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ANALYSIS OF FORMULATIONS CONTAINING PRALIDOXIME MESYLATE BY LIQUID CHROMATOGRAPHY

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SUMMARY

An improved liquid chromatography procedure has been devised for the analysis of pralidoxime mesylate (P2S) and its degradation products in solution. An additional degradation product, 2-hydroxyaminoiminomethyl-1-methylpyridinium, has been positively identified and its route of formation and that of another recently identified product, 2-hydroxymethyl-1-methylpyridinium, has been established. Previous problems with the chromatography of 2-formyl-1-methylpyridinium have been resolved and the new method will resolve P2S and eight of its degradation products.

INTRODUCTION

Pralidoxime (2-hydroxyiminomethyl-1-methylpyridinium) salts have been used for the treatment of anticholinesterase poisoning for many years with the drug delivered as an intramuscular injection in aqueous solutions. Problems have been encountered with the stability of the formulation and analytical methods for the determination of the pralidoxime salt and of its decomposition products are therefore required. In a previous paper[1] a method using liquid chromatography (LC) was described which gave good quantitation of pralidoxine mesylate (P2S) and could determine most of the then known decomposition products to the 0.1% level. Furthermore, a previously unreported degradation product was identified as the *anti*-isomer of P2S and its role in the degradation pathway was described. The route of decomposition at the pH of maximum stability, pH 3.2, was shown to be via an initial isomerisation step to *anti*-P2S followed by dehydration to the cyanide and subsequent hydrolysis to the amide and acid. At the acid pH of the formulation little of the cyanide was converted to the pyridone, a major decomposition product in alkaline solution, although traces could be detected in highly degraded solutions.

The method did however have some limitations. Firstly, P2S-aldehyde (2-formyl-1-methylpyridinium) was not satisfactorily chromatographed but was eluted as a very broad peak covering much of the chromatogram. Although this material is known to be the major product of decomposition in strongly acid solutions[2] its presence has never been reported in solutions around pH 3 either in early work[3] or in more recent studies[4]. It would nevertheless be desirable to be able to monitor the

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aldehyde in any system designed to give a complete picture of P2S decomposition. Secondly, an unknown peak was detected in the front of the amide peak. The separation from the amide was unsatisfactory and the identity of the peak was not determined. Finally a recent paper[4] has indicated that P2S-alcohol (2-hydroxymethyl-1-methylpyridinium salt) is a degradation product at pH 2.5 although in the method described it was co-eluted with what appeared to be the *anti*-isomer of P2S. P2S-alcohol was not included in the evaluation of the previous method but subsequent experiments have shown the same co-elution problem exists.

This paper indicates how these limitations have been overcome, describes a more satisfactory method for the analysis of P2S formulations, and discusses how some of the degradation products arise.

EXPERIMENTAL

Apparatus

The LC comprised a Model 6000A pump, WISP automatic injection system, Model 480 variable-wavelength detector, Model 730 data module (all from Waters Assoc.), Model 2140 diode array detector (LKB), Hypersil 3-μm ODS column (Hichrom), column oven (Jones Chromatography).

Materials

Pralidoxime mesylate (P2S), 2-formyl-1-methylpyridinium (P2S-aldehyde), 2-cyano-1-methylpyridinium (P2S-cyanide), 2-carbamoyl-1-methylpyridinium (P2S-amide), 2-hydroxymethyl-1-methylpyridinium (P2S-alcohol) as their iodide salts and 2-hydroxyaminoiminomethyl-1-methylpyridinium (P2S-amidoxime) as the methanesulphonate were prepared at CDE. 1-Methyl-2-pyridone (P2S-pyridone) and 2-carboxy-1-methylpyridinium chloride (P2S-acid) were purchased from Aldrich.

Chromatographic conditions

Mobile phase: 25% trimethylamine solution was added to orthophosphoric acid (98 g in 800 ml of water) to give a solution of pH 3.0. Volume was made up to 1 l to give a phosphate concentration of 1.0 *M* (solution A). Molar solutions of sodium dihydrogenphosphate and orthophosphoric acid were mixed to give a solution of final pH 3.0 (solution B). The mobile phase was made from solution A (10 ml), solution B (90 ml), acetonitrile (100 ml), sodium lauryl sulphate (2.66 g) and water to 1 l. The solution was filtered through a 0.2-μm filter before use.

A 15 cm × 4.6 mm I.D., Hypersil ODS, 3 μm, column was used. The injection volume was 5 μl, the column temperature 30°C, the detection wavelength 262 nm, the flow-rate 1.5 ml/min, the column pressure 3000 p.s.i. and the run time 20 min.

Analysis of samples

Injection solutions originally containing 250 mg/ml of P2S in pH 3.2 0.1 *M* citrate buffer were diluted with mobile phase. A volume of 1 ml of the solution was transferred to a tared 25-ml flask and weighed to give an accurate and precise measure of volume using the density of the solution, 1.076 g/ml at 20°C. LC injections of the diluted solution (5 μl) were used for analysis. Calibration solutions containing 0–15

mg/ml of P2S and 0–10 mol% of each of the nominated degradation products were prepared in the mobile phase.

