CHROM. 5802

A convenient chromatographic method to investigate the *in vivo* metabolism of 1,1'-trimethylene bis(4-aldoximinopyridinium)dibromide by cation exchangers*

The efficacy of 1,1'-trimethylene bis(4-aldoximinopyridinium)dibromide (TMB-4) in antagonizing alkylphosphate intoxication has received considerable interest, as it has been reported to be the most efficacious of a series of 1,1'-polymethylene bis(4-aldoximinopyridinium)halides[1,2]. Although TMB-4 is superior in many respects to the more widely used pralidoxime (2-PAM)[2-5], the biological disposition of TMB-4 and related compounds has received very little attention, primarily because it is very difficult to isolate bisquaternary compounds from biological tissues, especially as under normal conditions the urine would contain large amounts of unchanged TMB-4 and only small amounts of metabolites[6]. This should not infer that the metabolism of TMB-4 is of minor consequence, as the biotransformation of TMB-4 is of great toxicologic importance[7].

These studies describe rapid chromatographic procedures to remove very large amounts of this antagonist from its probable metabolites, and to resolve each of these biotransformation products in very high purity.

Methods

The chromatographic basis of the separation of TMB-4 from its potential metabolites by ion-exchange column chromatography is based on the existence of this bispyridinium oxime ion in five possible chemical species, depending on the pH of the solution (Fig. 1): the enolic form with a net electrical charge of +2 (A); the combined enolic and zwitterionic form (B) in resonance with (C) with a net electrical charge of +1; and the zwitterionic form (D) in resonance with (E) with a net electrical charge of zero.

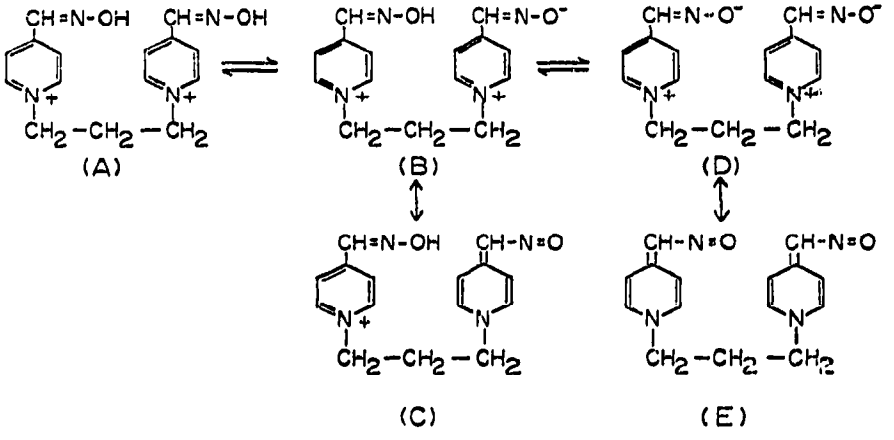


Fig. 1. Five chemical species of TMB-4: enolic form (A); combined enolic-zwitterionic form (B) in resonance with (C); zwitterionic form (D) in resonance with (E).

* This work was supported in part by funds from the National Institutes of Health, U.S. Public Health Service (NBO7855, GM 17530 and MH 14719) and Office of Naval Research (NONR-1202).

The pK_E of the two aldoximino groups of TMB-4 have been reported to be 8.0 and 8.7, respectively[8,9]. Therefore, adjustment of the sodium: hydrogen ratio in the cation exchangers to a pH above 9.7 permits the TMB-4 to exist predominantly as the zwitterion and its resonance stabilized form (D and E). The zwitterionic species of TMB-4 would carry a net electrical charge of zero and is not adsorbed on the cation exchangers to any appreciable extent. However, once the oxime group is metabolized to a nitrile, the zwitterion can no longer be formed; therefore, the bispyridinium ions will exist as cations at these high pH values and are adsorbed quite strongly to the cation exchanger (Fig. 2). At pH 9.9, a proton is removed from the enolic form (F) of trimethylene-1-(4-aldoximinopyridinium)-1'-(4-cyanopyridinium) ion (TACN) to form the zwitterionic form (G), which is resonance-stabilized (H) and would possess a net electrical charge of +1, whereas the 1,1'-trimethylene bis(4-cyanopyridinium) ion (TBCN) would retain a net electrical charge of +2. As one would expect the cyanobispyridinium ions to be quite labile at pH 9.9 (refs. 10–12), the exposure of these compounds to pH 9.9 with these chromatographic procedures must be minimized.

