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High-Performance Liquid Chromatographic Determination of Pralidoxime Chloride and Its Major Decomposition Products in Injectable Solutions

DAVID G. PRUE ˣ, RAYMOND N. JOHNSON, and BOEN T. KHO

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Abstract □ A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of pralidoxime chloride (I) and its major decomposition products in an injectable formulation is described. I and its decomposition products were detected and quantitated by their UV absorbances at 270 nm, after being separated from related compounds and formulation excipients on a reverse-phase C-18 column using a mobile phase consisting of 52% acetonitrile and 48% of an aqueous solution containing 0.005 *M* phosphoric acid and 0.001 *M* tetraethylammonium chloride. The major decomposition products of I in the injectable formulation were identified by their retention times and stop-flow spectroscopy as 2-carboxy-*N*-methylpyridinium chloride, *N*-methyl-2-pyridone, 2-carbamoyl-*N*-methylpyridinium chloride, 2-hydroxymethyl-*N*-methylpyridinium chloride, and 2-cyano-*N*-methylpyridinium chloride. A substance of unknown identity also was detected in degraded solutions of I. Stop-flow spectroscopy, employing the spectral discrimination technique, showed that the method is specific for I. Recovery of I from a spiked placebo formulation averaged 99.9%. The accuracy of the method was also demonstrated for the decomposition products over a range of concentrations representing 1–50% decomposition. Replicate determinations of I in degraded solutions gave coefficients of variation of 1.0 and 1.5%, while the precision of determining the decomposition products range from 1.3 to 6.5%. Regression lines with correlation coefficients >0.9999 were obtained for I and its decomposition products, and solutions of these compounds were shown to be stable in the mobile phase for several days. Results for I by the HPLC and USP procedures are compared.

Keyphrases □ Pralidoxime chloride—decomposition products in aqueous solutions, concurrent high-performance liquid chromatographic determination □ High-performance liquid chromatography—concurrent determination of pralidoxime chloride and its decomposition products in aqueous solutions, comparison to USP procedure □ Degradation products—of pralidoxime chloride, concurrent high-performance liquid chromatographic determination

Pralidoxime chloride, 2-[(hydroxyimino)methyl]-1-methylpyridinium chloride (I), is a reactivator of organophosphate-inhibited cholinesterase. It has therapeutic value as an antidote to poisoning by organophosphate agricultural chemicals, chemical warfare agents, and drugs acting as cholinesterase inhibitors. Compound I is typically formulated as an aqueous injectable solution which is administered intramuscularly immediately after the onset of anticholinesterase poisoning.

Unfortunately, aqueous solutions of the various salts of pralidoxime have been shown to be unstable. The instability of pralidoxime in both acidic and basic media was first described in a series of reports (1–4) which proposed the existence of several decomposition products of pralidoxime iodide: the iodide salts of the 2-carboxy-, 2-formyl-, 2-carbamoyl-, and 2-cyano-*N*-methylpyridinium ions and *N*-methyl-2-pyridone. In addition, two major decomposition products were detected in solutions of pralidoxime

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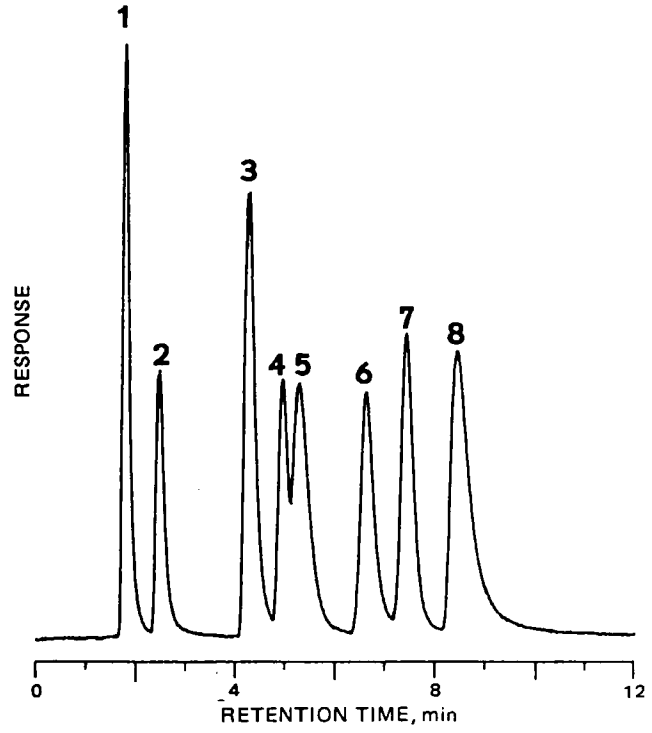

