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Note

Determination of N,N'-trimethylene-bis(pyridinium-4-aldoxime) dibromide by ion-pair high-performance liquid chromatography

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The administration of N,N'-trimethylene-bis-(pyridinium-4-aldoxime) dibromide (TMB-4) to mammals as an effective reactivator of organophosphate-inhibited cholinesterase has been reported extensively[1–6]. Sim[7] and Li[8] have studied the antidotal effects of oximes in the treatment of poisoning by insecticides. TMB-4 has been shown to be more active therapeutically, than either pyridine-2-aldoxime methiodide (2-PAM) or its methanesulfonate salt, P2S, for reversing neuromuscular blocks due to anticholinesterase activity[9,10]. Several other investigators[11,12] have indicated significant therapeutic effects of TMB-4 in raising the LD_{50} of sarin, tabun and tetraethyl pyrophosphate in rats and rabbits.

In view of this extensive documentation supporting the therapeutic values of TMB-4, few reports have been published dealing with the analytical quantification of TMB-4 and some of its degradative by-products by more specific methods. Current methodology utilizes colorimetric and spectrophotometric techniques[13–15]. In many cases, the procedures are tedious and time consuming.

In this paper, we describe a relatively simple and specific analytical method for quantifying TMB-4 and some of its degradative by-products by ion-pair high-performance liquid chromatography (HPLC). Quantitative levels of TMB-4 in amounts of less than 1 ng are detectable by this method. Analysis time is 3 min per sample. The method is highly accurate and reproducible. As an improved technique, the method offers a convenient alternative to the conventional methodologies presently employed.

EXPERIMENTAL*

Apparatus

This study was performed utilizing a Waters Assoc. (Milford, Mass., U.S.A.) Model ALC/GPC-204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a Model 660 solvent programmer, a U6K loop injector, a 254-nm UV detector, a Houston Instrument Omni-Scribe A5000 dual-pen recorder and a Columbia Scientific Supergrator-3 integrator.

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

Reagents

Spectroquality acetonitrile (Eastman-Kodak, Rochester, N.Y., U.S.A.) mixed with PIC B-7 reagent (1-heptane sulfonic acid; Waters Assoc.) was used as the mobile phase. TMB-4, trimethylene-bis(pyridinium-4-aldoxime) dibromide and 4-pyridine aldoxime were obtained from Research Lab. (Edgewood Arsenal, Md., U.S.A.).

Procedure

A prepacked 30 cm × 3.9 mm I.D. μBondapak C_{18} column was used for all separations performed in this study. Micro Bondapak C_{18}, which constitutes the stationary phase of the column is an octadecylsilyl bonded-phase packing material. The solid support media is capable of operating within a pH range of 2–8. It is thermally stable over a wide temperature gradient (< 300°). Because of its excellent hydrolytic stability, various aqueous–organic mixed solvent systems are compatible with the column.

The mobile phase used in this separation, consisted of 0.01 *M* 1-heptane sulfonic acid mixed with acetonitrile. PIC B-7 reagent was prepared by dissolving 20 ml of the prepackaged reagent into 480 ml of glass-distilled water. The pH of the solution was 3.40. Acetonitrile–PIC B-7 reagent (35:65) was pumped isocratically through the column, utilizing both pumps of the system, along with the 660 solvent programmer. The flow-rate was 1.5 ml/minute. The column pressure ranged between 1200 and 1500 p.s.i. All separations were performed at ambient temperatures. Sample (2 μl) was introduced into the column through a continuous-flow loop injector. Detection limit of the method was 1 ng on-column. Peak areas were measured by an on-line computing integrator.

RESULTS AND DISCUSSION

A great deal of attention has been focused recently on reports pertaining to pesticidal poisoning among workers employed in the manufacturing of organophosphates[16]. Many of the compounds cited, constituted a series of chemicals which caused spastic paralysis, severe nervous disorders or other permanent disabilities similar to symptoms observed in poisoning by biological or chemical nerve agents. Recently, several new oximes have been tested for their therapeutic properties and were found to provide protection against many anticholinesterase compounds. TMB-4 was among the compounds tested.