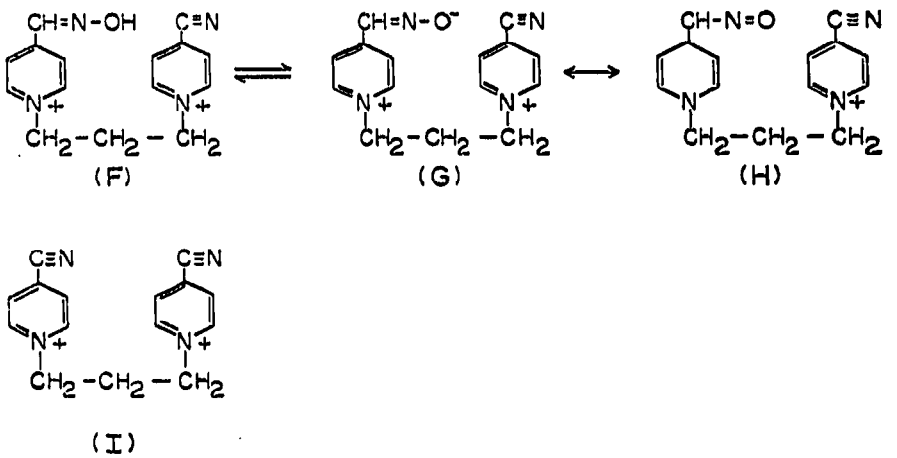


Fig. 2. Two chemical species of TACN: enolic form (F); zwitterionic form (G) in resonance with (H). One chemical species of TBCN (I).

Experimental

Materials. The SE Sephadex C-25, which is a strongly acidic polysaccharide type of cation exchanger containing sulphoethyl functional groups, was purchased from Pharmacia Fine Chemicals, Inc.

Chromatographic procedures. These procedures were conducted at 0–4° using a Gilson Medical Electronics Volume Fractionator, Model V-10, with an accessory ultraviolet absorption meter. Elution of ultraviolet-absorbing material was determined by the use of an interference filter which absorbed at approximately 280 nm and the elution patterns were recorded with an Esterline-Angus Model 424-A Recorder.

Solutions containing 5–500 mg of TMB-4 and 5 mg each of TACN and TBCN were prepared in 5–25 ml of 0.002 *M* sodium carbonate buffer, pH 9.9 and loaded on the cation-exchange columns. When a SE Sephadex C-25, short column (0.8 cm × 0.63 cm^2) was used, it was washed rapidly and profusely with 0.002 *M* sodium carbonate buffer, pH 9.9, until no ultraviolet-absorbance at 280 nm was noted in the effluent. The washed cation exchangers were removed subsequently from the

short column and carefully layered on top of a new long column of SE Sephadex C-25 pH 9.9 (14.2 cm × 0.63 cm²). This cation-exchange column was then developed at an operating flow-rate of 2.0 ml/min by linear gradient elution, with 1.0 l of water in a mixing flask and 1.0 l of 0.2 *M* sodium carbonate buffer, pH 9.9, in a reservoir flask. The effluent was collected in 10-ml fractions in tubes containing 0.5–2.0 ml of 1.0 *N* hydrochloric acid to minimize the alkaline hydrolysis of the cyanobispyridinium ions. In other chromatographic experiments, 5 mg each of TMB-4, TACN and TBCN were prepared in 5–25 ml of water, and the solutions were then loaded on columns of SE Sephadex C-25 (15 cm × 0.63 cm²) prepared as previously described using 0.2 *M* sodium phosphate buffer, pH 6.7, 0.2 *M* sodium borate buffer, pH 8.35, or 0.2 *M* sodium carbonate buffer, pH 9.9. These cation-exchange columns then were developed at a flow-rate of 2.0 ml/min by linear gradient elution, with 1.0 l of water in a mixing flask and 1.0 l of the appropriate buffer in a reservoir flask.

