Analysis and Quantitation of Insect Juvenile Hormones Using Chemical Ionization Ion-Trap Mass Spectrometry

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A method for identification and quantitation of insect juvenile hormones (JH) has been developed using capillary gas chromatography–chemical ionization (isobutane)–ion-trap mass spectroscopy. The method does not require derivatization of samples or use of selected ion monitoring. Analysis over a mass range of 60–350 u allowed for identification of as little as 0.01 pmol of individual JH homologs. Quantitative analysis was based on the ion intensities of six diagnostic ions and the summed intensities of these ions for each homolog. The ratio of diagnostic ions did not vary significantly over a range of concentrations from 2.7 to 200 pg. The technique was used to identify and quantify the amounts of JH homologs secreted by individual retrocerebral complexes from the moth Manduca sexta maintained in tissue culture and to identify JH III from hexane extracts of hemolymph of the Caribbean fruit fly. No discrimination due to disparate abundance ratios of the individual homologs was found when analyzing natural product samples differing in concentration by at least fivefold. The technique allows for facile, concrete identification and quantitation of biologically relevant amounts of JH. The ability to analyze samples without derivatization or fractionation by chromatographic methods, coupled with data acquisition over a broad mass range, provides levels of accuracy and confidence greater than those of other methods.

Key Words: juvenile hormones; corpora allata; Caribbean fruit fly; Manduca sexta; mass spectroscopy.

Juvenile hormones (JH) of insects are critical for regulation of a number of processes including development, polymorphism, diapause, coordination of reproductive maturity, and pheromone production. Consequently, accurate determination of the structures and quantities of JHs produced by insects in different physiological states is critical for determination of their roles. However, these sesquiterpene epoxide methyl esters of dodecadienoate and tridecadienoate (Fig. 1) are difficult to extract from biological samples and analyze because they are hydrophobic, usually present in picogram quantities, and labile. Several techniques including bioassays, immunological analysis, and radiochemical methods have been developed for measuring synthesis and determining titers of JH (1–5). Although each method has been used effectively for specific purposes, all have been criticized for various reasons and none allows for concrete identification of JH. For example, analysis of JH using bioassays is severely limited by the sensitivity of the system and inability to distinguish between various JH homologs. Immunological analyses suffer from a lack of precision and accuracy because of cross-reactivities of antibodies developed against specific JHs with other homologs and metabolites. Radiochemical methods that rely on incorporation of L-[3H-methyl]methionine into JH during the terminal step in biosynthesis by isolated corpora allata are not amenable to analysis of JH present in hemolymph or tissue extracts.

Physicochemical methods of analysis of JH by gas chromatography–mass spectroscopy (GC–MS) are considered the most accurate methods for analysis of JH because they allow for unequivocal identification and quantitation (6, 7). The most commonly adopted technique for GC–MS analysis is that developed by Bergot et al. (8) in which the monoepoxide JH homologs are converted to their corresponding methoxyhydrin derivatives and analyzed by electron impact MS using selective ion monitoring (SIM). Identification and quantification using GC–MS–SIM rely on determination of the relative retention index for each JH derivative and detection of the base peak for each homolog (i.e., m/z 76.
for JH III and m/z 90 for JH I and II). While this method is usually reliable, identification and quantitation based on analysis of a single fragment ion for each homolog can be misleading, particularly when JHs have not been identified for an insect species that is expected to produce two or more homologs. For example, in a recent study on endogenous levels of JH present in the tomato moth, inconsistencies in data prompted Edwards et al. (7) to reevaluate samples using an “accurate mass” method in which JH III was quantified using m/z 76.084. As many less-expensive GC–MS systems are incapable of such accuracy, confidence in identification and quantitation based on detection of a single fragment ion is reduced. Confidence can be improved by monitoring multiple ions by SIM, though derivatization of JH to the methoxyhydrin derivative is facile, this reaction reduces recovery rates by 15–25% (8). To overcome these problems we have developed a method which allows for direct analysis of JHs without derivatization, using an ion-trap MS operated in the chemical ionization mode with isobutane as reagent gas.

**METHODS AND MATERIALS**

Chemicals. Synthetic samples of JH I, II, and III (Fig. 1) were a gift from D. A. Schooley (University of Nevada, Reno, NV) and trans,trans-farnesyl acetate was purchased from Aldrich Chemical. These synthetics were used without further purification because GC–MS analysis indicated that all were ≥98% pure. Capillary GC/GC–MS-grade hexane and methanol (B & J GC) were obtained from Burdick and Jackson and 18 MΩ water was obtained from a Milli Q UVplus water purification system. Tissue culture medium 199 containing Hanks’ salts and glutamine was obtained from Gibco. All other chemicals were obtained from Sigma and were of the highest purity available.

