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ISOLATION FROM HUMAN URINE AND PRELIMINARY CHARACTERIZATION OF A METABOLITE OF "PYRIDINE-2-ALDOXIME METHIODIDE"

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Abstract—A study of the metabolic alteration of pyridine-2-aldoxime methiodide (PAM) in man has involved analysis of human urine following the oral administration of the drug to healthy volunteers. This report describes the isolation of the intact drug and a fluorescent metabolite of it from human urine.

From preliminary chemical, ultraviolet and infrared spectral studies it is concluded that: (1) the metabolite is a highly polar molecule; (2) it possesses a carboxyl function and a weakly acidic, ionizable group; and (3) it is not a conjugate of glycine or glucuronic acid, and it is not an oxime. Spectral behavior of the metabolite of PAM, in comparison with known pyridine derivatives, suggests that it is a derivative of N-methyl picolinic acid.

INTRODUCTION

PAM IODIDE† and P_2S‡, in combination with atropine sulfate, have proved to be effective prophylactic agents against poisoning by such organophosphorous cholinesterase inhibitors as Parathion, DFP, TEPP, etc.[1,2] The only metabolic studies of PAM iodide that have been reported previously were those of Creasey and Green,[3] who determined the percentage degradation of P_2S by rat liver homogenates, and of Way *et al.*,[4] who studied the metabolism of pyridine-2-aldoxime ^{14}C-methiodide by perfusion of isolated rat livers. Isolation and identification of the metabolic products found in the above studies have not yet been reported. The objective of the present investigation was the determination of the nature of the metabolites of PAM iodide in man by analysing urine obtained from healthy volunteers to whom the drug had been administered orally.

EXPERIMENTAL

Materials

PAM iodide, of 100 per cent purity and for drug use, was made available by the Borden Chemical Company of Philadelphia, Pennsylvania. Most of the reference pyridinium compounds used in this study were synthesized in these laboratories by standard chemical procedures.

Procedures

Paper chromatography. Descending paper chromatography in a Chromatocab was used for the isolation and purification from urine of the drug and its metabolite.

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† 2-Formyl-1-methylpyridinium iodide oxime (PAM iodide):

‡ 2-Formyl-1-methylpyridinium methane sulfonate oxime (P_2S):

Whatman 3 MM paper (18 × 22 in.) was used with four solvent systems: (1) the organic phase of butanol:acetic acid:water (4:1:5), as described by Dalgliesh;[5] (2) phenol–water and butanol:propionic acid:water of Gadsden and Edwards;[6] (3) water-saturated butanol; and, in some instances, (4) 80 per cent *iso*propanol in water. The papers were examined under short- and long-wave ultraviolet light for selection of the appropriate areas for elution with distilled water. Pure PAM iodide was used as a reference compound. Ultraviolet spectra of the compound in distilled water, 1 N HCl, and 1 N NaOH were determined in a Beckman DK-1 spectrophotometer at three different levels of pH. The spectra of fluorescence of the metabolite, using these same solvents, were determined in a spectrophotofluorometer (Aminco–Bowman). Preliminary infrared spectral studies of the metabolite were carried out with the samples as a liquid film between two NaCl plates in a double-beam infrared spectrophotometer (Perkin–Elmer model 21).

Urine collections and drug administration. Five healthy volunteers were fed a regular Army diet, but the use of tobacco, chocolate, coffee, tea, vitamins and drug preparations was excluded for 1 week prior to this study. Then, while under the same dietary regimen with normal fluid intake, the volunteers were given a total of 4 g of PAM iodide (2 g in gelatin capsules daily for two consecutive days); the administration of the drug occurred 2 hr prior to breakfast. A control urine was collected 24 hr before drug administration, and urine was collected during a 24-hr period following each dosage with PAM iodide. Urine was collected under toluene and frozen until used.

