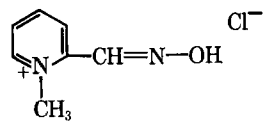
Technical Requirements and Identification of Pralidoxime Chloride and Its Determination in Biological Material

By JON R. MAY, PETER ZVIRBLIS, and ALBERT A. KONDRITZER

Pralidoxime chloride, commonly known as 2-PAM chloride, has been classified as standard type by the Medical Services, Department of Defense, for use in the therapy of nerve agent casualties. Chemical and physical data, ultraviolet and infrared absorption spectra, identification tests, and acute toxicity data of highly purified pralidoxime chloride are presented; analytical procedures for the quantitative determination of the oxime in blood plasma, feces, and urine are outlined. A safety test, developed for the item to insure the absence of toxic impurities, is given.

CHEMICAL agents that are organic esters of phosphoric acid derivatives are toxic because they inhibit cholinesterases throughout the animal body. The search for satisfactory antidotes to these agents has led to a number of oxime derivatives that have the ability to restore the activity of the enzymes so inhibited. Pralidoxime chloride, chemically described as 1-methyl-2-pyridinium aldoxime chloride, $C_7H_9N_2OCl$, mol. wt. 172.62, and commonly referred to as 2-PAM chloride, has been suggested as the reactivator of choice because of its physiologic compatibility, high water solubility, and favorable ratio of oximate moiety to molecular weight (1). Its structural formula may be represented as:



The importance of pralidoxime chloride as an adjunct to atropine in the field therapy of nerve agent casualties led to the recommendation by the Defense Medical Materiel Board that this oxime be classified as standard type for inclusion in the Federal Catalog, Department of Defense Section, Medical Materiel. The item[1] that has been type classified consists of 5 Gm. of the oxime as a powder in a 20-ml. bottle with a rubber diaphragm closure. A solution of 2-PAM chloride for parenteral use by medical personnel is made by the addition to the bottle of 13 ml. of sterile diluent, *e.g.*, U.S.P. sterile water for injection, sodium chloride injection.

Highly purified samples of 2-PAM chloride were prepared; the physical and chemical constants and the ultraviolet and infrared spectra of these samples were determined. The *Experimental* of this report contains information generated during this effort. *Determination of Pralidoxime Chloride in Biological Material* is a description of the methods we used for determining pralidoxime chloride in biological materials. This is included because the authors have received numerous requests for these procedures.

EXPERIMENTAL

Preparation of Pralidoxime Chloride.—Two lots of purified 2-PAM chloride were used to establish the standards that follow. One lot, designated M-1/161, was prepared by recrystallization of 2-PAM chloride obtained commercially; the other lot, M-2/59, was prepared by conversion of 2-PAM methane sulfonate, commonly referred to as P-2-S, to the chloride salt.

To free the commercial pralidoxime chloride of colored impurities, 200 Gm. was ground to a fine powder and shaken with ice-cold absolute ethanol until the alcoholic filtrate was pale yellowish-green; residual alcohol was removed by an acetone wash. The air-dried oxime salt was dissolved in boiling 75% isopropanol containing 5 ml. of 0.1 *N* hydrochloric acid. The hot solution was filtered, cooled to room temperature, and then stored at −5°. The copious white precipitate was collected on a sintered-glass filter, washed with cold isopropanol and acetone, and air-dried. The material was ground to a fine powder to release any solvent

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[1] Federal Stock No. 6505-082-2456 pralidoxime chloride, 5 Gm.

trapped in the crystal structure and placed in a hot-air oven at 65° for 3 hr., after which it was stored over phosphorus pentoxide in a vacuum desiccator.

The preparation of 2-PAM chloride by the conversion of P-2-S was based on a procedure for the synthesis of pralidoxime chloride (2). The P-2-S, after treatment to remove colored impurities, was dissolved in a twofold excess of concentrated hydrochloric acid; 10 vol. of isopropanol was slowly added with stirring to precipitate the chloride salt, which was washed on the filter with successive portions of cold 90% isopropanol until acid-free. The product was recrystallized by the procedure already described.