The applicability of the method was demonstrated by analyzing solutions that had been rapidly degraded at 80°C for periods up to twenty days and by the analysis of solutions that had been stored at unspecified room temperatures for four years. The precision of the chromatographic procedure was determined by the multiple analysis ($n = 5$) of a solution that had been stored at 80°C for two days.

Identification of the pre-amide unknown

The previous study had indicated that this material was present at low levels, about 1% of the P2S concentration, when the solution had been heated at 80°C for two days. Because of the low concentration and the necessarily complex mobile phase to achieve separation it was impracticable to isolate the material for conventional spectroscopic identification. A deductive approach was therefore adopted involving heating pairs of known products (10 mg/ml) in citrate buffer at 80°C and analysing the solution directly by the method here described to indicate how the unknown arose.

Chromatographic behaviour of P2S-aldehyde using Mk I conditions

P2S-aldehyde (2.5 mg/ml) in both the mobile phase used in the previous method (the Mk I conditions) and in the mobile phase of the method described in this paper (Mk II conditions) were chromatographed using the Mk I procedure[1] with flow-rates of 1 ml/min and 5 ml/min. Spectra of the eluted material were recorded using a diode array detector.

Formation of P2S-alcohol

The route of formation of P2S-alcohol was deduced in a similar way to the identification of the pre-amide unknown by observing the interaction between pairs of known degradation products. In particular P2S-aldehyde (10 mg/ml) in citrate buffer containing 0.01 *M* and 0.001 *M* potassium cyanide were heated at 80°C and also allowed to react at room temperature. The products were analysed by the standard procedure and by a system using the same column and a similar mobile phase containing 40% acetonitrile in order to elute late running peaks.

RESULTS AND DISCUSSION

Chromatographic behaviour of P2S-aldehyde

The behaviour of P2S-aldehyde using the Mk I conditions is shown in Fig. 1a. The peak had a broad "sugar loaf" profile some 8 min wide. If the aldehyde had been present in degraded solutions at low levels it would have been completely obscured although high concentrations may have been detected using this procedure. When the flow-rate was increased to 5 ml/min the chromatogram resolved into two peaks separated by a continuum (Fig. 1b). The spectra of the two peaks and of the continuum were identical (Fig. 2).

P2S-Aldehyde is known to exist in aqueous solution as a gem-diol and in methanol as the hemi-acetal with identical UV spectra[5]. The observations in this study are consistant with a slow equilibrium between these compounds (Fig. 3) with

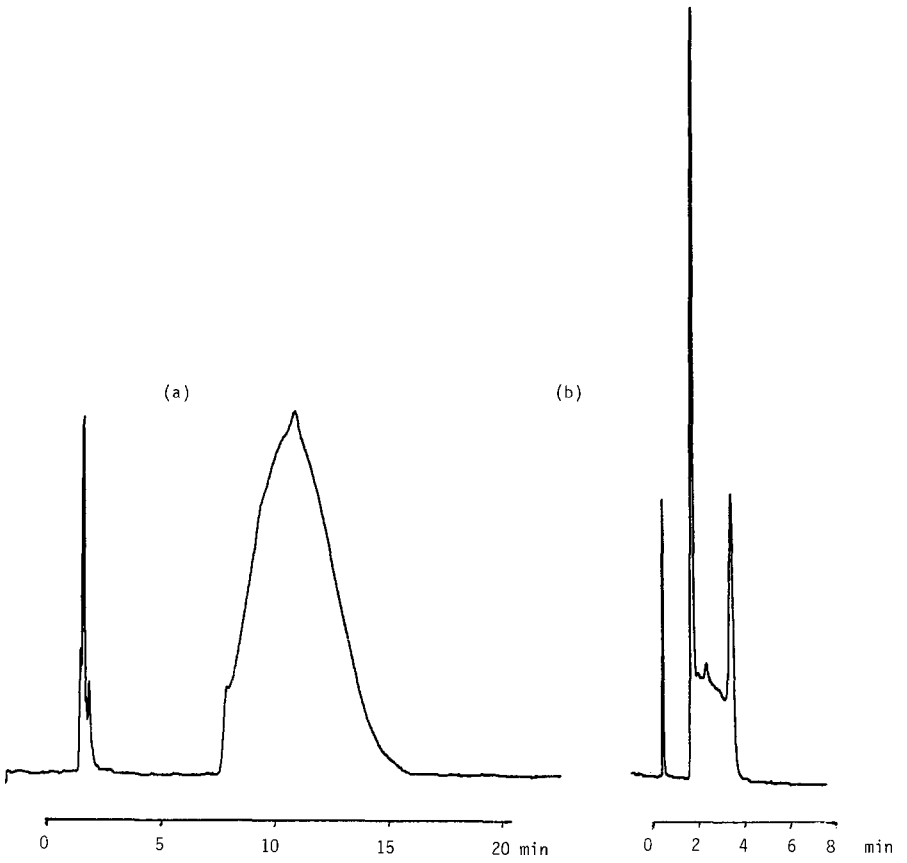

Fig. 1. (a) P2S-Aldehyde (2.5 mg/ml) Mk I conditions (see text). Flow-rate, 1 ml/min; 0.05 a.u.f.s. (b) P2S-Aldehyde (2.5 mg/ml) Mk I conditions (see text). Flow-rate, 5 ml/min; 0.05 a.u.f.s.