Isolation and purification of PAM iodide from urine. The control and post-drug urines of each volunteer were separately concentrated to one-tenth their original volume in a flash-evaporator at 40 °C, and, for comparison, 0·1 ml of each concentrate was chromatographed side by side in the butanol:acetic acid:water system. In addition, on each chromatogram, 0·1 ml of an aqueous solution of PAM iodide (1 mg/ml) was run as a standard and as an aid to the localization of unaltered PAM in the samples of post-drug urines. The PAM-spot in the post-drug chromatograms was cut out, eluted with distilled water, and evaporated to dryness under reduced pressure. The residue was dissolved in distilled water and purified by paper chromatography with one of the above-mentioned solvent systems. The material that separated on the paper chromatogram was extracted with hot acetone. As soon as the solution had cooled to room temperature, contaminating cellulose fibers appeared as an amorphous fluffy material; these were removed immediately by centrifugation and the supernatant fraction was refrigerated overnight. White needle-shaped crystals usually appeared within 1 hr. Recrystallization from acetone was repeated three times. These crystals were used for identification.

Identification of PAM. The isolated product was identified by (*a*) the R_f-values obtained with paper chromatography, (*b*) ultraviolet spectra in water, 1 N HCl, and 1 N NaOH,[7] and (*c*) a specific color test for the oximino group.[8]

Isolation of the fluorescent metabolite. When observed under long-wavelength ultraviolet light (3660 Å), chromatograms of post-drug urines showed a bright blue fluorescent zone that was not present on chromatograms of control urines; this zone was cut out of ten chromatograms and eluted with distilled water. The pooled eluates were evaporated to dryness under reduced pressure at 40 °C. The residue were redissolved in distilled water and purified by one- and two-dimensional paper chromatography with one of the mentioned solvent systems. The blue fluorescent material

that separated on the chromatogram was eluted with distilled water. After pooling and concentrating the eluates under reduced pressure, the flocculent impurities from the paper were removed by filtration through a sintered-glass funnel. The solution was then evaporated to dryness *in vacuo* at 40 °C; the residue was dark yellow, viscous, very hygroscopic and water-soluble. Attempts at recrystallization have thus far been unsuccessful, since the material separates as an oily layer when in contact with organic solvents.

Incubation of PAM iodide with human urine. To determine whether the fluorescent compound is a true metabolite or had resulted from the action of urinary constituents on the drug, 0·5 g of PAM iodide was dissolved in 500 ml of control urine (pH 5–6). This solution, after division into five 100-ml portions, was kept under one of the following conditions: (1) — 10 °C, pH 5–6, for 7 days under 10 ml of toluene; (2)–(3) 24 °C, pH 5–6, for 7 days with and without toluene; (4)–(5) 37 °C, pH 5–6, for 24-hr with and without toluene. At the end of each of the time-periods, the urine specimens were concentrated ten-fold, and 0·1-ml aliquots of each were chromatographed, using the above-mentioned solvent systems. The chromatograms were examined under long-wave ultraviolet light in order to detect the fluorescent metabolite.

Preliminary characterization studies of the fluorescent metabolite. Solubilities in polar and nonpolar solvents and the extractability by organic solvents from aqueous solutions at three pH-values was ascertained. R_f-values were determined by paper chromatography in four solvent systems. Ultraviolet and fluorescent studies were carried out at three pH-values. An ultraviolet spectrophotometric determination of the pK_a was made by measurement of optical density at 270 mμ at various levels of pH.[9] The absorption pattern in the infrared region was examined for the tentative identification of functional groups. Color reactions for the presence of certain functional groups were performed as spot tests with reagents prepared as described by Feigl[8] or Block.[10]

RESULTS

The R_f-value (Table 1) of one of the isolated compounds was identical to that of the reference PAM iodide chromatographed simultaneously. This isolated material gave a positive color test for an oxime group,[8] and the shifts in the ultraviolet region of the absorption maxima at acid and alkaline levels of pH were identical with those of PAM, as described in the literature.[7]

Paper chromatograms of solutions of PAM iodide in normal human urine, incubated under various conditions, were compared under long-wave ultraviolet light with chromatograms of urine collected after the administration of the drug. All chromatograms were run in the same solvent systems. The blue-fluorescing metabolite of PAM did not appear in chromatograms of the incubated urines; however, it always appeared as a zone of fluorescence (PAM metabolite) on the chromatograms (Table 1) of urines excreted after the administration of PAM.