Figure 1—*Chromatogram of a synthetic mixture of I–XII. Key: (1) II, (2) III and IV, (3) V, (4) VI and VII, (5) VIII, (6) IX and X, (7) I, and (8) XI. Compound XII is not shown (retention time = 27.4 min).*

methanesulfonate (5), which were identified as the methanesulfonate salts of the 2-carbamoyl- and 2-cyano-*N*-methylpyridinium ions. The kinetics of the hydrolysis of the cyano-pyridinium ions were studied (6), and it was shown that 99.1% of the material from the alkaline hydrolysis of the 2-cyano-*N*-methylpyridinium ion could be accounted for as 2-carbamoyl-*N*-methylpyridinium ion and *N*-methyl-2-pyridone. A fluorescent product was isolated from the alkaline hydrolysis of I (7). On the basis of physical and physicochemical properties, it was concluded that this product was 2-cyano-1-methyl-4-pyridone.

Although a complete decomposition profile of I has not been described in the literature, it was anticipated that the decomposition of I would lead to the same decomposition products as reported for the corresponding iodide and methanesulfonate salts. The procedure in the USP XX (8) for determining I does not adequately separate and isolate these decomposition products before analysis. I is quantitated in the presence of its decomposition products at the UV wavelength of 336 nm, which is sensitive for I but has only minimal sensitivity for the decomposition products. A number of high-performance liquid chromatographic (HPLC) procedures (9–11) for determining pyridinium aldoximes have been reported; however, not one is suitable for studying the stability of I.

In this present study, a specific HPLC method has been developed which separates and simultaneously quantitates I and its major decomposition products. In addition, this chromatographic system separates I from its primary synthetic precursor, pyridine-2-aldoxime, and a number of other structurally related compounds. This method was used to study the stability of an injectable formulation of I and two previously unreported decomposition products were detected.

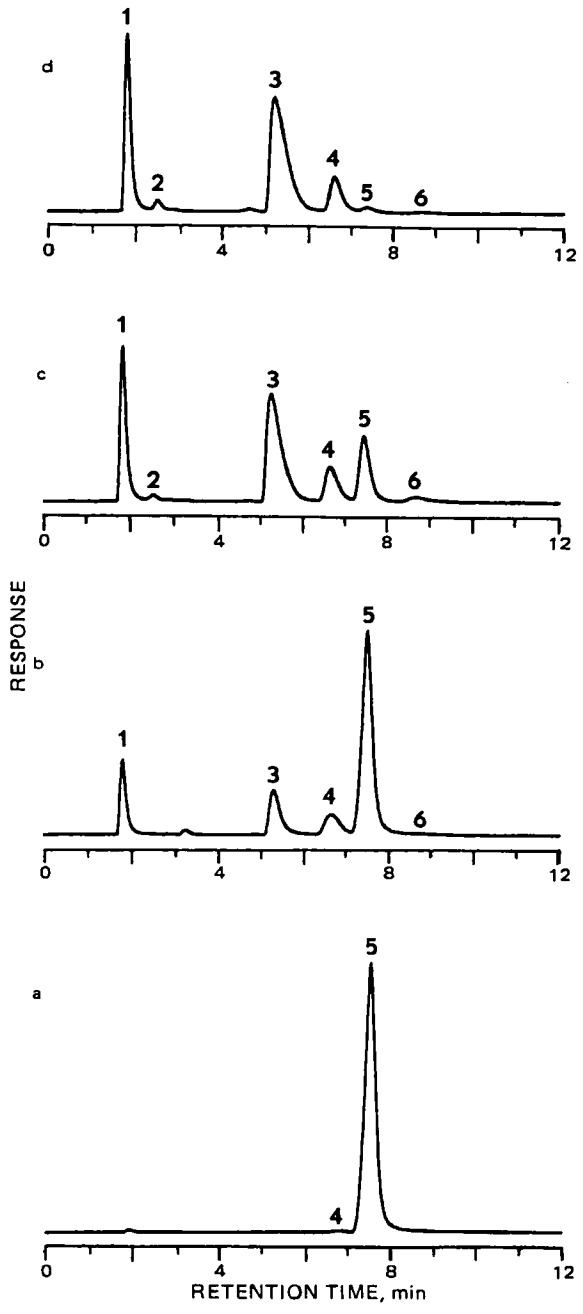

Figure 2—*Chromatograms of the injectable formulation at day zero (a) and after being stored at 80° for 2 (b), 4 (c), and 8 (d) weeks. Key: (1) II, (2) III, (3) VIII, (4) X and the unknown compound, (5) I, and (6) XI.*

EXPERIMENTAL

Reagents—Phosphoric acid[1] was reagent grade; tetraethylammonium chloride[2] was used without further purification; acetonitrile[3] was UV grade, distilled in glass. The following reference compounds were used: pralidoxime chloride[4] (I), 2-carboxy-*N*-methylpyridinium chloride[5] (II), *N*-methyl-2-pyridone[5] (III), 2-cyano-1-methyl-4-pyridone[6] (IV), pyridine-2-aldoxime[5] (V), *syn*-pralidoxime chloride[7] (VI), 2-formyl-*N*-methylpyridinium chloride[8] (VII), 2-carbamoyl-*N*-methylpyridinium