reaction times of the same order as the chromatographic time of separation. At high flow-rates some material travels through the column entirely as gem-diol or hemi-acetal giving rise to the two peaks while material which converts between the two forms on the column produces the continuum. At lower flow-rates and longer analysis times little material traverses the column entirely in one form and the different fraction of time spent in each form produces the broad peak. Increasing the column temperature increased the rates of the equilibrium reactions and produced a narrower single peak but not sufficiently discrete to form the basis of an analytical method for P2S-aldehyde. Injections of a solution of P2S-aldehyde in the acetonitrile containing Mk II solvent led to a suppression of the later eluting peak when high flows were used (Fig. 4) indicating that the first peak was the gem-diol and the second peak the hemi-acetal.

Replacement of methanol in the mobile phase by acetonitrile gave a single sharp peak for P2S-aldehyde. However it was then eluted with exactly the same retention times as P2S-amide. Other LC methods described using acetonitrile as the organic modifier also showed either the same retention time for aldehyde and amide[6]

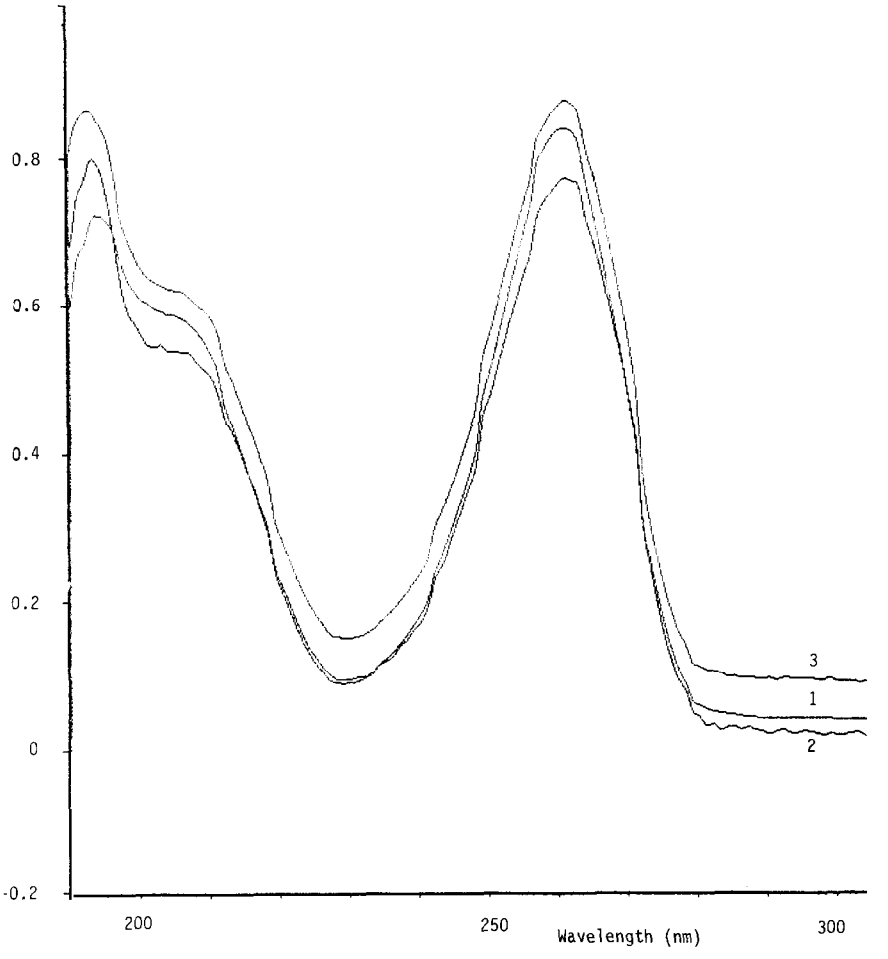

Fig. 2. Spectra of P2S-aldehyde obtained under the conditions in Fig. 1b. Spectrum 1 at 1.9 min; spectrum 2 at 3.0 min; spectrum 3 at 3.8 min. Spectra are normalized and zeros offset.

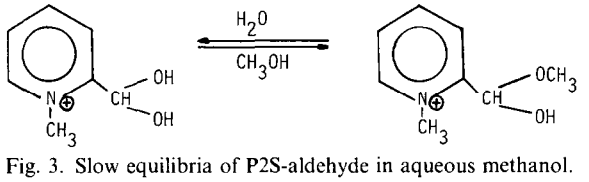

Fig. 3. Slow equilibria of P2S-aldehyde in aqueous methanol.

or inadequate resolutions[4]. Furthermore, the pre-amide unknown was no longer separated from the amide but was co-eluted with the other two compounds. The new method described here shows good separation of all three materials.

