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Quantification of methyl farnesoate levels in hemolymph by high-performance liquid chromatography

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ABSTRACT

Methyl farnesoate (MF) is an acyclic sesquiterpenoid that has been detected in hemolymph and other tissues of crustaceans and insects. This paper describes a rapid and sensitive method for measuring MF in crustacean hemolymph. Extracts of hemolymph samples were separated by normal-phase high-performance liquid chromatography (5-μm silica, 250 × 4.6 mm I.D., 1.3% diethyl ether in hexane) and detected by UV (220 nm). The limit of detection with this method was less than 250 pg/ml. This method should be useful for studying the physiological functions of MF in crustaceans and other arthropods.

INTRODUCTION

Arthropods produce a number of sesquiterpenoids that have important physiological functions. One of these is methyl farnesoate (MF), a compound that is structurally related to insect juvenile hormone III (JH III). MF has been detected in hemolymph from embryos of the cockroach *Nauphoeta cinerea*, but its role in embryogenesis in unclear [1,2]. This compound is also an intermediate in JH III synthesis in many insects [3]. MF has been detected in the hemolymph and other tissues of crustaceans [4,5]. Recent reports suggests that MF has important roles in crustacean development and reproduction [6–10], roles that are analogous to those of JH in insects [11]. Such studies would be aided by measurements of MF levels in animals at different physiological states.

The most sensitive and specific method for quantifying MF levels uses gas chromatography–mass spectrometry (GC–MS) with selected ion-monitoring (SIM) [4]. This method has proven useful for the identification of MF in hemolymph and other tissues of several crustaceans [4, 5, 12]. However, the preparation and analysis of samples by GC–SIM-MS is time-consuming and cumbersome, and does not lend itself to the routine analysis of many samples.

Other methods, such as high-performance liquid chromatography (HPLC), also hold potential for quantifying MF. For example, reversed-phase HPLC was used to measure MF in embryos of the cockroach *Nauphoeta cinerea* [1]. However, this procedure was not particularly sensitive, requiring MF levels over 75 ng/ml. Since the MF levels in many crustaceans are lower than this amount [4], a more sensitive HPLC

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method is needed. We found that normal-phase HPLC, when coupled with a triphasic extraction procedure is a simple and reliable means of quantifying MF, with a limit of detection of less than 250 pg/ml. During the past two years, we have used this approach to quantify MF levels in hundreds of hemolymph samples from several crustaceans.

EXPERIMENTAL

Experimental animals

Male lobsters (*Homarus americanus*), female spider crabs (*Libinia emarginata*) and female green crabs (*Carcinus maenas*) were obtained from the Department of Marine Resources at the Marine Biology Laboratory, Woods Hole, MA, U.S.A. Animals were held in running sea water until used. The eyestalks of some lobsters were removed to elevate hemolymph levels of MF [13].

Chemicals

Unlabeled MF was obtained from Dr. D. A. Schooley (Zoecon Research Institute, Palo Alto, CA, USA) as a mixture of two isomers (approximately 70% 2*E*,6*E* and 30% 2*Z*,6*E*). The 2*E*,6*E* isomer was purified by normal-phase HPLC using the conditions decribed below for MF analysis. Radiolabeled [10-^{3}H]MF (2*E*,6*E*) with a specific activity of 1.44 Ci/mmol [14] was obtained from Dr. G. D. Prestwich (Dept. of Chemistry, SUNY, Stony Brook, NY, USA). HPLC grade acetonitrile and diethyl ether, iso-octane (99 mol% pure), and Optima-grade hexane were obtained from Fisher Scientific (Pittsburgh, PA, USA). Ethyl farnesoate (EF: used as an internal standard) was prepared from all *trans*-methyl farnesoate as described [4].

