RAPID METHOD FOR INVESTIGATING THE *IN VIVO* METABOLISM OF 1-METHYL-2-ALDOXIMINOPYRIDINIUM IODIDE BY ION EXCHANGE COLUMN CHROMATOGRAPHY*

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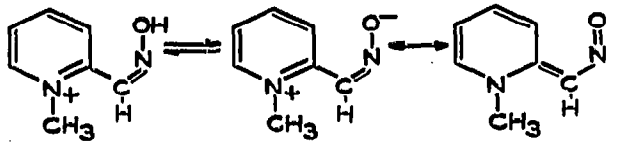
One of the most effective oximes in antagonizing alkylphosphate intoxication is 1-methyl-2-aldoximinopyridinium iodide (2-PAM)[1,2]. Although the mechanism of action of 2-PAM has received considerable attention[3] and the biotransformation of 2-PAM *in vitro* has been extensively investigated in the isolated perfused rat liver system[4–7], there is a paucity of information concerning the metabolism of this antagonist in the intact animal. This can be attributed primarily to the polarity of this compound which enhances the rapid urinary excretion of large amounts of unchanged 2-PAM and thereby permitting only minimal amounts of metabolites to be formed[8,9].

The present studies describe a convenient method for investigating the *in vivo* metabolism of 2-PAM by column chromatography. Chromatographic procedures have been developed which permit not only the rapid removal of large concentrations of this antagonist from most of its probable metabolites, but also the resolution of each of these biotransformation products.

METHODS

The application of ion exchange column chromatography to separate 2-PAM from its metabolites is based on the existence of the aldoximinopyridinium ion in 3 possible chemical species (Fig. 1): the enolic form (A), and the zwitterionic form (B)



A B C

Fig. 1. The three forms of 2-PAM: The enolic form (A) and the zwitterionic form (B) in resonance with (C).

in resonance with (C)[4]. Since the pK_E of the oximino group of 2-PAM is 8.0, adjustment of the sodium to hydrogen ratio in the ion exchange resin to a pH value of

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9.0 allows 2-PAM to exist primarily as the zwitterion, which is not adsorbed on the resin to any appreciable extent. On the other hand, most of the other pyridinium ions still exist as cations and are adsorbed to the resin. Because some of the 2-PAM metabolites are quite labile at pH 9.0, a very short resin column is employed initially so that 2-PAM can be rapidly washed from the resin. The metabolites which are adsorbed on the resin are then removed from the column and layered on long chromatographic columns. Development of the column is initiated by gradually decreasing the pH value while concomitantly increasing the ionic concentration of the eluant.

EXPERIMENTAL

Materials

Pyridine-2-aldoxime, 2-cyanopyridine, and 1-methyl-2-aldoximinopyridinium ion were obtained from the Aldrich Chemical Co. All these chemicals were carefully recrystallized at least 2 times. The 1-methyl-2-cyanopyridinium ion was prepared by the method of KOSOWER *et al.*[10] with minor modifications[6] and 2-methoxypyridine was prepared by the method of PECHMANN AND BALTZER[11]. The 1-methyl-2-methoxypyridinium ion was prepared by the interaction of 2-methoxypyridine with methyl iodide. All other chemicals were of the highest purity commercially available.

Amberlite CG-50 is a weakly acidic, polymethacrylic type of cation exchange resin which contains carboxylic groups as the functional sites. This resin was purchased from Rohm and Haas Company.

Procedures

Preparation of resins. Amberlite CG-50-Na^+ (200–400 mesh) was cleaned with 1.0 *N* HCl, 1.0 *N* NaOH and 50% ethanol, and the resin was then adjusted to pH 8.9 by treating the resin repeatedly with 0.5 *M* sodium borate buffer, pH 8.9, until the pH value of the supernatant fluid remained constant. The resin was then placed in glass chromatographic columns and 1.0 to 5.0 bed volumes of borate buffer were passed through these columns to ascertain that the pH value of the effluent fluid remained at 8.9. These chromatographic columns subsequently were washed with 10 bed volumes of water to remove excess buffer. The resin was now fully regenerated to the desired combination of ionic forms and was ready for use.

Chromatographic procedures. All operations were carried out at 0–2° in a Gilson Medical Electronics Volume Fractionator, Model V-10, with an accessory ultraviolet absorption meter. The elution patterns were monitored at approximately 280 mμ by the use of an interference filter with an Esterline-Angus Model 424-A Recorder.

