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Note

Separation and identification of substituted pyridine analogues in heat labile solutions of HI-6 dichloride

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Previous work[1,2], describing the chemical break-down of HI-6 dichloride [4-carbamoyl-2'-hydroxyiminomethyl-1,1'-oxydimethylene-di-(pyridinium chloride)] in aqueous solution has produced greater insight into the reaction kinetics of this compound during the degradation process. Earlier studies showed that the fate of HI-6 in solution is directly related to pH, temperature and concentration of the formulation during preparation and storage. Slight variation of the established guidelines required for maintaining a stable anticholinergic antidote reduces the desired effectiveness of HI-6 to act as a therapeutic agent in reactivating organophosphorus-inhibited acetylcholinesterase.

Thus, recognizing the early signs of an unstable or partially degraded formulation is an essential requirement for being assured that a safe and efficacious product is available for human use.

Using ion-pair high-performance liquid chromatographic (HPLC) and desorption chemical-ionization mass spectrometric (DCI-MS) direct-probe techniques, degradative by-products formed during HI-6 exposure to elevated temperatures were separated, collected and later analyzed by mass spectrometry (MS) for their chemical identities.

EXPERIMENTAL*

Apparatus

All separations were made using a Waters Model APC/GPC 204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a 660 solvent programmer, U6K loop injector, a Model 440 UV detector, set at 254 nm, a Houston Omni-Scribe A5000 dual-pen recorder and a Columbia Scientific Industries Supergrator-3 integrator. A Finnigan Model 4500 quadrupole mass spectrometer, using

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desorption chemical ionization with methane as the reactant gas, was used to identify the pyridine by-products.

Spectroquality acetonitrile (Fisher Scientific, Pittsburgh, PA, U.S.A.) mixed with PIC-B7 reagent (1-heptanesulfonic acid, Waters Assoc., Milford, MA, U.S.A.) constituted the mobile phase. Isonicotinic acid, isonicotinamide, pyridine-2-aldehyde, 4-pyridinecarboxaldehyde, 2-pyridinealdoxime and 4-cyanopyridine were purchased from Pfaltz and Bauer (Stamford, CT, U.S.A.). HI-6 dichloride was synthesized by Dr. Hagedorn's laboratories (University of Freiburg, Freiburg, F.R.G.).

Procedure

A μBondapak C_{18} column (Waters Assoc.), 30 cm × 3.9 mm I.D., was employed to chromatograph all compounds used in this study. The mobile phase consisted of a 0.01 *M* solution of PIC-B7 reagent in water mixed with acetonitrile. The ion-pairing reagent was prepared by dissolving 20 ml of the pre-packaged solution into 480 ml of glass-distilled water. The pH of the solution was 3.4. Acetonitrile–PIC reagent (20:80) was isocratically pumped through the column. The flow-rate for the dual pumping system was 1.5 ml/min. Column pressures ranged between 76 and 92 bar. Separations were performed at ambient temperature. Samples were introduced into the column through a continuous flow loop injector. All peaks were collected for later analyses by DCI-MS techniques. An on-line computing integrator was used to measure area and peak heights of the eluting compounds.

RESULTS AND DISCUSSION

The guidelines established for maintaining a chemically stable HI-6 formulation have been shown to be of extreme importance in producing an antidote that will have a shelf-life of more than two years. However, in certain instances where conditions cannot be adequately controlled, such as in the storage of the antidote in various types of warehouses, the stability of HI-6 becomes questionable. As such, the characterization and identification of degraded by-products formed in HI-6 formulations are the key elements in determining the fate of this reactivator after prolonged periods of storage.

Previous work by various investigators[3–5] has shown that the fate of the mono- and bis-pyridinium oximes is determined by monitoring the HI-6 molecule and noting changes in the oxime moiety. In specific cases involving trimedoxime dibromide, 1-(2-hydroxyiminoethylpyridinium)-1-(3-carboxy-amidopyridinium)-dimethyl ether (HS-6) and pralidoxime chloride, both pH and temperature gradients produce change at the oxime site. However, with HI-6, we have observed that less than ideal pH and temperature conditions cause the breaking of the methylene carbon bond at both ends of the molecule. The pyridine analogues of the cleaved methylene bridges of HI-6 produced the degradation scheme shown in Fig. 1.

Chemical changes were also noted at the isonicotinamide group, as well as at the oxime site.

Incorporating HPLC and DCI-MS techniques, the separation and identification of the substituted pyridine were accomplished. Three different sample preparations were tested in these studies. Temperature was the only variable used to produce the chemical changes observed in this investigation. All aqueously prepared HI-6

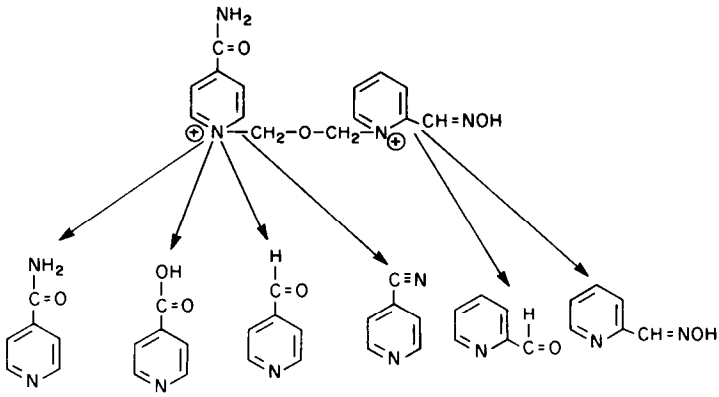

Fig. 1. Degradation scheme for HI-6 in a pH 4 acetate buffer at 40°C.

solutions were maintained in a pH 4.0, 0.01 *M* acetate buffer during the experimental time frame. Samples were stored in sealed glass ampules for six weeks at 4°C, 18°C and 40°C. The concentration of the HI-6 solution was 12.5 mg/ml.

