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LIQUID CHROMATOGRAPHIC PROCEDURES FOR THE ANALYSIS OF COMPOUNDS IN THE SEROTONERGIC AND OCTOPTAMINE PATHWAYS OF LOBSTER HEMOLYMPH

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SUMMARY

High-performance liquid chromatography, with serial electrochemical and ultraviolet detectors, was used with a reduced activity catecholamine C_{18} column to separate and quantify compounds important in the serotonergic and octopamine pathways in lobster hemolymph. The chromatographic mobile phase was composed of potassium dihydrogenphosphate buffer, trichloroacetic acid, sodium dodecyl sulfate, the sodium salt of ethylenedinitrilotetraacetic acid and the organic solvents, acetonitrile and methanol. The compounds serotonin, 5-hydroxyindoleacetic acid, tryptophan, 5-hydroxytryptophan, tryptamine, melatonin, octopamine and tyrosine were well resolved within 13 min. Good electrode maintenance, the use of a silica gel precolumn and careful sample preparation were necessary to give a stable baseline, high resolution of these compounds and reproducibility of retention times and peak heights. The electrochemical detector extended the range of detection to the picogram level. Because of the instability of the solutes and of the chromatographic baseline, sample preparation procedures were investigated. Deproteinization with ammonium sulfate gave the best recovery of the compounds of interest and the most stable baseline with the electrochemical detector. Peaks in the hemolymph were characterized by addition of standards, dual detection (electrochemical and ultraviolet) and the enzyme peak shift technique. With this methodology, important endogenous neurohormones in the hemolymph of lobsters can be quantitatively determined with respect to the molt cycle.

INTRODUCTION

In order to define the chemical basis for the molt-related changes in the behavior of lobsters, it is necessary to monitor changes in the concentrations of a unique group of compounds in the hemolymph of these crustaceans. Among the compounds which can act as neurotransmitters are the amines, serotonin and octopamine[1–5]. Other compounds of interest are the precursors or catabolites of serotonin and octopamine: 5-hydroxyindoleacetic acid, tryptophan, 5-hydroxytryptophan, melatonin, tryptamine and tyrosine. (Figs. 1 and 2).

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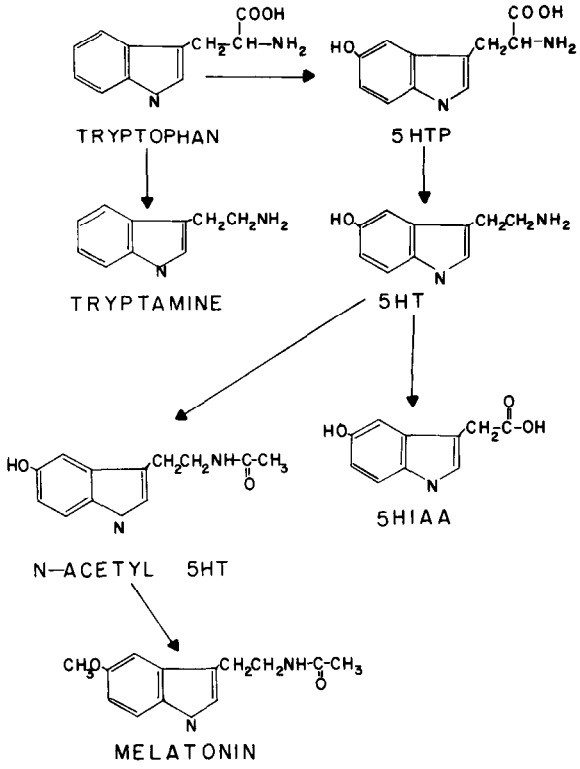

Fig. 1. Metabolic pathway of serotonin in lobster. 5HTP = 5-hydroxytryptophan, 5HT = serotonin and 5HIAA = 5-hydroxyindoleacetic acid.

