table 2).

Applications to the rat in vivo study

The analytical method developed in this study was applied to the rat ovaries obtained from the previously described subacute in vivo study. The results are summarized in Table 4 and Fig. 6. See Electronic supplementary material for examples of chromatograms.

Tissue dosimetry

Table 4 shows the ovarian concentrations of ATZ, BPA, HPTE, and M2. After the recovery period, the chemicals could not be detected.

Table 2 MS/MS conditions for the analytes and their internal standards; extraction recoveries of the analytes

Mass detection mode	Analytes/internal standards	Precursor ion (*m/z*)	Product ion (*m/z*)	Declustering potential (V)	Collision energy (V)	Transition ratios	Retention time (min)	Matrix effect (%)	Extraction recovery (%)	Extraction repeatability (recovery RSD %)	Intermediate precision (RSD %)	Bias (%)
Positive	Atrazine (ATZ)	*216.1* 216.1	*174.1* 104.0	*161* 161	*25* 37	2.6±0.1	2.7±0.2	12	83	3	15	20
	Atrazine-d_5(ethyl-d_5)	221.0	179.1	81	27		2.7±0.2					
	Testosterone (T)	*289.1* 289.1	*97* 109	*71* 71	*27* 29	1.1±0.03	3.1±0.2	5	84	3	9	16
	Testosterone-1,2-d_2	291.0	99.0	56	31		3.1±0.2					
	Androstenedione (A)	*287.1* 287.1	*97.1* 109	*66* 66	*31* 33	1.2±0.1	3.4±0.2	0	80	2	7	13
	4-androsten-3,17-dione-2,2,4,6,6,16,16-d_7	294.0	100.1	56	32		3.4±0.2					
	Ethoxyphenacetine C13	181.0	110	106	27		1.5±0.2					
Negative	Bisphenol A (BPA)	*226.9* 226.9	*133.0* 116.9	*−115* −115	*−34* −66	9.8±1.1	3.9±0.2	16	73	6	26	NA
	BPA d_4	231.1	216.1	−80	−26		3.9±0.2					
	17B estradiol (E_2)	*271.0* 271.0	*183.2* 144.8	*−55* −55	*−56* −60	1.6±0.1	4.2±0.2	8	80	8	16	18
	E_2 d_2	273.0	146.8	−65	−50		4.2±0.2					
	HPTE	*314.9* 314.9	*242.9* 279.0	*−30* −30	*−8* −6	1.3±0.2	4.5±0.2	3	56	10	13	29
	Estrone (E_1)	*268.9* 268.9	*145.1* 159.0	*−130* −130	*−50* −48	7.7±0.6	4.5±0.2	10	88	6	11	9
	E_1 d_2	271.1	147.0	−120	−50		4.5±0.2					
	Vinclozoline M2 (VM2)	*258.0* 258.0	*159.8* 34.9	*−65* −65	*−24* −66	44.7±3.9	5.0±0.2	4	86	3	16	24
	Ethoxyphenacetine C13	179.0	148.6	−30	−18		2.7±0.2					

Entries in red present quantitation transitions used for quantification. The percentage matrix effect was then calculated according to the following equation: matrix effect $(\%) = \left(\frac{A_{\text{extract}}}{A_{\text{solvent}}} - 1\right) \times 100$.

Extraction repeatability: intra-day precision expressed in recovery relative standard deviation (RSD; %). Intermediate precision, inter-day precision (6 days) expressed in RSD (%). Bias, averaged bias between the theoretical and calculated concentrations of a methodological Quality Control (6 days, 2 replicates/day)

NA non-applicable

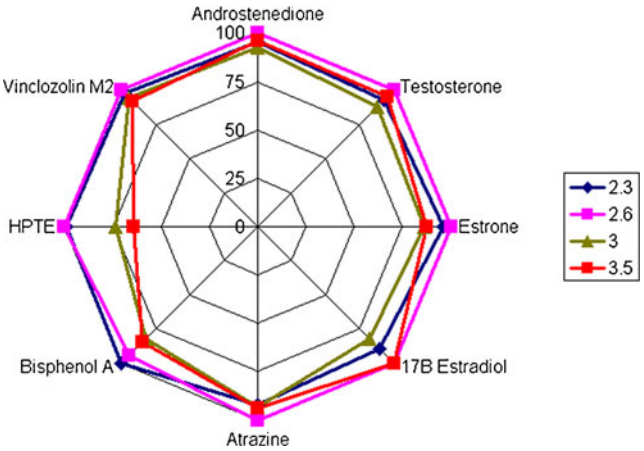

Fig. 3 Comparison between the different ratios V_{MeCN}/V_{water} for the extraction step. The figure represents relative extraction efficacy of the most intense signal for every compound

