

MULTIPLE SPOT PHENOMENA OF 2-PAM AND METABOLITE ON PAPER CHROMATOGRAMS*

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(Received August 24th, 1965)

Subsequent to the development of 1-methyl-2-aldoximinopyridinium iodide (2-PAM iodide) as an agent which would antagonize anticholinesterase alkylphosphate intoxication¹, there has been a considerable interest in the metabolic disposition of this antagonist. Owing to the rapid urinary excretion of large amounts of unchanged 2-PAM and only small amounts of metabolites, most studies of the biotransformation of 2-PAM have employed isotopically labeled 2-PAM of high specific activity and, unfortunately, paper chromatography as the primary means to "resolve" various radioactive components which were presumed to be "metabolites" of 2-PAM. The report herein describes the alteration of the chromatographic properties of the 2-aldoximinopyridinium ion and its major metabolite, 1-methyl-2-cyanopyridinium ion, in the presence of different anions, which may result in the formation of multiple spot phenomena by a single compound.

MATERIALS AND METHODS

The 1-methyl-2-aldoximinopyridinium iodide and 2-cyanopyridine were products obtained from the Aldrich Chemical Company. The 1-methyl-2-cyanopyridinium iodide was prepared by the method of Kosower *et al.*². Different salts of these two pyridinium ions were prepared by interacting approximately equimolar amounts of the silver chloride, phosphate and perchlorate respectively, with 1-methyl-2-aldoximinopyridinium iodide and 1-methyl-2-cyanopyridinium iodide. The products subsequently were passed through Dowex-1 ion exchange columns with the resin in the desired anionic form. All these compounds were subsequently carefully recrystallized at least two times with absolute ethanol. When a 1% solution of TCA was employed to dissolve the 2-PAM iodide, equimolar concentrations of trichloroacetic acid and 2-PAM iodide were employed. As the concentration of the trichloroacetic acid was increased or decreased, the molar ratios of TCA:2-PAM iodide were proportionately altered. Paper chromatography was carried out on Whatman No. 1 paper at 0-2° for 24 h. The solvent systems employed were: (a) propanol-water (7:3, v/v), (b) *n*-butanol-ethanol-water (4:1:1, v/v), and (c) *n*-butanol-1 *N* acetic acid (10:1, v/v).

* This work was supported in part by funds from the National Institutes of Health, United States Public Health Service (NB-04541, MH-10109).

** Research Career Development Awardee (GM-K3-4148) of the National Institutes of Health, United States Public Health Service.

The pyridinium ions were visualized on the paper chromatograms by (1) ultra-violet absorption and contact photography, employing a Mineralight Ultraviolet Lamp (approximately 254 m μ) Model S 2537 and Kodabromide F-5 photographic paper and (2) iodoplatinic acid spray reagent³. The iodide anion was localized with a 1% palladous chloride solution.

RESULTS AND DISCUSSION

Most of the investigative efforts on the *in vivo* metabolism of the 2-aldoximinopyridinium ion^{4,5} have been carried out with paper chromatographic methods and relatively nonspecific spot tests to "isolate" and "characterize" "metabolites" of 2-PAM. Interpretation of these results may be open to serious question, since the chromatographic property of some pyridinium ions can be altered considerably according to the anions present. This has been well established in these studies, as 2-PAM in the presence of various anions was shown to possess markedly different chromatographic properties. Furthermore, in the presence of a solution containing two different anions, it can be clearly demonstrated that this single pyridinium ion was "resolved" into two components with vastly different R_F values. Similar alterations of the chromatographic properties of one of the major metabolites of 2-PAM, the 2-cyanopyridinium ion, were also noted under these conditions.