However, many problems have been encountered in analyzing biological samples for this particular set of compounds. Some of the compounds are extremely labile to light or heat, others to acid. The sample volumes available are usually very small and may contain relatively large amounts of one of the compounds and only trace amounts of others; for example some of the endogenous neurohormones are present in the hemolymph in amounts as low as 10^{-10}. Moreover, some of these

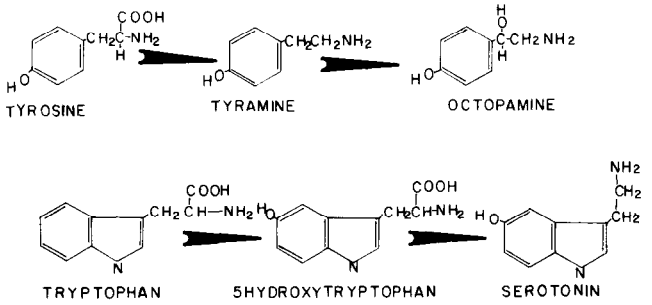

Fig. 2. Precursors in the biosynthesis of octopamine (top). Precursors in the biosynthesis of serotonin (bottom).

compounds may be present in the hemolymph in the free form and some bound to a high-molecular-weight protein or a low-density lipoprotein[6–8].

Although good procedures for reversed-phase liquid chromatography using electrochemical detection (RPLC–ED) have been reported for the analysis of serotonin and its metabolites[9–13], no analytical procedures have been published for the determination of trace amounts of these compounds together with octopamine. Therefore, we developed an RPLC–ED method which can be used for the analysis of any or all of these compounds. Using a solution of standards, this method gave us the required sensitivity and resolution. However, when the method was applied to samples of hemolymph, the sensitivity of the assay was reduced because of noisy baselines. In addition, some of the compounds of interest were lost in the sample preparation steps. Therefore, methods of sample preparation were also investigated in order to find the method which was most compatible with the RPLC–ED analysis. The requirements for the sample preparation method are maximum solute recovery, high degree of deproteinization and baseline stability.

EXPERIMENTAL

Instrumentation

The chromatographic instrumentation consisted of an M6000 reciprocating pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a Rheodyne 7125 injection port and 6-μl injection loop. All separations were performed on a 15 cm $\times$ 4 mm I.D. reduced activity catecholamine analytical column of 5 μm particle size (Perkin-Elmer, Norwalk, CT, U.S.A.) prededed by a guard column containing 30–38 μm C_{18} packings (Whatman, Clifton, NJ, U.S.A.). A pre-column, uniformly packed with silica gel, 37–53 μm (Whatman), was incorporated in the system between the pump and the injector. Detection was based on a Perkin-Elmer LC15 UV detector at 254 nm in series witha Perkin-Elmer LC-17 electrochemical detector. The electrochemical detector utilized an LC-4B amperometric system with Ag/AgCl reference electrodes and glassy carbon working electrodes. A strip chart recorder and an integrator were used to obtain simultaneous signal recordings from both detection devices. The integration system used was a 3380A Hewlett-Phackard (Avondale, PA, U.S.A.).

Reagents and standards

Chromatographic standards were octopamine (*dl*-aminomethyl-4-hydrobenzyl alcohol), serotonin (5-hydroxytryptamine), tryptophan, 5-hydroxyindoleacetic acid, tryptamine, melatonin and 5-hydroxytryptophan. They were obtained from Sigma (St. Louis, MO, U.S.A.). The retention times of the phenylalanine and tyrosine were also determined in an attempt to identify peaks in the lobster hemolymph. The enzymes monoamine oxidase (MAO), pyridoxal-5′-phosphate apotryptophanase and phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28), used in peak identification, were purchased from Sigma. Trichloroacetic acid (TCA) was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.), dibasic anhydrous sodium phosphate from Matheson, Coleman and Bell (Norwood, OH, U.S.A.), ethylenedinitrilotetraacetic acid disodium salt (EDTA) from Mallinckrodt (St. Louis, MO, U.S.A.) and sodium

dodecyl sulfate (SDS) from Sigma. HPLC-grade acetonitrile and methanol were purchased from EM Science (Gibbstown, NK, U.S.A.). All the water for preparation of the eluents and standard solutions was doubly distilled, deionized and then filtered through 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A.).