Results

The variation in the resolution of approximately equimolar amounts (5 mg each) of TMB-4, TACN, and TBCN at pH 6.7, 8.35 and 9.9 by cation-exchange chromatography is shown in Figs. 3a, 3b and 3c, respectively. The bispyridinium compounds were placed on a cation-exchange column (15 cm × 0.63 cm²) and were developed immediately with the appropriate buffer solutions. At pH 6.7, the ion exchange pattern showed only one fraction (Fig. 3a), which contained a mixture of TMB-4, TACN, and TBCN. At pH 8.35, two fractions were observed (Fig. 3b) Fraction 1 was TMB-4, and fraction 2 was TACN and TBCN. At pH 9.9, the cation exchange elution pattern showed three fractions (Fig. 3c). Fraction 1 was TMB-4 fraction 2 was TACN; and fraction 3 was TBCN. A much better separation is obtained

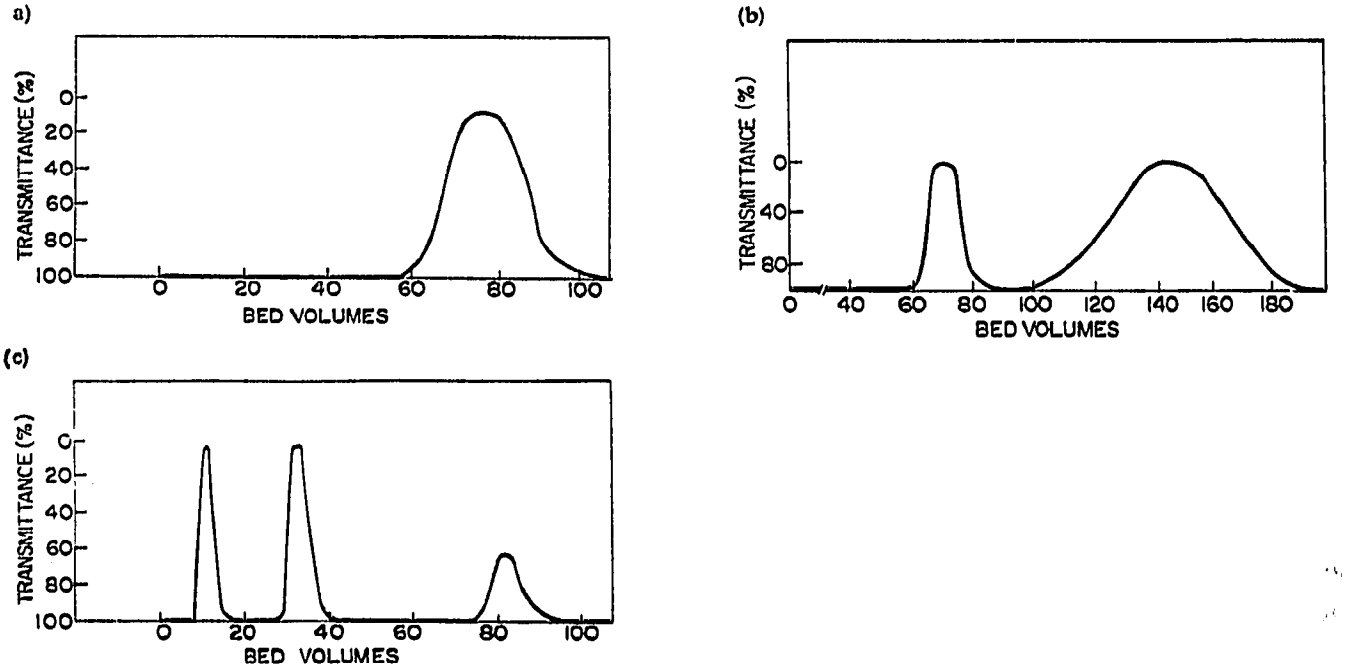
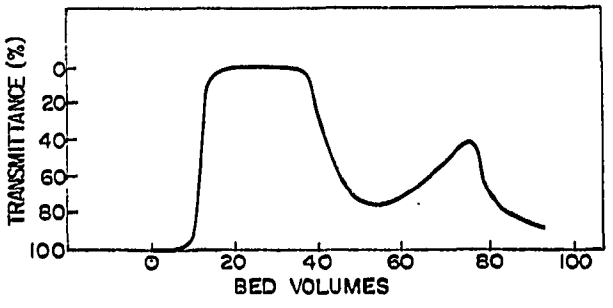


Fig. 3. Ion-exchange elution pattern of 5 mg each of TMB-4, TACN and TBCN on SE Sephadex C-25 column (15 cm × 0.63 cm²) developed with (a) 0.2 *M* sodium phosphate buffer, pH 6.7 (b) 0.2 *M* sodium borate buffer, pH 8.35; (c) 0.2 *M* sodium carbonate buffer, pH 9.9.

at pH 9.9, as the three bispyridinium compounds were separated into three very distinct fractions. If the bispyridinium ions were present in approximately equimolar amounts, the chromatographic procedures used at pH 9.9 would be quite satisfactory. However, in the investigation of the *in vivo* metabolism of quaternary ammonium compounds, such as the bispyridinium ion, this would certainly not be the case. Under *in vivo* conditions, the overwhelmingly predominate bispyridinium ion would be TMB-4 and only small amounts of the metabolites would be present[13].