Anal.—Calcd. for C, 48.7; H, 5.3; Cl, 20.5; N, 16.2; O, 9.3. Found for lot M-1/161: C, 48.6; H, 5.3; Cl, 20.5; N, 16.2; O, 9.5. For lot M-2/59: C, 49.0; H, 5.2; Cl, 20.6; N, 16.2; O, 9.3.

Melting points, when determined in accordance with the U.S.P. method for substances of class I, were 220–222° dec. (Thomas-Hoover capillary melting point apparatus).

Physical Properties.—Pralidoxime chloride is a white, odorless or nearly odorless, crystalline powder. One gram dissolves readily in 2 ml. of water. At 25°, 100 ml. of a saturated aqueous solution contains approximately 65 Gm. of 2-PAM Cl; at 0°, the amount is approximately 54 Gm. The solubility in absolute ethanol is approximately 1 Gm./100 ml. at 25° and 0.5 Gm. at 0°. The pH values of 1, 10, and 50% aqueous solutions of 2-PAM chloride at approximately 25° are 4.5–4.7, 4.1–4.3, and 3.6–4.0, respectively.

Neutralization Equivalent.—The neutralization equivalent of pralidoxime chloride shall not be less than 167 and not more than 178 when determined by the following nonaqueous titration.

Dissolve accurately weighed 50–100-mg. samples of pralidoxime chloride in 40 ml. of glacial acetic acid (reagent grade) with slight warming. Add 15 ml. of a 5% solution of mercuric acetate in glacial acetic acid and 3 drops of a 1% solution of crystal violet indicator in glacial acetic acid. Titrate with 0.1 *N* perchloric acid solution (prepared and standardized in accordance with U.S.P. XVI, p. 1080) to the emerald-green end point. Titrate a reagent blank solution to the end point color and subtract the blank value from the sample value.

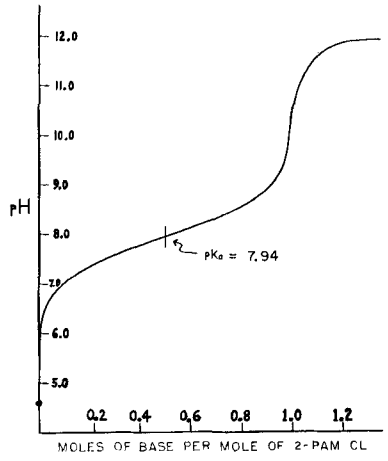

Fig. 1.—Titration curve of neutralization of pralidoxime chloride, lot M-2/59.

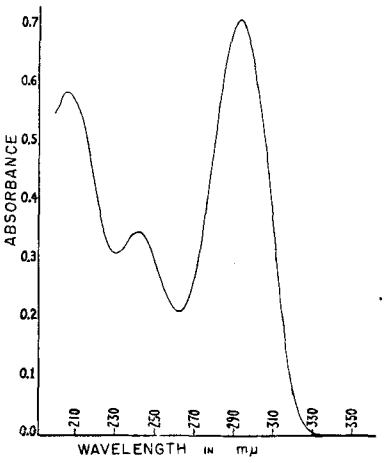

Fig. 2.—Ultraviolet absorption spectrum of pralidoxime chloride, lot M-1/161, 10.0 mcg./ml. in 0.1 *N* hydrochloric acid.

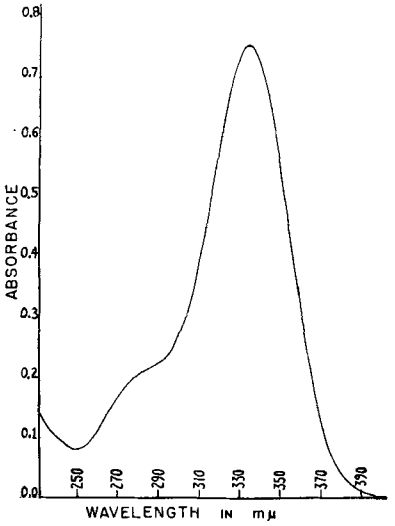

Fig. 3.—Ultraviolet absorption spectrum of pralidoxime chloride, lot M-1/161, 7.0 mcg./ml. in 0.1 *N* sodium hydroxide.