Chromatographic conditions

All separations were performed isocratically at ambient temperature. The mobile phase consisted of 0.02 *M* TCA, 1.5 μM EDTA, 2.2 m*M* SDS, 0.070 *M* dibasic anhydrous sodium phosphate and 20% acetonitrile and 12% methanol as organic modifiers; a modification of the mobile phase reported by Martin *et al.*[8]. The pH of the mobile phase was adjusted to 7.4 with acetic acid–water (50:50). This solvent system was degassed with a stream of helium and pumped at a flow-rate of 0.8 ml/min.

Samples of the standard mixture, the hemolymph and the hemolymph with internal standards, respectively, were injected onto the column in 7-μl aliquots to insure that the injection loop was filled. The UV absorbance was monitored at a wavelength of 254 nm and sensitivity was varied from 0.004 to 0.064 a.u.f.s. to achieve simultaneous quantitation of the hormones present in widely varying concentrations in the hemolymph. The electrochemical oxidation was held at an applied potential of 0.90 V and sensitivity varied from 5 to 500 nA according to detection requirements of different compounds.

Standard curve and detection limits

A standard curve was prepared using both peak height and peak area *vs.* concentration of serotonin and octopamine. Concentrations ranged from 10^{-12} to 10^{-3} *M*. The lower limits of detection were determined; a signal was defined as detected if it was two times the square root of the noise.

Degradation study

To determine the stability of serotonin and octopamine to light and temperature respectively, a standard mixture of the two compounds (10^{-3} *M* in each one) was allowed to stand at room temperature and in indirect sunlight for intervals of 10 min, 20 min, 30 min, 2 h, and 24 h. After each interval, a 7-μl sample was injected onto the column. The observed peak shape was qualitatively and quantitatively compared to a control chromatogram where the sample was iced and shielded from all light. Peak height, for each interval, was also determined and compared to the control.

Hemolymph collection

Lobsters were agitated for 60 s to control effects due to variability in handling. They were then chilled for 30 min. The reduced temperature simultaneously slowed the blood clotting while minimizing degradation of the compounds of interest. Blood was drawn with claws elevated to drain blood posteriorly. A cold 18-gauge needle was inserted in the dorsal abdominal vessel between the cephalothorax and the first abdominal segment (pericardial cavity). Care was taken to draw the blood from only the immediate pericardial region, not the digestive area anteriorly adjacent. Blood was centrifuged in a Sorvall refrigerated centrifuge with a SS-34 rotor, 2500 rpm (600 *g*) for 5 min at 4°C to remove cell membranes. The supernatant (the hemolymph) was decanted off, divided into labeled vials and stored at −50°C until HPLC analysis.

Sample preparation

In preparing hemolymph for chromatographic analysis, all compounds with a molecular weight greater than 25 000 were removed. Several methods of hemolymph deproteinization were investigated to obtain optimal separation, high recovery of the compounds of interest and baseline stability. The methods investigated included deproteinization with strong acid, strong acid and heparin to facilitate precipitation of lipoproteins and stabilize the baseline[6,7], and "salting out" with ammonium sulfate[14]. Since deproteinuzation with saturated ammonium sulfate gave the best baselines and recovery of all the compounds of interest, only this method of sample preparation will be described.

Hemolymph (1 ml) was added to 1 ml of saturated ammonium sulfate (25°C); the tube was covered with parafilm and immediately iced (5°C). Samples were shielded from light in a black, ice container to inhibit the breakdown of serotonin, a light sensitive compound, and of octopamine, which breaks down rapidly at temperatures exceeding 10°C. Tubes were vortexed for 2 min. The samples were then centrifuged at 600 *g* in a Clini-Cool general purpose refrigerated bench top centrifuge (Damon, IEC Division, Needham Heights, MA, U.S.A.) at 4382 rpm for 20 min to insure complete removal of the protein precipitate and full recovery of the neurohormones. The rotating radius was 12.5 cm and the temperature of operation was 4°C.