Sample preparation

Culture tubes with PTFE-lined screw caps were used to extract MF from hemolymph. The tubes were filled with 2.5 ml of acetonitrile, 0.5 ml hexane, 5 ng of EF and sufficient saline (0.9% NaCl) to give a final aqueous volume of 2.0 ml after the addition of hemolymph. Hemolymph (0.3 to 2 ml) was collected from individual animals, added to a culture tube, rapidly mixed, and chilled on ice. After a brief centrifugation (1000 g × 5 min), three phases were observed in each tube: a clear, upper layer containing hexane; a middle yellow-colored layer containing primarily acetonitrile; and a bottom aqueous layer. In samples from animals in late premolt, the middle phase was occasionally small or absent. Acetonitrile was added to these sample until the middle and lower layers were approximately equal in volume. The top (hexane) layer was removed, a second volume of hexane added, and the extraction repeated. The second hexane layer was removed and added to the first. In some cases the volume of the combined hexane layers was reduced with a Savant Speed-Vac concentrator so the entire sample could be analysed in a single injection.

High-performance liquid chromatography

Samples were injected manually or with an autosampler (Spectra-Physics 8780) and analyzed by normal-phase HPLC using a silica column (Econosil SI: 5 μm, 250 × 4.6 mm I.D.; Alltech, Deerfield, IL, USA) with a silica precolumn. Eluting material was detected with a Beckman 166 programmable detector (220 nm) and the output

analyzed by either a Beckman System-Gold or a Jandel JCL6000 chromatography data program. The solvent (1.3% diethyl ether in hexane, 2.5 ml per min) was selected to give a retention time for MF of about 5 min. The retention times of MF and EF were determined every 2 or 3 h by injecting MF and EF standards. Because this method has a low limit of detection, small amounts (usually 2 ng each) of MF and EF were used to calibrate the column. This avoided the contamination of unknown samples that can occur when large amounts of standard are used for calibration [15]. After each sample, the column was rinsed with an additional 50 ml of the eluting solvent to remove contaminants.

The amount of MF in each sample was determined by comparing either the peak height or the peak area of the sample to that of EF. In some cases, the eluate was collected from the beginning of the EF peak to the end of the MF peak. After reducing the solvent volume to <50 μl with a Speed-Vac, each sample was dried manually with nitrogen and resuspended in 5 μl of octane for analysis by GC–MS.

Gas Chromatography–mass spectroscopy

The GC–MS analysis was similar to that described previously [4]. An aliquot (0.5 μl) of the resuspended sample was injected into a Hewlett-Packard MSD (Model 5790A/5970) with an Alltech RSL-150 (0.25 μm film, 0.25 mm I.D., 25 m) capillary column. EF was used as an internal standard. MF levels were determined by selected ion monitoring (SIM) using an ion common to both MF and EF (m/z 69) and the analogous ion pair (m/z 114 and 128).

RESULTS

Initial studies indicated that normal-phase HPLC could detect and quantify low levels of MF standards. However, the quantification of MF in total lipid extracts [16] of hemolymph proved difficult due to the presence in interfering (*i.e.* UV-absorbing) compounds. As a solution to this problem, other extraction procedures were tested. We found that the treatment of hemolymph with acetonitrile, saline, and hexane formed a triphasic solution which removed these interfering compounds from the upper (hexane) phase containing MF.

The effectiveness of this triphasic extraction (TE) procedure could be monitored by observing the distribution of yellow organosoluble pigments found in many of the samples. When the ratio of water to acetonitrile was high (3:1), the solution was biphasic and the yellow pigments were found in the upper (hexane) phase. When the ratio of water to acetonitrile was lower (4:5), the solution was triphasic and the yellow pigments were in the middle (acetonitrile) phase. Chilling the extract also helped reduce the amount of interfering materials in the hexane phase. The presence of methanol in the extraction increased the amount of interfering materials.

The TE procedure was compared with the total lipid extraction procedure [16] using samples ($n=4$) of lobster hemolymph. The hexane phase of the TE procedure contained 65.7% [$\pm$ 6.4 (S.E.M.)] of the extractable mass and 64.9% [$\pm$ 9.9 (S.E.M.)] of the UV-absorbing material (at 220 nm) found in total lipid extracts. Thin-layer chromatographic analysis of the hexane phase indicated that the TE procedure removed fatty acids and sterols and reduced the level of triglycerides, all of which are quantitatively recovered in total lipid extracts. MF recovery in the hexane phase (assessed by