Titration Curve.—The titration curve for the neutralization of pralidoxime chloride, given in Fig. 1, was obtained by the following procedure.

An accurately weighed sample of the oxime salt, approximately 200 mg., was dissolved in 10 ml. of carbon dioxide-free, triple-distilled water. An electrode probe assembly (Beckman No. 39176) was immersed in the solution; the surface of the solution was flushed continuously with nitrogen gas. The solution was titrated with 1.0 *N* sodium hydroxide solution from a microburet (Aminco-Koegel Menisco-Matic Buret), the tip of which was kept beneath the surface of the solution. The hydrogen-ion activities were measured with a Beckman model GS pH meter. Approximately 1.5 ml. of base was added during the titration.

An average molecular weight of 172.4 ± 1.5 and a pKa of 7.94 ± 0.02 were calculated for lot M-2/59 pralidoxime chloride from five determinations.

Identification.—*A.*—To 1 ml. of a 20% aqueous solution of pralidoxime chloride add 1 ml. of a 20% aqueous solution of potassium iodide. A yellow crystalline precipitate of pralidoxime iodide will appear almost immediately.

B.—To 0.1 ml. of a 20% aqueous solution of pralidoxime chloride add 1 ml. of a 0.6% aqueous ferric chloride solution. The color will change from pale yellow to amber brown, indicating the presence of the oxime function.

C.—To 0.5 ml. of 2 *N* sodium hydroxide solution add 1 ml. of a 20% aqueous solution of pralidoxime chloride. The color will change to a bright yellow. This color change can be reversed by acidification with hydrochloric acid.

D.—To 1 ml. of a 20% aqueous solution of pralidoxime chloride add 1 ml. of a 10% silver nitrate solution. A white curdy precipitate of silver chloride is formed.

E.—The ultraviolet absorption spectra of acid and alkaline solutions of 2-PAM chloride, determined with a Cary 14 spectrophotometer, are given in Figs. 2 and 3. Typical ultraviolet spectrophotometric constants of the principal wavelengths of maximum and minimum absorption are listed in Table I.

F.—The infrared absorption spectra of pralidoxime chloride in a potassium bromide disk and in a Nujol mull are given in Fig. 5. The infrared

TABLE I.—ULTRAVIOLET SPECTROPHOTOMETRIC CONSTANTS[a]

Soln.	λ Max., mμ	λ Min., mμ	*M* Absorptivity (ε)
0.1 *N* HCl	293	...	1.25×10^4
	...	262	3.68×10^3
0.1 *N* NaOH	336	...	1.84×10^4
	...	246	1.65×10^3

[a] Isosbestic point: ultraviolet absorption curves of 2-PAM chloride at various hydrogen-ion activities (Fig. 4) indicate that the isosbestic point of the oxime is at 307 mμ.

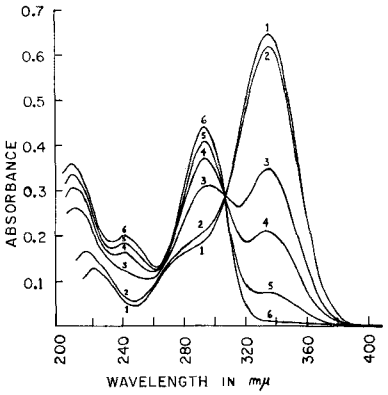

Fig. 4.—Ultraviolet absorption spectra of pralidoxime chloride at various hydrogen-ion activities. Key: 1, pH 10.0; 2, pH 9.1; 3, pH 8.0; 4, pH 7.6; 5, pH 7.0; 6, pH 6.0.