[1] Mallinckrodt Inc., Paris, Ky.
[2] Eastman Kodak Co., Rochester, N.Y.
[3] Burdick and Jackson Laboratories, Muskegon, Mich.
[4] House reference standard strength *versus* USP reference standard is 100.3%.
[5] Aldrich Chemical Co., Milwaukee, Wis.; used without further purification.
[6] Synthesized by the procedure of Spoljarić *et al.* (7).
[7] Synthesized by the procedure of Ginsburg and Wilson (12).
[8] Synthesized by the procedure of Ellin and Kondritzer (2).

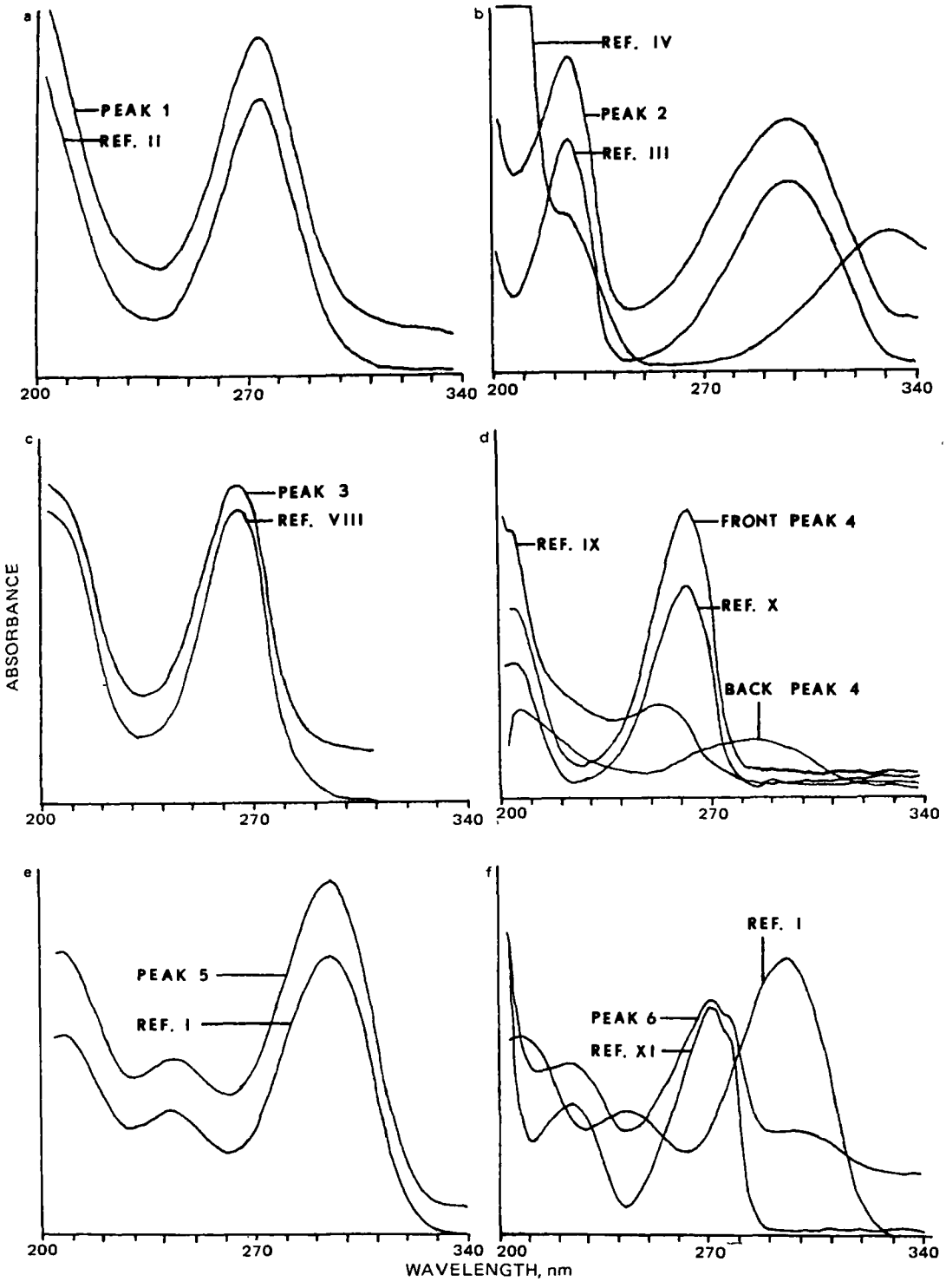

Figure 3—*Stop-flow UV absorption spectra of peaks 1–6 in Fig. 2c (injectable formulation stored at 80° for 4 weeks) and reference compounds of corresponding retention time.*

chloride[9] (VIII), 2-aminomethyl-*N*-methylpyridinium chloride[9] (IX), 2-hydroxymethyl-*N*-methylpyridinium chloride[9] (X), 2-cyano-*N*-methylpyridinium chloride[10] (XI), and *N*-methylpyridinium chloride[9] (XII).