Identification of the pre-amide unknown

The interaction experiments showed that the pre-amide unknown was formed when P2S was heated with hydroxylamine and when P2S-cyanide was allowed to

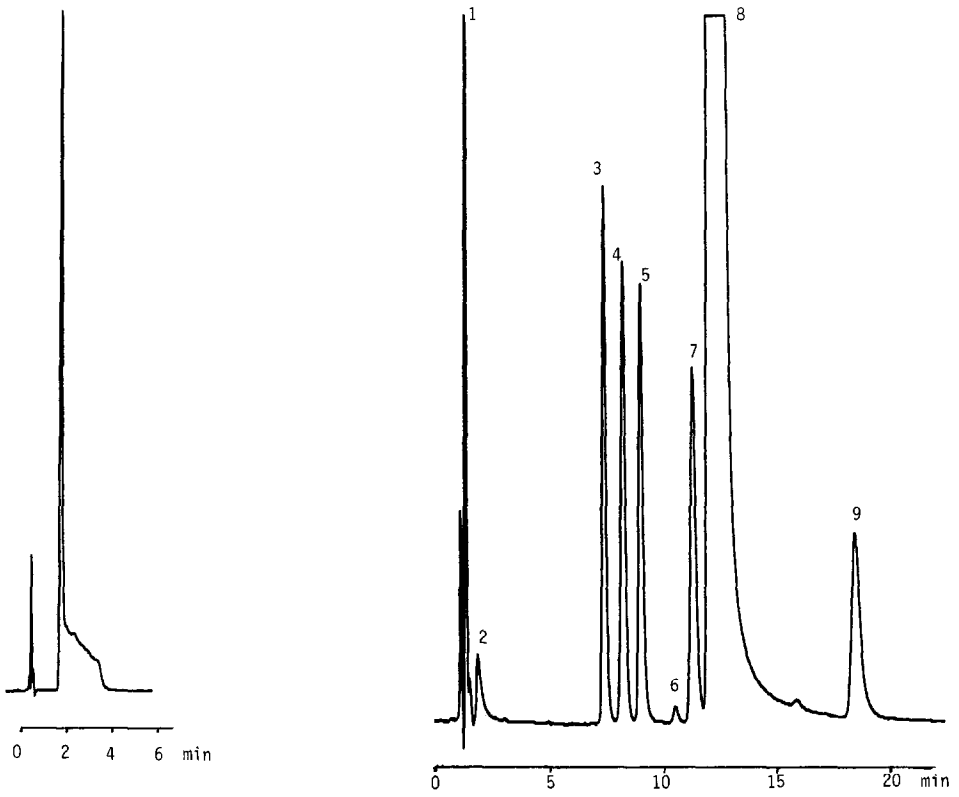

Fig. 4. P2S-aldehyde (2.5 mg/ml) in Mk II solvent (see text). Mk I conditions: flow-rate, 5 ml/min, 0.05 a.u.f.s.

Fig. 5. Calibration chromatogram containing P2S (10 mg/ml) and 1 mol% of degradation products. Column: 3-μm Hypersil ODS, 15 cm × 4.6 mm I.D. Mobile phase: see text. Flow-rate: 1.5 ml/min. Column temperature: 30°C. Detector wavelength: 262 nm, 0.05 a.u.f.s. Peaks: 1 = P2S-acid; 2 = P2S-pyridone; 3 = P2S-amidoxime; 4 = P2S-aldehyde; 5 = P2S-amide; 6 = *anti*-P2S; 7 = P2S-alcohol; 8 = *syn*-P2S; 9 = P2S-cyanide.

react with hydroxylamine at room temperature. P2S-amide was formed as the major product in these experiments at a rate much greater than would be observed from the hydrolysis of P2S-cyanide.

It has long been known that nitriles react with hydroxylamine to form amidoximes[7]. If the nitrile is attached to a strongly electron withdrawing group mixtures of amides and amidoximes result from nucleophilic attack by the hydroxylamine oxygen or nitrogen respectively[8]. It therefore seemed likely that the unknown was the amidoxime. Attempts to synthesise P2S-amidoxime from the unquantanised material, pyridine-2-amidoxime and methyl iodide produced a complex mixture from which the desired material could not be isolated. Reaction of P2S-cyanide with hydroxylamine always gave mixtures of amide and amidoxime where the former predominated and again a pure product could not be isolated. However, by heating P2S itself with hydroxylamine a pure product free from amide was produced. Details of the synthesis will be published elsewhere. Comparison of the chromatographic and

UV spectrophotometric characteristics of the unknown and of an authentic sample of P2S-amidoxime confirmed the identification.

