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A rapid quantitative assay for juvenile hormones and intermediates in the biosynthetic pathway using gas chromatography tandem mass spectrometry

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ABSTRACT

A method for rapid quantitation of insect juvenile hormones (JH) and intermediates in the biosynthetic pathway, both *in vitro* and *in vivo* (hemolymph and whole body), has been developed using GC–MS/MS. This method is as simple as the radiochemical assay (RCA), the most commonly used method for measurement of JH biosynthesis *in vitro*, without need for further purification and derivatization, or radioactive precursors or ligands. It shows high sensitivity, accuracy and reproducibility. Linear responses were obtained the range of 1–800 ng/mL (approximately 4–3000 nM). Recovery efficiencies for farnesol, farnesal, methyl farnesoate and JH III were approximately 100% *in vitro* and over 90% *in vivo*, with excellent reproducibility at three different spike levels. Titer of JH III in the hemolymph was relatively low at day 0 (adult female emergence) (79.68 ± 5.03 ng/mL) but increased to a maximum of 1717 ng/mL five days later. In whole body, JH III quantity reached a maximum on day 4 (845.5 ± 87.9 ng/g) and day 5 (679.7 ± 164.6 ng/g) and declined rapidly thereafter. It is in agreement with the hemolymph titer changes and biosynthetic rate of JH *in vitro*. Comparison with the results of inhibition of JH biosynthesis by two known inhibitors (allatostatin (AST) mimic H17 and pitavastatin) using RCA and GC–MS/MS, showed that there was little difference between the two methods In contrast to other methods, the present method with GC–MS/MS can be used to elucidate the mechanism of inhibition by inhibitors of JH biosynthesis without any derivatization and purification. This method is applicable to screening of JH inhibitors and the study of inhibitory mechanisms with high sensitivity and accurate quantification. It may also be useful for the determination of JH titer in other Arthropods.

1. Introduction

Juvenile hormones (JHs) are acyclic sesquiterpenoids that play a central role in insect life. They are synthesized and released by the paired corpora allata (CA). [1]. There are 11 JH homologues known in insects and the structures have been elucidated and confirmed [2]. These JH homologues were identified in different insect species and show different actions and activity. The most common JH in insects is JH III [3]. The biosynthesis of JH III is divided into two biosynthetic pathways: the mevalonate (MVA) pathway and the JH specific pathway. The MVA pathway is responsible for the conversion of acetyl-CoA to farnesyl pyrophosphate (FPP) [4], and the JH specific pathway (Fig. 1) involves the farnesyl diphosphate hydrolysis to farnesol, oxidation to farnesal and then farnesoic acid, followed by a methyl transfer and epoxidation [5]. In some species, particularly the Lepidoptera, epoxidation often precedes methylation [6].

Because of its instability, physicochemical characteristics and low concentration in insects, the quantification of JHs has proven difficult. Four methods have been utilized to quantify JH: 1) Radio-

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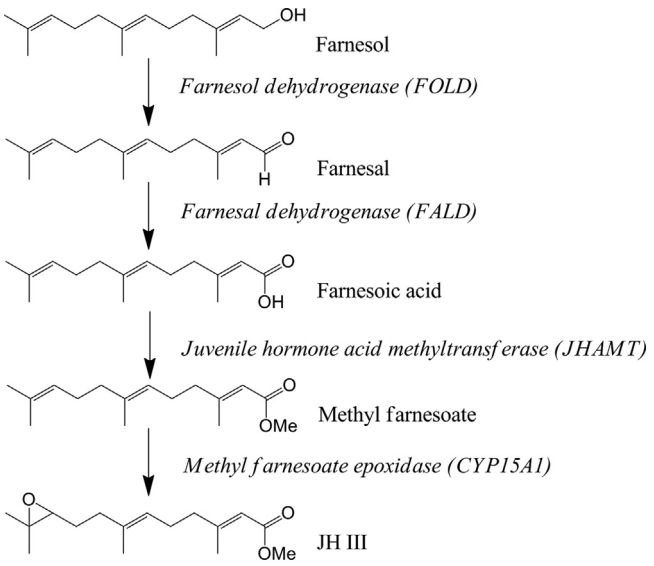

Fig. 1. Scheme of the JH specific biosynthetic pathway. Adapted from Belles et al. [4].

chemical Assay (RCA), 2) Chromatographic methods, including gas chromatography coupled with mass spectrometry (GC–MS), high performance liquid chromatography (HPLC), liquid chromatography tandem mass spectrometry (LC–MS/MS) and HPLC coupled to fluorescence detection (HPLC-FD), 3) Radioimmunoassay (RIA), 4) JH Binding Protein Assay (JHBP).

Radiochemical Assay (RCA) is the most commonly used method for determination of JH biosynthesis *in vitro*. It does not measure JH concentration, but rather measures the rate of incorporation of the methyl moiety from [^{3}H]- or [^{14}C]-methyl methionine into JH by freshly dissected CA *in vitro* [7]. The use of RCA is confined to assays *in vitro* and must be accompanied with a blank control.

