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Note

Stability study of HI-6 dichloride in various anticholinergic formulations

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Mono- and bispyridinium oximes have been shown to be effective anticholinergic compounds for reversing the toxic effects of organophosphorus poisoning in mammals. As reactivators of organophosphorus-inhibited cholinesterases, these compounds play a limited, but important role in ameliorating peripheral and central nervous systems dysfunctions caused by highly toxic insecticides. Compounds, such as trimedoxime dibromide (TMB-4), toxogonin chloride and pralidoxime chloride (2-PAM) are known reactivators of organophosphorus-inhibited acetylcholinesterase (AChE). However, some of these oximes are ineffective against more persistant organophosphates. A relatively new group of compounds, designated the H-oximes, synthesized by Hagedorn *et al.*[1,2] has proved to be highly effective against the more toxic organophosphorus compounds.

HI-6 [4-carbamoyl-2′-hydroxyiminomethyl-1,1′-oxydimethylen-di(pyridinium chloride)] is a drug that falls within this category. While being an excellent anticholinergic intramuscular parenteral antidote, it has several limiting factors which must be observed for maintaining its required therapeutic potency in aqueous solution during formulation and storage. Brown *et al.*[3] and Markov *et al.*[4,5] have shown that both pH and temperature conditions contribute directly toward the instability of HI-6 in aqueous solutions. Data obtained from these studies show that pH values greater than 4, or less than 3, along with a temperature higher than 37°C causes HI-6 to degrade rapidly. Christenson[6] has also shown that HS-6, a congener of HI-6 is subject to chemical hydrolysis of the oxime moiety when pH and temperature constraints, established in her studies were disregarded.

In this study, we describe a series of experiments which will show that a stable HI-6 antidotal preparation with an extended shelf-life is possible when proper pH and temperature parameters are used.

An ion-pair high-performance liquid chromatographic (HPLC) method described previously[3], along with a modification of the procedure were used to assay HI-6 in the various formulations during this 18-month study. Spectrophotometric analyses were also performed to observe and compare differences occurring in the resulting profiles.

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MATERIAL AND METHODS*

A Waters Assoc. (Milford, MA, U.S.A.) Model 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-3A-integrator was used to separate all compounds employed in this study.

Ultra-violet spectrophotometric analyses of HI-6 were performed using a Beckman Model 5320 spectrophotometer. A Hewlett-Packard Model 5840A gas chromatograph was used to assay atropine sulfate concentration in the multicomponent sample. Sample preparations and analyses were carried out according to the method of the United States Pharmacopeia[7].

Reagents

All chemicals and solvents used in the study were either of analytical grade or of spectroquality. Acetonitrile was obtained from Burdick and Jackson Lab. (Muskegon, MI, U.S.A.). PIC-B7 reagent (1-heptanesulfonic acid) was purchased from Waters Assoc. HI-6 dichloride was acquired from Dr. Hagedorn's Labs. (University of Freiburg, Freiburg, F.R.G.). Atropine sulfate, methyl *p*-hydroxybenzoate (methyl paraben) and propyl *p*-hydroxybenzoate (propyl paraben) were obtained from United States Pharmacopeial Convention (Rockville, MD, U.S.A.). Aprophen hydrochloride was synthesized in our laboratory, using the method of Zuagg and Horrom[8]. Its identification was confirmed by its melting point (161–162°C), infrared, nuclear magnetic resonance spectroscopic properties and by chemical ionization mass spectrometry.

Procedure

A prepacked 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc.) was used to chromatograph HI-6 and other components present in the various formulations. The mobile phase consisted of 0.01 *M* 1-heptanesulfonic acid combined with acetonitrile. PIC-B7 reagent was prepared by mixing 20 ml of the pre-packaged reagent with 480 ml of glass distilled water. The pH of the solution was 3.4. An acetonitrile–PIC-B7 mixture with a percent ratio of 20:80 was used in an isocratic mode to separate HI-6 in the simple formulation. The multicomponent sample was chromatographed using a 50:50 solvent system. Flow-rates for both separations were 1.5 ml/min. Column pressures ranged between 72 and 92 bar. All separations were performed at ambient temperatures. Samples were introduced into the column through a continuous-flow loop injector. Detection limits for the method was 1 ng on-column. Peak areas were measured by an on-line computing integrator.