A summary of the chromatographic properties of 2-PAM and the 2-cyanopyridinium ions in the form of the iodide, chloride, phosphate, perchlorate and trichloroacetate salt is shown in Table I. It is readily observed that the R_F values of 2-PAM varies considerably depending on the anionic constituent associated with the pyridinium cation. In solvent system (a) the R_F values of 2-PAM were between 0.15–0.52,

TABLE I

R_F VALUES OF 1-METHYL-2-ALDOXIMINOPYRIDINIUM CATION WITH DIFFERENT ANIONS

	R_F values	
	Solvent system a*	Solvent system b**
2-PAM iodide	0.24	0.55
2-PAM chloride	0.15	0.49
2-PAM phosphate	0.16	0.48
2-PAM perchlorate	0.28	0.54
2-PAM TCA***	0.52	0.70
2-CN-Py iodide****	0.17	0.49
2-CN-Py chloride	0.10	0.39
2-CN-Py perchlorate	0.12	0.42

* *n*-Butanol–EtOH–H₂O (4:1:1, v/v).

** *n*-Propanol–H₂O (7:3, v/v).

*** 2-PAM iodide dissolved in 1.0% TCA.

**** 2-CN-Py = 1-methyl-2-cyanopyridinium ion.

while in solvent system (b) the R_F values were between 0.48–0.70. In both solvent systems the trichloroacetate salt of 2-PAM possessed the greatest mobility of the 2-PAM salts while the chloride and phosphate salts possessed the least mobility.

Therefore, when animals are administered ^{14}C 2-PAM and the urine and tissue extracts of these animals are resolved into various components by paper chromatography, the comparison of the chromatographic properties of these radioactive components, with authentic synthetic compounds, presumed to be metabolites of 2-PAM, may lead to rather erroneous interpretations. For example, as shown in Fig. 1, an R_F value for a given anionic salt of 2-PAM probably can be found which would correspond with that of one of several potential metabolites of 2-PAM. This variability in mobility of 2-PAM is highly likely to occur when urine or crude tissue extracts containing a mixture of anions are employed. Similar variations in the chromatographic property of the 2-PAM metabolite, 1-methyl-2-cyanopyridinium ion, were also noted (Table I).

Since different anionic salts of 2-PAM will greatly alter the chromatographic behavior of the 2-aldoximinopyridinium and 2-cyanopyridinium ions, the corollary would be that each of these pyridinium cations in the presence of a solution containing different anions would resolve into separate areas in a single paper chromatogram. Separation of 2-PAM into two widely discrete areas in the presence of two different anions is best illustrated in Fig. 2. Those areas containing the pyridinium ions were visualized by the iodochloroplatinate spray reagent. In order to ascertain that the two areas resolved by paper chromatography were 2-PAM rather than a degradation product, the corresponding spots localized by an ultraviolet light source were eluted with cold methanol (2°) and the ultraviolet spectral properties of both of these areas were compared and found to correspond with those of the ultraviolet absorption spectra of 2-PAM. The absorption maximum of 2-PAM was at $293\text{ m}\mu$ in water and 0.01 N HCl and a bathochromic shift to $333\text{ m}\mu$ occurred in alkaline solution, presumably due to the formation of the 2-PAM zwitterion and its resonance stabilized form⁶. Furthermore, when the ultraviolet-absorbing area containing the 2-PAM TCA salt was eluted with 0.01 N HCl , extracted repeatedly with diethyl ether, and rechromatographed; the R_F value of the compound contained in the acidic layer was greatly decreased and now corresponded with those of 2-PAM chloride, as would be expected, since TCA would be extracted into the organic phase under these conditions.

It is to be noted in Fig. 2 that as the 2-PAM iodide is dissolved in a solution containing increasingly higher concentration and total amounts of TCA, there is a gradual disappearance of the spot corresponding with 2-PAM iodide which is concomitantly followed by the appearance of increased amounts of 2-PAM TCA. Additional evidence which would indicate that the resolution of 2-PAM ion into two components is due to the displacement of iodide ion is shown in Fig. 3. When palladium chloride is employed to visualize the iodide ion, it can be clearly demonstrated that the iodide ion is associated primarily with 2-PAM iodide when 2-PAM iodide is dissolved only in water. As the 2-PAM iodide was placed in increasingly higher concentrations of TCA, the iodide ion gradually was displaced from the 2-PAM iodide into a separate area, presumably hydrogen iodide, with a lower mobility than 2-PAM iodide. These observations on the simultaneous appearance of 2-PAM TCA and hydrogen iodide, and the disappearance of 2-PAM iodide in these paper chromatograms are consistent with the conclusion that trichloroacetate anion can displace iodide from 2-PAM iodide forming hydrogen iodide and 2-PAM trichloroacetate which are readily resolved into separate discrete areas by paper chromatography. The formation of multiple spot phenomena by 2-PAM with TCA can also be demonstrated in solvent