Radioimmunoassays (RIA) have high accuracy, but the cross-reactivity of antibodies against other JHs or precursors makes it inappropriate in the presence of JH homologues [8]. JH Binding Protein Assay (JHBP), using binding protein to replace antiserum, has similar precision to the RIA [9]. Moreover, the procedure for this assay can be more straight-forward than RIA, but both RIA and the JHBP assay lack universal applicability [10].

With the development of physicochemical methods, more modern analytical techniques have been used to quantify JH [11–13]. Each method has advantages and disadvantages. In addition to the above methods, HPLC-FD is a sensitive method for the detection of picomolar or femtomolar concentrations of JH [14]. However, it does not directly measure the JH concentration, because JH lacks natural fluorescence. Therefore, derivatization employing fluorescent tags is essential using this approach. However, time-consuming derivatization and the expense of fluorescent labeling reagents limit the applicability of this method [15]. To date, chromatography coupled to MS is increasingly used for of JH analysis because it provides unequivocal identification and quantification [16].

Until 1955, mass spectrometry (MS) was most commonly used for the direct analysis of volatiles. Gas chromatography (GC) was coupled to MS for the first time with the aim of expanding the analytical capabilities of MS to cover complex mixtures of unknowns [16]. Subsequent technological developments involved the introduction of hybrid mass analyzers such as the triple stage quadrupole (TSQ) mass spectrometers and the use of tandem MS (MS/MS) as a high specificity technique for routine quantitative analysis of complex mixtures [17]. The TSQ mass spectrometers can sometimes be used to quantify low levels of target compounds in the presence of a high sample matrix background. GC–MS/MS has quickly become established as the technology of choice for bioanalytical applications. At present, it is widely used in environmental analysis, and in the determination of pesticide residues.

Quantitative assays for JHs, JH degradation products and farnesoic acid have been reported [18]. In this paper, we report the development of a quantitative assay for JHs and their biosynthetic pathway, using GC–MS/MS. Using both GC–MS/MS and RCA, we have compared the results of JH biosynthesis *in vitro* and *in vivo*.

2. Materials and methods

2.1. Animals

Diploptera punctata, kept at 27 ± 0.5 °C and relative humidity 50 ± 2% were fed with standard lab cockroach food and water. Day 0 (newly emerged) mated female *D. punctata* were isolated, placed in containers with food and water.

2.2. Reagents and chemicals

JH III was purchased from Toronto Research Chemicals (Toronto, ON, Canada). (*E, E*) Farnesoic acid and methyl farnesoate were obtained from Echelon Biosciences (Salt Lake City, UT). Farnesol, farnesal, citronellol, geranylgeraniol, clotrimazole, pitavastatin, HPLC-grade *n*-hexane, isooctane and acetonitrile were from Sigma-Aldrich (St. Louis, MO). Allatostatin mimic H17 was a gift from Dr. Xin-ling Yang, (China Agricultural University, Beijing, China) and was synthesized from Rink Amide-AM resin using the standard Fmoc/tBu chemistry and HBTU/HOBt protocol [19].

Stock standard solutions of citronellol, JH III and its biosynthetic precursors were prepared in hexane and stored at −20 °C (1000 mg/L). The working multi-standard solutions and internal standard solution at the appropriate concentrations were prepared daily by dilution with hexane.

2.3. GC–MS/MS analysis

The GC–MS/MS system consisted of a Thermo Trace 1300 series GC coupled with an AI/AS 1310 autosampler and a TSQ 8000 triple-quadrupole mass spectrometer. The system was controlled by TraceFinder software, version 3.1 for data acquisition and processing.

(a) GC Parameters. Citronellol, JH III and its biosynthetic precursors were separated with Agilent HP-5 MS UI capillary columns (0.25 mm i.d. × 30 m, 0.25 μm film thickness). Helium (purity = 99.999%) was used as the carrier gas with a constant flow rate 1.2 mL/min. Inlet temperature was 230 °C, 2.0 μL pulsed splitless injection volume with the purge flow rate at 50 mL/min for 1 min. The column oven was initially held at 60 °C for 1 min, then increased to 160 °C at a rate of 25 °C/min, finally by a 12 °C/min ramp to 280 °C, the total analysis time was 15 min.

(b) Triple-quadrupole MS Parameters: The temperature of transfer line and ion source were held at 280 °C. The mass spectrometer was working in EI mode (70 eV), and the filament current was 50 μA. Electron multiplier voltage (EMV) was gained by automatic MS/MS tuning, and argon gas were used as the collision gas for default instrument settings in the collision cell. The optimal quantitation and confirmation transitions from parent ions to daughter ions and collision energy for SRM (selected-reaction monitoring) of each compound were achieved with Auto-SRM study tests furnished by the software.

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