Evaluation of the new analytical method

The new method gave good peak shape and separation for P2S and all eight degradation products. The inadequate separations of amidoxime, aldehyde and amide and of alcohol and *anti*-P2S in previous studies has now been improved to give baseline resolution. Furthermore the resolution was such that the degradation products at low levels could be separated from the large excess of the major component, P2S. Fig. 5 shows P2S at 10 mg/ml with 1 mol% of each of the degradation products. The peak for the *anti*-isomer was just visible in freshly prepared standard solution, increasing with time even in solutions stored at 0°C. The results of the 80°C storage experiment are summarised in Table I. The aldehyde and *anti*-isomer of P2S were formed early in the degradation and were visible after 0.5 h heating. After 5 h P2S-cyanide was present and the amidoxime and amide were just detectable. At two days (Fig. 6) the acid, amidoxime and aldehyde were present around the 0.5% level and the P2S-alcohol was detectable in the tail of the *anti*-isomer. An unknown peak was detected in the P2S tail. After five days (Fig. 7) the pyridone appears, the alcohol concentrations became significant and the amide became the major degradation prod-

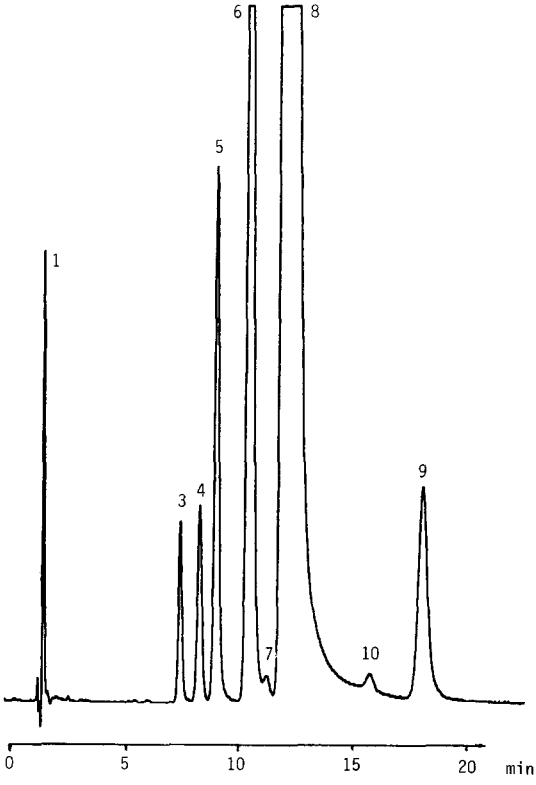

Fig. 6. Degraded P2S solution (250 mg/ml in 0.1 *M* citrate buffer, pH 3.2) after two days at 80°C, sample diluted 25:1. Conditions and peak identification as in Fig. 5, peak 10 = unknown.

TABLE I

DECOMPOSITION OF P2S (250 mg/ml IN 0.1 *M* CITRATE BUFFER, pH 3.2) AT 80°C

Amounts are in mol% relative to the P2S starting concentrations. Unknown are expressed in area% relative to starting P2S area.

	Time									
	0.5 h	*1 h*	*2 h*	*5 h*	*8 h*	*1 day*	*2 days*	*5 days*	*12 days*	*15 days*	*20 days*
P2S-Acid							0.46	2.74	8.52	11.2	13.2
P2S-Pyridone								0.48	3.94	4.59	5.44
P2S-Amidoxime						0.15	0.34	0.53	0.32	0.32	0.43
P2S-Aldehyde	0.09	0.12	0.16	0.18	0.18	0.31	0.45	0.02	0.03	0.06	0.11
P2S-Amide					0.02	0.31	1.26	6.69	24.6	29.4	34.1
anti-P2S	1.71	2.26	3.25	4.04	4.06	4.17	4.14	3.82	2.61	2.29	1.61
P2S-Alcohol							0.06	0.67	1.35	1.18	2.96
syn-P2S	97.8	97.3	96.1	94.8	94.7	93.5	91.4	81.4	52.3	44.1	33.1
P2S-Cyanide				0.17	0.23	0.84	1.20	2.53	4.48	3.37	1.62
Unknowns							0.04	0.53	0.24	0.24	–
Total	99.6	99.7	99.5	99.2	99.2	99.2	99.4	99.4	98.4	96.8	92.6

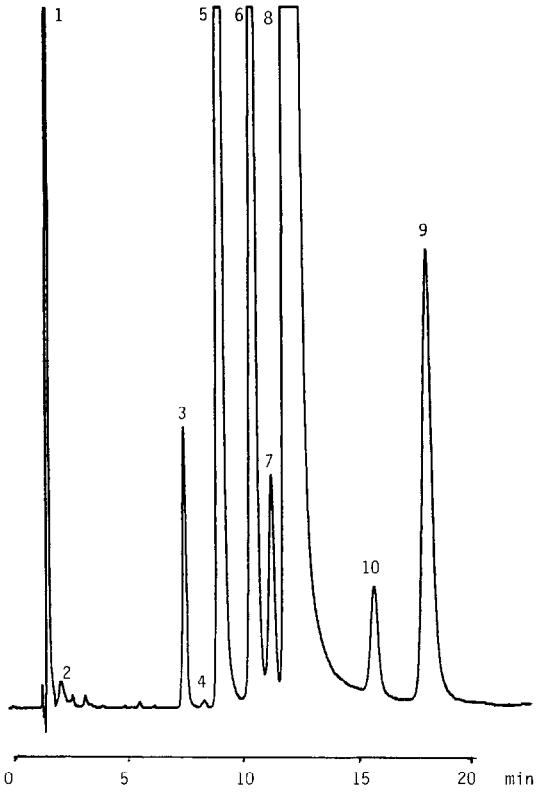

Fig. 7. Degradation of P2S solution (250 mg/ml in 0.1 *M* citrate buffer, pH 3.2) after five days at 80°C, sample diluted 25:1. Conditions and peak identification as in Fig. 5, peak 10 = unknown.