The supernatant was poured into ultrafiltration cones (Amicon, Danvers, MA, U.S.A.) 25 000 MW, 5 ml volume capacity, dimensions of 137 × 32 mm, and operating limit of 5000 rpm. The cones were soaked in doubly distilled, deionized water for at least 1 h before use. The samples were then recentrifuged for 15 min. The filtrates were poured into labeled glass tubes which were immediately placed in the ice container. Only one sample was prepared at a time to minimize degradation of the solutes of interest.

Aliquots of the standards alone (10^{-6} *M*), hemolymph alone and a hemolymph-standards mixture were chromatographed before and after the sample preparation procedures to determine the effects of the ammonium sulfate treatment on the baseline and the percent loss of the compounds of interest caused by the sample preparation steps.

Peak identification

Peaks in the chromatogram of the hemolymph sample were tentatively identified by the comparison of the retention time (t_R) of the peak with that of a known compound. Second, peaks were characterized by adding sequentially known amounts of standard compounds to the hemolymph. Next, the chromatographic peaks were characterized on the basis of dual detection using both UV and electrochemical detection.

Finally serotonin, octopamine, tryptophan and 5-hydroxyindole acetic acid were identified by the enzyme peak shift technique[15] which is based on the incubation of a compound with its respective degradation enzyme; thus the peak in question is shifted to its enzymatic end product. For all enzyme peak shift studies, the following samples were chromatographed: (1) standard solution, (2) standard solution plus enzyme mixture, (3) deproteinized hemolymph and (4) deproteinized hemolymph plus enzyme mixture. For the identification of serotonin, the enzyme monoamine oxidase was used; for tryptophan, apotryptophanase; for octopamine, phenylethanolamine N-methyl transferase; and for 5-hydroxyindole acetic acid, pyridoxal-5′-phosphate.

TABLE I

RETENTION TIMES AND ABBREVIATIONS OF POSSIBLE COMPOUNDS OF INTEREST

Peaks at 4.08 min (peak X), and 6.47 min (peak S) were found in the chromatograms of the samples of hemolymph from lobsters in some of the molt stages. These peaks have not yet been identified.

Compound	*Abbreviation*	*Retention time (min)*
5-Hydroxyindole acetic acid	HIAA	2.03
5-Hydroxytryptophan	HTP	2.12
Tyrosine	TYR	2.37
Tryptophan	TRP	3.13
Octopamine	OA	5.46
Melatonin	MEL	6.31
Phenylalanine	PHE	10.45
Tryptamine	TRY	11.52
Serotonin	5-HT	12.37

RESULTS

Good resolution of all the compounds of interest was achieved using isocratic elution (Table I). Temperature control was critical for the maintenance of good resolution and reproducibility of retention times. Fig. 3 shows a chromatogram of serotonin, octopamine, tryptophan, 5-hydroxytryptophan, 5-hydroxyindole acetic

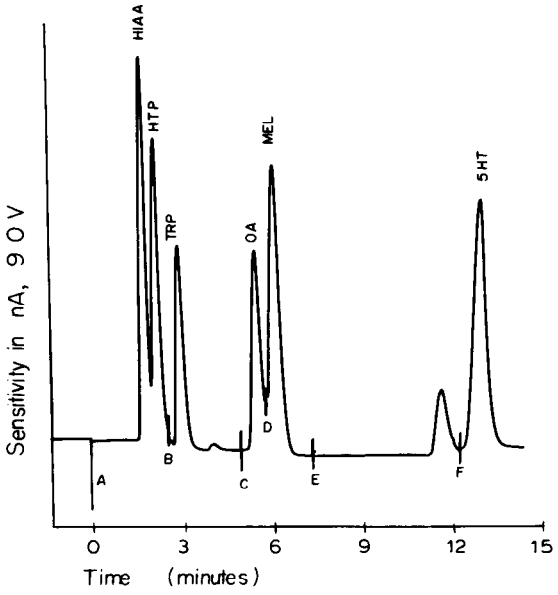

Fig. 3. HPLC–ED separation of a standard 10^{-6} *M* mixture of 5-hydroxyindole acetic acid (HIAA), 5-hydroxytryptophan (HTP), tryptophan (TRP), octopamine (OA), melatonin (MEL), tryptamine (TRY) and serotonin (5HT). Chromatographic conditions as described in Experimental. Sensitivity for sections of the chromatogram are: (A) 100 nA f.s., (B) 50 nA f.s., (C) 20 nA f.s., (D) 50 nA f.s., (E) 100 nA f.s., (F) 50 nA f.s.