Chromatographic System—The high-performance liquid chromatograph consisted of a mobile-phase pump[11] operated at 0.8 ml/min (1600 psi), a sample injector[12] set for a 15-μl injection volume, a variable-wavelength UV detector[13] at 270 nm (0.08 AUFS), a precolumn[14] positioned between the pump and the injector, and an analytical reverse-phase C-18 column[15]. Areas under the chromatographic peaks were measured by electronic integration[16]. The mobile phase consisted of 52% acetonitrile and 48% of an aqueous solution of 0.005 *M* phosphoric acid and 0.001 *M* tetraethylammonium chloride. Chromatographic data were acquired at ambient temperature.

Analytical Procedure—*Preparation of Sample and Standard Solutions*—A volume equivalent to 330 mg of I was transferred to a 250-ml volumetric flask and diluted to volume with water. A 1.0-ml aliquot of this solution was pipetted into a 50-ml volumetric flask and diluted to volume with the mobile phase.

Stock solutions were prepared by accurately weighing ~25, 50, and 75

[9] Synthesized by C. E. Orzech and F. Q. Gemmill, Jr., Ayerst Laboratories, Inc., Rouses Point, N.Y.

[10] Synthesized by the procedure of Ellin (1).

[11] Model 740-P, Spectra Physics, Santa Clara, Calif.

[12] WISP 710B, Waters Associates, Milford, Mass.

[13] Model LC-75 with autocontrol, Perkin-Elmer Corp., Norwalk, Conn.

[14] 250 × 4.6-mm i.d. column packed with LiChrosorb Si-60 (10 μm), MC/B Manufacturing Chemist Inc., Cincinnati, Ohio.

[15] 250 × 3.2-mm i.d., column packed with Spherisorb-ODS (5 μm), Phase Separations, Hauppauge, N.Y.

[16] Model 3354B, Hewlett-Packard, Avondale, Pa.

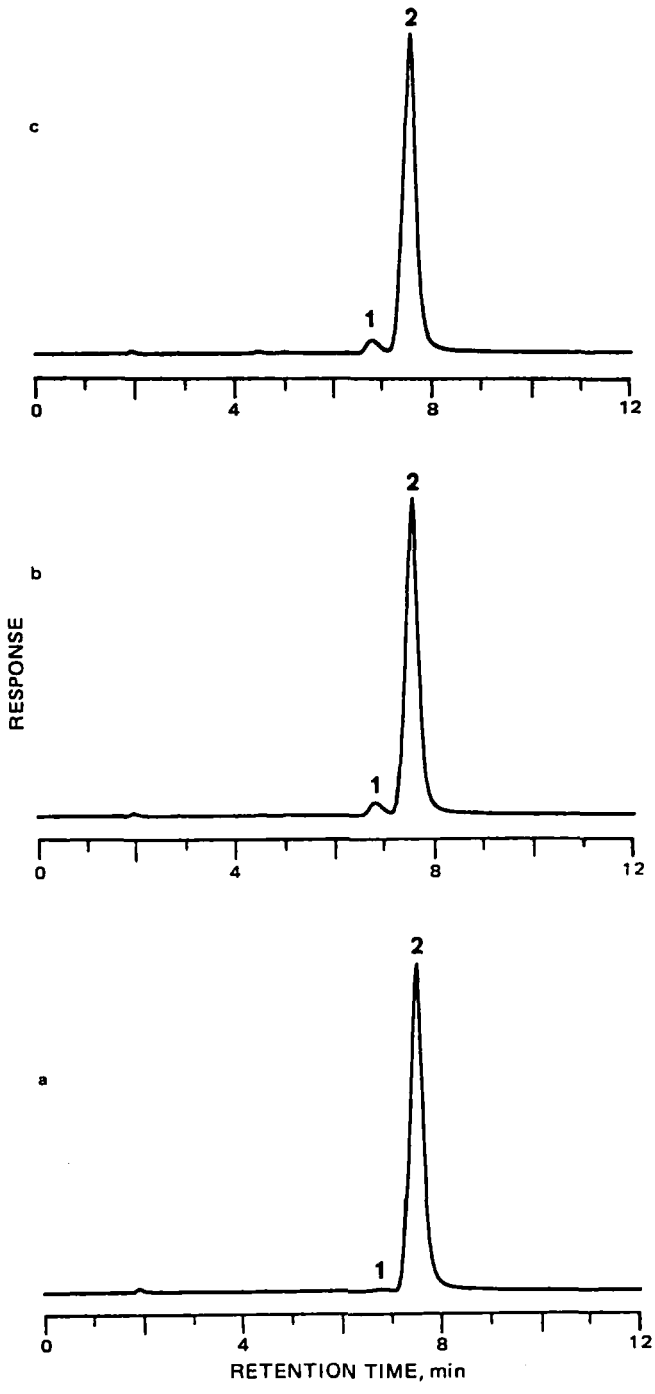

Figure 4—*Chromatograms of the injectable formulation at day zero (a) and after storage at room temperature for 8 (b) and 48 (c) weeks. Key: (1) unknown compound; (2) I.*