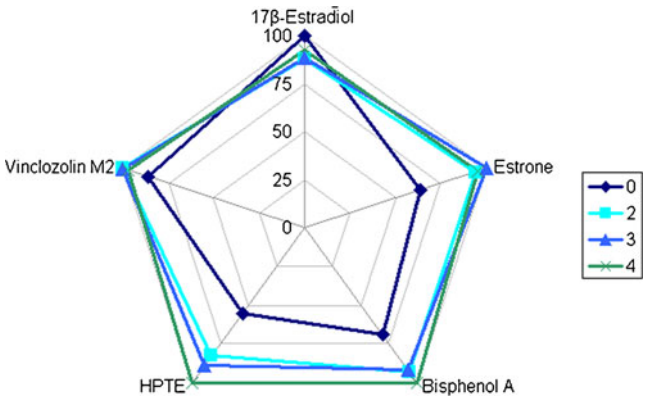

Fig. 5 Comparison between the different clean-up ratios V_{Hexane}/V_{MeCN}. The figure represents relative clean-up efficacy of the most intense signal for every compound

Steroid dosages

Sex steroid concentrations in the ovaries are presented in Fig. 6. After a 2-week treatment with ATZ, females showed slightly increased ovarian estrone. VCZ treatment significantly increased ovarian androstenedione and estrone. MXC-treated females showed a decrease in androstenedione and testosterone concentrations in the ovaries. BPA treatment showed no significant effect. After the recovery period, there was no more significant effect on sex steroid levels. As far as estrogen-to-androgen ratios are concerned, ATZ and MXC treatments significantly increased the ovarian estrone-to-androstenedione ratio.

Discussion

An over- or under-secretion of one or more steroids can cause a steroidal imbalance that results in various disorders.

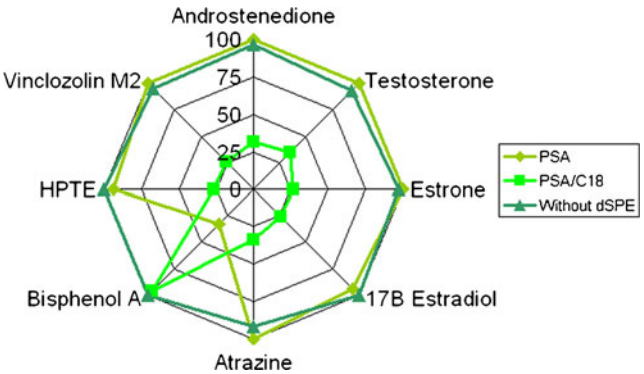

Fig. 4 Comparison between the different purification steps, with or without dSPE. The figure represents relative purification efficacy of the most intense signal for every compound

Precise steroid analysis is very important, not only to predict pathological alterations, but also to understand toxicity mechanisms. Indeed, small variations in steroid concentrations can induce major toxicological changes, particularly when exposure occurs during a critical window [35]. There are many challenges when analyzing steroids, due to their low concentrations and their structural similarities. The method developed herein aimed to simultaneously quantify sex steroids and EDCs in rat ovaries.

The excellent recoveries obtained for the sex hormones (Table 2), as compared with the recoveries reported in the literature (higher than 80 %) [10], highlight the efficiency of the "QuEChERS" approach for EDCs as well as for hormone extraction. Moreover, the biases of the recoveries as determined by the RSDs are sufficient and acceptable because they are lower than 20 % (Table 2). As ovaries are complex matrices, it was important to thoroughly study the matrix effect aspect. Even if ion suppression was observed, the low variations of recovery, illustrated by low values of matrix effect (Table 2), show the efficiency of the extraction process.

To the best of our knowledge, this is the first time that "QuEChERS" sample preparation was applied to rat ovaries with analyte concentrations reported in the nanogram range per gram of tissue and the first time that the simultaneous analysis of hormones and EDCs was conducted. LODs and LOQs are similar to those obtained with tissues from other species [10]. Furthermore, the method was sufficiently sensitive and precise to accurately quantify ovarian steroid and EDCs levels in female rats.

