Journal of Chromatography, 212 (1981) 361–365
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,927

Note

Determination of N-methyl pyridinium-2-aldoxime chloride and its hydrolytic by-products by ion-pair high-performance liquid chromatography

NESBITT D. BROWN*, M. PATRICIA STRICKLER, H. KENNETH SLEEMAN and BHUPENDRA P. DOCTOR

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20012 (U.S.A.)

(Received April 27th, 1981)

During the past two decades, much work has been conducted by various research groups to develop effective cholinesterase reactivators for use as therapeutic agents in reversing the deleterious effects of organophosphate poisoning of the nervous system in mammals[1–5]. As a result of these efforts, several groups of compounds have been tested in animals in an attempt to understand better their modes of action. N-Methyl pyridinium-2-aldoxime chloride (2-PAM) is an oxime that falls within this area of research. Although much research has been performed on this compound, many questions remain unanswered; this is particularly true with respect to the pharmacokinetic actions of 2-PAM in humans.

In studies conducted by Ellin and co-workers[6,7], 2-PAM was shown to degrade via two routes when exposed to various hydrogen and hydroxyl ion concentrations *in vitro* (Fig. 1). The results obtained from these studies are relevant in view of the fact that 2-PAM may be subject to these conditions when administered to human subjects, either orally or intramuscularly as a therapeutic drug. It has been determined that parenteral solutions containing pyridinium oximes are most stable at pH values

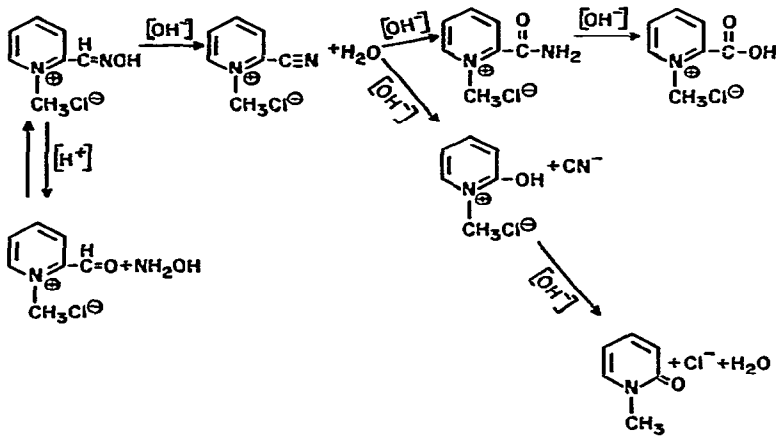


Fig. 1. Decomposition mechanism for 2-PAM in acid and alkaline solutions at 95°C.

0021-9673/81/0000–0000/$02.50

below 5. Within an *in vivo* system, where the normal pH value is approximately 7.4, the reaction kinetics for 2-PAM should follow the reaction scheme of the hydroxyl ion degradation.

Specific and sensitive methods are required to establish the decomposition mechanism for 2-PAM *in vivo*. In this paper, we describe a relatively simple procedure for separating and quantifying major degradative by-products of 2-PAM in both basic and acidic solutions. The method involves the separation of 2-PAM and its by-products by employing ion-pair reversed-phase high-performance liquid chromatography (HPLC). Amounts as low as 25 ng are detected by this method. Analysis time requires less than 6 min per sample. In addition, high accuracy and reproducibility are obtained by using this procedure. The method offers an excellent alternative to many of the previously described procedures used for analyzing the pyridinium oximes.

EXPERIMENTAL*

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) Model ALC/GPC 204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a 280-nm UV detector and a Model 730 data module was used in this study.

Reagents

All solvents and chemicals used in the study were either of spectroquality or of analytical grade. Acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). PIC-B7 reagent (1-heptane sulfonic acid) was purchased from Waters Assoc. Stock standard solutions (5 μg/μl) of 2-PAM, 1-methyl-2-pyridone (Aldrich, Milwaukee, WI, U.S.A.), 2-formyl-N-methyl pyridinium chloride hydrate, 2-carbamoyl-1-methyl pyridinium chloride monohydrate, and 2-carboxy-N-methyl pyridinium chloride (Ash Stevens, Detroit, MI, U.S.A.) were prepared in methanol–water (1:1).

Procedure

A pre-packed 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc.) was employed to chromatograph all compounds used in this study. The mobile phase consisted of a 0.01 *M* solution of PIC-B7 mixed with acetonitrile. PIC-B7 reagent was prepared by dissolving 20 ml of the pre-packaged reagent into 480 ml of glass-distilled water. The pH of the solution was 3.4. A PIC-B7–acetonitrile ratio of 80:20 was used in an isocratic mode to separate each compound. The flow-rate was 1.5 ml/min. Column pressures ranged between 1500 and 1700 p.s.i. All separations were performed at ambient temperature. Samples were introduced into the column through a continuous-flow loop injector. Peak areas and heights were measured and computed with an on-line integrator.