mg of I, transferring these amounts to separate 50-ml volumetric flasks, and dissolving them in water. A 1.0-ml aliquot of each stock solution was diluted separately to 50 ml with mobile phase. A standard solution containing II, III, VIII, and XI was prepared by accurately weighing ~33 mg of each compound, transferring these amounts to a single 50-ml volumetric flask, and dissolving and diluting them to volume with water. A 10.0-ml aliquot of this solution was diluted to 50 ml with water. A 1.0-ml aliquot of this diluted solution was pipetted into a 50-ml volumetric flask, which contained 1.0 ml of the stock solution containing 50 mg of I. The mixture was diluted to volume with the mobile phase.

System Suitability Test—The detector wavelength was set to 270 nm, 15 μl of the middle (50 mg) standard preparation was injected, and the flow rate of the mobile phase and sensitivity of the detector were adjusted so that the I peak eluted in ~7.5 min (~0.8 ml/min) and was at least 50% of full scale (~0.08 AUFS). After the chromatographic conditions stabilized, 15 μl of the middle (50 mg) standard preparation and 15 μl of the

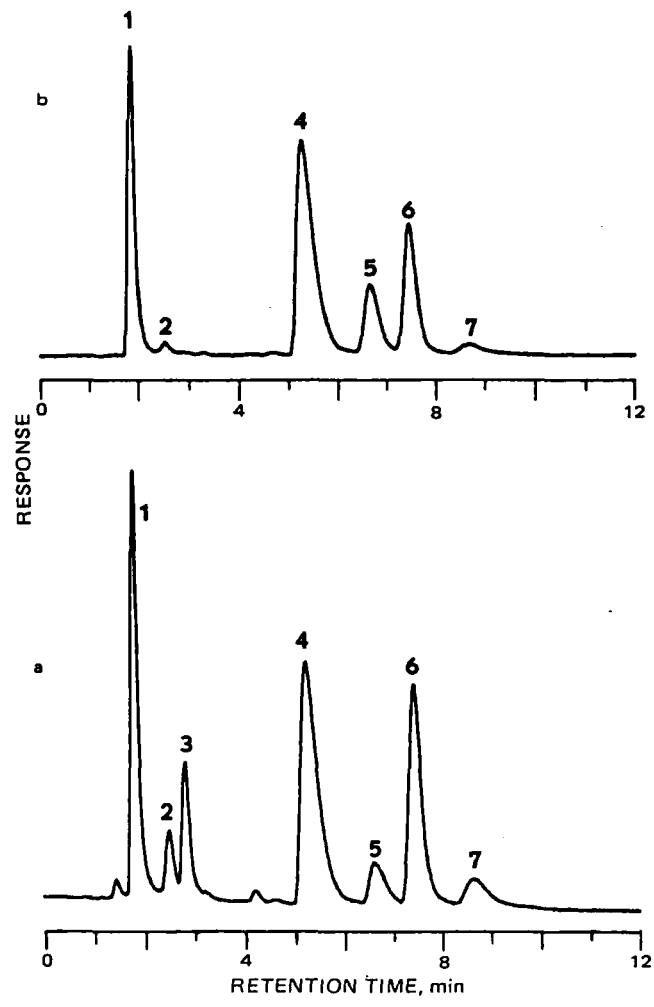

Figure 5—*Chromatograms of the injectable formulation stored at 80° for 4 weeks, using a detector wavelength of 200 nm (a) or 270 nm (b). Key: (1) II, (2) III, (3) benzyl alcohol, (4) VIII, (5) X and the unknown compound, (6) I, and (7) XI.*

standard preparation containing II, III, VIII, and XI were injected. The areas of the I peak in each chromatogram were obtained and compared; the chromatographic system was considered suitable for use if the areas were within 2%.

Injection Procedure—A 15-μl aliquot of each standard preparation was injected and the areas of the peaks for I, II, III, VIII, and XI were obtained. The same volume of each sample preparation was injected and the corresponding peak areas were determined.