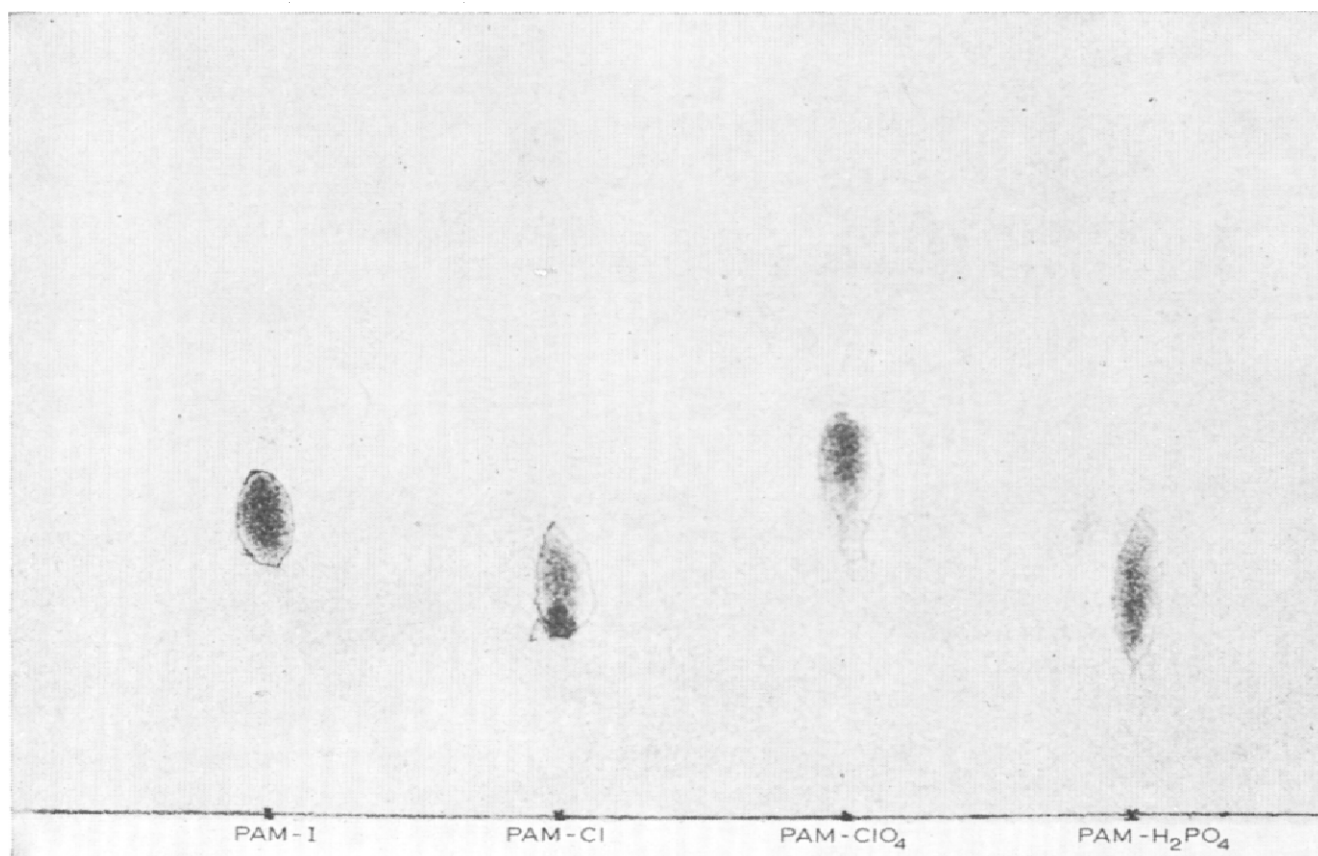


Fig. 1. Paper chromatography of 2-PAM (0.04 μ mole) iodide, chloride, perchlorate and phosphate in *n*-butanol-ethanol-water (4:1:1, v/v) solvent system. The pyridinium ion was developed with iodochloroplatinate.