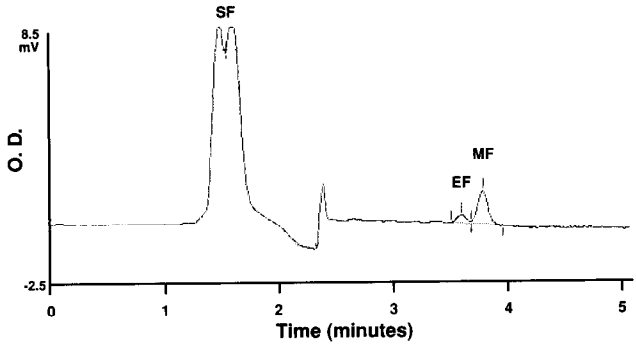

Fig. 1. HPLC detection of methyl farnesoate (MF) in lobster hemolymph. An amount of 5 ng of ethyl farnesoate (EF) was added to a hemolymph sample (0.5 ml) as an internal standard. The quantity of MF detected in this figure is approximately 1.8 ng; the concentration of MF in this animal was 18.4 ng/ml.

the addition of [^{3}H]-MF) was >95% using the TE procedure. Most important, TE coupled with normal-phase HPLC provided a sensitive method for quantifying MF in hemolymph samples (Fig. 1). MF and EF were resolved by normal phase HPLC, allowing the latter to be used as an internal standard.

The quantification of MF by this method was tested in several ways. Its linearity was examined by extracting multiple samples (from 0.1 to 2 ml) of hemolymph from each of three lobsters. The amount of MF detected increased linearly with increasing amounts of hemolymph, indicating that the quantification of MF was not affected by the volume of hemolymph used (Fig. 2). This method was further validated by comparing the amount of MF detected by normal-phase HPLC with the amount detected by GC–MS. As shown in Fig. 3, an excellent correlation (r = 0.963) was obtained between these two methods for hemolymph samples from three crustacean species.

The accuracy of this method was determined by measuring MF standards over a wide range of levels. MF was accurately measured between 250 pg and 25 ng per

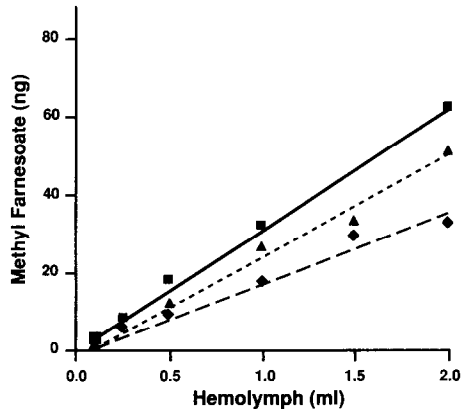

Fig. 2. Linearity of methyl farnesoate detection. Increasing volumes of hemolymph from each of three lobsters were analyzed separately by normal-phase HPLC.

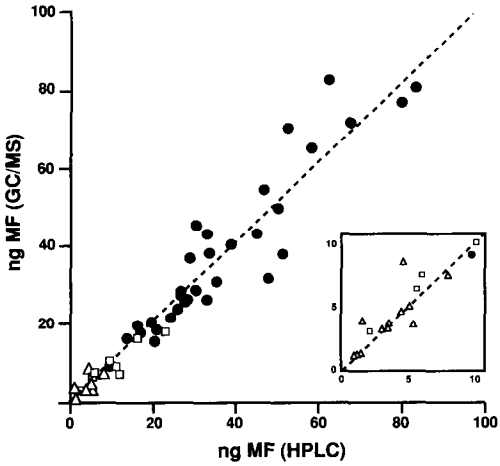

Fig. 3. Correlation between methyl farnesoate values determined by HPLC and by GC–MS. Hemolymph samples from *Homarus americanus* ($n=32$; ●), *Libinia emarginata* ($n=12$; △), *and Carcinus maenas* ($n=8$; □) were analyzed by normal-phase HPLC and by GC–MS. The correlation coefficient was 0.983, and the slope of the line was 1.03.

injection (Table I). In all cases, the amount of MF injected and detected differed by less than 10%. The precision of this method was examined by collecting hemolymph from three lobsters with low, intermediate and high levels of MF (*ca.* 2, 15, and 43 ng/ml, respectively). The hemolymph from each animal was divided into three equal aliquots, which were separately extracted and analyzed for MF. As shown in Table II, the data showed small coefficients of variation (1.2 to 5.3) for the replicate aliquots from each animal.