Calculations—A least-squares linear regression line of the peak area *versus* weight of I (W_p) was obtained. The weight (W) corresponding to the peak area of I in the sample preparation was obtained from the regression line. The concentration of I in the sample was calculated from:

$$[\text{mg/ml}] = (W)(5)(P)(1/V) \qquad \text{(Eq. 1)}$$

where W is the weight of I corresponding to the area of I in the sample preparation chromatogram, P is the strength of the I reference standard (decimal), V is the volume (ml) of sample solution assayed, and 5 is the dilution factor (250/50). The concentration of each decomposition product detected in the sample was calculated from:

$$[\text{mg/ml}] = (A_2/A_1)(W_d)(P)(1/V) \qquad \text{(Eq. 2)}$$

where A_1 and A_2 are the areas of the appropriate decomposition product peaks (II, III, VIII, or XI) in the standard and sample preparations, respectively; W_d is the weight (mg) of the decomposition product; and P is the strength of the decomposition product standard (decimal). If a peak(s) was observed eluting between that of VIII and I (~6.7 min), a response equal to the latter was assumed and the concentration was calculated accordingly.

Table I—Wavelength Absorbance Ratios for Reference I and Injectable Formulation Stored for 2 Weeks at 80° [a]

Wavelength (λ), nm	Absorbance Ratios (Q) Peak Identity Reference/Sample	Peak Homogeneity Middle/Front	Middle/Back	Front/Back
210	0.74	3.10 (min)	3.70	1.20 (max)
220	0.75 (max)	3.24	3.51 (min)	1.09
240	0.75	3.47	3.81	1.10
250	0.75	3.54	3.82	1.08
270	0.74	3.87 (max)	4.30 (max)	1.11
280	0.74	3.56	3.68	1.04
290	0.75	3.40	3.53	1.04
300	0.74	3.48	3.59	1.03
310	0.72 (min)	3.85	3.85	1.00 (min)
$D = Q_{max}/Q_{min}$ =	1.04	1.25	1.23	1.20

[a] Front, middle, and back of peak 5 in Fig. 2b.

RESULTS AND DISCUSSION

Specificity and Identification of Decomposition Products—Previous reports (1–7) have indicated that the degradation of I can proceed *via* hydrolysis or dehydration to form a number of decomposition products. Among those decomposition products previously reported are II, III, IV, VII, VIII, and XI. Since pyridinium compounds readily form decomposition products, some of which may not have been described in these original studies, additional compounds including V (the primary synthetic precursor of I), VI (the *syn*-isomer of I), IX, X, and XII were also studied.

To accurately monitor the stability of I in the injectable formulation, it is mandatory that the chromatographic system be capable of separating I from all its possible decomposition products. Because of the quaternary structure of the pyridinium aldoximes, ion-pair HPLC was expected to provide the required separation. However, various mobile phase combinations employing classical ion-pairing reagents of opposite charge, *e.g.*, *N*-heptanesulfonic acid and sodium dodecanesulfonate, produced only partial separations and tailing of some peaks. Mobile phases employing tetraethylammonium chloride, a modifier with the same charge as the pyridine aldoximes, produced superior separations and more symmetrical peaks. Control of retention and peak shape by a modifier of the same charge as the sample is not considered to be the classical ion-pairing phenomenon, but is better explained by the broader-scoped concept of ion interaction (13).

The separation of I from II–XII using the aforementioned HPLC system is shown in Fig. 1. Compound XII is not shown in the chromatogram due to its excessively long retention time of 27.4 min. Experience with this chromatographic system has shown that different batches of Spherisorb-ODS have slightly different separation characteristics. These differences are attributed to variations in the amounts of free silanols present on the bonded silica. These differences can be minimized by modifying the composition of the mobile phase. The retention time of I can be shortened or lengthened by increasing or decreasing, respectively, the concentrations of tetraethylammonium chloride. Modifiers such as tetraethylammonium chloride minimize the interactions of the sample with residual silanols on the surface because the modifier can cover, or mask, these silanol groups. This coverage of accessible silanols is the basis of the competing base concept (14).

Table II—Wavelength Absorbance Ratios for Reference I and the Injectable Formulation Stored for 4 Weeks at 80° [a]

Wavelength (λ), nm	Absorbance Ratios (Q) Peak Identity Reference/Sample	Peak Homogeneity Middle/Front	Middle/Back	Front/Back
210	0.83 (min)	1.90 (min)	3.04 (min)	1.60
220	0.85	1.98	3.21	1.62
240	0.86 (max)	2.15	3.80	1.77
250	0.86	2.24 (max)	4.3 (max)	1.94 (max)
270	0.85	2.21	4.22	1.90
280	0.86	2.02	3.24	1.60
290	0.86	2.01	3.10	1.54
300	0.86	2.00	3.06	1.53 (min)
310	0.85	2.10	3.52	1.68
$D = Q_{max}/Q_{min}$ =	1.04	1.18	1.43	1.27

[a] Front, middle, and back of peak 5 in Fig. 2c.

