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DETERMINATION OF 2-HYDROXYIMINOMETHYL-1-METHYL-PYRIDINIUM METHANESULPHONATE (PRALIDOXIME MESYLATE, P2S) AND ITS DEGRADATION PRODUCTS IN SOLUTION BY LIQUID CHROMATOGRAPHY

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SUMMARY

Pralidoxine mesylate (P2S) and its degradation products in solution can be analysed by ion-pair high-performance liquid chromatography. The accuracy and sensitivity of the methods are high and allow a mass balance for the decomposition to be obtained. The decomposition is shown to proceed via an initial reversible isomerisation step to *anti*-P2S which has not been detected in previous studies and which is shown to be capable of giving misleading predictions of storage life if UV methods of analysis are used.

INTRODUCTION

Pralidoxime (2-hydroxyiminomethyl-1-methylpyridinium) salts have been used for many years for the treatment of organophosphate anticholinesterase poisoning. The materials have usually been formulated in solution with anticholinergic drugs such as atropine and delivered as an intramuscular injection[1,2]. The stability and storage life of such formulations has been studied extensively and there is some indications that the pralidoxime is most stable in the range pH 3–4. First order kinetics have been observed in most studies although the predicted life of formulations does seems to be variable and is still the source of debate. The stability of pralidoxime salts in solutions has most recently been reviewed by Boeke[3].

The pralidoxime content of formulations has usually been analysed by the measurement of the UV absorption of the pralidoxime anion in alkaline solution at 335 nm and under these conditions the principal degradation products have been shown not to interfere[4]. The degradation products have been identified by UV spectrophotometry[5] and by paper chromatography[6] but have not been analysed quantitatively in stability studies. The general degradation routes (Fig. 1) show that the pralidoxime hydrolyses to the aldehyde and hydroxylamine in strong acid solution and is dehydrated to the cyanide followed by conversion to the amide and acid or pyridone in alkaline solution. Studies of the reaction at the pH of maximum stability have been limited and only the presence of the cyanide and amide has been reported

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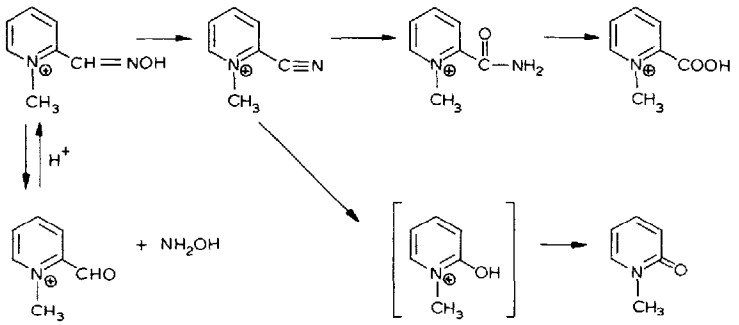

Fig. 1. Degradation routes of pralidoxime salts in acid and alkaline solution.

with no indication of acid, pyridone or aldehyde[6]. A recent paper using a high-performance liquid chromatographic (HPLC) method confirmed the presence of the above reaction at the pH extremes but did not detect the presence of the cyanide[7].

In order to characterise fully the formulation chosen for use in treatment a comprehensive study was required at the pH of maximum stability rather than at the extremes of the pH range. The method was required to be sufficiently accurate and precise, especially for the pralidoxime, and sufficiently selective with respect to the degradation products to allow a mass balance to be made for the reaction and to give some assurance that all the major degradation products were being accounted for. This paper describes such a method using HPLC.

During the development of the method an unidentified peak consistently appeared in the chromatograms of both standard solutions and degraded samples of pralidoxime mesylate (P2S). The identification of this component is described and its involvement in the degradation mechanism of P2S is discussed. The limitations of the UV method used in previous studies in the presence of this material is also discussed.

The method has been specifically developed for the analysis of the contents of an injection device containing 2 ml of a solution of P2S (250 mg/ml) and atropine sulphate (1 mg/ml) in 0.1 *M* citrate buffer, pH 3.2.

EXPERIMENTAL

Apparatus

The HPLC system used in this study comprised a Model 6000A pump (Waters Associates), 7125 injection valve (Rheodyne), Hypersil ODS column (Shandon), column oven (Jones Chromatography), variable wavelength UV detector with Autocontrol Model LC75 (Perkin-Elmer) and Model CRS 304 computing integrator (Infotronics).

UV studies were made on a Model 551S spectrophotometer (Perkin-Elmer).

NMR identification was performed with a Model MH-100 spectrometer (Jeol). Sample irradiation was by a continuous xenon source from a spectrofluorimeter (Aminco-Bowman).