acid, melatonin and tryptamine. There was baseline resolution of all peaks except for the octopamine and melatonin peaks.

In order to achieve optimal detection limits of all the peaks in different biological samples, the attenuation in the UV and the sensitivity of the electrochemical detector were altered throughout the separation as noted in the lettered chromatogram (Fig. 3). The stability study showed that degradation of the octopamine started within 10 min upon exposure to 25°C (room temperature) and that serotonin began to deteriorate within 10 min upon exposure to indirect sunlight. After 10, 30, and 120 min and 24 h exposure, 91.6, 95.3, 31.8 and 2.9% of the octopamine concentration remained after a standard solution was held at room temperature. When serotonin was placed in indirect sunlight, 56.0, 58.7, 32.3 and 14.7% of the control remained after 10, 20, 30, and 120 min intervals, respectively (Fig. 4).

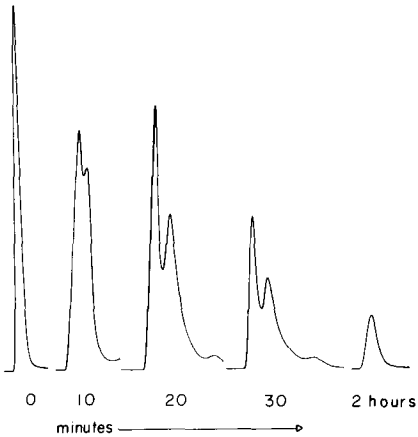

Fig. 4. Degradation of the serotonin peak upon exposure to indirect sunlight. The peak height decreased progressively with time of exposure in comparison to the peak height of the control. 500 nA f.s., applied potential 90 V. Chromatographic conditions as given in Experimental.

There was good linearity of response. Linearity is maintained down to 9 pg for octopamine when electrochemical detection was used and to 0.9 ng with UV detection ($r = 0.9996$). With the serotonin, the detection limit was 0.1 pg with ED. The limiting factor in detection with the electrochemical detector is not only the peak response but also the loss in baseline stability (Fig. 5).

The recovery of the neurohormones, octopamine and serotonin, after undergoing sample preparation and handling procedures was 98% for the octopamine and 98% for the serotonin. The recoveries were calculated on the basis of the percent difference in peak height before and after sample preparation procedures were carried out on the hemolymph samples to which known amounts of the model compounds were added. With our RPLC–ED method the relative concentrations of tryptophan, serotonin, 5-hydroxyindole acetic acid, and octopamine were monitored in seven molt stages. These peaks were identified by the enzyme peak shift technique (Fig. 6). A chromatogram of the hemolymph of a lobster in a different stage of the molt cycle