DISCUSSION

This paper describes and validates a sensitive normal-phase HPLC method for measuring MF levels in the hemolymph of crustaceans. This method is simple and rapid, allowing the MF level of a single sample to be determined within an hour of

TABLE I

ACCURACY OF METHYL FARNESOATE DETECTION

Samples of methyl farnesoate standard were analyzed by HPLC. The amount of MF detected (± S.E.M., $n=3$) is indicated.

Injected (ng)	Detected (ng)	% Difference
0.25	0.27 ± 0.01	8.0
0.50	0.52 ± 0.04	4.0
1.00	1.07 ± 0.01	7.0
5.00	5.30 ± 0.13	6.0
25.00	23.55 ± 0.34	6.0

TABLE II

PRECISION OF METHYL FARNESOATE DETECTION

Hemolymph samples were collected from three lobsters (A, B and C), and each sample divided into three 1-ml aliquots. Each aliquot was analyzed once by HPLC, and the amount of MF in each hemolymph sample (± S.E.M., $n=3$) calculated.

Sample	MF detected (ng/ml)	Coefficient of variation
A	2.1 ± 0.03	1.5
B	15.3 ± 0.8	5.3
C	43.3 ± 0.5	1.2

collection. When used with an autosampler and an automatic data collection system, over 40 samples a day can be quantified. In hemolymph samples from three crustacean species, the data obtained by HPLC were comparable to those obtained by the more selective and rigorous method of GC–MS. In addition, the accuracy and precision of HPLC were similar to GC–MS methods for related compounds [17].

Part of the precision of this method results from the use of an internal standard in each sample, a strategy that has also been used in a GC–MS method for MF [4]. Though we used EF in our studies, it seems likely that other compounds such as the 2*Z*,6*E* isomer of MF, would also be useful. The addition of internal standard to each sample allowed us to correct for occasional losses that occurred during sample preparation as well as possible changes in detector sensitivity. However, the efficiency of the extraction procedure (>95% recovery of MF) and the stability of the detector usually meant that these corrections were small. The internal standard was also useful in samples with low levels of MF (<0.5 ng), where the EF peak helped identify the MF peak.

The HPLC conditions used caused MF and EF to elute rapidly (< 5.0 min) and resolved the two compounds satisfactorily for purposes of quantification. Better resolution of MF and EF can be obtained with solvents containing a lower percentage of diethyl ether in hexane. However, this approach increases the cycle time and raises the limit of detection of the procedure, making it difficult to detect MF in samples with low levels of this compound.

The internal standard (5 ng of EF) was added to each sample. This amount allowed the reliable quantification of MF at levels between 0.25 ng and 25 ng of MF per injection. Since the retention times of EF and MF are close, higher quantities of MF tend to obscure the EF peak. For samples that have consistently high levels of MF, this problem can be resolved by using larger amounts of EF. Alternately, the quantity of MF can be calculated by comparing the size of the MF peak in an unknown sample to that of the MF standard. Of course, this approach does not allow correction for losses during sample preparation or for changes in detector sensitivity.

The low limit of detection achieved by this HPLC method is partly the result of the extinction coefficient of MF, which is sufficiently high to allow small amounts (<0.25 ng) of this compound to be detected after separation on normal-phase HPLC. In addition, the entire sample can be analyzed by normal-phase HPLC. Thus, the overall detection limit of this method is similar to that of GC–MS where only a small fraction of the sample is usually injected.

Probably the most important factor in achieving high sensitivity by this method was the elimination of interfering compounds. This was accomplished using the TE procedure. Samples prepared by other extraction methods were unsatisfactory due to these compounds. Although the reduction in the extractable mass and UV-absorbing materials was modest (30% and 40%, respectively), the TE procedure succeeded in removing those contaminants from hemolymph that interfered with MF quantification.