Chemical analysis. GC–MS analysis was carried out using a Finnigan-MAT ITQ 40 ion-trap MS operated in chemical ionization (CI) mode and interfaced to a Varian Star 3400 GC. The GC was equipped with both cool-on-column and split/splitless injectors and a CTC Analytics A 200 S autosampler. The 30 m × 0.25-mm (i.d.) analytical column used in the GC was a DB5-MS (0.1-μm film thickness) (J & W). When the cool-on-column injector was used the analytical column was interfaced to a 10 m × 0.25-mm (id) uncoated, deactivated fused silica retention gap and a 10 cm × 0.5-mm (i.d.) length of uncoated, deactivated fused silica in the injector to allow for injection of large volumes of sample without loss of resolution (9, 10). Conditions of chromatography were initial injector temperature, 40°C for 30 s; injector temperature increased at 170°C/min to 270°C; initial column temperature, 40°C for 5 min; column temperature increased at 5°C/min to 210°C; He carrier gas linear flow velocity, 24 cm/s; GC–MS transfer line temperature, 230°C. Under these conditions farnesyl acetate eluted at 32.3, JH III at 33.8, JH II at 35.4, and JH I at 37.3 min, respectively. Prior to use, the mass spectrometer was tuned in the electron-impact mode to establish proper peak relative intensities, peak widths, and peak resolution using perfluorotributyl amine (fc-43). MS operating conditions were multiplier voltage, 1900 V; manifold temperature, 130°C; emission current, 16 μA; mass acquisition range, 60–350 u; scan rate, 1 s; scan mode, chemical ionization; isobutane reagent gas (partial pressure = 2.6 × 10⁻³ Pa). Chemical ionization time was 1.5 ms and reagent reaction time was 12 s. The ionization RF level was 10 u and the reaction RF level was 20 u. Observed reaction reagent ions were m/z 43 and m/z 57 in a 1:2 ratio. Automatic gain control (AGC) RF levels for each segment were calculated as 123 digital analog converter steps with the AGC scale factor equal to 100% for each segment.

Initially, we analyzed a mixture containing 200 pg of each JH and the internal standard (farnesyl acetate), using the cool-on-column injector, determined cleavages that resulted in production of specific ions, and six selected diagnostic ions for each compound for use in quantitative analyses. Confirmation of cleavage assignments was validated using spectra obtained from analysis of synthetic samples by CI–MS/MS using a GCQ plus GC–MS/MS system [courtesy of ThermoQuest (Finnigan) Corp.]. We then compared the effects...
of using the cool-on-column injector or a split/splitless injector on quantitative analysis of JH III. For split/splitless injector studies the analytical column was attached directly to the injector. In this case the injector temperature was maintained at 250°C throughout the chromatographic run. Samples were injected in the splitless mode with the injector being purged 60 s after injection. Other conditions of chromatography were the same as those used for analyses conducted when using the cool-on-column injector. Equal amounts (1 ng/µl each) of octadecane, farnesyl acetate, and JH III were injected using either the split/splitless injector or cool-on-column injector and amounts of farnesyl acetate and JH III recovered were determined by comparison to octadecane which does not degrade when flash vaporized in the splitless injector. Subsequently, we conducted a series of analyses using the cool-on-column injector in which the amounts of the JH homologs were decreased. The minimum amount of each JH homolog analyzed was 0.01 pmol. Intensities of each diagnostic ion, determined after subtraction of background ions, were plotted against concentration and regression equations were calculated. Relative abundances of the diagnostic ions, as percentages of the most intense ion (base ion), were calculated for each compound at each concentration and were compared to the mean relative abundances calculated for all concentrations using a t test (P = 0.05).