Materials

Pralidoxime mesylate (P2S) 2-formyl-1-methylpyridinium (P2S-aldehyde), 2-cyano-1-methylpyridinium (P2S-cyanide) and 2-carbamoyl-1-methylpyridinium (P2S-amide) as their iodide salts, and 1-methyl-2-pyridone (P2S-pyridone), were prepared at Chemical Defence Establishment. 2-Carboxy-1-methylpyridinium chloride (P2S-acid) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and nicotinamide from BDH (Poole, Great Britain).

Chromatographic procedure

Preparations of mobile phase. About 40 ml of trimethylamine as a 25% aqueous solution were added to 1 l of 0.111 *M* orthophosphoric acid and the solution thoroughly mixed. Further phosphoric acid solution was added until the pH was 3.00. This solution (900 ml) was added to methanol (100 ml) and sodium lauryl sulphate (2.88 g) dissolved in the mixture. The mobile phase was degassed before use with a stream of helium passed through a metal frit and maintained air-free during use with a slow helium bleed. The trimethylamine was used in the mobile phase to reduce tailing and improve peak shape and resolution of organic ammonium ions which are known to be troublesome[8]. Mobile phase was pumped through the column at 2 ml/min until the sodium lauryl sulphate breakthrough occurred and a stable baseline was obtained. This took typically 30 min.

Chromatographic conditions. Mobile phase: 0.1 *M* trimethylamine phosphate, pH 3.0, in methanol–water (10:90) + 10^{-2} *M* sodium lauryl sulphate. Column: 12.5 cm × 4.6 mm Hypersil ODS. Injected volume: 2 μl by syringe into 20-μl loop. Column temperature: 30°C. Analytical wavelength: 262.0 nm. Flow-rate: 1.0 ml/min. Column pressure: 800–1000 p.s.i. Run time: 1000 sec.

Calibration. P2S was recrystallised from ethanol, washed and dried at 60°C under vacuum over freshly dried silica gel, cooled and weighed. The drying process was repeated until the weight loss was less than 0.1% and the standard solutions prepared immediately. The P2S purity was checked by spectroscopic methods, HPLC and titration. Nicotinamide was recrystallised from acetone, washed and dried and a stock solution of 2.000 mg/ml in mobile phase prepared. The degradation products were dried at room temperature before use. Standard solutions were prepared containing 1.0 mg/ml of nicotinamide as an internal standard and 2, 5, 10, 12.5 and 15 mg/ml of P2S. The degradation product standards contained 1.0 mg/ml nicotinamide, 12.5 mg/ml P2S and each of the products except P2S-aldehyde over the concentration range 25 μ*M* to 10 m*M* corresponding to 0.05–20% of the amount of P2S. Calibration curves were prepared for P2S and the degradation products.

Analysis of samples. A 0.5-ml aliquot of a formulation containing a starting concentration of 250 mg/ml of P2S was transferred to a weighed 10-ml flask and the volume determined by calculation from the density of the solution, 1.076 g/ml. This approach was used because of the inadequate accuracy and precision of volumetric measurement in this situation. 5 ml of the internal standard stock solution were added and the solution made up to 10 ml with mobile phase. This solution was analysed using 2-μl injections.

Evaluation of the method

Linear range and detection limit. The linear range and detection limit of the method were determined from the calibration process.

Precision. The precision of the chromatography was determined by multiple injections of standard solutions. For P2S the precision was determined at a level corresponding to 100% and for the degradation products at levels corresponding to 1% of the P2S concentration. The overall precision of the method was determined by the multiple analysis of a sample of P2S solution (250 mg/ml in 0.1 *M* citrate buffer, pH 3.2) which had been stored at 80°C for 2 days.

Interferences. The interference from atropine was assessed by the direct injection of 2 μl of a freshly prepared solution of atropine sulphate (2 mg/ml) in citrate buffer and injection of the same solution that had been maintained at 80°C for 2 days and 14 days.

The presence of impurity peaks from the internal standard was determined by injection of the diluted stock solution containing 1.000 mg/ml of nicotinamide.

P2S solution which had been maintained at 80°C for 2 days and 7 days was diluted 20:1 as in the general method but without the internal standard in order to assess the interference of degradation products with the internal standard peak.

Comparison of UV and HPLC methods. Samples of freshly prepared P2S solution and solution which had been heated at 80°C for 2 days were analysed by the HPLC and UV methods. Interference with the UV method was assessed by observation of the spectra of the decomposition products in alkaline solution.