RESULTS AND DISCUSSION

A class of compounds referred to as the H-oximes has been effectively em-

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

TABLE I

COMPOSITION OF HI-6 DICHLORIDE IN A SIMPLE AND MIXED ANTICHOLINERGIC FORMULATION (mg/ml)

Compound	*0.015 M citrate*	*0.015 M acetate*	*0.001 M HCl**	*0.015 M citrate*
HI-6 dichloride	12.5	12.5	12.5	12.5
Aprophen hydrochloride	50.0	–	–	–
Atropine sulfate	1.19	–	–	–
Methyl paraben	0.50	–	–	–
Propyl paraben	0.25	–	–	–

* Adjusted to pH 4 with dilute sodium hydroxide.

ployed during the past five years as a therapeutic agent to reverse the toxic effects of organophosphate poisoning in mammals exposed to soman[9,10]. HI-6, one of the many bispyridinium oximes synthesized by Hagedorn *et al.* has been shown to be, by far, the most potent reactivator of this group of compounds. While results pertaining to the therapeutic qualities of HI-6 were encouraging, data concerning the stability of this oxime were not too promising. In an attempt to gain more insight into this problem, we initiated a study to establish some of the parameters required for maintaining a chemically stable formulation for an extended period of time.

Four different preparations were developed for this study. Each formulation contained a HI-6 concentration of 12.5 mg/ml. The pH of these HI-6 solutions was adjusted to 4.0 with a series of buffers. Table I shows the chemical composition of the formulations.

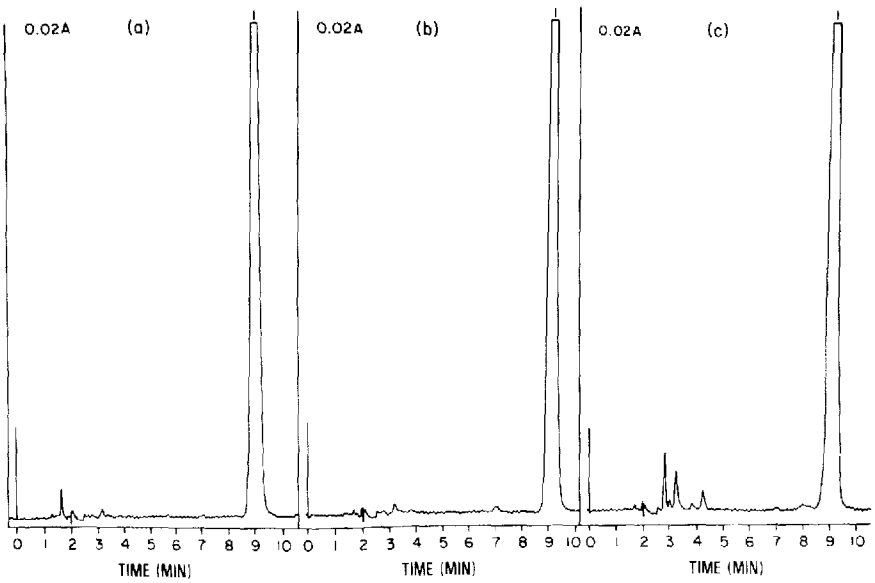

Fig. 1. Chromatogram of 750 ng of HI-6 in pH 4 solutions of (a) citrate, (b) acetate and (c) hydrochloric acid. Column, 30 cm × 3.9 mm I.D. μBondapak C_{18}; mobile phase, 0.01 *M* PIC-B7–acetonitrile (80:20); flow-rate, 1.5 ml/min; column temperature, ambient; detection wavelength, 254 nm.