However, the development of new analytical methods for assaying TMB-4 and some of its degradative by-products by simpler and more specific techniques have been lacking. Ellin *et al.*[17] have reported TMB-4 to be unstable when exposed to acids, bases and elevated temperatures. As such, one can only speculate on the efficacy of the altered compound when administered as a therapeutic agent. Ion-pair HPLC is a unique tool for observing the fate of TMB-4 under these circumstances.

A series of standard solutions and experimental samples were separated and quantified by this new procedure. The method was excellent in producing linearity for the various concentrations of TMB-4 quantified.

Fig. 1 represents the separation of a 100 ng standard of TMB-4. The aqueous standard was stable for 2 weeks when refrigerated at 4°. Even after a 2-month period, 98% of the original compound was present in the standard. However as the sample

continued to age, traces of a break-down product were observed. From the preliminary data, 4-pyridine aldoxime was suspected as the primary by-product. Using a combined sample of 4-pyridine aldoxime and TMB-4, an identical chromatographic profile was obtained.

Fig. 2 shows the separation of 4-pyridine aldoxime and TMB-4 in an aqueous standard. The sensitivity of 4-pyridine aldoxime was 4 times greater than TMB-4 on mole/mole basis; 200 pg of the compound were detectable by this method.

The fate of TMB-4 in bases and acids at various temperatures tends to show a different picture. TMB-4 dissolved in 0.1 *N* NaOH at room temperature, immediately produced 4 peaks. The amounts of the new compounds were low in concentration, 98% of the original TMB-4 was present in the sample. As we increased the temperature of the solution, greater amounts of the impurities formed.

Fig. 3 is a chromatogram showing the formation of 4-pyridine aldoxime and several other unknown compounds. In this particular case, TMB-4 was dissolved in

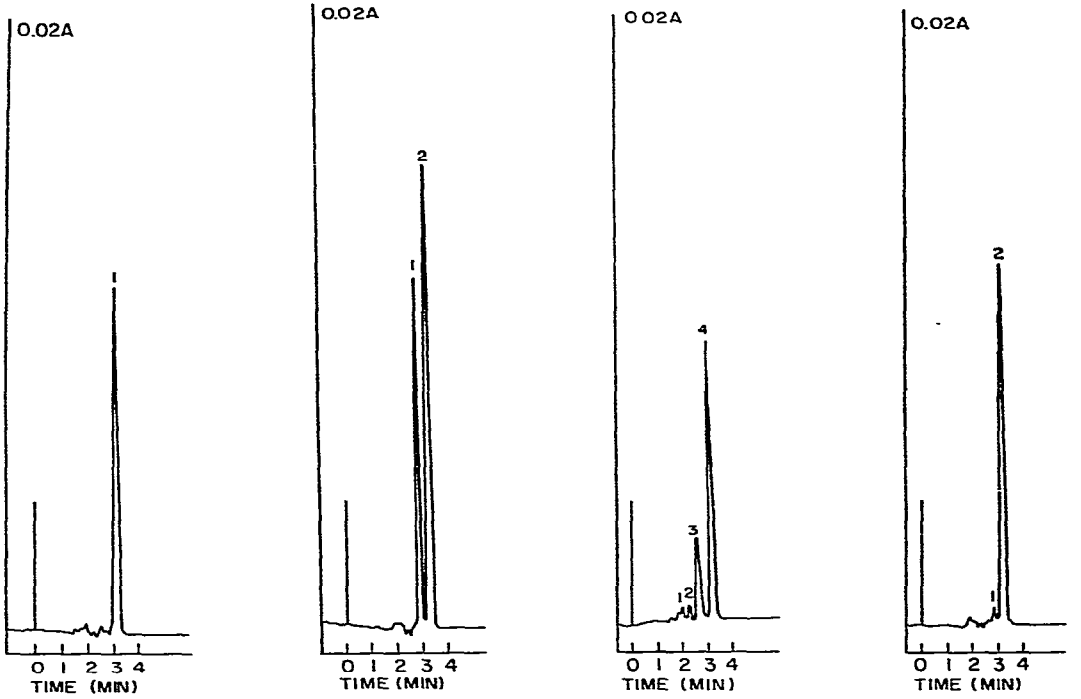

Fig. 1. Chromatogram of a 100-ng sample of TMB-4 detected at 254 nm, 0.02 A. Column, 30 cm × 3.9 mm I.D. μBondapak C_{18}.