Application of the method to stability studies

In order to conveniently evaluate the method samples arising from accelerated storage conditions were used. P2S solution was stored at 80°C for up to 14 days in sealed tubes. Samples were removed periodically, cooled to room temperature and analysed. In a more realistic parallel study P2S (250 mg/ml) and atropine sulphate (1 mg/ml) were stored in an injection device (Combopen-Duphar BV, Amsterdam, The Netherlands) at 0–5, 20, 30 and 40°C and the contents analysed after 6 and 11 months.

Identification of the major unknown peak

The major unknown peak was collected from 10-μl injections of a P2S solution that had been heated at 80°C for 8 h. Under these conditions the unknown peak was near its maximum size while the concentration of other degradation products was low. The UV spectrum of this solution was recorded at pH values over the range 3.0–12.0 in order to detect the presence of any ionisable groups. Attempts were made to isolate the unknown material from the mobile phase by solvent extraction and chromatographic adsorption on ion-exchange, silica, reversed-phase and polymer material for identification. The effect of heating the collected unknown to 80°C was observed by direct analysis of the solutions by HPLC in order to observe its role in the degradation process.

The unknown material was prepared by UV irradiation of dilute solutions of P2S (10 mg/ml) in water using a high intensity xenon lamp from a spectrofluorimeter. The solution in a 1-cm fluorimeter cell was cooled below 15°C with a standard spectrophotometer thermostat block. Samples for NMR analysis were prepared in ^2H_2O solution. The course of the photochemical reaction was monitored by the HPLC method over 24 h.

RESULTS AND DISCUSSION

Chromatographic performance

Typical test chromatograms (Figs. 2 and 3) showed good separation of the major degradation products of P2S. The sensitivity of detection of the pyridone at 262 nm was low but it could be improved by an order of magnitude by using the wavelength of maximum absorption at 298 nm. However in this study it did not appear as a major degradation product. The P2S-aldehyde is formed only in strongly acid solution from P2S and was eluted as a broad symmetrical peak and was not satisfactorily handled by this method although in the present study over the range pH 3–4 the P2S-aldehyde was not observed. A sharper peak could be obtained by using a higher concentration of organic modifier in the mobile phase but the other major products were not then adequately separated.

In the context of the analysis of pharmaceutical formulations the ability to detect low concentrations of degradation products in the presence of a large excess of the major component is an essential requirement. Figs. 2 and 3 show that this can easily be achieved for the major degradation products at the 1 % and 0.05 % level. It was possible to detect and measure products as low as 0.01 % of the P2S. Calibration curves were routinely drawn from 0.05 % to 30 % of the P2S level and were linear over this range. For P2S itself the calibration was linear over the range 0–120 % relative to an undiluted starting concentration of 250 mg/ml. The absorbance at the top of the P2S peak was 1.7 at 12.5 mg/ml which is well within the linear range of the detector. Both major component and trace levels of degradation products could therefore be determined in a single analysis.

The precision of the chromatographic step and the overall analysis for P2S were the same with a relative standard deviation of 0.6 %. For the degradation products at the 1 % level a relative standard deviation overall of 2 % or better, depending on the particular compound, was obtained. The method is therefore sufficiently precise to allow a reasonable mass balance for the degradation reaction to be obtained. The overall accuracy would be limited by the purity of the material used for calibration and although no certified reference material was available the P2S was considered to be more than 99 % pure (see Experimental section). P2S is extremely hydroscopic so a rigorous drying procedure was employed before its use in the calibration process.

No peaks were detected in the chromatogram from fresh or degraded atropine even at levels an order higher than would be found in a formulation, nor were there any peaks that would be integrated together with the internal standard peak from P2S degraded at 80°C for 2 days. At 7 days two small peaks appeared near the internal standard peak with a combined area of 0.7 % of the internal standard but under these conditions the P2S was extensively degraded. The nicotinamide had an impurity peak of 0.1 % relative area at a retention time of 140 sec that was eluted after the pyridone peak.

Application to degradation studies

The results of the trial at 80°C are summarised in Fig. 4. The unknown peak was clearly visible in the starting solution and was detected in all calibration solutions (peak 5 in Figs. 2 and 3). After only 15 min at 80°C it was greater than 1 % of the P2S

