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Quantification of ecdysteroids and retinoic acids in whole daphnids by liquid chromatography-triple quadrupole mass spectrometry

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ABSTRACT

Quantification of ecdysteroids and retinoic acids at picograms per individual is typically achieved with radioimmunoassay methods. However, those methods cannot identify individual types of ecdysteroids or provide an absolute concentration, which poses problems for comparative assays such as the metabolic profiling approach for toxicity testing. The method described in the present paper, based on liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry, was developed to allow the quantification in whole daphnids extracts of ecdysteroids (20-hydroxyecdysone, ecdysone, ponasterone A) and retinoic acid (sum of isomers). This approach avoids having to perform the difficult task of sampling the haemolymph on small organism (<5 mm). Recoveries, evaluated at three concentrations in matrix blank fortified samples, ranged from 83 to 119% for ecdysteroids and from 144 to 155% for retinoic acids. Precision (2.4–14.2%) and accuracy (−41.7 to 14.5%) were reproducible and stable over three quality control concentrations. The described liquid chromatography-triple quadrupole mass spectrometry method achieved quantification limits ranging from 210 to 380 pg mL^{-1} for ecdysteroids and 5 ng mL^{-1} for retinoic acids in spiked matrix blanks. 20-hydroxyecdysone was quantified in *Daphnia magna* adults (19 ± 8 pg ind^{-1}) and juveniles (3.6 ± 1.0 pg ind^{-1}), but was below the limit of quantification in neonates (≈0.19 pg ind^{-1}). Ecdysone was also detected in adult specimens (≈1.8 pg ind^{-1}).

1. Introduction

Contaminants of emerging concern such as pharmaceuticals, personal care products, flame retardants and plasticizers are transported into the aquatic environment mainly through municipal sewage. Although removal of these substances occurs in wastewater treatment plants, these facilities were not designed to eliminate them and therefore contaminants of emerging concern are continually released into the aquatic environment [1]. Effects of single contaminants of emerging concern on aquatic species at environmental concentrations (<100 ng L^{-1}) have been reported for a few compounds such as 17α-ethinylestradiol [2], ibuprofen and ciprofloxacin [3], however anthropogenic introduction of thousands of these compounds at nanogram-per-liter concentrations into surface waters still poses an unknown risk to the aquatic environment.

Current regulatory toxicity assessment of effluents and receiving waters that include endpoints such as mortality, behavioural effects and reproductive dysfunction are limited and are not able to detect differences between a control group and individuals exposed to contaminants of emerging concern at concentrations <1 μg L^{-1} [4]. Thus, more sensitive bioassays are needed to detect subtle changes in aquatic species caused by prolonged exposure to trace amounts of mixtures of these compounds.

A metabolic profiling approach to toxicity testing may bridge this gap between environmental levels of contaminants of emerging concern and bioassay toxicity levels. Among the different organisms suitable for a metabolic bioassay, the water flea *Daphnia magna* was chosen because it is already extensively used in laboratories, reproduces very quickly, and is relatively easy to culture. An adult *D. magna*, is able to reproduce every 3–4 days to an average of 6–10 neonates per clutch via cyclical parthenogenesis resulting in a clonal female population [5,6]. Studies have shown that ecdysteroids and terpenoids are potential candidates for a targeted metabolic bioassay since they are suspected to be involved in moulting, reproduction and stress response in *D. magna* and other crustaceans [7–10]. Therefore, they could be potential biomarkers for the detection of subtle toxic effects of contaminants of emerging concern in this organism. Quantification of ecdysteroids at the picogram-per-individual level in small organisms is usually

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done using radioimmunoassay methods [11,12], which although very sensitive, have several limitations: they cannot distinguish individual types of ecdysteroids, provide semi-quantitative values representing the sum of all cross-reacting substances (often called 'ecdysteroids equivalent') and do not account for the ecdysteroids that do not bind to available antisera due to different antisera specificity profile. As a result, data obtained using radioimmunoassay methods cannot be compared [13]. The relatively poor selectivity of radioimmunoassay methods quickly led to the development of selective gas chromatography–mass spectrometry methods using *N*-trimethylsilylimidazole derivatization [14,15]. Those methods required derivatization for 30 min to 60 h followed by purification by thin-layer chromatography before analysis by gas chromatography–mass spectrometry. Interestingly, significant structural information can be obtained using those methods by comparing hydroxyl groups' reactivity; however since ecdysteroid stability during derivatization is unknown, such long derivatization step makes routine application difficult and could affect method reproducibility.

More recently, methods focusing on the profiling and characterization of ecdysteroids have used liquid chromatography-triple quadrupole mass spectrometry with great success. A method using that technique achieved the detection of 20 pg per injection [16]. Further improvement was later done by another group using nano-liquid chromatography-quadrupole-linear ion trap mass spectrometry and achieving the detection of 4.81 pg per injection in *Drosophila melanogaster* larvae extracts [17]. A method of characterization and detection of ecdysteroids with liquid chromatography-tandem mass spectrometry using derivatization of ecdysteroids has also been published by Lavrynenko et al. in *D. melanogaster* with a detection limit of 10 pg per injection [18]. However, quantification methods of ecdysteroids using methods other than radioimmunoassay in small crustacean or insect such as *D.magna* or *D. melanogaster* could not be found in scientific literature.