Table III—Recovery of II, III, VIII, and XI from the Injectable Formulation

Spike Level[a], %	Recovery, % II	III	VIII	XI
1	114.7	115.4	100.0	114.7
2	105.9	109.1	92.6	106.0
5	99.4	98.8	97.6	95.8
10	100.9	101.2	99.1	100.3
20	99.4	100.0	101.0	102.2
50	96.8	97.6	100.0	100.8

[a] Concentration (in mg/ml) of II, III, VIII, or XI divided by 330 mg/ml, expressed as percent.

Table IV—Precision of the Concentrations of I and Its Major Decomposition Products Found in the Injectable Formulation Stored at 80° for 7 and 26 Days

Compound	7 Days @ 80° Average Concentration[a], mg/ml	*CV*	26 Days @ 80° Average Concentration[a], mg/ml	*CV*
I	276	1.0	93.3	1.5
II	15.7	3.9	83.7	1.3
III	—[b]	—	3.0	3.5
VIII	17.9	3.9	103	2.2
XI	—	—	4.3	6.5
Other[c]	15.5	5.4	22.3	3.0

[a] n = 10, five determinations on each of 2 days. [b] — None detected. [c] Composite of X and an unknown compound; assumes a response equal to I.

Figure 2 depicts the loss of I and the formation of its decomposition products in the injectable formulation which was stored at 80°. After 2, 4, and 8 weeks at 80°, the amounts of I remaining were 65, 20, and 3% of the initial concentration, respectively. Use of another mobile phase, which shortened the retention time of XII to 8 min, indicated that XII was not formed in measurable quantities in any of the degraded samples of the formulation.

The peaks corresponding to the decomposition products in Fig. 2 were tentatively identified by comparison of their respective retention times with those of reference compounds I–XII. Confirmation of the identity of each peak was made by comparison of its stop-flow UV absorption spectra with the stop-flow spectra of the reference compound of corresponding retention time. Stop-flow UV spectra of peaks 1–6 in Fig. 2c (injectable formulation stored for 4 weeks at 80°) are given in Fig. 3.

Other investigators (11) have recently studied the degradation of I under both acidic and basic conditions using ion-pair HPLC. Unfortunately, in the chromatographic system they employed, VII and VIII have the same retention time. Nevertheless, based on retention time data, it was reported that the only hydrolytic byproduct of I in acid solution (0.1 *N* HCl) was VII. Under basic conditions (0.1 *N* NaOH) it was reported that II, III, and VIII were formed. Although acid hydrolysis was the expected mode of decomposition in the injectable formulation (which is buffered at pH 2.5), it is evident from Fig. 2 that primarily products usually associated with base hydrolysis are formed. It is readily seen that the major decomposition products of I in the injectable formulation are II and VIII; III and XI are also formed, but in relatively small amounts. Compound VII, which is separated from VIII in Fig. 1, is not observed in any of the chromatograms in Fig. 2.

In addition to peaks corresponding to decomposition products previously reported, another peak (peak 4) with a retention time of 6.7 min was observed in the chromatograms in Fig. 2. The retention time of this peak corresponds exactly to X, since compound IX has a retention time of 6.5 min. As shown in Fig. 3d, the frontside of peak 4 has a stop-flow UV spectra consistent with the reference spectra of X, but the backside of

Table V—Stability of I, II, III, VIII, and XI in Mobile Phase

Compound	Days at Room Temperature[a] 2	5	6	8	12
I	99.1	100.5	—	—	100.2
II	102.5	—	104.2	102.2	101.4
III	99.1	—	98.1	100.6	99.9
VIII	100.8	—	101.1	103.8	104.5
XI	99.0	—	100.1	100.9	108.8

[a] Results are expressed as percent of initial input.

Table VI—Comparison of HPLC and USP Results For I in the Injectable Formulation

Storage Conditions	Concentration of I, mg/ml HPLC	USP
Initial	324	328
2 Days, 80°	309	316
6 Days, 80°	285	294
9 Days, 80°	272	281
13 Days, 80°	249	255
21 Days, 80°	156	160
28 Days, 80°	62.6	66.6

the peak has a spectra which does not match any of the reference compounds. These differing spectra suggest that peak 4 is a composite of X and another substance. This was later confirmed when peak 4 was resolved into two components using a different mobile phase. The first component was identified as X. The identity of the second component is unknown, but is of major interest because it has been detected in solutions of I when no other decomposition peaks are observed (Fig. 4). This substance seems to be formed soon after the dissolution of I in aqueous media, then gradually increases to an equilibrium concentration of ~3% (area percent relative to I). Similar observations have been made by other investigators[17] using a method based on ion-pairing HPLC. As of this writing, the presence of X and the unknown compound in degraded solutions of I has not been reported in the literature.

The formulation that was decomposed at 80° for 4 weeks (Fig. 2c) was chromatographed at the nonspecific wavelength of 200 nm to observe any other peaks that might appear and are not detected at 270 nm. Figure 5 demonstrates that no other decomposition peaks of significant intensity were detected at 200 nm, and that the analytical wavelength of 270 nm provides adequate sensitivity for I and the corresponding decomposition products. However, a formulation excipient, benzyl alcohol, was detected at 200 nm (Fig. 5a).