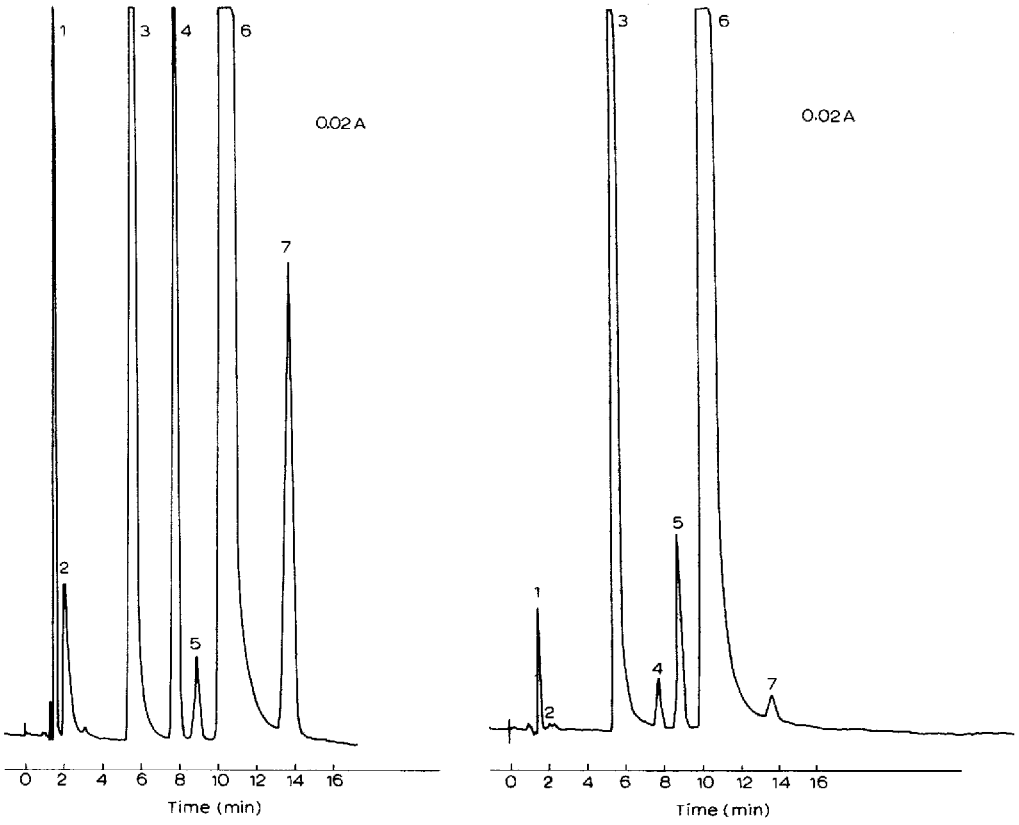

Fig. 2. Test chromatogram of a solution containing degradation products at the 1 % molar concentration relative to P2S. Peaks: 1 = P2S-acid; 2 = P2S-pyridone; 3 = nicotinamide internal standard (1.0 mg/ml); 4 = P2S-amide; 5 = unknown (*anti*-P2S), see Results and discussion; 6 = P2S (12.5 mg/ml); 7 = P2S-cyanide.

Fig. 3. Test chromatogram of a solution containing degradation products at the 0.05 % molar concentration relative to P2S. Peaks as in Fig. 2.

area and reached a plateau of about 7.5 % of the starting P2S area after 5 h. The P2S-cyanide and P2S-amide were just detectable at 2 h (Fig. 5). When the P2S was extensively decomposed the major product was the P2S-amide. The general appearance of Fig. 4 indicates that the unknown was formed early in the degradation pathway with perhaps an initial equilibrium between P2S and the unknown followed by reaction to the P2S-cyanide. After 2 days at 80°C when the P2S content had fallen to 88 % of the starting level further unknown peaks appeared (Fig. 6), the largest eluted in front of the amide peak with a relative peak area of 1 % of the original P2S level. At 7 and 14 days numerous small peaks and unresolved shoulders were observed although under these conditions the P2S was so extensively decomposed to be unusable for all practical purposes.

Table I shows the degradation of P2S under more realistic storage conditions in the temperature range 0–40°C. The major products were the same as at higher temperatures although the major unknown peak reached a lower equilibrium level

than at 80°C. The other unknown peaks were also observed in the extensively degraded samples. A good overall mass balance was achieved within the accuracy and precision of the method.

Identification of the major unknown peak

Attempts to isolate the unknown material from the mobile phase in which it had been collected failed because of its extreme water solubility. In this respect it was similar to P2S and the other products and indicated that the quaternary nitrogen was still present. The UV spectrum in the mobile phase at pH 3.0 (Fig. 7) was quite different from P2S (Fig. 8) but gave little structural information. However the change in the spectrum with varying pH showed that the material has an ionisable proton with a pK_a of 8.7 in the mobil e phase compared with 8.0 for P2S. On reacidification the original UV spectrum was observed indicating that the material was not the carbinolamine structure observed in early studies on P2S related compounds[9] although its UV spectrum was similar.