The metabolite of PAM had lower R_f-values than those of PAM iodide, when run in the four solvent systems mentioned (Table 1). The fluorescent material was soluble in water, less soluble in alcohol, and insoluble in all other organic solvents. It was not extractable by organic solvents from aqueous solutions which had been adjusted to pH 1, 7 or 12. The infrared absorption spectrum showed strong absorption peaks

TABLE 1. RESULTS OF PAPER CHROMATOGRAPHY OF PAM IODIDE (R)*, PAM (U), AND PAM METABOLITE (U) FROM NORMAL HUMAN URINE

Solvent system	PAM i (R)	PAM i (U)	PAM metabolite (U)
Butanol–acetic acid–water (4:1:5)[5], R_f	0·42	0·42	0·17
Butanol–propionic acid–water, [6] R_f	0·60	0·60	0·30
Water-saturated butanol, R_f	0·10	0·10	0·02
80% *iso*Propanol, R_f	0·55	0·55	0·12
Absorption in ultraviolet light	Strong	Strong	—
Fluorescence in ultraviolet light	None	None	Bright blue

* The abbreviations used are: R, the reference compound; U, compound isolated from urine.

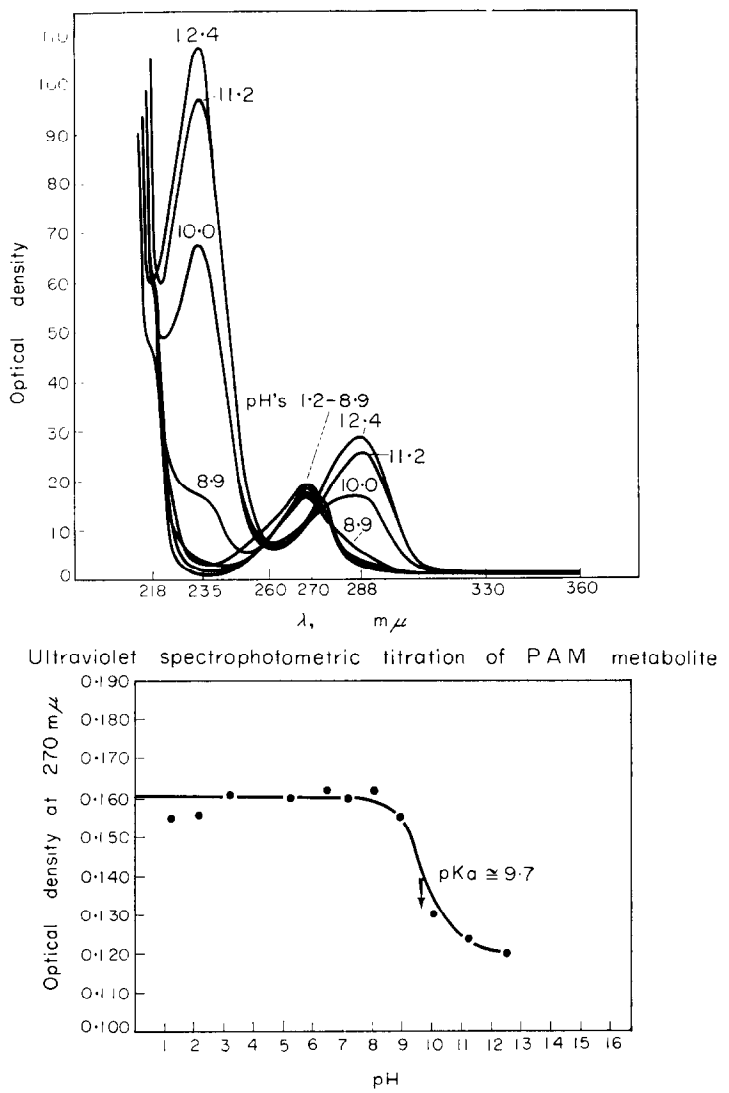


FIG. 1. Absorption spectra of the metabolite of PAM at different levels of pH. pK_a, as determined by optical density measurements at different levels of pH; derived from upper graph.