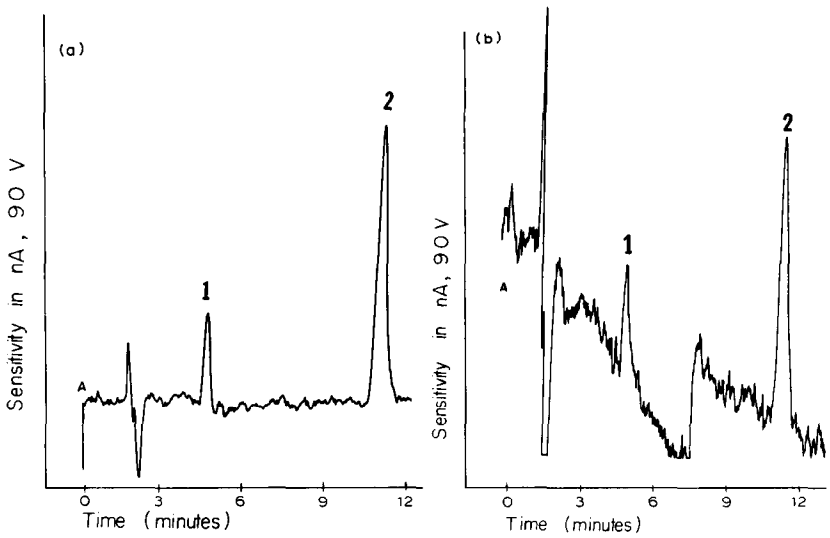

Fig. 5 (a) Detection limits for octopamine (peak 1) and serotonin (peak 2) obtained at 10 nA f.s. and a 10^{-9} *M* solution. (b) Chromatogram obtained using a 10^{-12} *M* solution when at a sensitivity of 5 nA f.s.

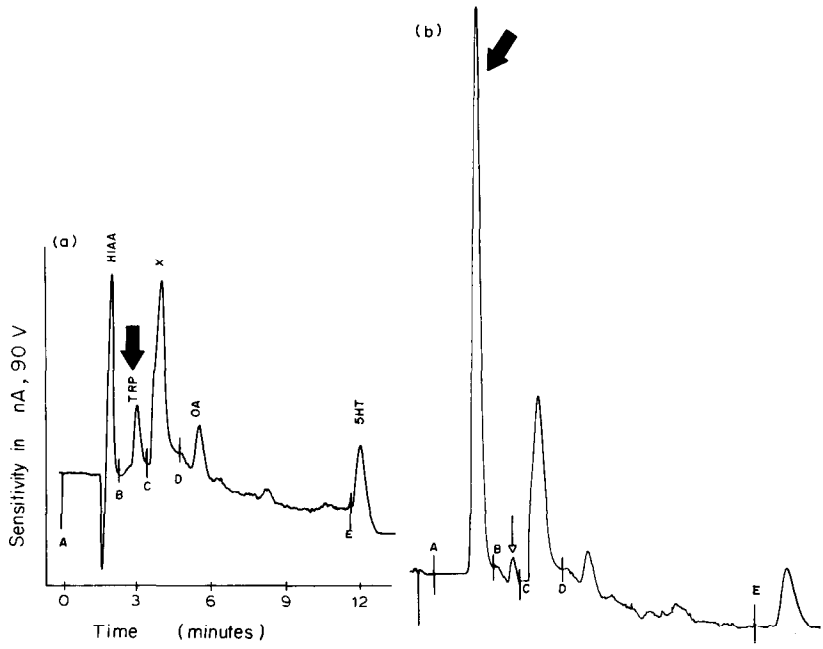

Fig. 6. Peak identification of tryptophan using the enzyme peak shift technique with the enzyme apotryptophanase. (a) Chromatogram of hemolymph. (b) Chromatogram of the hemolymph after incubation for 13 min with apotryptophanase. Note the increase in the HIAA and the decrease in TRP. The letters denote the sensitivity for the designated section of the chromatogram as described in Fig. 3.

