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A FLUOROMETRIC METHOD FOR DETERMINATION OF 1-METHYL-2-ALDOXIMINOPYRIDINIUM ION IN BIOLOGICAL FLUIDS*

STANLEY L. GIBBON† and JAMES L. WAY

Department of Pharmacology, Marquette University School of Medicine, Milwaukee, Wis., U.S.A.

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Abstract—A rapid, sensitive method has been developed to measure 1-methyl-2-aldoximinopyridinium ion (2-PAM) in biological fluids. This method is based on the alkaline hydrolysis of 2-PAM to 1-methyl-2-pyridone and subsequent determination of the fluorescent emission spectrum of this latter compound in chloroform. The fluorometric method is applicable over a wide range of concentrations, and as little as $1{\cdot}0 \times 10^{-6}$ M can be determined by this procedure. It has been employed to measure the disappearance of 2-PAM from the blood of dogs.

STUDIES by this laboratory of the metabolic disposition of the anticholinesterase alkylphosphate inhibitor, 1-methyl-2-aldoximinopyridinium ion (2-PAM), prompted the investigation of a better method to measure this compound in biological fluids. The early method for estimation of 2-PAM by the bound oxime method of Czaky[1] is based on the acid hydrolysis of oximes to hydroxylamine and subsequent oxidation to nitrite, which is determined colorimetrically by a coupling reaction with sulfanilic acid and α-naphthylamine. Although this method has been useful, it is slow, inconvenient, and lacks specificity. Subsequently a more rapid method for 2-PAM was described by Creasy and Green[2] and Way,[3, 4] which is based on the bathochromic shift of the ultraviolet spectrum of 2-PAM in alkaline solution. The molecular basis for the bathochromic shift of 2-PAM in alkaline solution was investigated by Way,[4] and it was attributed to the formation of the 2-PAM zwitterion and its resonance-stabilized form. Although this u.v. spectral analysis is usually adequate for concentrations as low as $1{\cdot}0 \times 10^{-5}$ M 2-PAM, tissue blank interference and clouding become a problem, especially at lower concentrations.

The report herein describes a new, rapid, sensitive method for the determination of 2-PAM in biological fluids. This method is based on the formation of the 1-methyl-2-pyridone,[5] whose fluorescent properties are described by Miranda and Way,[6, 7] which is liberated by the alkaline hydrolysis of 2-PAM at elevated temperatures.[8]

EXPERIMENTAL AND RESULTS

Reagents. The 1-methyl-2-aldoximinopyridinium iodide was obtained from the Aldrich Chemical Co. Recrystallization from ethanol yielded yellow platelets, m.p.

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224–26° (reported m.p. 224–25°[9]). The 1-methyl-2-pyridone was purchased from the Aldrich Chemical Co. The commercial 1-methyl-2-pyridone was purified by fractional distillation prior to conversion and recrystallization as the hydrochloride salt. Chloroform, reagent grade, was obtained from Fisher Scientific Co. The chloroform was washed repeatedly with 1·0 N NaOH and 1·0 N HCl prior to use.

Analytical method. To a 15-ml glass-stoppered centrifuge tube was added a 5-ml sample of plasma and 0·56 ml of 3·0 N NaOH. The mixture was heated in a boiling water bath for 3 hr and then rapidly cooled to room temperature with water. A 5-ml aliquot of chloroform was added with a pipette, and the tube was shaken for 5 min. The mixture was centrifuged to separate the phases, and the fluorescence of the chloroform layer was determined in an Aminco-Bowman spectrophotofluorometer with an excitation wavelength of 315 mμ and an emission wavelength of 370 mμ (Fig. 1).

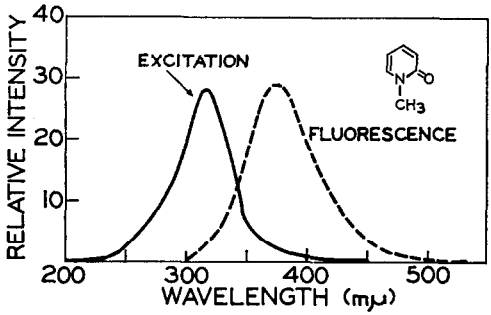

FIG. 1. Excitation and emission spectra of 1-methyl-2-pyridone in chloroform.

Effect of NaOH. The alkaline hydrolysis of 2-PAM was carried out as described under analytical methods in a boiling water bath for 3 hr with the addition of different concentrations of sodium hydroxide. Although maximal formation of 1-methyl-2-pyridone could occur with concentrations of 1×10^{-4} N NaOH (plasma pH, 7·7), the use of 0·3 N NaOH was dictated by theoretical considerations to adjust the final pH value to 13·0.[10] Also, the ease of extraction of 1-methyl-2-pyridone was facilitated when 0·3 N NaOH was employed.