In order to simulate conditions that would occur during *in vivo* metabolism of TMB-4, an excess of this antagonist (500 mg) relative to its potential metabolites (5 mg each) was loaded on a long chromatographic column (15 cm × 0.63 cm²) and immediately developed with sodium carbonate buffer at pH 9.9. The ion-exchange elution pattern (Fig. 4) showed two overlapping ultraviolet-absorbing fractions; fraction 1 contained TMB-4 and TACN, while fraction 2 was TBCN. If the amount of TMB-4 were increased further, the two fractions would be expected to merge even more into a single fraction. The large amount of TMB-4 under these conditions would prevent the resolution of the various bispyridinium derivatives of TMB-4.



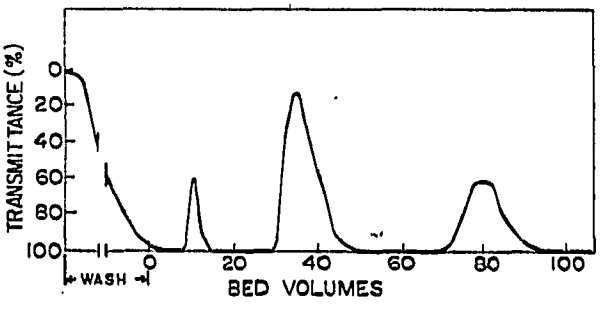


Fig. 4. Ion-exchange pattern of 500 mg of TMB-4 and 5 mg each of TACN and TBCN on SE Sephadex C-25 Column (15 cm × 0.63 cm²) without prior buffer wash.

Fig. 5. Ion-exchange elution pattern of 100 mg of TMB-4 (Fraction 1), 5 mg each of TACN (fraction 2) and TBCN (fraction 3) after washing with 0.002 *M* sodium carbonate buffer, pH 9.9 on SE Sephadex C-25 column (0.8 cm × 0.63 cm²) and transfer of cation exchanger to SE Sephadex C-25 column (14.2 cm × 0.63 cm²) and developed with 0.2 *M* sodium carbonate buffer, pH 9.9

Chromatographic separation of an excessively large amount of TMB and small amounts of other bispyridinium ions can be accomplished by passing the mixture through a short cation-exchange column (0.8 cm × 0.63 cm²) and washing profusely with 0.002 *M* sodium carbonate buffer, pH 9.9, to remove TMB-4. The cation exchanger containing the remaining bispyridinium ions is then layered on a long cation-exchange chromatographic column (14.2 cm × 0.63 cm²) and developed with sodium carbonate buffer as previously described. Under these conditions, the ion-exchange elution pattern showed three distinct fractions similar to that observed when only small amounts of each compound were used (Fig. 5). This method permits the removal of over 97 % of TMB-4 by the short column, and an over-all recovery of approximately 50 % of TACN and TBCN by the entire procedure.

Discussion

Since TMB-4, TACN and TBCN possess a net electrical charge which varies from 0 to +2, depending on the pH value of the solution, a procedure was developed which would rapidly remove large amounts of TMB-4 so that the smaller amounts

of bispyridinium metabolites could be resolved by conventional chromatographic methods. The chemical basis of the chromatographic procedure was to maintain the TMB-4 primarily as the zwitterion so that its adsorption on the cation exchangers would be minimized. Removal of the small amount of TMB-4 which adsorbed on the column was facilitated by washing the column with 0.002 *M* sodium carbonate buffer, pH 9.9. This buffer was of a sufficient concentration to elute TMB-4, but not the potential metabolites, as they could possess a net electrical charge of +1 to +2 under these conditions, and therefore would be much more strongly adsorbed on the cation exchangers.