Insects. One-day-old females of Manduca sexta were obtained from a laboratory colony maintained at the Division of Neurobiology, University of Arizona (Tucson AZ). The retrocerebral complex, containing the corpora allata, which synthesizes JH, and corpora cardica, was dissected from the head and placed in tissue culture medium 199 containing 2% Ficoll 400, 72 mg/ml CaCl2, and 0.6 mM sodium acetate and sodium propionate as described by Cusson et al. (11). The tissue was transferred to 100 µl of fresh medium in 1-ml amber glass conical vials (Chromacol Ltd.). After transfer, Teflon-lined caps were applied and the samples were usually incubated for 4 or 8 h with shaking (120 rpm) on an orbital shaker at 25°C. Incubations were stopped by addition of 100 µl of hexane containing 10 pg/µl of farnesyl acetate as internal standard and vortexed at 3200 rpm for 2 min. The emulsion was broken by centrifugation at 10,000g for 5 min and the organic layer was removed with a 250-µl syringe. The aqueous layer was extracted an additional two times as above with 100-µl aliquots of hexane. The organic extracts were combined in a clean vial and a Teflon-lined crimp cap was applied prior to shipment on dry ice (to Gainesville, FL) for analysis.

Sexually mature female adults (8 days old) of the Caribbean fruit fly were obtained from colonies maintained at our laboratory. Hemolymph was collected from flies held in a wax-bottom dish with pins. A small slit was made down the dorsal midline of the thorax and the tip of a fused silica needle (0.15 mm, o.d.) held in a Hamilton 10-µl gas-tight syringe was inserted into the wound. Hemolymph was withdrawn and placed in a conical vial held on ice. After 15 µl of hemolymph had been collected (ca. 0.5 µl/fly), 135 µl of methanol and 100 µl of hexane containing 10 pg/µl of farnesyl acetate as internal standard were added and crimp caps were applied. The sample was vortexed and extracted as above. Although methyl-6,7,10,11-bisepoxy-3,7,11-trimethyl-(2E)-dodecenoate (JH IIIB), the bisepoxide analog of JH III, has been identified from extracts of in vitro incubations of the corpora allata from other cyclorrhaphous Diptera (12), we did not have pure synthetic samples of the diasteriomers of JH IIIB and chose not to analyze for the presence of JH IIIB. This is the subject of ongoing research.

Analysis of naturally produced JH. Prior to MS analysis, aliquots of samples were transferred to new vials and concentrated to appropriate volumes under a fine, gentle stream of purified N2. We routinely analyzed a series of dilutions of the JH–farnesyl acetate synthetic blend and calculated the regression equations for each compound based on intensities of the diagnostic ions prior to analyzing natural product samples. For samples from moths we analyzed initially 1–2 µl of each extract from 4-h incubations, representing approximately 1/10 of each sample. The remaining portions of these samples were combined and concentrated and an aliquot representing about 5-h equivalents of the pooled sample was analyzed. Then we analyzed about 1/4 of each sample which contained 8-h equivalents and 1/4 of the sample obtained by pooling these extracts (three replicates). We analyzed aliquots of the fruit fly hemolymph that represented about 1 µl of hemolymph.

RESULTS AND DISCUSSION

Analysis of synthetic compounds. The isobutanate CI spectra obtained for the JHs were significantly different from isobutanate CI spectra published elsewhere (13, 14). For example, ions at m/z 267 (M + H) and m/z 249 (M + H – HOH) for JH III were reported as major diagnostic ions by Mauchamp et al. (13) and Carlson and Borovsky (14) but had intensities of only 2 and 8% of the base ions in our analyses, making them of limited value for quantitative purposes. Additionally, the base ions of the JHs (M + H – CH3OH-HOH), determined from our analyses, were not evident in isobutanate CI spectra published by Mauchamp et al. (13) or Carlson and Borovsky (14) but were present in methane CI spectra obtained by Mauchamp et al. (13).

We selected six diagnostic ions for each JH homolog and the internal standard farnesyl acetate for quanti-
tative purposes (Table 1). Ions considered to be diagnostic for farnesyl acetate included m/z 81, 109, 121, 135, 149, and 205. Cleavages resulting in production of most of the diagnostic ions for the JHs have been reported elsewhere (13–16). However, spectra of the JHs obtained from our analyses also contained an intense fragment ion at m/z 111 (see Fig. 5). This novel ion has not been reported from either CI–MS (13, 14) or electron-impact mass spectrometric (EI–MS) analyses (15, 16). The intensities of m/z 111 were 45, 75, and 95% of the intensities of the base ions for JH I, JH II, and JH III, respectively, and its presence in spectra obtained from all three homologs indicated that it evolved from fragmentation before carbon 7 of the skeleton. Given these restrictions, the only possible formula for this fragment was C₇H₁₁O which would result