Stop-flow spectroscopy, employing the spectral discrimination technique (15), was performed on the peaks corresponding to I in the injectable formulations that were decomposed at 80° for 2 (Fig. 2b) and 4 weeks (Fig. 2c). These storage conditions produced 35 and 80% decomposition of I, respectively. Absorbance readings at nine discrete wavelengths across the UV spectrum were obtained on the front, middle, and back of each I peak. Similarly, absorbances at each wavelength were obtained on a reference peak of I from a separation injection. If the ratio (Q) of absorbance values at each wavelength between the reference and sample peaks remains constant ($D = Q_{max}/Q_{min} \leq 1.5$), evidence is strong that the sample peak has the same identity as the reference peak. Likewise, if the ratio of absorbances taken from different portions of a single peak are constant, then the peak is considered homogeneous and is, therefore, the response of a single molecular species. For all cases, the test for peak identity and peak homogeneity was positive, *i.e.*, the values of the spectral discriminator (D) were between 1 and 1.5 (Tables I and II). These data strongly suggest that the chromatographic system separates I from all UV-absorbing components in the degraded formulation.

Accuracy—Known amounts of I corresponding to 80, 100, and 120% of the label claim (330 mg/ml) were added to a placebo of the injectable formulation and assayed. The recoveries of I from the spiked formulations were 100.2, 99.9, and 99.6%, respectively.

The accuracy of determining various concentrations of the decomposition products (those actually detected in the decomposed formulation) was determined by adding known amounts of II, III, VIII, and XI to the formulation at levels corresponding to 1, 2, 5, 10, 20, and 50% decomposition. Therefore, the spiked formulation representing 1% decomposition contained ~3.3 mg/ml of each decomposition product, the spiked formulation representing 10% decomposition contained ~33 mg/ml of each decomposition product, *etc.* Recoveries of II, III, VIII, and XI were calculated from a single chromatogram that represented each spike level. The results are given in Table III. The accuracy of determining the concentration of X was not evaluated because its peak, being a composite of two substances in the degraded formulation, precludes such a determination.

Precision—Replicate assays were performed on the injectable formulation which was stored for 7 and 26 days at 80° to promote decomposition. Assays were performed 10 times over a period of 2 days using freshly prepared standards on each day of analysis. Between determinations the solutions were stored at 4°. The results are given in Table IV and indicate that the method has acceptable precision for determining all compounds of interest.

Linearity—The UV absorbance of I at 270 nm increased linearly (r = 0.9999874, y-intercept = −338) with concentration in the range corresponding to 125–375 mg/ml in the injectable formulation. Although the analytical method does not require that a calibration curve be prepared for each decomposition product, the responses of II, III, VIII, and XI at 270 nm are linear over the range 3.3 (limit of detection) to 165 mg/ml in the injectable formulation. The correlation coefficients for curves in this range were: II, r = 0.9999504, y-intercept = 452; III, r = 0.9999574, y-intercept = 243; VIII, r = 0.9999806, y-intercept = −73; and XI, r = 0.9999516, y-intercept = 62. An evaluation of the y-intercepts of each curve indicated that they were not statistically different from zero at $p < 0.05$.

Stability of I and Decomposition Products in Mobile Phase—The stability of I and its decomposition products during the time required to perform the analytical procedure is an important consideration. During the determination, I and its decomposition products should not be subjected to conditions of acid or base hydrolysis other than that which has actually occurred in the formulation. The high pH requirements of the UV procedure in USP XX for determining I in the presence of its decomposition products promotes base hydrolysis and requires that the analytical measurements be performed as rapidly as possible. The UV method does not easily lend itself to unattended operation because of this requirement.

The stability of I and its decomposition products during the time required to perform the HPLC procedure was assessed by preparing solutions of I, II, III, VIII, and XI in accordance with the analytical procedure, and assaying them at various intervals after storage at ambient laboratory conditions. The results (Table V) indicate that solutions of these compounds are stable in the mobile phase not only for the time required to perform the procedure, but for extended times up to several days. Consequently, with the use of an autoinjector, unattended chromatographic separation and measurement may be performed.

Comparison of HPLC and USP Procedures on Degraded Formulations—A comparison of the concentrations of I in degraded formulations found by the HPLC and USP procedures is given in Table VI. Although good agreement is observed, there is a consistent bias of ~2–3% between the two procedures. The higher values obtained by the USP procedure may be the result of interference, since the USP procedure requires that the measurement of I be made in the presence of its decomposition products. To its advantage, the HPLC procedure separates the decomposition product prior to the determination of I.

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[17] L. Ernerot, Astra Lakemedal AB, S-151 85 Sodertalje, Sweden, personal communication.