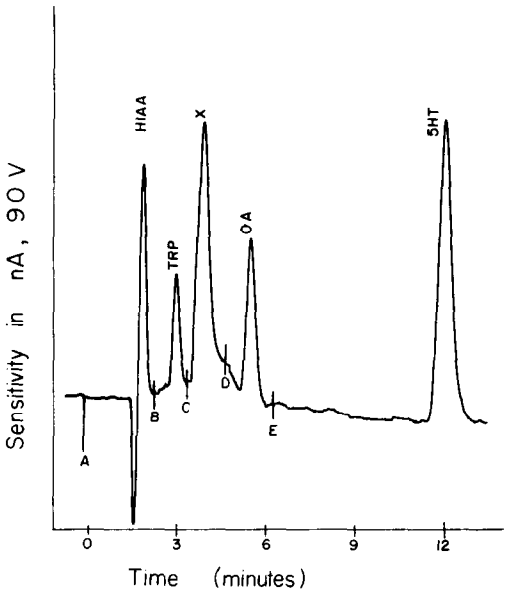

Fig. 7. Chromatograms of hemolymph from lobsters in a different molt stage from that in Fig. 6a. Note the differences in the relative amounts of TRP, 5-HT, OA and peak X in the two chromatograms. For abbreviations of the compounds, see Fig. 3. The chromatographic conditions are given in Experimental. The letters denote the sensitivity for the designated section of the chromatogram as described in Fig. 3.

than that in Fig. 6a is shown in Fig. 7. Note the relative differences in the peak heights of tryptophan, octopamine, serotonin and peak X. No tyrosine, 5-hydroxytryptophan, tryptamine, phenylalanine or melatonin were detected in any of the samples analyzed. Changes in two peaks (called peak X and peak S), which are not yet identified were observed in the hemolymph from lobsters in some of the stages of the molt cycle. Peak X had a retention time of 4.08 min and peaks S of 6.47 min. The endogenous levels of the compounds in each stage with respect to the behavioral aspect most relevant for that molt stage will be reported in the near future.

DISCUSSION

Three primary factors were found to influence the quantitation, resolution, and reproducibility of the HPLC–ED chromatograms of the compounds in the serotonergic and octopamine pathways: (1) electrode maintenance, (2) sample preparation, and (3) mobile phase composition. Electrode maintenance for both the glassy carbon working electrode and Ag/AgCl reference electrode is critical to insure peak height and shape reproducibility. Peak height is directly correlated with the oxidative properties of a compound. These properties are altered when the electrode is not properly maintained. The glassy carbon electrode must be polished weekly with aluminum oxide to prevent accumulation of contaminants which can reduce the available oxidation surface and hence cause peak height variance. The Ag/AgCl electrode must be stored in 3 *M* sodium chloride to prevent leakage of the chloride solution through

the seals. Leakage quickly ensues if electrodes are left overnight in the acetonitrile mobile phase. In addition the baseline is more stable and the background noise reduced when a small amount of EDTA is added to the mobile phase.

Sample preparation was also a key factor which determined the precision of the concentrations of endogenous octopamine and serotonin. Our protocol provides a high recovery of trace amounts of circulating neuroactive compounds. In order to provide better recovery than that previously reported for octopamine and serotonin, which are very labile, special care must be taken to maintain the samples at 4°C and to shield the samples from light. If these precautions are taken, good reproducibility in peak height and shape can be obtained. Even a slight delay time between processing the samples and injection of aliquots onto the column can introduce variability due to decomposition of octopamine and serotonin.

Lastly, because a complex mobile phase consisting of phosphate buffer, TCA, SDS, and organic solvents is needed to provide good peak shape as well as adequate resolution in minimum time, the column had to be washed well daily to prevent degradation of the column. In addition EDTA was required in the mobile phase to maintain electrode performance in the electrochemical detector.

Two problems were encountered when regular C_{18} columns were used with this mobile phase: (1) strong adherence of compounds to the analytical column which produced peak tailing, and (2) breakdown of the column or reduction in column lifetime producing non-reproducible retention times and high back pressures. To alleviate these problems, the Perkin Elmer reduced activity catecholamine column was used. It provided symmetrical, reproducible peak shapes for the strongly retained compounds. Secondly, a silica gel precolumn was placed between the pump and the injector to protect the analytical column. This precolumn was repacked every two weeks with fresh silica.

The RPLC–ED method described proved to be an excellent technique to determine serotonin and its metabolites along with octopamine at the picogram level in the hemolymph of lobsters. Despite the presence of only trace amounts of the neuroactive hormones in physiological samples as well as the inherent instability of these compounds, endogenous levels of these hormones can be measured with excellent precision in mininal time if careful attention is paid to electrode and column maintenance and if chromatographic conditions as well as sample preparation and storage procedures are carefully controlled.

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