When the collected unknown was heated to 80°C P2S was regenerated as the major product together with smaller amounts of the P2S-amide, P2S-cyanide and P2S-acid decomposition products. No new peaks were observed. This indicated that the unknown material was closely related to P2S and could be reconverted to it using only the components of the mobile phase. In P2S solution there is probably an equilibrium between P2S and the unknown.

Pralidoxime salts are known to exist completely in the *syn*-form from X-ray crystal structure determination[10] and from ^{15}N NMR studies[11], but the *anti*-isomer has been prepared by the UV irradiation of *syn*-pralidoxime in solution[11]. In the present study the irradiation of P2S solution showed that a compound was formed with the same chromatographic and UV absorption properties as the unknown peak in the degradation studies. The chromatogram obtained from a 10 mg/ml solution irradiated for 24 h (Fig. 9) showed that some P2S-amide and P2S-cyanide were formed even though the sample was cooled and that further degradation can proceed

TABLE I

DEGRADATION OF P2S

The figures are percentages relative to the starting concentration of P2S on a molar basis. Unknown 5 is the major unknown peak number (5) in the chromatograms subsequently identified as *anti*-P2S. The percentage has been corrected for response as described in the section on its identification. The other unknowns are assumed to have the same molar response as P2S.

Storage temperature (°C)	*Stored for 6 months*				*Stored for 11 months*			
	0–5	*20*	*30*	*40*	*0–5*	*20*	*30*	*40*
P2S	95.5	95.8	94.4	83.5	95.8	95.2	92.9	68.8
Unknown 5 (*anti*-P2S)	3.1	3.3	3.6	3.7	3.3	3.3	3.2	2.8
P2S-cyanide		0.09	0.31	0.4	0.02	0.1	0.25	1.6
P2S-amide		0.16	1.0	7.6	0.03	0.26	2.3	17.7
P2S-acid			0.32	3.6		0.05	0.81	5.9
Other unknowns				1.0			0.32	2.4
Total	98.6	99.4	99.6	99.8	99.1	98.9	99.8	99.2

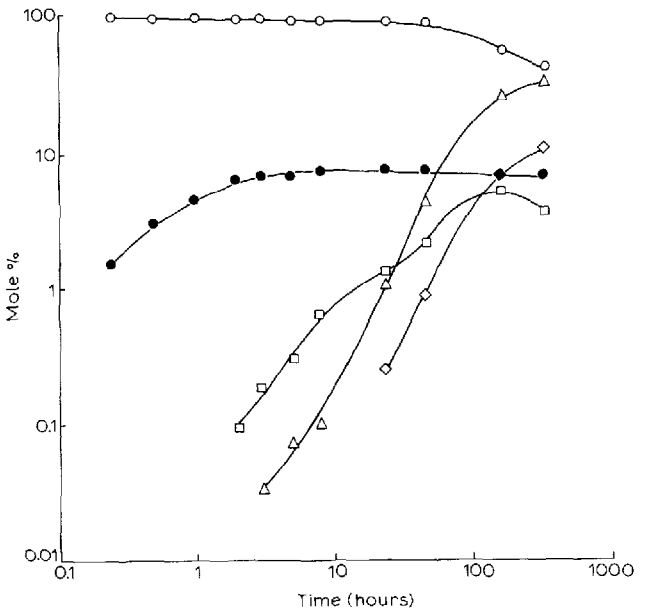

Fig. 4. Degradation of P2S solution (250 mg/ml in 0.1 *M* citrate buffer, pH 3.2) at 80°C. Curves: ○, P2S; ●, unknown (*anti*-P2S), see Results and discussion ; □, P2S-cyanide; △, P2S-amide; ◇, P2S-acid.