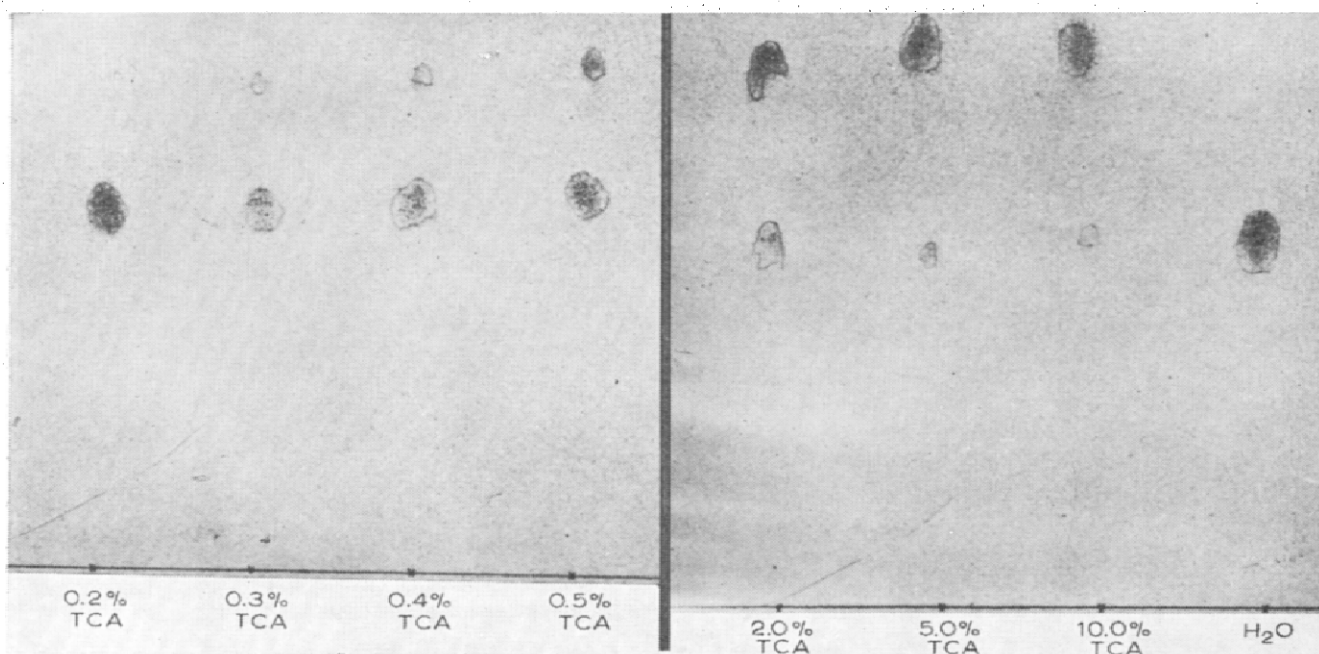


Fig. 2. Paper chromatography of 2-PAM iodide (0.04 μ mole) with TCA in *n*-propanol-water (7:3, v/v). The 2-aldoximinopyridinium ion was developed with iodochloroplatinate.