---

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

RESULTS AND DISCUSSION

The therapeutic efficacy of 2-PAM to reactivate organophosphorus-inhibited cholinesterases is completely dependent upon the potency of the antidote, based on the stability of the active compound in the formulation, prior to its administration. In certain cases where the anticholinergic activity of the antidote is unknown due to the deterioration of the parent compound caused by prolonged storage conditions, specific methods are needed to characterize the formulation.

In a series of recently published reports[8–10] we studied the degradative fate of several anticholinergic compounds after exposure to various pH and temperature gradients. The data compiled from these studies were used to predict the shelf life of these compounds when stored in different climatic regions. In this paper, we studied the reaction kinetics of 2-PAM by subjecting it to elevated temperatures in acidic and basic solutions, thus simulating long-term storage.

In order to identify the hydrolytic by-products of 2-PAM, a series of standard solutions containing the major degradation products of this oxime were prepared and chromatographed (Figs. 2 and 3). Concentrations ranged from 100 to 1500 ng/μl. Linearity was established for each compound chromatographed. Experimental samples of 2-PAM were prepared in 0.1 *N* hydrochloric acid and 0.1 *N* sodium hydroxide.

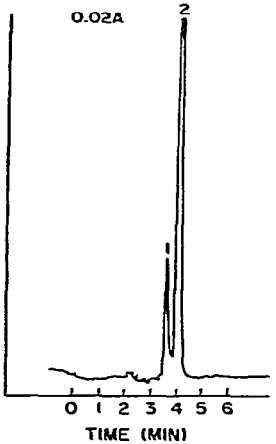


Fig. 2. Separation of a standard solution containing (1) 100 ng of 2-formyl-N-methyl pyridinium chloride and (2) 1.25 μg of 2-PAM. Column: 300 × 3.9 mm I.D. μBondapak C_{18}. Mobile phase: PIC-B7–acetonitrile (80:20). Flow-rate: 1.5 ml/min. Column temperature: ambient.

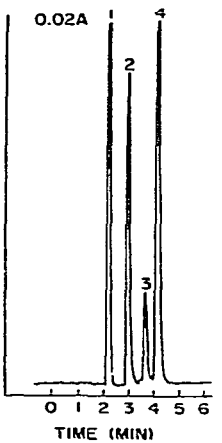
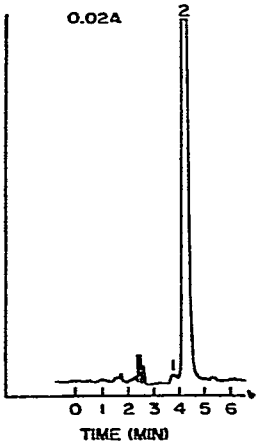


Fig. 3. Separation of a standard solution of (1) 2-carboxy-N-methyl pyridinium chloride (560 ng), (2) 1-methyl-2-pyridone (560 ng), (3) 2-carbamoyl-1-methyl pyridinium chloride (280 ng) and (4) N-methyl pyridinium-2-aldoxime chloride (560 ng).

Each group was heated at 95°C for various time periods. The chromatograms below depict the results of this experiment. Chromatograms of the acid hydrolysates showed a pattern of degradation with 2-formyl-N-methyl pyridinium chloride as the principal hydrolytic by-product. The first signs of degradation were observed after 5 min of heating. Heating was continued for 60 min. Samples were collected every minute during the first 5 min, followed by 5-min intervals during the next 30 min and in 10-

min time periods, thereafter. During the 60-min hydrolysis, only 10% of the 2-PAM was degraded to 2-formyl-N-methyl pyridinium chloride. No other by-products were observed in the chromatographic separations. Figs. 4 and 5 represent two time frames of the reaction kinetics for the hydrolytic break-down of 2-PAM in acid.