Solutions containing from 3 to 1000 mg of 2-PAM and 3 mg each of 1-methyl-2-methoxypyridinium ion and 1-methyl-2-cyanopyridinium ion were prepared in 5 to 50 ml of water. When a short Amberlite CG-50, pH 8.9, column (0.8 cm $\times$ 0.63 cm^2, 200 to 400 mesh) was employed, it was washed with water to remove the 2-PAM. The short chromatographic column was washed rapidly and profusely with water until no absorbance at 280 mμ was noted in the effluent. The washed resin subsequently was removed from the column and carefully layered on top of a new column of Amberlite CG-50 pH 8.9 (10.2 cm $\times$ 0.63 cm^2, 200 to 400 mesh). 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 0.001 *M* sodium phosphate buffer, pH 6.7, in a mixing

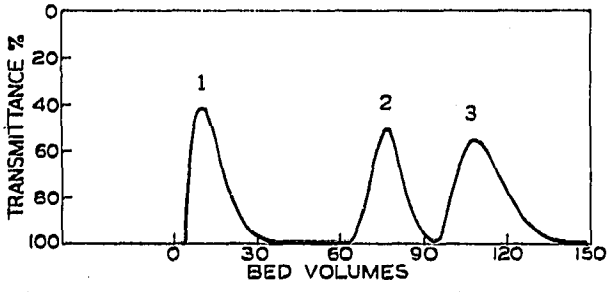
flask and 1.0 l of 0.005 *M* sodium phosphate buffer, pH 6.7, in a reservoir flask. The effluent was collected in 10 ml fractions in tubes containing 0.5 ml of 1.0 *N* HCl.

Analysis of eluant. The effluent fractions which were shown to contain ultraviolet-absorbing material were analyzed further on a Beckman DB Spectrophotometer with a Sargent SRL Linear Log Recorder to ascertain that the fractions recovered were the original material and that no chemical degradation had occurred.

RESULTS

Elution profiles by spectrophotometric analyses

The chromatographic resolution of approximately equimolar amounts (3 mg each) of 2-PAM, 1-methyl-2-methoxypyridinium ion and 1-methyl-2-cyanopyridinium is shown in Fig. 2. The mixture was placed on a single, long chromatographic column (11.0 cm × 0.63 cm²) and immediately developed without a prior water wash. The ion exchange elution patterns show 3 ultraviolet absorbing fractions (Fig. 2). Fraction 1 appearing between 5 and 30 resin bed volumes is 2-PAM, fraction 2 appearing at 65–90 bed volumes is 1-methyl-2-methoxypyridinium ion, while fraction 3 appearing from 95–145 resin bed volumes is 1-methyl-2-cyanopyridinium ion. Although this procedure would be satisfactory if all of these pyridiniums were present in small and approximately equimolar amounts, this would not be the case in investigating the *in vivo* metabolism of a quaternary ammonium compound such as 2-PAM[8,9]. The predominant pyridinium ion in *in vivo* conditions would be 2-PAM, while only minute amounts of metabolites would be present.



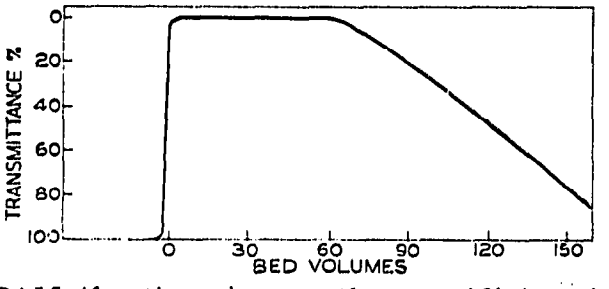


Fig. 2. Ion exchange pattern of 3 mg each of 2-PAM (fraction 1), 2-methoxypyridinium ion (fraction 2) and 2-cyanopyridinium ion (fraction 3) on Amberlite CG-50, pH 8.9, column (11 cm × 0.63 cm²) without prior water wash.

Fig. 3. Ion exchange pattern of 100 mg of 2-PAM and 3 mg each of 2-methoxypyridinium ion and 2-cyanopyridinium ion on Amberlite CG-50, pH 8.9, column (11 cm × 0.63 cm²) without prior water wash.

Fig. 3 shows the ultraviolet absorbing elution pattern when a solution containing an excess of 2-PAM (100 mg) relative to its metabolites (3 mg each) is placed on a long chromatographic column (11.0 cm × 0.63 cm²) and immediately developed without a water wash. The excessive amount of 2-PAM under these conditions prevents the satisfacory resolution of the various pyridinium derivatives from 2-PAM.

In Fig. 4 the solution containing the excessively large amount of 2-PAM is initially passed through a short resin column (0.8 cm × 0.63 cm²), washed with water and then the resin is layered on a long cation exchange chromatographic column (10.2 cm × 0.63 cm²) and developed. With the present modification of conventional

chromatographic procedure, it was possible to separate the metabolites of 2-PAM which otherwise would have been obscured by the large amount of 2-PAM present (Fig. 3). This method permits the recovery of 82 to 93 % of 2-cyanopyridinium ion and 1-methyl-2-methoxypyridinium ion. No attempt was made to quantitate the recovery of 2-PAM, as this substance was being removed to allow resolution of the other two compounds.

