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Note

Separation of HI-6 [4-carbamoyl-2′-hydroxyiminomethyl-1,1′-oxydimethylen-di(pyridinium chloride)] and its degradation products by ion-pair high-performance liquid chromatography

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Oximes, such as N-methylpyridinium-2-aldoxime chloride (2-PAM · Cl), obidoxime chloride and N,N′-trimethylene-bis(pyridinium-4-aldoxime) dibromide (TMB-4 dibromide) are clinically used as effective antidotes against toxicity cause by organophosphate insecticides[1–3]. They are known as reactivators of acetylcholinesterase. However, these oximes are ineffective against the more potent organophosphates, such as methylphosphonofluoridic acid 1,2,2-trimethylpropyl ester (soman), that age the acetylcholinesterase at a rapid rate.

Hagedorn *et al.*[4,5] synthesized a number of bispyridinium salts having a 2-aldoxime functional group, called H-Oximes[6]. A few of them are effective antidotes against soman. One of these oximes, HI-6, [4-carbamoyl-2′-hydroxyiminomethyl-1,1′-oxydimethylen-di(pyridinium chloride)] proved to be extremely effective[7]. However this compound appears to be unstable upon storage in aqueous solution for prolonged periods of time. The present study was initiated to investigate the effects of pH and temperature on its stability.

An ion-pair high-performance liquid chromatographic (HPLC) method was developed to separate and quantify HI-6 and some of its degradation products. Quantitative amounts, measuring 5 ng on-column are possible using this procedure. Analysis time is 10 min per sample. The use of an isocratic mode minimizes analysis time between runs.

Because of the accuracy and reproducibility of the method, the use of this technique offers a convenient alternative to the currently employed methodologies.

EXPERIMENTAL*

Apparatus

This study was performed using a Waters Assoc. (Milford, MA, U.S.A.) Model

* The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

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ALC/GPC-204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a Model 660 solvent programmer, U6K loop injector, a Model 440 detector, set at 254 nm, a Houston Instrument Omni-Scribe A5000 dual-pen recorder and a Columbia Scientific Supergrator-3-integrator.

Reagents

Spectroquality acetonitrile (Burdick & Jackson, Muskegon, MI, U.S.A.) mixed with PIC-B7 reagent (1-heptane sulfonic acid, Waters Assoc.) was used as the mobile phase. HI-6 dichloride was obtained from Dr. Hagedorn's Laboratories (University of Freiburg, Freiburg, F.R.G.).

Procedure

A prepacked 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc.) was employed to chromatograph all compounds used in this study. The stationary phase (μBondapak C_{18}) is an octadecylsilyl bonded-phase packing material. The solid support medium contained in the column is capable of operating within a pH range of 2–8. It is thermally stable over a wide temperature range (< 300°C). Because of its excellent hydrolytic stability, various hydrophilic–hydrophobic mixed solvent systems are compatiable with the column.

The mobile phase consisted of a 0.01 *M* solution of PIC-B7 reagent in water mixed with acetonitrile. The 0.01 *M* PIC-B7 solution was prepared by dissolving 20 ml of the pre-packaged reagent into 480 ml of glass-distilled water. The pH of the solution was 3.4. Acetonitrile–0.10 *M* PIC-B7 (20:80) was used in an isocratic mode. The flow-rate was 1.5 ml/min. Column pressure ranged between 76–92 bar. All separations were performed at ambient temperatures. Sample volumes (5 μl) were introduced into the column through a continuous flow loop injector. Detection limits of the method was 1 ng on-column. Peak areas were measured by an on-line computing integrator.

RESULTS AND DISCUSSION

While much attention has focused on the biochemical aspects of HI-6 in animals[8–10], very little information has been obtained on the degradative fate of this

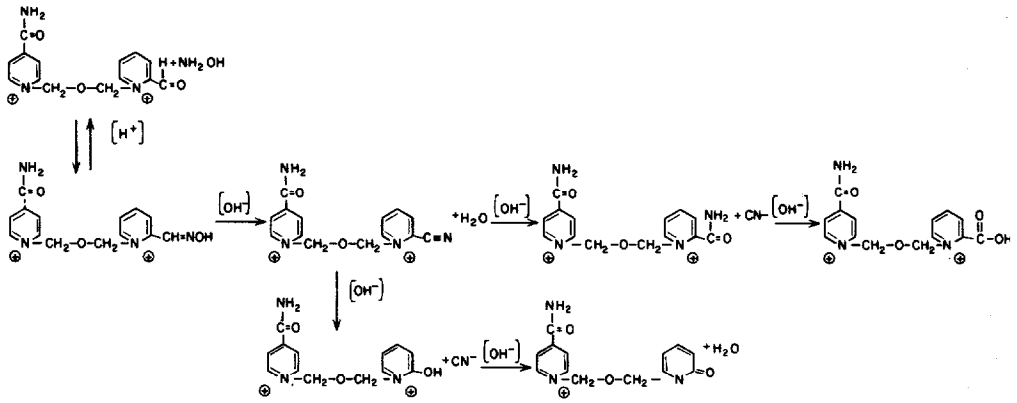

Fig. 1. Proposed degradation scheme for HI-6 in acidic and basic solutions.