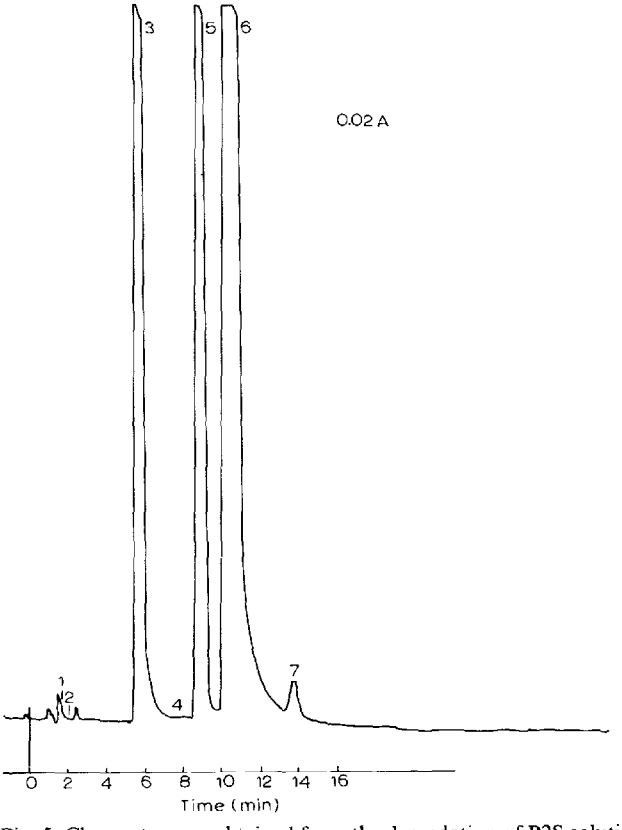

Fig. 5. Chromatogram obtained from the degradation of P2S solution (250 mg/ml in 0.1 *M* citrate buffer) after 2 h at 80°C. Peaks: 1 = P2S-acid; 2 = P2S-pyridone; 3 = nicotinamide (1.0 mg/ml), internal standard; 4 = P2S-amide; 5 = unknown (*anti*-P2S), see Results and discussion; 6 = P2S; 7 = P2S-cyanide.

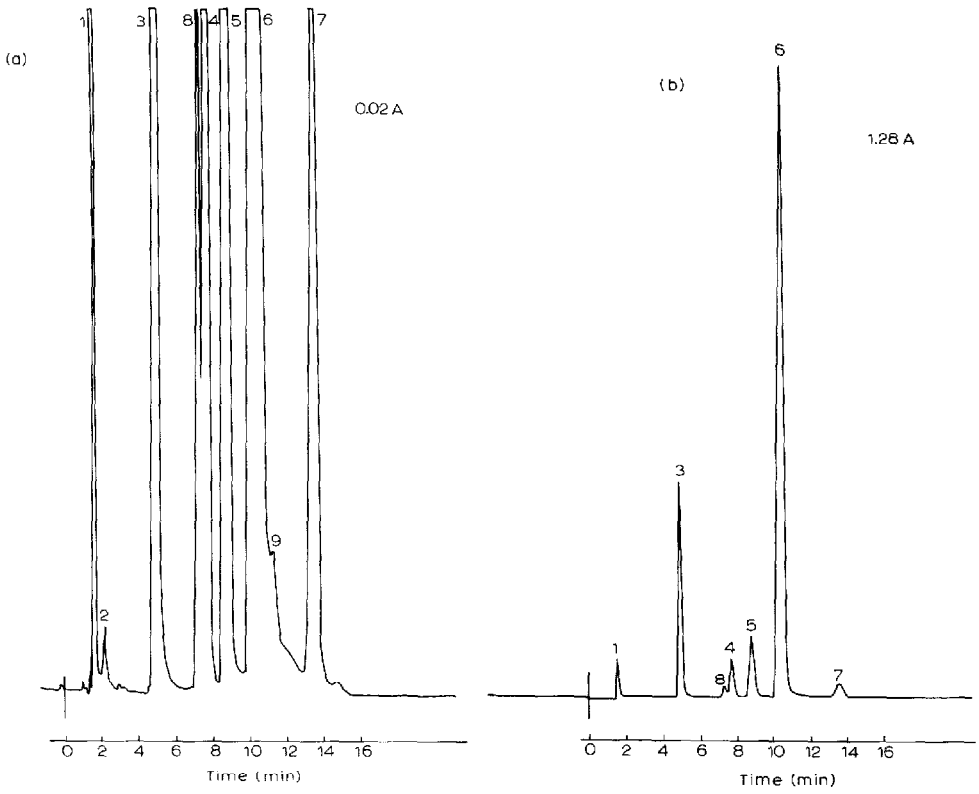

Fig. 6. Chromatograms obtained from the degradation of P2S solution (250 mg/ml in 0.1 *M* citrate buffer) after 2 days at 80°C: (a) 0.02 A; (b) 1.28 A full scale deflection. Peaks 1–7 as in Fig. 5; 8, 9 = unknowns.

by a photochemical as well as a thermal process. The failure of the chromatogram to reach baseline between the peaks indicated that a small amount of P2S-aldehyde was also formed in the unbuffered solution. It is therefore suggested that the unknown compound was the *anti*-isomer of P2S. The maximum yield of *anti*-P2S that could be obtained was 55 % after 24 h. Further irradiation led to more decomposition. The use of more dilute P2S solutions allowed the maximum yield of *anti*-isomer to be obtained more quickly although higher percentage yields could not be achieved.