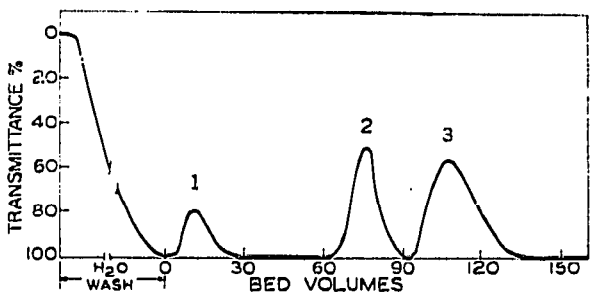


Fig. 4. Ion exchange pattern of 100 mg of 2-PAM (fraction 1), 3 mg each of 2-methoxypyridinium ion (fraction 2) and 2-cyanopyridinium ion (fraction 3) after water wash on Amberlite CG-50, pH 8.9, column (0.8 cm × 0.63 cm^2) and transfer of resin to Amberlite CG-50, pH 8.9, column (10.2 cm × 0.63 cm^2).

DISCUSSION

The development of this column chromatographic procedure for investigation of the *in vivo* metabolism of 2-PAM was prompted by several factors. Firstly, under conditions of *in vivo* studies, large quantities of the administered compound would be expected to be excreted unchanged, while minimal amounts of the metabolites would be present[8,9,15,16]. Under these circumstances the methods employed for the *in vitro* studies of 2-PAM by conventional cation exchange chromatography would be inadequate. Secondly, the metabolites are chemically unstable, especially in alkaline solution; therefore, a rapid procedure was necessary to minimize alkaline degradation. Thirdly, 2-PAM and its *in vitro* metabolites, 1-methyl-2-methoxypyridinium and 1-methyl-2-cyanopyridinium ions, have a net charge of + 1, which greatly complicates the separation and isolation of these metabolites. These factors dictated the development of a procedure which would rapidly separate large amounts of unaltered 2-PAM from much smaller amounts of pyridinium metabolites.

The establishment of a mechanism for the spectral properties of 2-PAM greatly facilitated the development of satisfactory chromatographic procedures to separate 2-PAM and its metabolites. The pK_E of the oximino group of 2-PAM was found to be 8.0[4,12,13]; thus at pH values above 8.0, the 2-PAM would be present primarily as the zwitterion (B) and its resonance stabilized from (C) (Fig. 1). The chemical basis of the chromatographic procedure was to maintain the 2-PAM predominantly in its zwitterionic form in order to limit its adsorption on the ion exchange resin and to facilitate the removal of any adsorbed 2-PAM by merely washing the columns with water. Since most of the metabolites of 2-PAM have a net charge of + 1 under these conditions, they would adsorb quite avidly on the ion exchange resin

A cationic resin with weak-acid characteristics was employed for these studies to allow a controlled ratio of salt to free acid by the use of appropriate buffers. One of the resins which possessed this property was Amberlite CG-50. A pH value of greater

than 9.0 was not employed in these procedures due to the alkaline lability of the 2-cyano and 2-methoxypyridinium ions[5,14].

An attempt was made to use a single chromatographic column (11.0 cm × 0.63 cm²) to remove 2-PAM. However, the duration of time required to remove 2-PAM under these conditions caused substantial degradation of the 2-cyanopyridinium ion. Therefore, a more rapid method to remove 2-PAM was developed which was based on the use of a very short ion exchange column (0.8 cm × 0.63 cm²). Since the capacity of this resin is quite high, it was possible to pass a solution containing an excessive amount of 2-PAM and small amounts of metabolites rapidly through a short column (0.8 cm × 0.63 cm²) with almost quantitative adsorption of the metabolites, while retaining minimal amounts of 2-PAM that can be rapidly washed from the column. By this procedure 2-PAM can be removed entirely from the reaction mixture containing other pyridinium ions. However, under practical conditions the short resin column was washed with only a sufficient amount of water to remove most of the 2-PAM prior to transferring the resin to a long column (10.2 cm × 0.63 cm²). This procedure permits the rapid removal of 2-PAM from the solution under adverse alkaline conditions and the subsequent resolution of various pyridinium ions at a pH value which is more consistent with their stability. The principles involved in this procedure appear to have wide application as a convenient general procedure to separate large amounts of one substance from trace amounts of labile structural analogues. Preliminary studies on the successful application of this method to investigate the *in vivo* metabolism of 2-PAM in rat[15] and man[16] have been reported.

SUMMARY

A rapid, chromatographic method for the separation of large amounts of 1-methyl-2-aldoximinopyridinium ion (2-PAM) from small amounts of its pyridinium biotransformation products as would occur in *in vivo* conditions has been developed. This method is based on the conversion of 2-PAM to its zwitterionic form so that 2-PAM can be rapidly washed from a short Amberlite CG-50, pH 8.9 cation exchange column (0.8 cm × 0.63 cm²) with water. The water washed resin is subsequently carefully layered on a long Amberlite CG-50, pH 8.9 chromatographic column (10.2 cm × 0.63 cm²) to resolve these metabolites by linear gradient elution with sodium phosphate buffer, pH 6.7. These procedures permit almost complete removal of the 2-aldoximinopyridinium ion and a rapid separation of 1-methyl-2-cyanopyridinium ion and 1-methyl-2-methoxypyridinium ion.

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