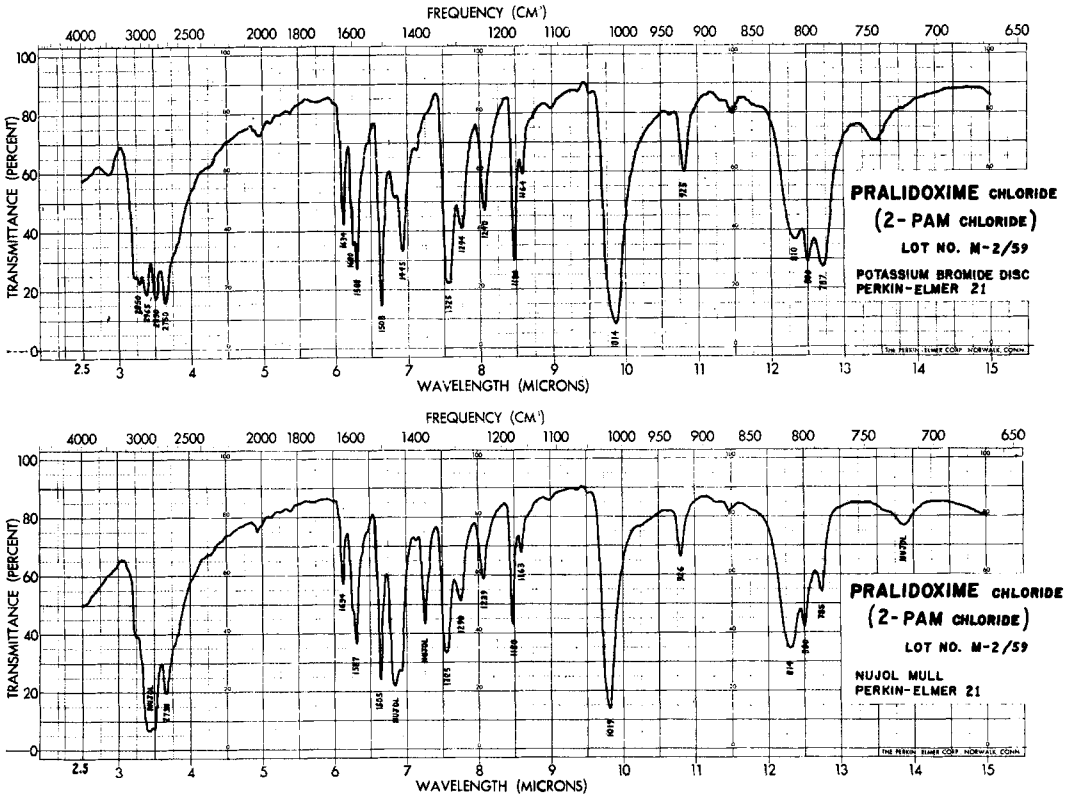

Fig. 5.—Infrared absorption spectrum of pralidoxime chloride in potassium bromide disk (0.4%). (Top.) Infrared absorption spectrum of pralidoxime chloride in Nujol mull (25%). (Bottom.)

spectrum of the disk should be obtained within 1 hr. after its preparation.

Safety Test

Five vials are selected at random from each lot of the commercially prepared item. The contents of each vial are diluted to 100 ml. in separate volumetric flasks with either sterile water for injection U.S.P. or sodium chloride injection U.S.P. For each of the diluted solutions, the following procedure is carried out.

Exactly 2.0 ml. of the solution is pipeted into a 10-ml. volumetric flask and diluted to volume with sodium chloride injection U.S.P. An amount of 100 mg./Kg. of the pralidoxime chloride is injected intravenously in two equally divided doses within a period of 1 hr. as follows: 0.1 ml., equivalent to 50 mg./Kg., is injected intravenously into the tail vein of each of ten mice of approximately 20 Gm. weight at a uniform rate during a period not exceeding 5 sec. Sixty minutes after the injection, each animal is injected again in the same manner and with the same volume of solution as described. If no deaths occur during a 24–48-hr. observation period, the sample meets the safety standard. If only one death occurs, the test is repeated on ten additional animals; if no deaths occur in the second group, the sample meets the safety standard. If more than one death occurs in the first group or if one or more deaths occur in the second group of mice, the sample is provisionally rejected. It is retested by determination of its LD_{50} along with that of a suitable standard sample of pralidoxime chloride, as given under *Toxicity*.