Effect of temperature and time. The reaction proceeded very slowly at room temperature and, as the temperature was increased, the rate of formation of 1-methyl-2-pyridone from 2-PAM in 0·3 N NaOH was concomitantly increased (Fig. 2). The

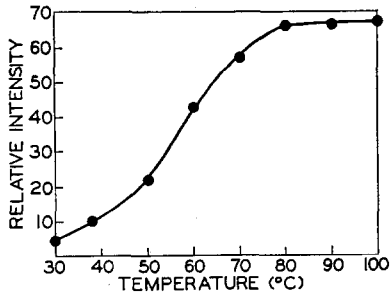

FIG. 2. Effect of temperature on the hydrolysis of 2-PAM.

maximal formation of product was attained at 80°; however, a 100° temperature was selected as the most convenient temperature to carry out the procedure. The effect of time at 100° on the reaction with 0·3 N NaOH is shown in Fig. 3. The maximal formation of product was attained at approximately 2 hr.

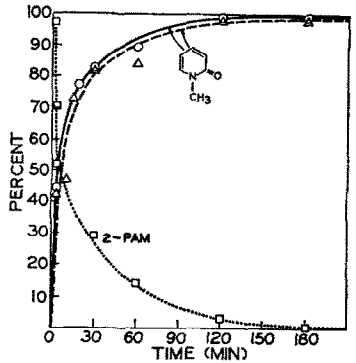

FIG. 3. Per cent disappearance of 2-PAM and appearance of 1-methyl-2-pyridone by alkaline hydrolysis (0·3 N NaOH) as determined by: ultraviolet absorbance of 2-PAM (340 mμ) ··□··; 1-methyl-2-pyridone (304 mμ) —△—; and fluorescent emission (370 mμ) of 1-methyl-2-pyridone —○—.

To establish that the derived product, 1-methyl-2-pyridone, was not degraded under these conditions, it was added to plasma and then subjected to alkaline hydrolysis with 0·3 N NaOH at 100°. Periodic measurement up to 4 hr by the spectrophotofluorometric method indicated that no degradation of 1-methyl-2-pyridone was observed.

*Sensitivity.*This spectrophotofluorometric method was approximately ten times more sensitive than the present u.v. spectral methods. A linear relationship between fluorescent intensity and concentration was obtainable with concentrations of 2-PAM ranging from $1{\cdot}0 \times 10^{-4}$ to $1{\cdot}0 \times 10^{-6}$ M 2-PAM (Fig. 4).

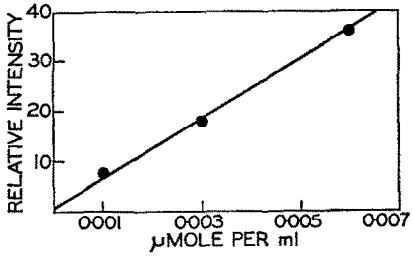

FIG. 4. Sensitivity of the fluorometric determination of 2-PAM.

Comparison of the fluorometric procedure with ultraviolet methods. The amount of 2-PAM remaining during the alkaline hydrolysis at different time intervals was measured by its absorbance at 340 mμ caused by the bathochromic displacement of 2-PAM in alkali as described by Way.[4] The amount of formation of 1-methyl-2-pyridone from the alkaline hydrolysis of 2-PAM was determined not only by the

absorbance of 1-methyl-2-pyridone in the chloroform extract at 304 mμ but also by its fluorescent emission spectrum. It is readily observed in Fig. 3 that both the measurement of the disappearance of 2-PAM by u.v. absorption and the appearance of 1-methyl-2-pyridone by fluorescent emission and by u.v. absorption gave compatible results.

Determination of the plasma level of 2-PAM *in the dog.* Dogs received 2-PAM at a dose of 30·0 mg/kg i.v., and blood was removed at various time intervals. The blood was centrifuged to obtain the plasma, which was then determined for 2-PAM content by both the highly specific u.v. absorption method and by the spectrophotofluorometric method, as shown in Fig. 5. Measurements of the plasma level of 2-PAM by

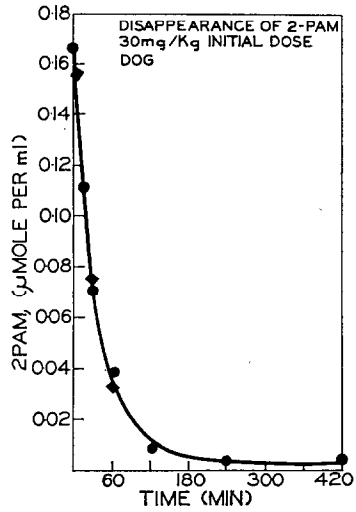

FIG. 5. Plasma level of 2-PAM in a dog receiving 30 mg 2-PAM/kg i.v. by the ultraviolet absorption (■) and the fluorescent (●) methods.

both the u.v. absorption and fluorescent method gave comparable results. It should be pointed out that the relatively low sensitivty of the u.v. method limited its application to determining 2-PAM at concentrations above 0·01 μmole/ml. These results indicate that 2-PAM rapidly disappears from the blood, with a half-life of approximately 0·75 hr.