uct. The aldehyde level however fell sharply to the detection limit, 0.02%. In highly degraded solution after twenty days when only 33% of the original P2S remained the alcohol and pyridone became important products, the amidoxime remained stable at the 0.5% level and numerous small unidentified peaks appeared. However, with this degree of decomposition the material would be unusable for all practical purposes. The total amount of material accounted for was greater than 99% even after five days degradation when only 81% of the original P2S remained. Beyond this the total mass balance was not quite so good. However in a real situation it is the decomposition of up to 10% of the active component and the ability to determine and account for the degradation products in this range to give a good mass balance that is important.

With the *anti*-isomer of P2S and P2S-alcohol now well resolved the measured concentration of *anti*-P2S is seen to fall in proportion to the remaining *syn*-P2S concentration; this is more consistant with an equilibrium between the two isomers than the previously reported results where *anti*-isomer concentrations apparently remained substantially constant[1]. The *anti*-isomer concentration was calculated using the derived conversion factor given in the earlier study[1], which indicated that at 262 nm the *anti*-isomer absorbed 1.24 times more strongly that the *syn*-isomer.

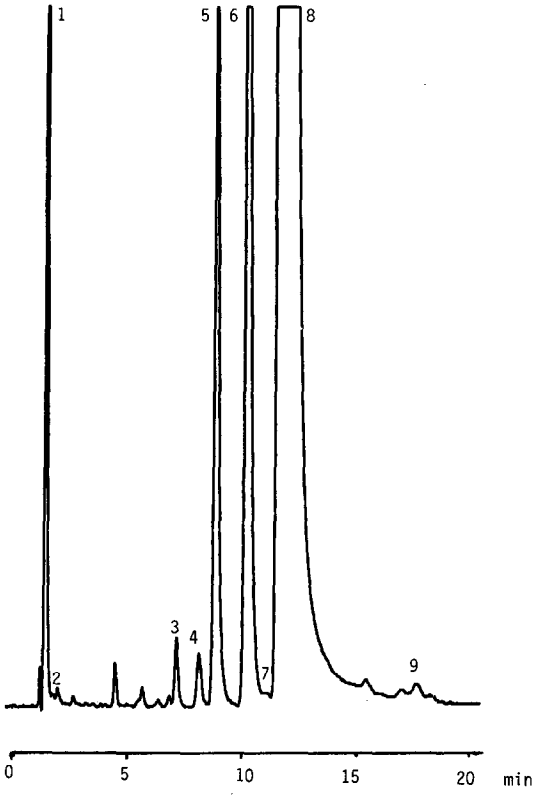

Fig. 8. Degradation of P2S solution (250 mg/ml in 0.1 *M* citrate buffer, pH 3.2) after four years at room temperature. Conditions and peak identification as Fig. 5.

Analysis of a solution that had been stored at room temperature for four years, Fig. 8, showed the presence of acid, amide and *anti*-isomer as major products but with much smaller amounts of amidoxime, aldehyde, cyanide and pyridone.

In the original method an internal standard, nicotinamide, was included to achieve adequate precision. In the present method using more modern equipment and with a dilution scheme using larger volumes good precision could be obtained using external standard quantitation. With samples run on the same day, the method gave coefficients of variation of 0.4% ($n = 5$) for the major component, P2S, and 0.8% ($n = 5$) for the degradation products at concentrations around 1 mol%. The internal standard could still be used if even higher precision was required and coefficients of variation approaching 0.1% ($n = 5$) could be achieved. With the Mk II chromatographic conditions the nicotinamide still eluted in the clear region ahead of the amidoxime peak.

Degradation products at 0.02% of the P2S concentrations gave peaks which could be easily seen and quantified above the baseline.