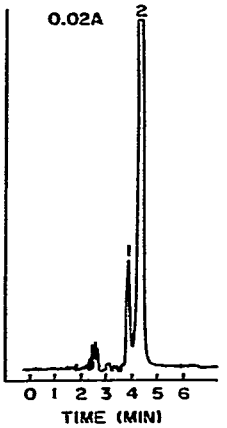
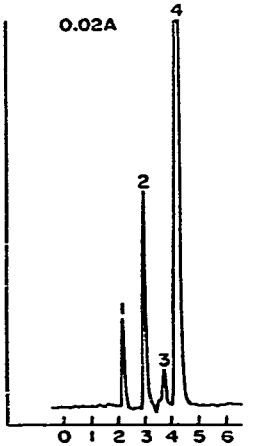


Fig. 4. Chromatogram of a 1.50-μg sample of 2-PAM heated in 0.1 *N* HCl at 95°C for 5 min. 1 = 2-Formyl-N-methyl pyridinium chloride; 2 = 2-PAM.

Fig. 5. Chromatogram showing the hydrolysis of 2-PAM in acid after 60 min. 1 = 2-Formyl-N-methyl pyridinium chloride; 2 = 2-PAM.

Chromatograms of the alkaline hydrolysates showed contrasting results for 2-PAM. Hydrolytic degradation occurred rapidly. Whereas one major by-product was formed during acid hydrolysis, a series of by-products were formed in the basic solution. The chromatograms in Figs. 6 and 7 show the rate of formation of break-down products occurring during hydrolysis.



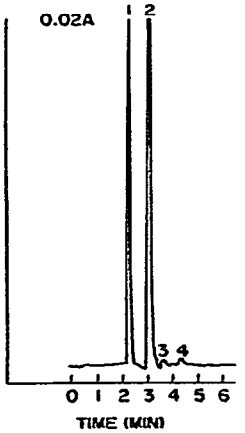


Fig. 6. Chromatogram of a 1.50 μg sample of 2-PAM hydrolyzed in 0.1 *N* NaOH at 95°C for 5 min. (1) 2-Carboxy-N-methyl pyridinium chloride, (2) 1-methyl-2-pyridine, (3) 2-carbamoyl-1-methyl pyridinium chloride and (4) 2-PAM.

Fig. 7. Chromatogram of the 60 minute alkaline hydrolysate of 2-PAM. (1) 2-Carboxy-N-methyl pyridinium chloride, (2) 1-methyl-2-pyridone, (3) 2-carbamoyl-1-methyl-pyridinium chloride, (4) and 2-PAM.

It was noted that after exposure of 2-PAM to alkaline hydrolysis for 5 min, three degradative by-products were observed in the mixture. As the reaction time increased, the rate of degradation of 2-PAM was accelerated. Upon completion of the 60-min hydrolysis, it was shown that 1-methyl-2-pyridone (61.4%) and 2-carboxy-N-methyl pyridinium chloride (37.8%) were the major by-products formed during the hydrolysis. A small amount of 2-carbamoyl-1-methyl pyridinium chloride was also formed.

Studies conducted by Kramer[11], from analysis of human urine, following oral administration of 2-PAM showed that a metabolite possessing a carboxyl function and weak acidic group was produced. The trend observed by Kramer is similar to what we saw during basic hydrolysis *in vitro*.

In light of the evidence, which indicates that the degradative fate of 2-PAM *in vivo* is similar to *in vitro* degradation, the method described in this paper may possibly be used to answer some of the questions pertaining to the metabolism of 2-PAM in human subjects.

ACKNOWLEDGEMENTS

We thank Dr. Jurgen von Bredow for his technical support in helping us to complete this study. We also express our sincere appreciation to Mr. Stewart Galloway for his excellent secretarial assistance.

REFERENCES

1 F. Hobbiger and P. W. Sadler, *Brit. J. Pharmacol.*, 14 (1959) 192.
2 W. D. Erdman, *Arzneimittel-Forsch.*, 15 (1965) 135.
3 J. H. Fleisher, L. W. Harris and E. F. Murtha, *J. Pharmac. Exp. Ther.*, 156 (1967) 345.
4 E. Meeter and O. L. Wolthuis, *Eur. J. Pharmacol.*, 2 (1968) 377.
5 L. W. Harris, J. H. Fleisher and H. I. Yamamura, *Eur. J. Pharmacol.*, 14 (1971) 38.
6 R. I. Ellin, *J. Amer. Chem. Soc.*, 80 (1958) 6588.
7 R. I. Ellin, J. S. Carlese and A. A. Kondritzer, *J. Pharm. Sci.*, 51 (1962) 141.
8 N. D. Brown and H. K. Sleeman, *J. Chromatogr.*, 138 (1977) 449.
9 N. D. Brown and H. K. Sleeman, *J. Chromatogr.*, 150 (1978) 225.
10 N. D. Brown and H. K. Sleeman, B. P. Doctor and J. P. Scovill, *J. Chromatogr.*, 195 (1980) 146.
11 B. Kramer, *Biochem. Pharmacol.*, 11 (1962) 299.