Acute Toxicity Test (LD_{50})

The systemic toxicity of commercially manufactured lots of pralidoxime chloride powder shall not differ by more than ±10% from that of a standard pralidoxime chloride sample, as determined by measuring its potency relative to that of the standard. The relative potency shall be determined by intravenous injections into young adult rats or mice of the sample and the standard pralidoxime chloride. A minimum of four logarithmically spaced dosages shall be used for the sample and four for the standard pralidoxime chloride, with a minimum of ten animals for each dosage.

A partial mortality response must be obtained for at least 3 of the points. The relative potency factor, R, relating the lethality of the standard to that of the sample pralidoxime chloride shall be calculated by the bioassay method of either Bliss (3) or Finney (4) and shall lie between 0.9–1.11 ($p = 0.05$).

Examples of LD_{50} values reported for 2-PAM Cl when administered to the mouse by various routes and to the rabbit intravenously are given in Table II.

TABLE II.—TOXICITY OF PRALIDOXIME CHLORIDE

Species	LD_{50}, mg./Kg. i.v.	i.p.	i.m.	Oral
Mouse	115 (5)	143 (1)	...	...
Mouse	...	205 (6)	...	4100 (6)
Mouse	90 (7)	155 (7)	180 (7)	...
Mouse	74 (8)	...	...	...
Mouse	86 (8)	...	...	...
Rabbit	95 (8)	...	...	...

DETERMINATION OF PRALIDOXIME CHLORIDE IN BIOLOGICAL MATERIAL

Concentrations of oxime in plasma, urine, and feces are estimated by ultraviolet spectrophotometry based on procedures previously described (9).

Plasma.—Whole blood is withdrawn by venipuncture, heparinized, centrifuged, and the plasma separated. To 2 ml. of plasma in a centrifuge tube, 4 ml. of water and 1 ml. of 0.26 M barium hydroxide are added, and the contents are mixed by shaking. After 2–3 min., 1.0 ml. of 0.30 M zinc sulfate is added, the tube is stoppered, and the contents are shaken thoroughly. The precipitate is centrifuged down and the supernatant solution is filtered through a pledget of glass wool. To the filtrate, which is approximately 5 ml. in volume, 0.2 ml. of 20% sodium hydroxide is added, and the absorbance is determined at 336 mμ against a distilled water blank. The absorbance due to the plasma is determined on the control sample, *i.e.*, the zero time sample, and is subtracted from the experimental values. Concentrations of oxime in the plasma samples are calculated by comparison with absorbance values obtained with standard solutions, 5 ml. of which are treated with 0.2 ml. of 20% sodium hydroxide.

Urine.—Exploratory studies showed that alkalized, protein-free extracts of urine from apparently normal individuals absorbed markedly at 336 mμ. The amount of this absorbing material varied from person to person as well as from sample to sample from the same individual. This wide variation in absorbance of the control urine samples made it impractical to subtract the absorbance of the control sample or even an average absorbance of many control samples from many individuals from the values of the experimental urine samples. A study of the U.V. spectra of the protein-free extracts of individual samples of urine from 28 apparently normal untreated individuals showed that the ratio of the absorbance in alkaline solution to that in acid solution at 336 mμ gave a constant value of 1.76 ± 0.19, with a standard error of the mean of 0.036.

The pralidoxime chloride content of urine is, accordingly, determined as follows. A suitable volume, usually 1 ml. or less, is diluted with water to 6 ml., and a protein-free filtrate prepared as described for plasma. To one portion of the filtrate 0.2 ml. of 5 N hydrochloric acid is added, and 0.2 ml. of 5 N sodium hydroxide is added to an equal portion of the filtrate. The absorbance at 336 mμ of the acidified filtrate is multiplied by 1.76 and the product subtracted as a blank from the absorbance at 336 mμ of the alkalized filtrate. Salicylate ion has been found to interfere markedly with the procedure; therefore, care must be taken to insure that the subject does not take aspirin or any other medication containing salicylate prior to the experiment.