Formation of P2S-alcohol

Heating binary mixtures of decomposition products showed that the P2S-al-

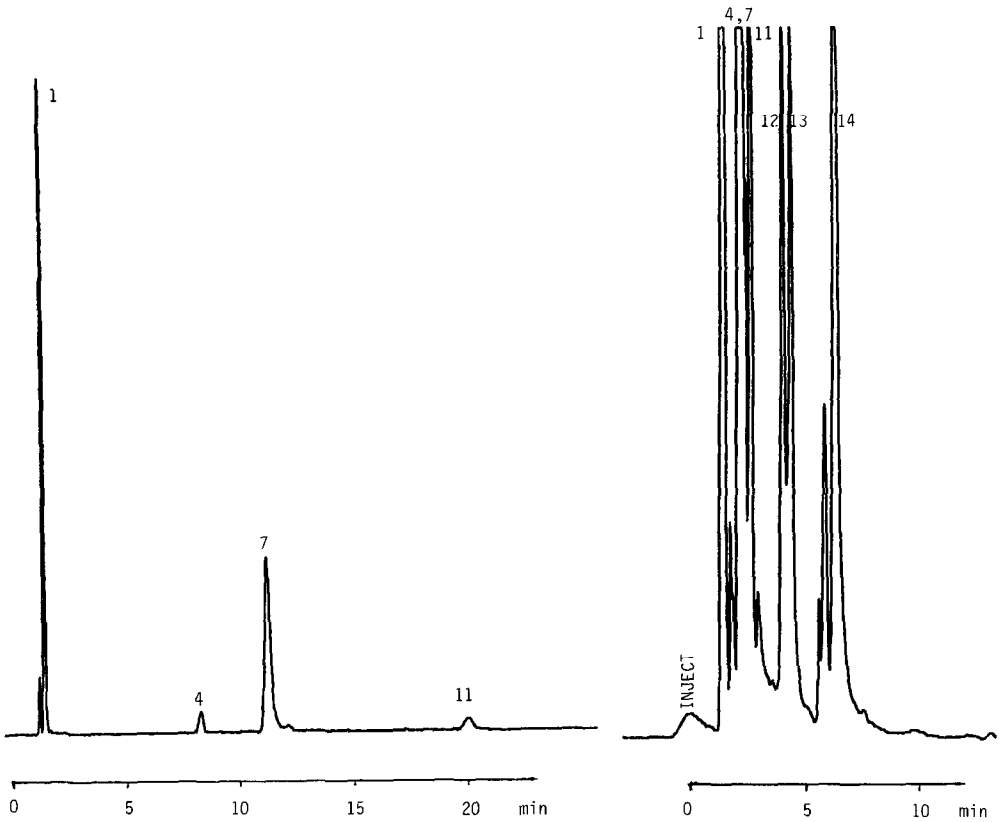

Fig. 9. Reaction between P2S-aldehyde (10 mg/ml in 0.1 *M* citrate buffer, pH 3.2) and potassium cyanide (0.01 *M*) at 80°C for 1 h. Conditions and peak identification as Fig. 5, peak 11 = unknown; 1.0 a.u.f.s.

Fig. 10. Reaction between P2S-aldehyde (10 mg/ml in 0.1 *M* citrate buffer, pH 3.2) and potassium cyanide (0.01 *M*) at room temperature for one day. Column: 3-μm Hypersil ODS, 15 cm × 4.6 mm I.D. Mobile phase: 40% acetonitrile, 60% trimethylamine–orthophosphoric acid (pH 3.0), 0.01 *M* sodium lauryl sulphate. Flow rate: 1.5 ml/min. Column temperature: ambient. Detector wavelength: 262 nm, 0.05 a.u.f.s. Peaks as Fig. 5. Peaks 11 and 14 are unknown; peaks 12 and 13 are unknowns with long wavelength absorption (Fig. 11).

cohol was formed by reaction of P2S-aldehyde with inorganic cyanide. When P2S-aldehyde alone was heated at 80°C for 1 h no reaction took place. However when trace amounts of cyanide were present the conversion to alcohol and acid readily took place. Fig. 9 showed that with a cyanide concentration of 0.01 *M*, that is about 20 mol% of the P2S-aldehyde level, 3.2% of the original aldehyde concentration remained and 32% alcohol and 39% acid were formed. An unidentified peak appeared at about 20 min retention time. Cyanide concentrations an order lower, 10^{-3} *M*, yielded 16% alcohol and 28% acid with 28% of the aldehyde remaining. At room temperature the reaction occurred more slowly and the solution developed a pronounced orange colour after two days. When this solution was analysed by the standard method late eluting peaks with retention times greater than 2 h were detected. Analysis of the solution using a mobile phase containing 40% acetonitrile showed

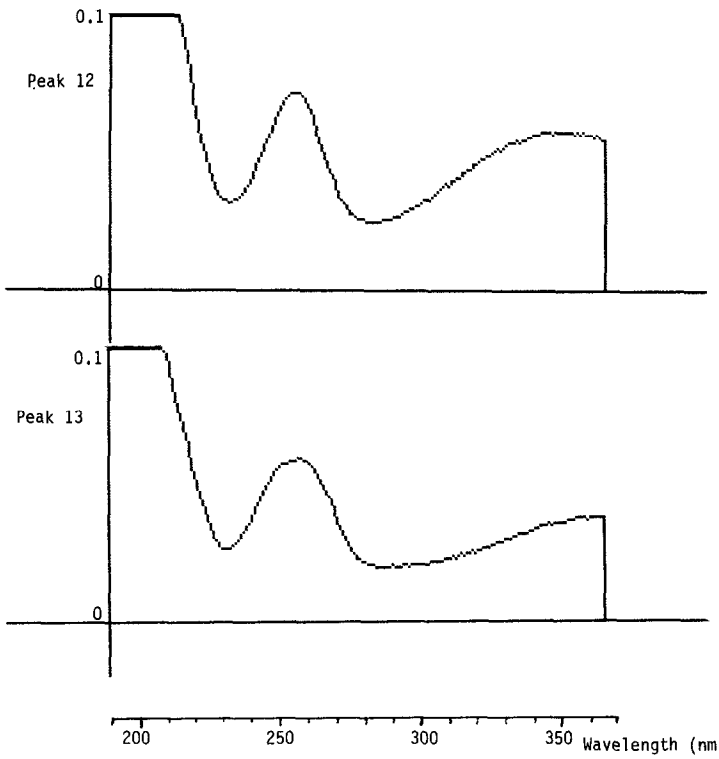

Fig. 11. Spectra of peaks 12 and 13 (Fig. 10) showing long wavelength absorption. Conditions as Fig. 10.

several of the late running materials (Fig. 10). The spectra of the two peaks centred on 4 min were similar and showed absorptions extending towards the visible region (Fig. 11). These are presumably responsible for the orange colour.