From each of the four HI-6 stock preparations 100-μl aliquots were removed, sealed in glass ampules and stored at room temperature (18–20°C) and 40°C, for periods up to 18 months. At the time of the initial preparation, samples were analyzed by HPLC and spectrophotometric techniques to establish normal profiles for the unaltered HI-6 content. Fig. 1 shows chromatograms of HI-6. No apparent differences were observed between samples. Similarly, the multicomponent solution was chromatographed to establish its profile for the study. In this particular case, the acetonitrile–PIC-B7 (20:80), mixture as well as the 50:50 solvent system were used to characterize the mixture. From our earlier work, it was shown that HI-6 and its degradation products are best resolved when a more polar mobile phase (20:80) is employed. However, when organic compounds, such as aprophen, methyl and propyl parabens are to be separated by this ion-pair HPLC procedure, a higher concentration of acetonitrile is required to elute these compounds from the column. Fig. 2 shows the separation of the mixed formulation using the 50:50 solvent system. All samples were diluted 1:80 with 0.002 *M* hydrochloric acid. Using this dilution, HI-6, aprophen and the parabens were all quantifiable by HPLC. Gas chromatography was used to analyze atropine sulfate in the multicomponent formulation. HPLC and spectrophotometric analyses were performed bi-weekly during the 18-month study. Atropine sulfate was analyzed monthly.

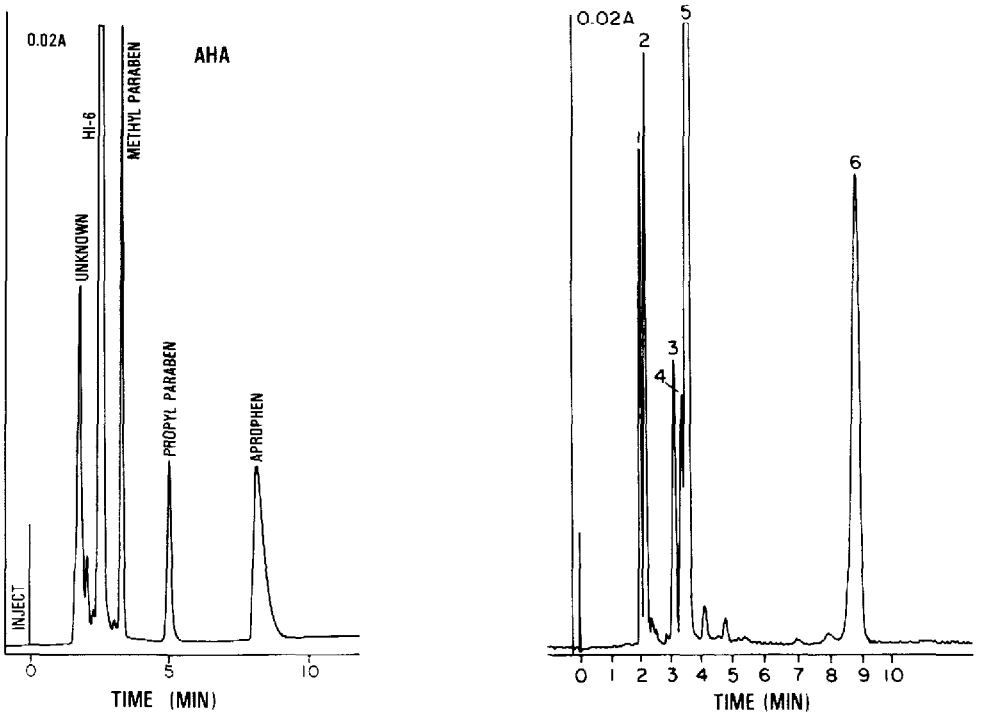

Fig. 2. Chromatogram of a multicomponent mixture containing HI-6, methyl paraben, propyl paraben and aprophen. Mobile phase: 0.01 *M* PIC-B7–acetonitrile (50:50).

Fig. 3. HPLC profile of HI-6 sample stored at 40°C for six weeks. Peaks: 1–5 = unknowns; 6 = HI-6.