<table>
<thead>
<tr>
<th>Ion No.</th>
<th>Ion description</th>
<th>JH I</th>
<th>JH II</th>
<th>JH III</th>
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<tr>
<td>1</td>
<td>M + 1 – CH₃OH (from methyl ester)</td>
<td>263&lt;sup&gt;13,14&lt;/sup&gt;</td>
<td>249&lt;sup&gt;13,14&lt;/sup&gt;</td>
<td>235&lt;sup&gt;13,14&lt;/sup&gt;</td>
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<td>2</td>
<td>Ion 1 – HOH (from ring cleavage of epoxide)</td>
<td>245&lt;sup&gt;13,13&lt;/sup&gt;</td>
<td>231&lt;sup&gt;13,13&lt;/sup&gt;</td>
<td>217&lt;sup&gt;13&lt;/sup&gt;</td>
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<tr>
<td>3</td>
<td>Ion 2 – CO (from methyl ester)</td>
<td>217&lt;sup&gt;13,13&lt;/sup&gt;</td>
<td>203&lt;sup&gt;13,13&lt;/sup&gt;</td>
<td>189&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td>4a</td>
<td>Ion 1 – C₆H₄O₂ (from methyl ester) – C₆H₄O (from epoxide terminus)</td>
<td>161&lt;sup&gt;13&lt;/sup&gt;</td>
<td>147&lt;sup&gt;13&lt;/sup&gt;</td>
<td>147&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td>4b</td>
<td>Ion 1 – C₆H₄O₂ (from methyl ester) – C₆H₄O (from epoxide terminus)</td>
<td>153&lt;sup&gt;13&lt;/sup&gt;</td>
<td>139&lt;sup&gt;13&lt;/sup&gt;</td>
<td>125&lt;sup&gt;13&lt;/sup&gt;</td>
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<tr>
<td>5</td>
<td>M – C₆H₄O₂ (cleavage between C6 and C7)</td>
<td>111&lt;sup&gt;ms&lt;/sup&gt;</td>
<td>111&lt;sup&gt;ms&lt;/sup&gt;</td>
<td>111&lt;sup&gt;ms&lt;/sup&gt;</td>
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<sup>a</sup> Superscript numbers in parentheses refer to references for cleavage assignments.

<sup>b</sup> Ions having (ms) as superscript indicate that the cleavage assignment was determined from CI–MS/MS studies conducted by Finnigan Corp.

<sup>c</sup> Base ion.

![FIG. 2. Linear regression lines of ion intensity vs concentration of analyte obtained for each of six diagnostic ions and the sum of the ions for each JH homolog (a–c) and for farnesyl acetate (FA) (d). For all regressions, r² = 0.98.](image)
from scission between carbons 6 and 7 after loss of CH$_3$OH from the methyl ester end of the molecules. This assignment was confirmed from CI–MS–MS experiments in which data on the fragmentation of ions resulting from loss of CH$_3$OH (m/z 263, 249, and 235 for JH I, II, and III, respectively) were obtained. The principal ion generated from fragmentation of m/z 235 for JH III was m/z 111. Similarly, m/z 111 was a major ion in spectra obtained when analyzing fragmentation of m/z 249 (JH II) and m/z 263 (JH I). Differences in the relative intensity of m/z 111 between JH III (95% of base ion), JH II (75% of base ion), and JH I (45% of base ion) appear to result from hindrance due to the presence of one or two ethyl moieties in JH II and JH I, respectively.

Studies to determine the effect of using the cool-on-column injector or split/splitless injector for injection of farnesyl acetate and JH III indicated no differences in the amount of farnesyl acetate recovered. However, significantly less JH III (21.5 ± 4.4%, n = 6, t = 4.924) was recovered when using the split/splitless injector operated at 250°C. We attribute the loss of JH III to theromolysis of the epoxide when flash vaporized in the split/splitless injector at 250°C (18, 19), although no attempt was made to identify degradation products.

Results of studies in which we reduced the concentrations of the JHs and farnesyl acetate (Fig. 2) by serial dilution indicated that the intensities for each of the diagnostic ions decreased in a linear fashion. The ratio of each of the ion intensities for the lowest concentration analyzed (0.01 pmol) was not significantly different from that of the mean ion intensities for all concentrations analyzed (Fig. 3). Studies in which the combined ion intensities of farnesyl acetate were plotted against those of the JHs over all concentrations for all runs showed linear relationships for each of the JH analogs (data not shown). The fact that the ratios of ions remained constant over all concentrations and that there was a linear decrease in ion intensities with decreasing amounts of analyte allowed for determination of concentrations of JHs and farnesyl acetate present in naturally produced samples.