Fig. 2. Separation of a standard solution of (1) 20 ng 4-pyridine aldoxime and (2) 125 ng TMB-4. Mobile phase, acetonitrile–PIC B-7 reagent (35:65). Flow-rate, 1.5 ml/min. Column temperature, 20°. Chart speed, 1 cm/min.

Fig. 3. Chromatogram of a 100-ng sample of TMB-4, heated in 0.1 *N* NaOH at 90° for 60 sec. 1 = Unknown, 2 = unknown; 3 = 4-pyridine aldoxime and 4 = TMB-4.

Fig. 4. Chromatogram of a 100 ng sample of TMB-4, heated in 0.1 *N* HCl at 90° for 60 sec. 1 = Unknown and 2 = TMB-4.

0.1 *N* NaOH and heated in a 90° water bath for 60 sec. A similar profile was produced at 37°, but the reaction rate was much slower.

Conversely, fewer peaks were observed when TMB-4 was prepared in 0.1 *N* HCl at room temperature and 37°. Even after heating TMB-4 at 90° for 60 sec in 0.1 *N* HCl, no noticeable change was observed in the TMB-4 concentration. One small peak did appear in the chromatogram, but it was not identified. Fig. 4 depicts the separation.

From this study, we were able to show an abbreviated kinetic picture of TMB-4 under various chemical and physical conditions. The development of an ion-pair HPLC method that specifically measures TMB-4 and some of its degradative by-products in an accurate and precise manner offers a convenient and practical analytical tool for better understanding of the chemical nature of TMB-4.

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REFERENCES

1 D. G. Sullivan and P. W. Sadler, *Nature (London)*, 182 (1958) 1948.
2 E. J. Poziomek, B. E. Hackley, Jr. and G. M. Steinberg, *J. Org. Chem.*, 23 (1958) 714.
3 E. Bay, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 18 (1959) 366.
4 J. H. Willis, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 18 (1959) 1020.
5 R. A. Lehman, H. M. Fitch, L. P. Bloch, H. A. Jewell and M. E. Nicholls, *J. Pharmacol. Exp. Ther.*, 128 (1960) 307.
6 B. Calesnick, J. A. Christensen and M. Richter, *Arch. Environ. Health*, 15 (1967) 599.
7 V. M. Sim, *J. Amer. Med. Ass.*, 192 (1965) 403.
8 K. M. Li, *Hawaii Med. J.*, 24 (1965) 358.
9 D. Grob and R. J. Johns, *Amer. J. Med.*, 24 (1958) 497.
10 W. D. Erdmann and M. S. Clarmann, *Deut. Med. Wochenschr.*, 88 (1963) 2201.
11 J. H. Fleisher, H. O. Michel, L. F. Yates and C. S. Harrison, *J. Pharmacol. Exp. Ther.*, 129 (1960) 31.
12 J. F. O'Leary, A. M. Kunkel and A. H. Jones, *J. Pharmacol. Exp. Ther.*, 132 (1961) 50.
13 B. M. Askew, Davies, A. L. Green and R. Holmes, *Brit. J. Pharmacol.*, 11 (1956) 424.
14 R. I. Ellin and A. A. Kondritzer, *Anal. Chem.*, 31 (1959) 200.
15 W. A. Groff and R. I. Ellin, *Clin. Chem.*, 15 (1969) 72.
16 A. F. Plant, *Chem. Eng. News*, 54 (1976) 6.
17 R. I. Ellin, D. E. Easterday, P. Zvirblis and A. A. Kondritzer, *J. Pharm. Sci.*, 55 (1966) 1263.