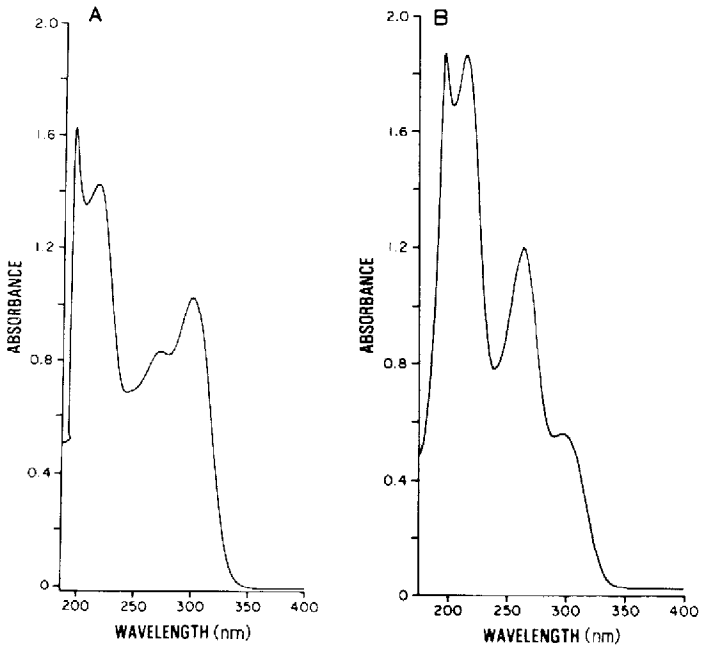

Fig. 4. Absorbance spectra of (A) HI-6 in citrate (day 1) and (B) HI-6 stored at 40°C for six weeks. Sample preparations: 1:320 dilution in 0.002 *M* hydrochloric acid.

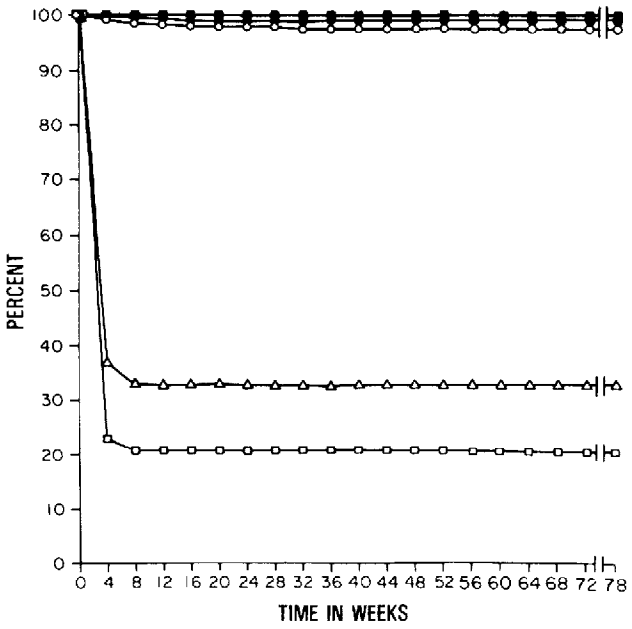

Fig. 5. Stability of Hi-6 (●), aprophen (○), methyl paraben (△), propyl paraben (□), and atropine (■) during 78 weeks at room temperatures in citrate buffer pH 4.0.

During the first two weeks of the study, analyses showed that HI-6 had not degraded at either the room temperature or at 40°C. However, this was not true after the fourth week. HPLC analyses showed that degradation had occurred in the 40°C incubated samples. A slight green coloration was also observed in these samples, indicating that chemical deterioration was taking place. The average percentage break-down for the four elevated temperature formulations was approximately 9%. Spectrophotometric results showed a slight spectra shift in the HI-6 profiles. At the same time no noticeable differences were observed in any of the room temperature samples.

After six weeks of storage at 40°C, more than 60% of the HI-6 content in both the simple and mixed formulations had degraded to its hydrolytic byproducts. Fig. 3 shows the altered HPLC profile of a representative sample taken from the citrate buffered solution. More than five major additional peaks can be seen in this heat labile sample. Spectrophotometric analyses showed similar results when the degraded samples were compared to stable HI-6 ones. Distinguishing differences can be seen in the spectra shown in Fig. 4.

Ten weeks of heating at 40°C reduced the HI-6 content to 18% of its original concentration. At that point, the experiments at 40°C were discontinued. The room temperature samples were analyzed on its bi-weekly schedule for the next 68 weeks. During that time, no more than a 4% loss in HI-6 concentration was observed in any of the prepared samples.

At the same time, no appreciable differences were noted in the concentrations of aprophen hydrochloride and atropine sulfate in the multicomponent formulation. The bacteriostatic and fungistatic compounds, methyl and propyl paraben showed a reduction in their concentrations during the first six weeks, but stabilized thereafter. Fig. 5 depicts the results of this study during the first 78 weeks.

From this investigation, it is evident that a stable HI-6 preparation can be maintained for extended periods of time when pH and temperature parameters established in this study are followed.

ACKNOWLEDGEMENT

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