A buffer solution of pH 9.9 was used with reluctance, as the cyanobispyridinium compounds are readily degraded in alkaline solutions[10]. The SE Sephadex C-25 was selected, as it possesses sufficient capacity so that a solution containing large amounts of TMB-4 and small amounts of TACN and TBCN could be rapidly passed through an extremely short column. The use of a short column permitted the rapid removal of TMB-4 and allowed subsequent resolution of these bispyridinium ions with no detectable chemical degradation of the labile cyanobispyridinium derivative. It was of theoretical and practical importance to investigate the cation-exchange elution pattern of these bispyridinium compounds, TMB-4, TACN and TBCN, under different pH conditions. One would predict that, at pH 6.7, all three of these compounds would possess a net electrical charge of +2, and the resolution of these bispyridinium ions would be minimal. This was verified in our studies at pH 6.7, as TMB-4, TACN and TBCN could not be resolved and appeared as a single fraction. The pH was then increased to 8.35, the isoelectric point of TMB-4. Although the net electrical charge of TMB-4 would be +1 at the isoelectric point, the molar ratio of the chemical species of TMB-4 as the enol (A):enol-zwitterion (B):zwitterion (D) would be 1:2:1. Under these conditions, the net electrical charge of TACN was closer to that of TBCN; therefore, only two fractions were observed. The first fraction contained TMB-4 and the second fraction TACN and TBCN. It was only when the alkalinity of the eluant was raised to pH 9.9 that the net electrical charges of these three bispyridinium ions were sufficiently different that they could be separated.

In summary a rapid chromatographic method for the separation of large amounts of 1,1'-trimethylene bis(4-aldoximinopyridinium) dibromide (TMB-4) from small amounts of its potential metabolites, trimethylene-1-(4-aldoximinopyridinium)-1-(4-cyanopyridinium) ion (TACN) and 1,1'-trimethylene bis(4-cyanopyridinium (TBCN) has been developed. The chemical basis of this chromatographic procedure is attributed to the conversion of TMB-4 to its zwitterionic form so that it can be rapidly washed from a very short SE Sephadex C-25, pH 9.9 cation-exchange column (0.8 cm × 0.63 cm^2) with 0.002 *M* sodium carbonate buffer. The buffer-washed cation exchanger was subsequently removed from the short column and carefully layered on a long SE Sephadex C-25, pH 9.9 chromatographic column to resolve the remaining bispyridinium compounds.

Washington State University, Colleges of Veterinary Medicine and Pharmacy, College Hall, Pullman, Washington, 99163 (U.S.A.)

GEORGE E. BURROWS
URSULA F. FEITKNECHT
PAULO M. MIRANDA
STANLEY L. GIBBON
JAMES L. WAY

1 E. J. POZIOMEK, B. E. HACKLEY, JR., AND G. M. STEINBERG, *J. Org. Chem.*, 23 (1958) 714.
2 F. HOBBIGER, D. G. O'SULLIVAN AND P. W. SADLER, *Nature*, 182 (1958) 1498.
3 I. B. WILSON AND S. GINSBURG, *Biochem. Pharmacol.*, 1 (1958) 200.
4 W. K. BERRY, D. R. DAVIS AND A. L. GREEN, *Br. J. Pharmacol.*, 14 (1959) 186.
5 J. H. FLEISHER, H. O. MECHEL, L. YATES AND C. S. HARRISON, *J. Pharmacol.*, 129 (1960) 31.
6 L. PETERS, *Pharmacol. Rev.*, 12 (1960) 1.
7 J. L. WAY AND E. L. WAY, *Annu. Rev. Pharmacol.*, 8 (1968) 187.
8 Y. ASHANI, N. DINAR AND S. COHEN, *J. Med. Chem.*, 11 (1968) 967.
9 D. BIEGER AND O. WASSERMAN, *J. Pharm. Pharmacol.*, 19 (1967) 844.
10 E. M. KOSOWER AND J. W. PATTON, *Tetrahedron*, 22 (1966) 2081
11 R. I. ELLIN, D. E. EASTERDAY, P. ZVIRBLIS AND A. A. KONDRITZER, *J. Pharm. Sci.*, 55 (1966) 1263.
12 C. N. CORDER AND J. L. WAY, *J. Med. Chem.*, 9 (1966) 638.
13 J. L. WAY, U. F. FEITKNECHT, C. N. CORDER AND P. M. MIRANDA, *Biochim. Biophys. Acta*, 121 (1966) 432.

First received October 6th, 1971; revised manuscript received October 26th, 1971