During the six-week period, aliquots were periodically removed and analyzed by HPLC for developing characteristic profiles of the reaction kinetics involved in the HI-6 breakdown. At the same time, peaks collected from the HPLC system were analyzed by MS to identify by-products.

Figs. 2a, 2b and 2c represent the HPLC separations of 750 ng of HI-6 at 4°C, 18°C and 40°C after a six-week storage period. As shown in the 4°C and 18°C profiles,

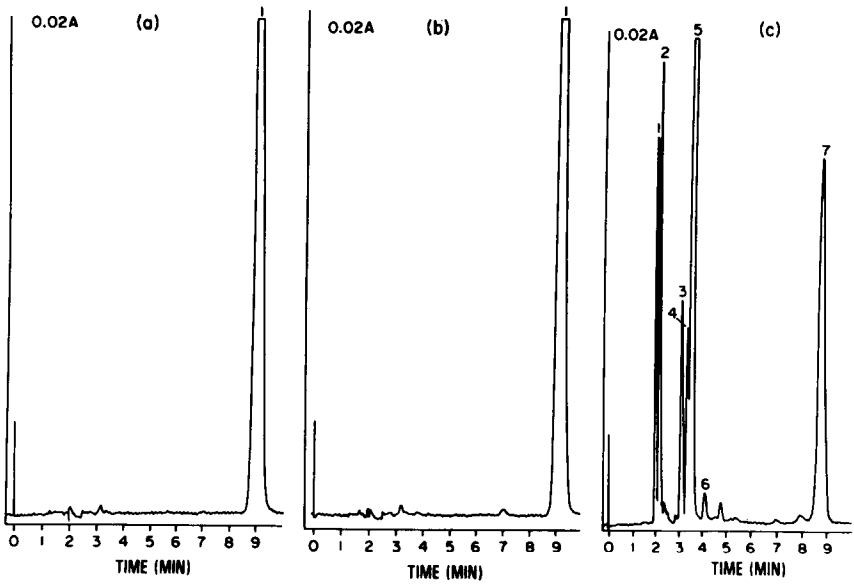

Fig. 2. Chromatograms of a pH 4 acetate buffered solution of HI-6 stored for six weeks at (a) 4°C, (b) 18°C and (c) 40°C. Peak 1 at 4°C and 18°C = HI-6; peaks at 40°C: 1 = isonicotinic acid; 2 = isonicotinamide; 3 = 4-pyridinecarboxaldehyde; 4 = pyridine-2-aldehyde; 5 = pyridine-2-aldoxime; 6 = 4-cyanopyridine; 7 = HI-6.

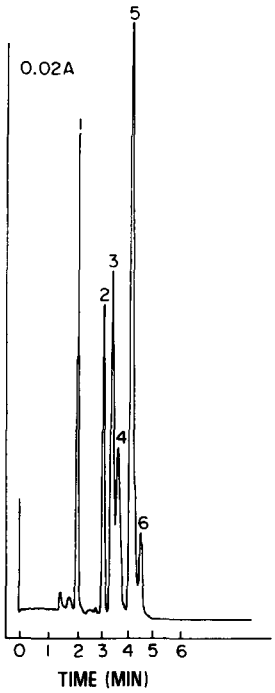

Fig. 3. Separation of a standard solution containing (1) isonicotinic acid, (2) isonicotinamide, (3) 4-pyridinecarboxaldehyde, (4) pyridine-2-aldehyde, (5) pyridine-2-aldoxime and (6) 4-cyanopyridine. Column: 300 × 3.9 mm I.D. μBondapak C_{18}; mobile phase: PIC-B7 (0.01 *M*)–acetonitrile (80:20); flow-rate: 1.5 ml/min; column temperature: ambient; detection wavelength: 254 nm.

no gross changes were observed in the samples, when compared to the original, one-day-old solutions.

In contrast, when the 40°C, six-week-old sample was compared with its two counterparts, significant changes in its chemical make-up were observed. Six identifiable peaks constituting the newly formed pyridine analogues were separated by HPLC. A standard solution, containing a combination of substituted pyridine compounds separated by HPLC, produced retention times that corresponded to those

TABLE I

MASS SPECTROMETRIC DATA OF SUBSTITUTED PYRIDINE ANALOGUES FROM HI-6 DEGRADATION

Compound	*Actual mass*	*Experimentally obtained mass*
Isonicotinic acid	123	123.1
Isonicotinamide	122	122.1
4-Pyridinecarboxaldehyde	107	107.0
Pyridine-2-aldehyde	107	107.0
Pyridine-2-aldoxime	122	122.1
4-Cyanopyridine	104	104.0

produced in the 40°C samples (Fig. 3). Peaks collected from the 40°C samples produced mass numbers identical to the actual molecular weights of the known standard compounds. Table I shows confirmational data obtained from mass spectrometry results.

As a result of these experiments, data obtained from these studies show that the mechanistic reactions involved in the degradation of HI-6 are different from that of the other bis pyridinium oximes. With this newly acquired information, chemical solvents and buffers will be developed which may slow the thermal degradation of HI-6.

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