In vitro production of JH by Manduca sexta. In initial studies we analyzed about 1/10 of each extract obtained from 4-h incubations of the retrocerebral complex of M. sexta. The precise amount of each extract was determined by calculating the amount of internal standard (farnesyl acetate) present using regression equations generated from serial dilution experiments.
All six diagnostic ions for JH II and JH III were clearly evident in all samples and the ratios were comparable to the mean ratios obtained from analyses of synthetic standards. Mean amounts of JH II and III produced per hour for each of the diagnostic ions showed limited variability within samples but the amounts recovered from different individuals showed substantial variability, despite the fact that the retention time windows for JH II and JH III were essentially free of interferences (Fig. 4). JH I could not be identified concretely or quantified in these samples because all of the diagnostic ions for this compound were not present. However, JH I was identified from analysis of the pooled sample and accounted for 50.3 and 48.7% of the total JH. The fact that JH I was present in such small amounts, relative to JH II and III, provided an opportunity to determine if quantitative discrimination was occurring. To assess this we first determined the amounts of JH I, II, and III produced per hour by analyzing 1/4 of each sample which contained 8-h equivalents. These samples were then pooled and the pooled sample was analyzed. JH I was identified in each of the individual samples and the ratio of diagnostic ions was comparable with that determined from analysis of synthetic standards (Fig. 5). JH I accounted for an average of only 1.07% (min = 0.69%, max = 1.25%) of the total JH secreted. The average amounts of JH I, II, and III were 0.023 pmol/h (min = 0.011, max = 0.043), 1.065 pmol/h (min = 0.761, max = 1.674), and 1.043 pmol/h (min = 0.78, max = 1.722), respectively (Fig. 4). These average amounts were not significantly different (t test, P = 0.05) from the amounts of 0.033 (±0.007), 0.975 (±0.078), and 0.98 (±0.13) pmol/h calculated for JH I, II, and III from analyses of the pooled sample (Fig. 3) and are comparable to amounts reported by others (20). The data indicated that no discrimination occurred due to disparate amounts of the different JHs in the individual or pooled samples and that quantitation was accurate over a large concentration range.

Identification of JH III from hemolymph of Caribbean fruit flies. Application of the GC–MS technique to analysis of extracts obtained from in vitro incubations.
tions of the corpora allata demonstrated that the technique was applicable to analysis of naturally produced JH. However, we were interested in determining if it could be applied to analysis of extracts obtained from hemolymph because this allows for determination of circulating levels of JH. Hexane extracts of hemolymph from female flies were analyzed directly, without further purification, because we wanted to determine if we could identify JH despite the presence of large amounts of other lipids in the hemolymph. Analysis of aliquots representing about 1 μl of hemolymph revealed the presence of 21.1 pg (±3.7, n = 5) of JH III (Fig. 5). Neither JH I nor JH II was detected in the samples.

We have also used the technique to identify and quantify amounts of JH recovered from in vitro culture of the corpora allata from other insect species including the corn earworm and tobacco budworm moths and German and American cockroaches as well as from HPLC-purified extracts from whole-body extracts of Caribbean fruit flies. Slight modification of the technique, to include an ethyl acetate extraction and single-step diazomethane esterification (21), also allows for identification of JH acids present in samples. The technique has several advantages over currently used methods including high sensitivity, the ability to concretely identify and quantify amounts of individual JH homologs based on spectra over a wide mass range (60–350 u) rather than monitoring single ions, and limited sample preparation with no requirement for derivatization, which improves recoveries.

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![FIG. 5.](image-url) Comparison of CI–MS spectra obtained from analyses of synthetic and naturally produced JH I and JH III from moths and flies. Diagnostic ions used for quantitation are indicated on the spectra. (a) Spectrum (m/z 100–300) obtained from analysis of 59 pg of synthetic JH I. (b) Spectrum (m/z 100–300) of naturally produced JH I obtained from analysis of ca. 2.2-h equivalents of an extract obtained after in vitro incubation of the retrocerebral complex of the moth M. sexta. The mean amount of JH I calculated from data for the six ions was 5.7 pg (±1.4 pg). (c) Spectrum (m/z 100–270) obtained from analysis of 53 pg of synthetic JH I. (d) Spectrum (m/z 100–270) obtained from analysis of ca. 1-μl equivalent of the hexane extract obtained from hemolymph of female Caribbean fruit flies. The mean amount of JH III calculated from data for the six diagnostic ions was 16.3 pg (±3.5 pg).
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REFERENCES