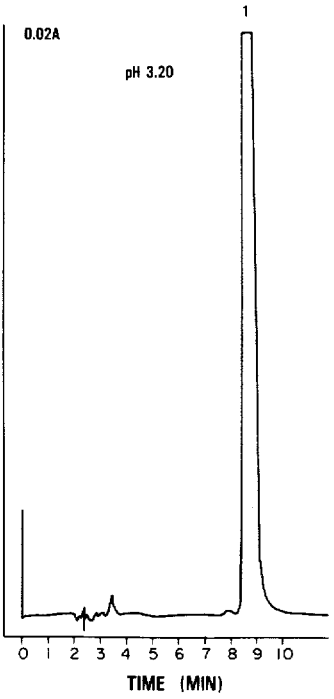

Fig. 2. Separation of a standard solution containing (1) 750 ng of HI-6. Column: 30 cm × 3.9 mm I.D. μBondapak C_{18}. Mobile phase: 0.01 *M* PIC-B7–acetonitrile (80:20). Flow-rate: 1.5 ml/min. Column temperature: ambient. Detection wavelength: 254 nm.

compound when subjected to various pH and temperature gradients or when HI-6 is stored in aqueous solutions over prolonged periods of time[11].

Studies conducted by Christenson[12] showed that HS-6, a congener of HI-6 degraded via two routes when subjected to various hydrogen and hydroxyl ion concentrations. The degradation scheme shown in Fig. 1 for HI-6, is similar to the decomposition scheme of HS-6. In these hydrolytic reactions, only the oxime moiety was affected during its conversion to alternate bis-quaternary pyridinium structures. Brown *et al.*[13] observed similar results with 2-PAM · Cl.

In an attempt to follow the reaction kinetics of HI-6, an ion-pair reversed-phase HPLC method was developed. The procedure was designed to measure the amounts of HI-6 degraded at the experimental pH and temperature conditions, as well as to separate and characterize the degradation products.

A series of standard solutions of HI-6 were separated and quantified using this new method. Linearity was obtained for the various concentrations of HI-6 (5–1000 ng). Correlation coefficient for HI-6 was 0.996.

Fig. 2 represents the chromatogram of a 750 ng standard of HI-6. A working standard of HI-6 was prepared in 0.001 *M* hydrochloric acid and was stable for more than six months when stored at 4°C. In order to evaluate the effects of various pH's and temperatures on aqueous solutions of HI-6, we designed a series of experiments to observe the stability of HI-6 for a 60-min period.