Further evidence for the identification of the unknown peak as *anti*-P2S was obtained by ^{1}H NMR. The proton spectrum showed two signals from the methine hydrogen consistent with a 40:60 mixture of isomers. The *syn*-signal at τ 1.4 and the *anti* at τ 2.1 indicated that the –OH group has a greater deshielding effect than the nitrogen lone pair and are consistent with the values obtained for the geometrical isomers of 4-pyridine oximes[12].

A revised degradation pathway for P2S at pH 3.2 is shown in Fig. 10.

Since a pure sample of *anti*-P2S could not be obtained the UV calibration factor was deduced from the conversion of *syn*-P2S to *anti*-P2S by UV irradiation and by the action of heat on the *anti*-isomer collected by chromatography which gave only *syn*-P2S, P2S-amide and P2S-cyanide. It was found that *anti*-P2S absorbed 1.24 times

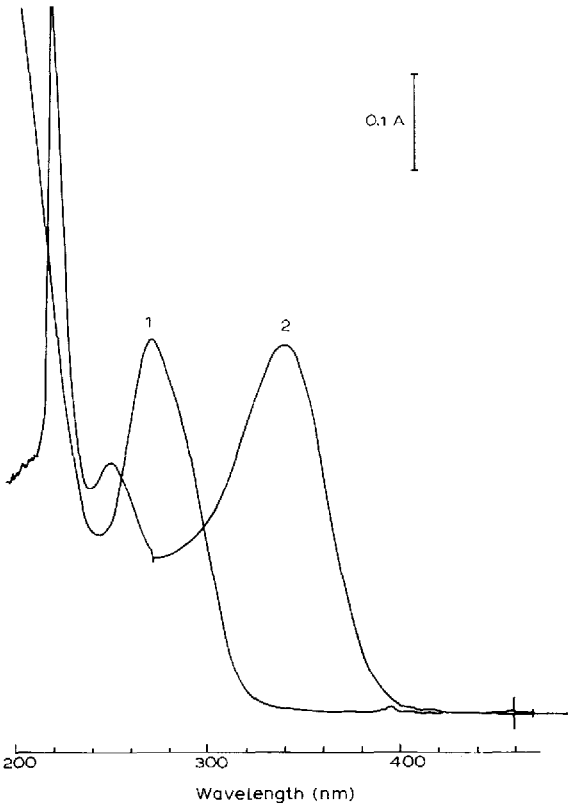

Fig. 7. Spectrum of unknown peak (peak 5 in the chromatograms) collected from degraded P2S: 1, diluted 5:1 with mobile phase, pH 3.0; 2, diluted 5:1 with 0.1 *M* NaOH (methanol–water, 10:90), pH 12.2, after dilution. Concentration after dilution estimated to be 15.2 μg/ml (see Results and discussion).

more strongly than *syn*-P2S at the analytical wavelength of 262 nm. This correction factor was applied to the peak area ratios obtained in the degradation studies (Table I) and was used in the measurement of the concentration of the *anti*-isomer in the solution collected from the column (Fig. 7).

Comparison with the UV method of analysis

The UV spectra of *syn*- and *anti*-P2S in alkaline solution (Figs. 7 and 8) are not identical. The *anti*-isomer has a broader absorption with an extinction coefficient only 30% of that of the *syn*-isomer and with a λ_{max} at 338 nm compared with 335 nm for *syn*-P2S. The UV method therefore measures accurately neither the *syn*-P2S nor the total, *syn* + *anti*-P2S, concentration but gives a value between the two. Furthermore, since the *anti*-P2S was found to be much less stable than *syn*-P2S in strongly alkaline solution the time between making up the solution and reading was critical. The precision of the HPLC method, 0.6%, was also much better than the 1.7% relative standard deviation that could be obtained with the UV method. Analysis of a degraded sample (80°C, 2 days) by UV gave a P2S value of 95.4% of the starting concentration compared with the HPLC value of 92.8% for the *syn*-isomer and 5.3%