Cyanide is known to catalyse the condensation of aromatic aldehydes to form benzoins[9]. Although the existence of the pyridoin of P2S-aldehyde has not been reported the non-quatanised pyridoin is known and exists entirely in the enediol form with an extended conjugation giving it an orange colour[10]. The similar orange colour of P2S solutions that have been stored at room temperature for long periods of time which has never been explained in terms of known decomposition products may be due to the formation of a pyridoin/enediol.

The disproportionation of aldehydes to give the corresponding acid and alcohol is well known as the Cannizzaro reaction in strongly alkaline solutions but there are no reports of the reaction proceeding in acid solution with cyanide catalysis. The mechanism of the reaction is therefore unclear and requires further investigation.

The cyanide catalysed reaction of P2S-aldehyde now explains why the aldehyde concentration falls rapidly in solutions heated at 80°C for times longer than two days for it was at this stage of the decomposition that the pyridone and therefore free cyanide began to appear.

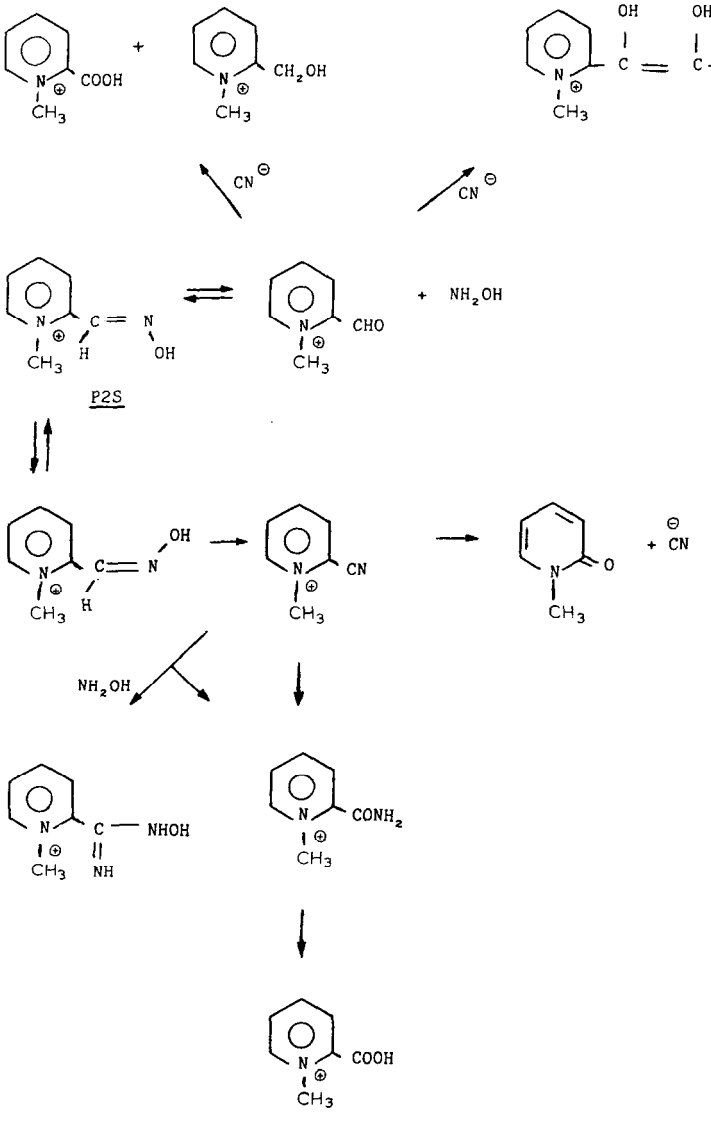

Fig. 12. Degradation pathways of P2S at pH 3.2.

Degradation pathways of P2S at pH 3.2

The results of this study indicate that the decomposition of P2S at its pH of maximum stability is more complicated than was originally thought and involves secondary reaction pathways between pairs of degradation products. An updated reaction scheme is shown in Fig. 12.

CONCLUSIONS

Slow equilibrium between the diol and hemi-acetal of P2S-aldehyde in aqueous

methanol solution can lead to broad peaks when methanol is used as the organic modifier in the reversed-phase chromatography of this material.

A previously unidentified degradation product has been shown to be the amid-oxime formed by the reaction between P2S-cyanide and hydroxylamine.

P2S-Alcohol has been shown to be formed by the catalytic reaction of free cyanide and P2S-aldehyde. The mechanism of the reaction is unclear.

An improved chromatographic procedure has been devised which will separate the degradation products of P2S at trace levels from the large excess of P2S itself.

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