in the region of 1410, 1562–1588, 1720, 3040, and 3180 cm^{-1}. The band at 1720 cm^{-1} became a shoulder when the material was exposed to NH_3 vapor, but the band reappeared upon re-exposure to HCl vapor.

In the ultraviolet region, the metabolite showed a maximum absorption at 270 mμ in water and in HCl, whereas in alkaline solution the absorption peaks were at 235 and 288 mμ (Fig. 1). The pK_a was 9·7 (Fig. 2), as determined by ultraviolet spectrophotometric analysis at 270 mμ.[9]

Fluroescent activation and emission spectra of the metabolite in aqueous solutions are given in Fig. 3. The emission spectrum reveals a single peak (λ_{max} 330mμ) at either acid or neutral pH-values. Fluorescence is completely eliminated in alkaline solution, but, upon re-acidification of the solution, reappears at 330 mμ.

Spot tests[8, 10] for the following functional groups were negative: free α-amino acid, aldehyde, ketone, oxime, amine, indole, amide, ester, glucuronide, and phenolic hydroxyl.

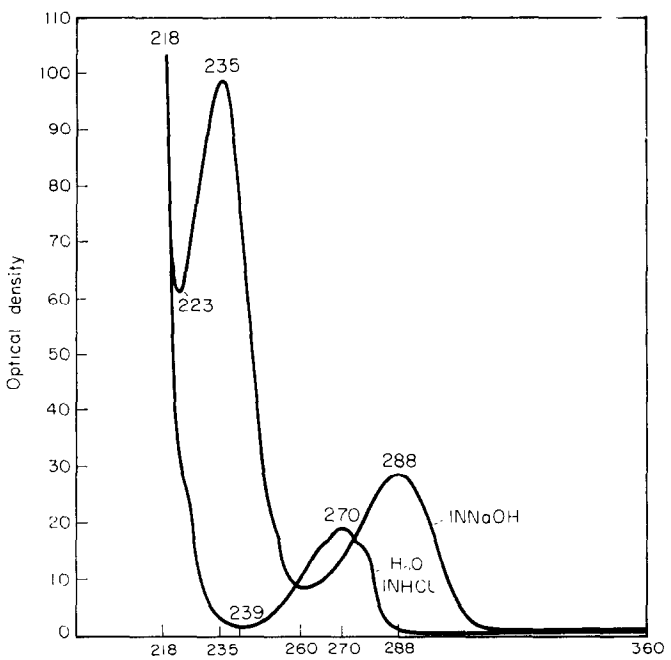


FIG. 2. Ultraviolet spectrum of the metabolite of PAM.