The chromatograms shown in Fig. 3, depict the chromatographic differences

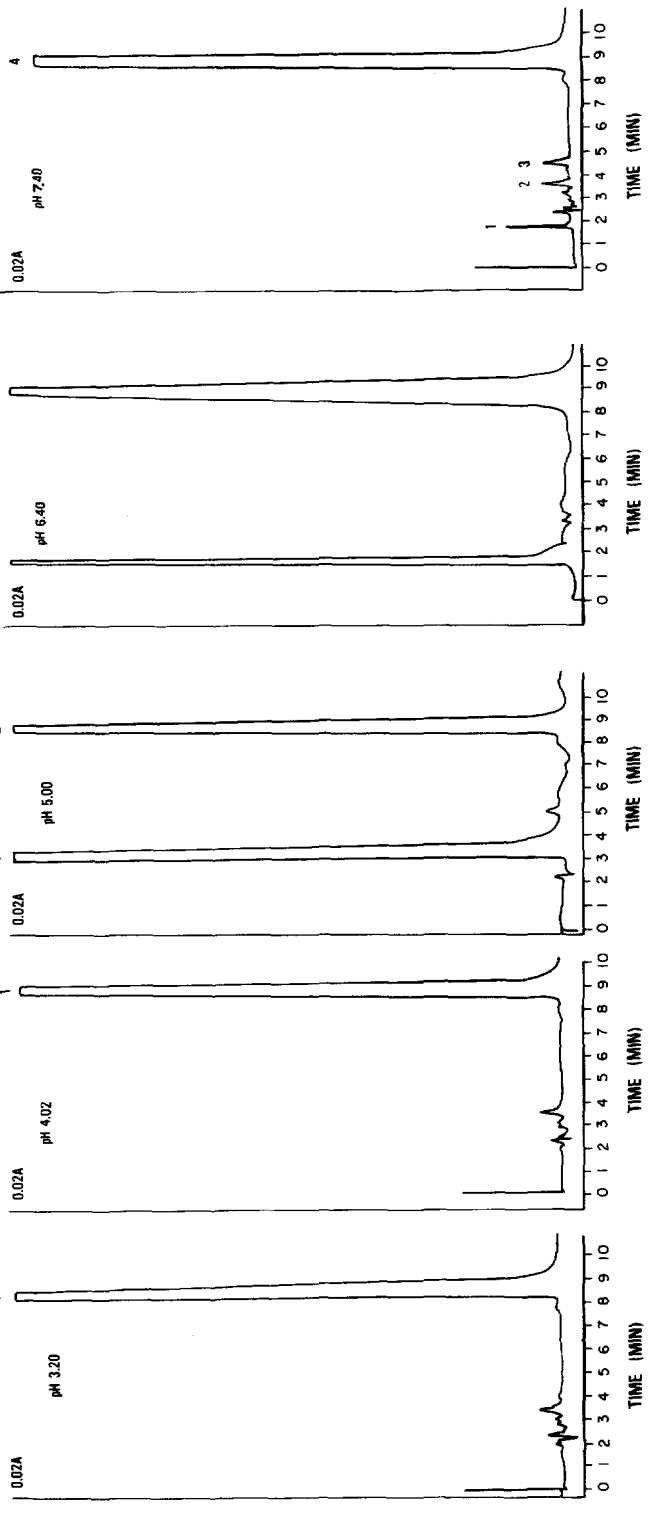

Fig. 3. Separation of standard solutions of HI-6 at pH values of 3.20, 4.02, 5.00, 6.40 and 7.40, incubated at 25°C for 60 min. Peaks at 9.5 minutes represent HI-6, all other numbered peaks are unknowns.