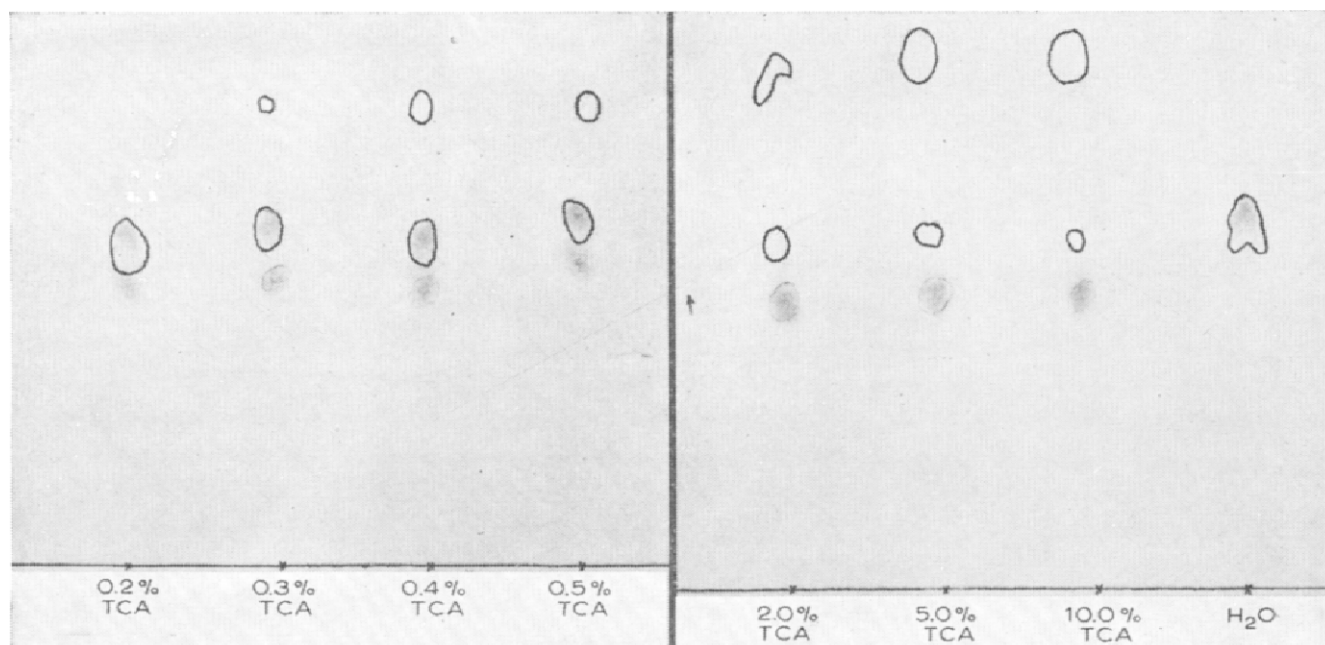


Fig. 3. Paper chromatography of 2-PAM iodide ($0.04 \mu\text{mole}$) with TCA in *n*-propanol-water (7:3, v/v). The iodide ion was developed with palladous chloride. Encircled spots represent ultraviolet-absorbing areas (2-PAM iodide and 2-PAM TCA).

systems containing other anions, for example, when *n*-butanol-acetic acid is employed as the solvent system.

These results on the chromatographic properties of 2-PAM and 2-cyanopyridinium ion should emphasize that certain precautions should be taken in the preparation of substances for paper chromatography and the interpretation of the results obtained by chromatographic analysis.

Multiple spot phenomena of pure organic compounds by paper chromatography have been reported for a variety of other compounds. Various amines⁷ and quaternary ammonium compounds⁸⁻¹¹, thyroxine¹², have been shown to either form multiple spots or artifacts and in some instances¹³⁻¹⁶ these spots probably have been erroneously presumed to be the "isolation" of a new product. The appearance of multiple spot phenomena can be prevented by conversion of all ionized compounds to a common salt. Such precautions would minimize fallacious interpretation with respect to paper chromatographic findings, particularly when the characterization of the compounds in question by other procedures is inadequate.

SUMMARY

The anticholinesterase alkylphosphate antagonist, 1-methyl-2-aldoximino-pyridinium ion (2-PAM), and its major metabolite, 1-methyl-2-cyanopyridinium ion, have been shown to possess different paper chromatographic properties, depending on the anion present. Each of these pyridinium cations in the presence of two or more different anions can elicit a multiple spot phenomenon.

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