DISCUSSION

The infrared spectrum of the metabolite is indicative of a highly associated system. The bands in the region of 1410 cm^{-1} and 1562–1588 cm^{-1} may be consistent with the presence of a Zwitterion structure. The disappearance and reappearance of the band at 1720 cm^{-1} at alkaline and acidic pH-values, respectively, are characteristic of a carboxylic acid.[11]

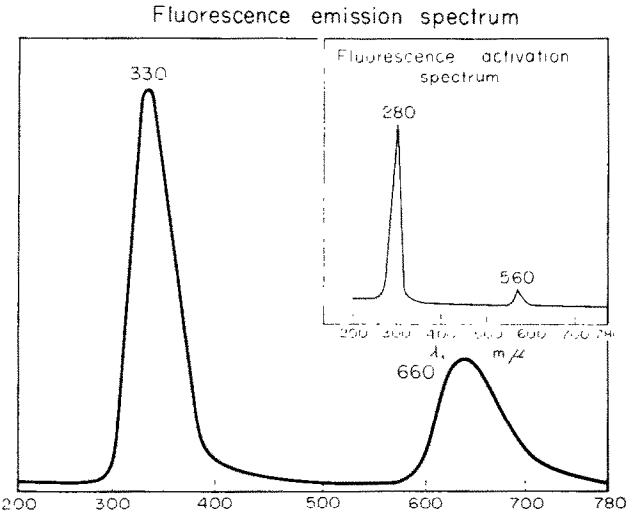
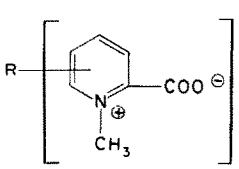


FIG. 3. Fluorescence activation and emission spectra of the metabolite of PAM.

There is a similarity of the metabolite to N-methyl picolinic acid; this compound has an absorption minimum at 240 mμ and a maximum at 272 mμ, at either neutral or acidic levels of pH, which shifts to a maximum of 275 mμ at alkaline pH.[12] The derivative of PAM has almost identical minimum and maximum points at neutral and acidic pH-values. However, in alkali, shifts occur to a new minimum (260 mμ) and maxima (235, 288 mμ). The metabolite, as determined by ultraviolet spectrophotometric titration,[9] has a pK_a value of about 9·7; such a value for pK_a is too high for a compound containing a carboxyl group.[13]

These findings suggest the presence in the molecule of two functional groups: (1) a weakly acidic, ionizable group (based on the ultraviolet data),[9] and (2) a carboxyl group (suggested by the infrared spectrum of the compound). The ultraviolet spectral shifts of the former function obscure the smaller shifts of the latter on changing the pH of the solution from an acidic to a basic level.

From these considerations, a tentative partial structure may be postulated for the metabolite of PAM, for the complete elucidation of which, however, additional study and synthesis will be required.



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REFERENCES

1. H. KEWITZ, I. B. WILSON and D. NACHMANSOHN, *Arch. Biochem. Biophys.* **66**, 271 (1956).
2. B. M. ASKEW, *Brit. J. Pharmacol.* **12**, 340 (1957).
3. N. H. CREASEY and A. L. GREEN, *J. Pharm., Lond.* **11**, 485 (1959).
4. J. L. WAY, H. TONG and R. RABIDEAU, *Fed. Proc.* **19**, No. 1, Pt. 1 (1960).
5. C. E. DALGLIESH, *Biochem. J.* **52**, 3 (1952).
6. E. L. GADSDEN, C. H. EDWARDS and G. A. EDWARDS, *Analyt. Chem.* **32**, 1415 (1960).
7. R. I. ELLIN and A. A. KONDRITZER, *Analyt. Chem.* **31**, 200 (1959).
8. F. FEIGL, *Spot Tests in Organic Analysis.* Elsevier Press (1956).
9. G. OSTER and A. W. POLLISTER, *Physical Techniques in Biological Research*, vol. I. New York (1955).
10. R. J. BLOCK, *A Manual of Paper Chromatography and Paper Electrophoresis.* Academic Press, New York (1955).
11. L. J. BELLAMY, *The Infra-red Spectra of Complex Molecules.* London (1954).
12. E. L. GASTEIGER, P. C. HAAKE and J. A. GERGEN, *Ann. N.Y. Acad. Sci.* **90**, 622 (1960).
13. G. K. BRANCH and M. CALVIN, *The Theory of Organic Chemistry.* Prentice-Hall, New York (1941).