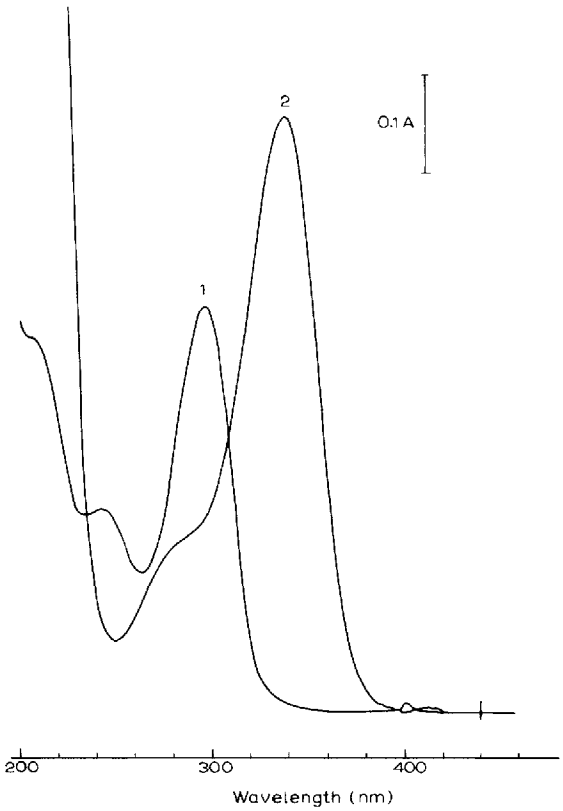

Fig. 8. Spectrum of P2S in mobile phase: 1, diluted 10:1 with mobile phase, pH 3.0; 2, diluted 10:1 with 0.1 *M* NaOH (methanol–water, 10:90), pH 12.2. Concentration after dilution: 7.5 μg/ml.

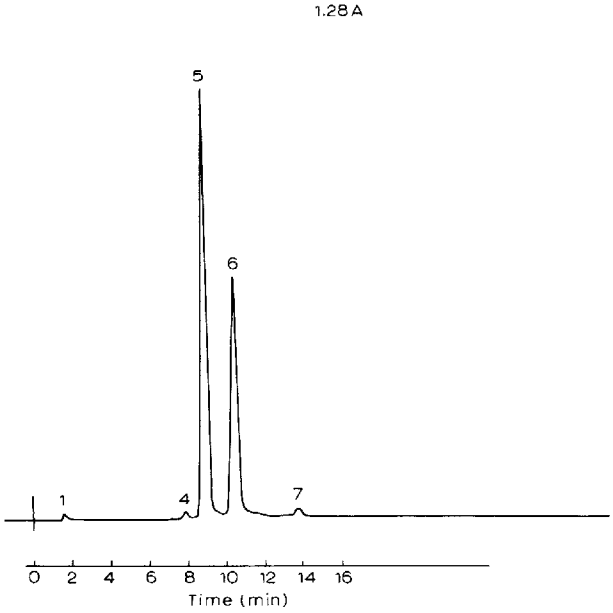

Fig. 9. Chromatogram obtained from the irradiation of P2S solution (10 mg/ml in ^2H_2O) by a xenon lamp for 24 h at 15°C. Peaks: 1 = P2S-acid; 4 = P2S-amide; 5 = *anti*-P2S; 6 = *syn*-P2S; 7 = P2S-cyanide.

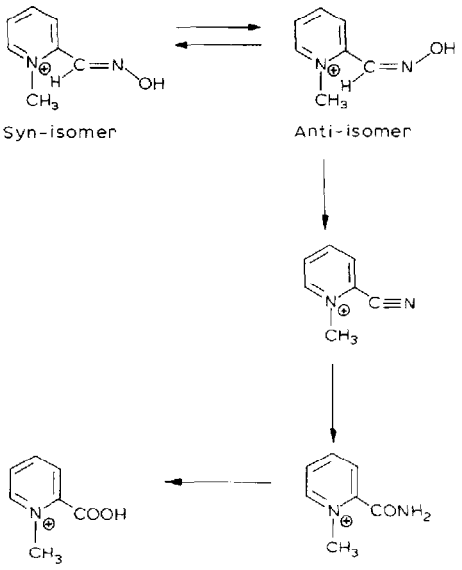

Fig. 10. Degradation route for P2S at pH 3.2.

for the *anti*-P2S. It was confirmed that the other degradation products do not interfere with the UV method to an extent greater than 0.1 %.

Most of the previous studies of the storage life of pralidoxime formulations have assumed a first order reaction but this work indicates that there is an initial equilibrium between the *syn*- and *anti*-isomers. Since the UV method measured predominantly the *syn*-isomer lifetime predictions based on the initial reaction rate when the equilibrium is being established could be erroneously short. The present HPLC method should allow more meaningful data to be acquired.

CONCLUSIONS

(1) A HPLC method for the analysis of P2S and its degradation products in formulations has been developed.

(2) The decomposition of P2S proceeds via the *anti*-isomer.

(3) Errors associated with the methods used and assumptions made in the stability studies on pralidoxime salts described in the earlier literature may have led to inaccurate predictions of storage life.

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