To better characterize the effects of EDCs in female rats, we focused our work on hormone production in the ovaries after in vivo EDC exposure. We took into account not only the effect on absolute sex steroid concentration, but also on the estrogen-to-androgen ratio and the reproductive side

Table 3 The method performance summary for EDCs and steroid hormones

	BPA	M2	ATZ	HPTE	E_2	E_1	A	T
LOD (ng/g)	0.22	0.09	0.04	4.90	0.62	0.20	0.20	0.20
LOQ (ng/g)	24.51	0.28	0.31	14.66	1.78	0.62	0.62	0.62

This table summarizes the LOD and LOQ for bisphenol A (BPA), vinclozolin metabolite M2 (M2), atrazine (ATZ), methoxychlor metabolite HPTE, estradiol (E_2), estrone (E_1), androstenedione (A), and testosterone (T). The LOD and LOQ data are for an organ with a mean weight of 55 mg, with a clean-up step with hexane

effects. We discuss below the potential mechanisms for altered hormone balance. Following EDC treatment, some females presented estrous cycle disruption and histopathological disorders of the uterus or ovaries (Table 1). These alterations were caused by hormonal balance disruption or altered receptor availability. Ovarian sex steroid concentrations were altered following ATZ, VCZ, and MXC treatments (Fig. 6). ATZ, MXC, and BPA are classified as estrogenic, according to in vitro studies [4, 5, 7]. However, the effects on hormonal balance in our study are more or less important. ATZ elevated the estrone-to-androstenedione ratio by increasing estrone. This effect was in accordance with several other studies, which associated ATZ with disturbances in the balance between androgens and estrogens in male rats [36]. This elevated estrogen-to-androgen ratio could be specifically linked to aromatase activity induction observed in in vitro studies [37]. Ovaries from females treated with MXC showed normal estrogen levels and decreased androgen levels. Alteration of steroidogenesis resulting in an androgen decrease has been previously described in male rats following MXC treatment [38]. Thus, MXC appears to exert an anti-androgenic activity in rats. As the effect of BPA on rat ovaries was not significant, we cannot conclude what effect BPA had on the sex steroid levels in our study. VCZ treatment has been associated with anti-androgenic effects in vitro, via the antagonistic binding of its metabolite M2 to the androgen receptor [6]. In our study, VCZ increased ovarian androstenedione and testosterone levels, an effect which is opposite to what is usually reported for this class of compound (anti-androgenic). This effect could account for a direct steroidogenic up-regulation, previously reported in rat and human cells [6], or a blockade of the androgen negative feedback in hypothalamus and pituitary mechanism [24].

These results show that EDC treatment can lead to hormonal balance disruptions that may or may not be in accordance with the described EDC in vitro mechanism of action. The advantage of measuring the sex steroid concentrations in ovaries is the possibility of evaluating the effects of EDCs on ovarian steroidogenesis. It is important to be aware that in female rats, sex steroids, and almost all estrogens, are produced and secreted by the ovaries. However, there can be external factors influencing hormonal balance; therefore, these gonadal dosages must be followed by a serum measurement of sex steroid concentrations.

The obtained ovarian EDC concentrations are of the same order of magnitude as those employed in most in vitro studies of these chemicals (Table 4; [39]). This concentration level provides an important link between our results on ovaries and the results of in vitro studies of corresponding cell types (granulosa and theca cells). Furthermore, because in vitro studies supply more useful mechanistic information when they are executed at relevant concentrations and with a good test chemical (parent product or metabolite), this method gives good data for further in vitro study design. However, in vitro studies cannot substitute for in vivo ones, especially because they lack metabolism and other external regulation like the hypothalamo-hypophyseal axis. The need for an integrative evaluation of in vivo and in vitro measurements is reinforced by the observation, in our study, of different in vivo patterns for EDCs with the same described in vitro mechanisms of action.

In conclusion, an analytical method was implemented for the determination of sex steroid hormone concentrations and EDC concentrations in rat ovaries. The QuEChERS sample preparation protocol, which is new for this type of

Table 4 Tissue dosimetry of the ovaries of female rats treated with atrazine, 200 mg kg^{-1}; vinclozolin, 100 mg kg^{-1}; methoxychlor, 200 mg kg^{-1}; or bisphenol A, 200 mg kg^{-1}daily for 2 weeks

Ovarian concentrations	Atrazine (ng/g)	Vinclozolin metabolite M2 (ng/g)	Methoxychlor metabolite HPTE (ng/g)	BPA (ng/g)
ng/g	109.88±95.56	45.00±22.24	26.48±15.29	54.84±23.26
μM	0.51±0.44	0.17±0.09	0.08±0.05	0.24±0.10

For each female, necropsy occurred at mean 24 h following the last administration. Vinclozolin and methoxychlor are followed according to their metabolites M2 and HPTE, respectively. The values are the mean±SEM for five animals