Nevertheless, the extracts still contained major contaminants which elute after MF. These contaminants were removed by rinsing the column between samples. Since the eluting solvent was used during this rinsing period, the retention times of MF and EF were not affected. Most contaminants appeared to be removed during the rinse, but some did remain on the column. These eventually decreased column resolution. However, most silica columns remained useful for over 1000 injections, especially when care was taken during sample preparation to avoid the underlying acetonitrile phase when the hexane phase was removed. Although this rinsing procedure lengthened the time required for the analysis of each sample, it required no further manipulation of the sample after pooling the two hexane extracts. An alternate approach would be to remove these contaminants with a mini-column separation prior to HPLC. In our experience, the time and expense required to remove the contaminants outweigh the advantages of a shorter analysis time.

It should be noted that this method does not provide the experimenter with a rigorous identification of MF, since UV detectors are relatively non-specific. We have found that this method gives good results for several crustaceans, but it may prove unusable for other species. Thus, it is advisable to validate this HPLC method by GC–MS or some other means for each new species. Nevertheless, we suspect that this HPLC method, with perhaps some minor modifications, will prove useful for many, if not most, crustaceans. We have also used a modification of this method to measure JH levels in insect hemolymph [18]. Thus, normal-phase HPLC coupled with the TE procedure proved to be a valuable analytical approach for the study of MF and related compounds in many arthropods.

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REFERENCES

1 E. Bruning, A. Saxer and B. Lanzrein. *Int. J. Invertebr. Reprod. Dev.*, 8 (1985) 269.
2 E. Bruning and B. Lanzrein, *Int. J. Invertebr. Reprod. Dev.*, 12 (1987) 29.
3 D. A. Schooley and F. C. Baker, in G. A. Kerkut and L. I. Gilbert (Editors), *Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Vol. 7, Endocrinology*, Pergamon Press, Oxford and Elmsford, NY, 1985, p. 363.
4 D. W. Borst, H. Laufer, M. Landau, E. S. Chang, W. A. Hertz, F. C. Baker and D. A. Schooley, *Insect Biochem.*, 17 (1987) 1123.

5 H. Laufer, D. W. Borst, F. C. Baker, C. Carrasco, M. Sinkus, C. C. Reuter, L. W. Tsai, and D. A. Schooley, *Science (Washington, D. C.)*, 235 (1987) 202.
6 C. R. Paulson and D. M. Skinner, *Am. Zool.*, 28 (1988) 83a.
7 B. Tsukimura and F. I. Kamemoto, *Am. Zool.*, 28 (1988) 63a.
8 M. D. Brody and E. S. Chang, *Am. Zool.* 29 (1989) 62a.
9 J. M. Vogel and D. W. Borst, *Am. Zool.*, 29 (1989) 49a.
10 D. W. Borst and H. Laufer, in A. P. Gupta (Editor), *Morphogenetic Hormones of Arthropods: Discoveries, Syntheses, Metabolism, Evolution, Modes of Action, and Techniques*, Rutgers University Press, New Brunswick, NJ, 1990, p. 35.
11 A. K. Kumaran, in A. P. Gupta (Editor), *Morphogenetic Hormones of Arthropods: Discoveries, Syntheses, Metabolism, Evolution, Modes of Action, and Techniques*, Rutgers University Press, New Brunswick, NJ, 1990, p. 181.
12 S. S. Tobe, D. A. Young, H. W. Khoo and F. C. Baker. *J. Exp. Zool.*, 249 (1989) 165.
13 E. F. Couch, D. W. Borst, H. Laufer and J. K. Butler, *Am. Zool.*, 27 (1987) 68a.
14 W.-S. Eng and G. D. Prestwich. *J. Labelled Compd. Radiopharm.*, 25 (1988) 627.
15 F. C. Baker, in A. P. Gupta (Editor), *Morphogenetic Hormones of Arthropods: Discoveries, Syntheses, Metabolism, Evolution, Modes of Action, and Techniques*, Rutgers University Press, New Brunswick, NJ, 1990, p. 391.
16 J. Folch, M. Lees and G. H. Sloane Stanley, *Biol. Chem.*, 226 (1957) 497.
17 B. J. Bergot, M. Ratcliff and D. A. Schooley, *J. Chromatogr.*, 204 (1981) 231.
18 D. W. Hunnicutt and D. W. Borst, unpublished results.