To the best of our knowledge, quantification of ecdysteroids including a complete validation and determination of the analytical precision and accuracy has been achieved for the *Bombyx mori* (silkworm) only [19] and no study has reported the quantification of retinoic acids in any crustacean or insect. Straightforward application of the method developed for silkworm was not possible due to different sample preparation requirements, the absence of optimization for retinoic acids in addition to ecdysteroids as well as the difference in analytical instruments at our disposal.

Our objective was to achieve detection of three ecdysteroids (20-hydroxyecdysone, ecdysone, ponasterone A) and two retinoic acids (9-*cis*-retinoic acid and all *trans*-retinoic acids) (Supplementary material, Fig. S1) at low pictogram levels in whole *D. magna* samples by optimizing analyte extraction, derivatization, and chromatographic separation. Such a method will allow the monitoring of the concentrations of these key metabolites in *D. magna* in order to study at the molecular level the effect on *D. magna* of exposure to mixtures of contaminants of emerging concern.

2. Materials and methods

2.1. Reagents and chemicals

20-Hydroxyecdysone, ponasterone A, makisterone A, ecdysone, 9-*cis* retinoic acid (9-*cis*) and all-*trans* retinoic acid (all >95% purity) were obtained from Santa Cruz Biotech (Dallas, TX, USA). Acitretin (>95% purity) was purchased from Cedarlane (Burlington, Ontario). Makisterone A and acitretin were used as internal standards (ISTD) for ecdysteroids and retinoic acids quantification, respectively. Additional purification of makisterone A to remove 20-hydroxyecdysone and ecdysone impurities was necessary and was done using ultra-performance liquid chromatography (UPLC) separation (Supplementary material, Fig. S2). Water, methanol, acetonitrile, methyl *tert*-butyl ether and liquid-chromatography mass spectrometry mobile phase additives formic acid, ammonium acetate and acetic acid are LC or LC–MS grade and were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The derivatization reagent hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$) was purchased from Sigma Aldrich (ReagentPlus, purity >99%). Frozen daphnids (Hikari Bio-Pure, Hayward, CA, USA), used as a matrix blank to prepare quality control samples, were purchased from a local aquarium store (Aquatica, Montreal, QC, Canada). Stock solutions were prepared at 0.1 mg mL^{-1} in methanol and stored at −20 °C. Working solutions were prepared in 1% formic acid in methanol and stored at −20 °C. The aqueous solution of the derivatization agent was prepared fresh before each experiment.

2.2. Culture of D. magna

Daphnids are cultured and maintained in ISO Standard Freshwater [20] at 25 °C under a 16 h:8 h light:dark photoperiod. Cultures are maintained at a density of 40 organisms per liter of culture medium. Culture medium wasrenewed once a week and daphnids are feed daily using a concentrated algal suspension of *Pseudokirchneriella subcapitata*. Daily rations were calculated to obtain 0.1–0.2 mg of organic carbon per daphnid per day using a nomograph plotting optical density versus total organic carbon in accordance with OECD 211 [21]. Algal cultures were grown in Bold's modified medium.

2.3. Sample preparation

2.3.1. Extraction and derivatization

Water fleas (*D. magna*) were sorted by size by filtering through a series of sieves (300 μm, 560 μm, 900 μm). Adults were collected on the 900 μm sieve, juveniles on the 560 μm sieve and neonates on the bottom sieve (300 μm). Between 25 (adults) and 100 (juveniles and neonates) *D. magna* individuals were collected on a 250 μm tissue strainer (Pierce, Thermo Scientific), washed with deionized 18 MΩ H_2O and sonicated in an ultrasonic bath (VWR symphony, model 97043-964) for 15 min in a volume of 1 mL 1% formic acid in methanol inside a 50 mL Falcon tube (Corning Life Sciences). Then, an 800 μL aliquot was evaporated to dryness under a $N_{2(g)}$ flow. Derivatization of ecdysteroids was done at 70 °C for 90 min using 1 mL of a 100 mg mL^{-1} hydroxylamine hydrochloride aqueous solution. Analytes were then extracted from the aqueous phase using 2 × 1.5 mL of methyl *tert*-butyl ether. The organic phase was transferred in another glass tube, evaporated to dryness and reconstituted in 150 μL of methanol.

2.4. Quantitative analysis by ultra-performance liquid chromatography-triple quadrupole mass spectrometry

UPLC was performed on an Acquity system from Waters Corp. using a solid-core particle column. Experimental conditions of the chromatographic method are summarized in Table 1.

The UPLC system was coupled to a triple quadrupole mass spectrometer (Quattro Premier, Waters Corp.) equipped with an electrospray ionization (ESI) source. The ESI source was operated in the positive mode from 0 to 10 min and in the negative mode from 10 to 30 min. Data acquisition was performed in the selective reaction monitoring (SRM) mode. The method developed used electrospray ionization instead of atmospheric pressure chemical ionization as it is more commonly used in laboratories. Mass spectrometry parameters are summarized in Table 2.