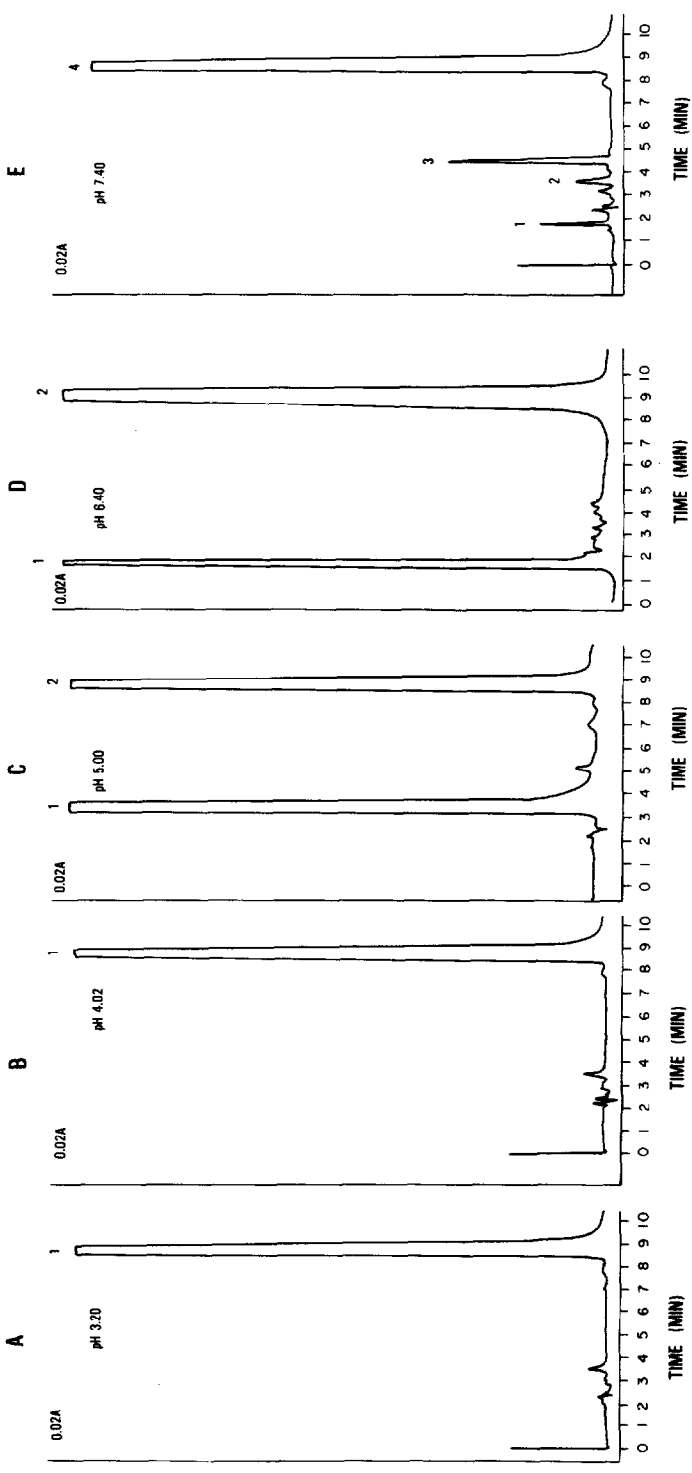

Fig. 4. Separation of standard solutions of HI-6 incubated at 37°C for 60 min. Experimental parameters and conditions were similar to those shown in Fig. 3.

occurring at 5 different values pH ranging from 3.20 to 7.40. Temperature was maintained at 25°C throughout the 60 min. incubation period. Buffers [acetate, citrate and phosphate (0.001 *M*)] with varying pH's were used to produce the pH values indicated in each chromatogram. The buffer molarity of each sample was maintained at 0.001 *M*.

Results from this study showed that when the pH of the sample was kept below 4.02, the stability of HI-6 did not change. However, when pH was increased to values ≥ 5.00 (Fig. 3C–3E), chromatographic profile changes were observed. This was especially true at pH 5.00 (Fig. 3C). At this pH, a large peak was observed at 3.5 min. It was noted that during the formation of this unknown peak, we observed a 38% decrease in the original amount of HI-6 present in the sample.

In the HI-6 sample prepared with the 6.40 buffer (Fig. 3D), a different chromatographic profile was observed. Instead of a peak at 3.5 min., a new unknown peak occurred at 1.5 minutes. Concomitantly, a reduction in the amount of HI-6 was observed at this pH value. However, in this case, it represented a loss of only 12% of HI-6.

As shown in Fig. 3E, the sample incubated at pH 7.4, showed again a different profile. At this pH, the differences were marginal. It was noted that there was only a 4% decrease in the HI-6. In this chromatogram, three peaks other than HI-6 were observed. Two of these peaks had retention times similar to the ones seen in Figs. 3C and 3D. A new unknown peak was also observed at 4.5 min.

In order to determine the effect of temperature on the stability of HI-6 in

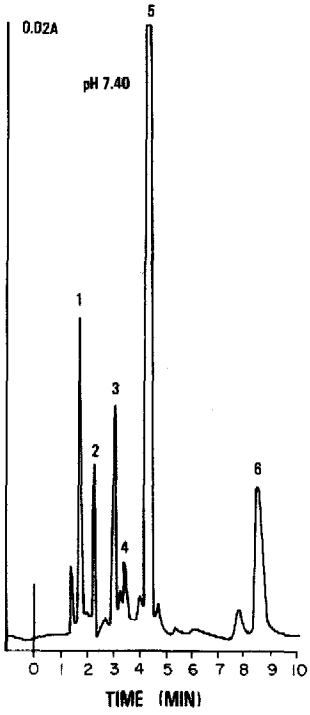

Fig. 5. Chromatogram of a 24-h HI-6 sample, incubated at 37°C and pH 7.4. Peaks: (1–5) unknowns and (6) HI-6.

aqueous solution at specific pH's, additional samples were prepared. Aliquots were incubated at 37°C for 60 min. All experimental parameters used in the 37°C study were similar to the 25°C study.

The results as depicted in Fig. 4 showed only slight differences in the chromatographic profile of HI-6 at 37°C when compared to 25°C. For each of the corresponding chromatograms, where the 25°C samples were compared to the identical pH's of the 37°C samples, no more than a 1–2% difference in HI-6 amounts was noted.

Finally, the effect of incubation of HI-6 sample at pH 7.4 and 37°C for 24 h was studied. The results are shown in Fig. 5. As can be seen in this chromatogram, 85% of HI-6 was degraded to at least five major products. Possibly, as many as thirteen peaks were observed. Using physico-chemical techniques, including mass spectrometry, these degradation products are being characterized.

The effect of long term storage on stability of HI-6 in aqueous solutions is being investigated at the present time.

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