DISCUSSION

A convenient method to measure 2-PAM has been developed, which is based on the conversion of this anticholinesterase alkylphosphate antagonist to a relatively less polar compound, 1-methyl-2-pyridone, which can then be extracted from biological fluids with organic solvents. The reaction presumably occurs via the formation of 1-methyl-2-cyanopyridinium ion and 1-methyl-2-pyridone cyanohydrin.[8, 10, 11] The ability to extract 1-methyl-2-pyridone from biological fluids with chloroform permits a quantiative determination of this antagonist in biological fluids in concentrations

previously not measurable. The present spectrophotofluorometric method is approximately ten times more sensitive than the u.v. measurement at 340 mμ, which is based on the bathochromic displacement of this antagonist.

It should be noted that the bathochromic shift method[4] is highly specific for 2-PAM, as the absorbance at 340 mμ is dependent upon the formation of the 2-PAM zwitterion and its resonance-stabilized form. This would mean that any change in the auxochromic oxime group would alter the absorbance at 340 mμ. Therefore, the close agreement between the plasma levels of 2-PAM by the u.v. absorption method and the spectrophotofluorometric method (Fig. 5) strongly suggests that the latter is a valid measurement of 2-PAM.

It is of interest to note that 90 to 95 per cent of the alkaline hydrolytic product of 2-PAM can be recovered as 1-methyl-2-pyridone by continuous chloroform extractions. By the present procedures, approximately 80 per cent of the product is recovered, but the recovery is consistent. The alkaline hydrolysis of 2-PAM to 1-methyl-2-pyridone via the intermediate, 1-methyl-2-cyanopyridinium ion, is consistent with the chemistry of oximes[12–15] and pyridinium ions.[5] Although 1-methyl-2-cyanopyridinium ion will readily form 1-methyl-2-pyridone and 1-methyl-2-carbamidopyridinium ion in alkaline solution under very mild conditions, a very high pH and/or elevated temperatures are necessary to convert 2-PAM preferentially to 1-methyl-2-pyridone. This would suggest that the elevated temperatures and alkaline conditions are necessary not only for the first reaction—that is, the conversion of 2-PAM to the 2-cyanopyridinium ion—but also for the second reaction with a preferential formation by the 2-cyanopyridinium ion to 1-methyl-2-pyridone rather than 1-methyl-2-carbamidopyridinium ion. The elevated temperatures and the alkaline conditions to convert 2-PAM to 1-methyl-2-pyridone would be consistent with the chemistry of this antagonist.

A rapid, sensitive, and versatile method has been developed to measure 2-PAM. The method has been found to be applicable to studies *in vivo* of the physiological disposition of 2-PAM in dogs. The present procedures measure not only 2-PAM but also its metabolic products; however, under normal circumstances the metabolites of 2-PAM are present in only negligible amounts. By carefully controlling the alkaline hydrolysis, not only 2-PAM but also each of its established metabolites probably can be measured separately. Details of this latter procedure will be published at a later date.

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REFERENCES

1. T. Z. Czaky, *Acta chem. scand.* **2**, 450 (1948).
2. N. H. Creasy and A. L. Green, *J. Pharm. Pharmac.* **11**, 485 (1959).
3. J. L. Way, H. Tong and R. Rabideau, *Fedn Proc.* **19**, 276 (1960).
4. J. L. Way, *J. Pharmac. exp. Ther.* **138**, 258 (1962).
5. H. L. Bradlow and C. A. Vanderwerf, *J. org. Chem.* **16**, 1143 (1951).
6. P. M. S. Miranda and J. L. Way, *Pharmacologist* **5**, 241 (1963).
7. P. M. S. Miranda and J. L. Way, *Molec. Pharmac.* **2**, 117 (1966).
8. R. I. Ellin, *J. Am. chem. Soc.* **80**, 6588 (1958).
9. S. Ginsberg and I. Wilson, *J. Am. chem. Soc.* **79**, 481 (1957).
10. E. M. Kosower, *Molecular Biochemistry*, p. 179. McGraw-Hill, New York (1962).

1. J. W. PATTON, Doctoral Dissertation, University of Wisconsin, Madison (1961).
12. A. HANTZSCH, *Ber. dt. chem. Ges.* **24**, 37 (1891).
13. A. HANTZSCH and A. LUCAS, *Ber. dt. chem. Ges.* **28**, 744 (1895).
14. C. R. HAUSER and E. JORDAN, *J. Am. chem. Soc.* **57**, 2450 (1935).
15. E. JORDAN and C. R. HAUSER, *J. Am. chem. Soc.* **58**, 1304 (1936).