Feces.—The entire sample of feces is mixed in a Waring blender for 3 min. with 3 to 4 times the sample weight of water to make a thin suspension. More or less water may be used as desired. An aliquot of this suspension is centrifuged, and portions of the supernatant, usually 0.1 ml., are used for the analysis by the method described under *Urine*. On seven samples of control feces, the

ratio between the absorbance at 336 mμ in alkaline solution to that found in acid solution averaged 1.8.

SUMMARY

Pralidoxime chloride, commonly known as 2-PAM chloride, has been recommended for classification as standard type by the Medical Services, Department of Defense, for use in the therapy of nerve agent casualties. The item to be cataloged and issued consists of 5 Gm. of the oxime salt in a 20-ml. bottle with a rubber diaphragm closure; the addition of 13 ml. of sterile diluent gives a solution for parenteral use by medical personnel. The preparation of highly purified pralidoxime chloride, physical and chemical constants, the ultraviolet and infrared absorption spectra, tests for identification, and analytical procedures for the quantitative determination of the oxime in blood plasma, feces, and urine are given. A safety test, developed for the standard item to insure the absence of toxic impurities, and a procedure for determining the acute toxicity of pralidoxime chloride are also presented.

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Analysis of Acetophenetidin in Dosage Forms by Nonaqueous Titration

By SONG-LING LIN and MARTIN I. BLAKE

A simple nonaqueous titrimetric procedure is presented for the determination of acetophenetidin in powder, tablet, and capsule dosage forms. Titration is effected with perchloric acid in acetic acid–acetic anhydride using an acetic anhydride–chloroform–benzene mixture as the titration solvent. The end point is determined potentiometrically with a Fisher titrimeter equipped with a modified calomel–glass electrode system.

PHENACETIN tablets U.S.P. XVII (formerly acetophenetidin tablets U.S.P. XVI) are assayed (1) by a gravimetric procedure involving extraction with chloroform. The "British Pharmaceutical Codex" procedure (2) is similar to the U.S.P. method, while the "British Pharmacopoeia," 1953 (3), utilized ethanol as the extracting solvent. The method of the Association of Official Agricultural Chemists (4) involves iodination and gravimetric determination of the periodide or volumetric measurement of standard iodine solution consumed in the iodination. A variety of procedures have been proposed which are based on volumetric titration, colorimetry, and ultraviolet or infrared spectrophotometry. These have been applied to acetophenetidin and combinations of acetophenetidin with other drugs, including aspirin, caffeine, acetanilid, and aminopyrine. Connors (5) has reviewed the literature on this subject.

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The authors acknowledge the following companies for supplying dosage forms containing acetophenetidin: Burroughs Wellcome and Co., Ives Laboratories, Inc., Eli Lilly and Co., A. H. Robbins Co., Inc., and Smith Kline & French Laboratories.

Because of the weakly basic nature of acetophenetidin, application of nonaqueous titrimetry has been limited. Wollish *et al.* (6) titrated acetophenetidin after hydrolysis with hydrochloric acid to liberate the free amine, *p*-phenetidin, which was extracted with chloroform after making the solution alkaline. Titration was effected potentiometrically with perchloric acid in *p*-dioxane. A glass–calomel electrode system was employed.

The present study reports a simple nonaqueous titrimetric procedure by which acetophenetidin may be determined without preliminary treatment. The method is applied to dosage forms and combinations with other active constituents.

EXPERIMENTAL

Apparatus.—A Fisher titrimeter, model 35, was used for all titrations in this study. A conventional glass electrode and sleeve-type calomel electrode were equilibrated by immersing in acetic anhydride for 24 hr. before use. The calomel electrode was modified by replacing the aqueous bridge in the calomel cell with a 0.1 *M* solution of anhydrous lithium perchlorate in acetic anhydride as the supporting electrolyte as described by Wimer (7).