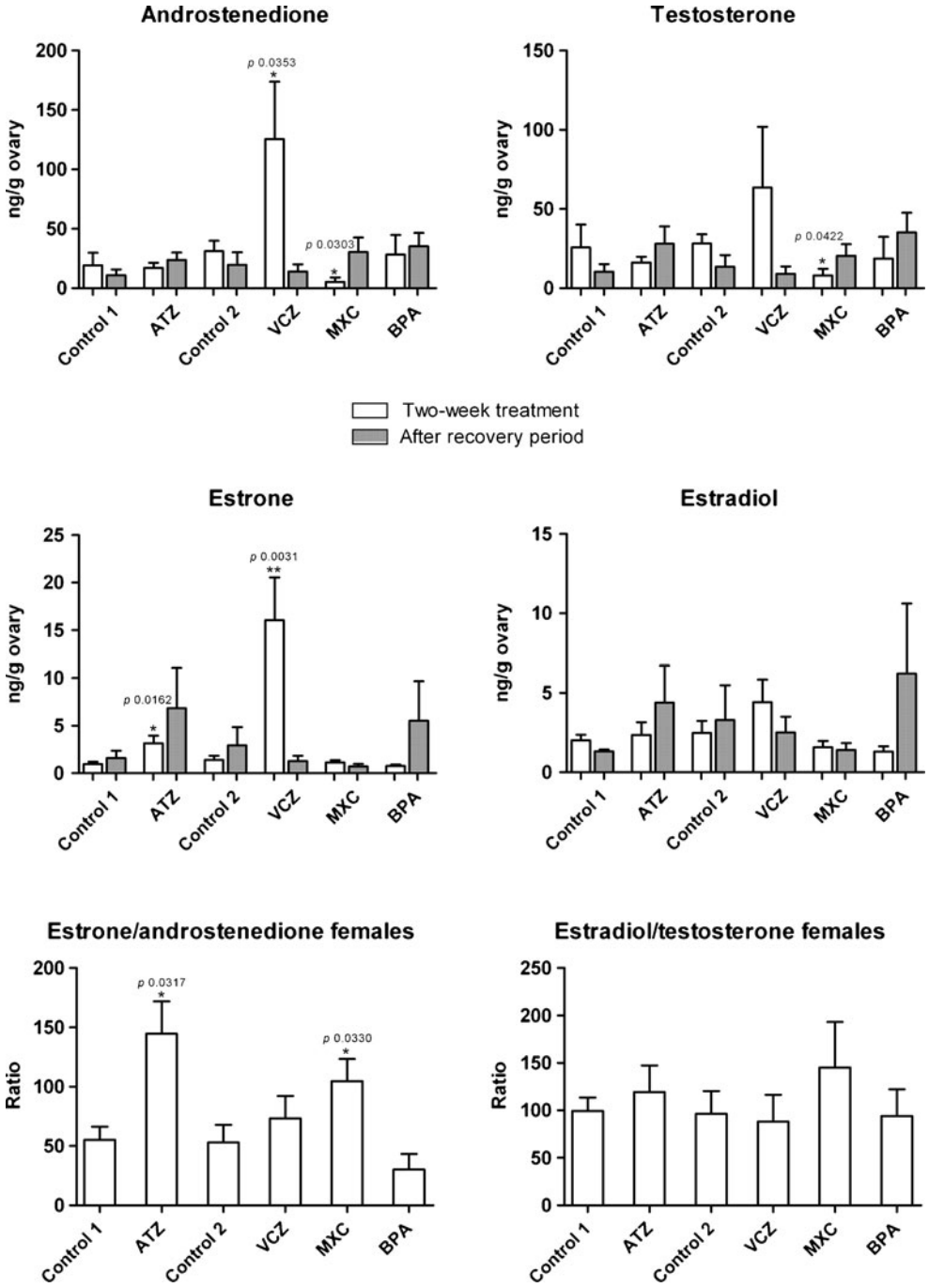

Fig. 6 Androstenedione, testosterone, estrone and estradiol concentrations and estrogen-to-androgen ratios in the ovaries of female rats treated with atrazine, vinclozolin, methoxychlor, and bisphenol A daily for 2 weeks. The figure represents: the mean ovarian steroid concentrations after a 2-week EDC treatment or after the recovery period following the 2-week EDC treatment and the ovarian estrone-to-androstenedione and estradiol-to-testosterone ratios (pg estrogens/ng androgens) in female adult rats treated or not with EDCs for 2-week. The values are the mean±SEM of 5 animals per group. *$p<0.05$; **$p<0.01$ versus the corresponding control (control 1 for atrazine treatment, control 2 for vinclozolin, methoxychlor, and bisphenol A treatment)

matrice, may be qualified as simple (a liquid–liquid extraction step followed by a cleaning step), rapid (extractions were performed in less than 15 min) and efficient (RSDs and recoveries were acceptable). The sensitivity and specificity of the LC-MS/MS analysis method allowed the simultaneous detection and quantification of low levels of two classes of molecules: steroids and EDCs. The relevance of this method, validated according to ICH criteria, was confirmed by the application of the methodology to an in vivo study. Thus, as well as providing a new tool in the endocrine toxicology field, this work provided new information about the toxicological patterns of EDCs.

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Conflict of interest The authors declare no conflicts of interest.

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