been reported previously (24). The methods seem appropriate for estimates of transdermal absorption if a specific analytical procedure is not available and percutaneous first-pass metabolism is not extensive.

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N-Acetyl-D-Mannosamine Analogues as Potential Inhibitors of Sialic Acid Biosynthesis

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Abstract □ The 1,3,6-tri-*O*-acetyl and 1,3,6-tri-*O*-acetyl-4-*O*-mesyl analogues of *N*-acetyl-D-mannosamine and the corresponding *N*-trifluoroacetyl derivative have been synthesized, and their effects on the proliferation of Friend erythroleukemia cells in culture have been evaluated. The acetamido series showed a dependency on the 4-substituent for optimum cytotoxicity while the trifluoroacetamido series did not. Thus, the 1,3,4,6-tetra-*O*-acetyl and 1,3,6-tri-*O*-acetyl-4-*O*-mesyl analogues of *N*-acetyl-D-mannosamine were 10-fold and 42-fold more active, respectively, than 2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-mannopyranose as inhibitors of cellular replication. The corresponding trifluoroacetamido analogues were essentially equiactive and had a potency equivalent to that of the 4-*O*-mesyl derivative in the acetamido series.

Keyphrases □ *N*-Acetyl-D-mannosamine—synthesis of acetamido and trifluoroacetamido analogues, effects on the proliferation of Friend leukemia cells □ Analogues—of *N*-acetyl-D-mannosamine, acetamido and trifluoroacetamido series, synthesis, effects on the proliferation of Friend leukemia cells □ Antileukemic agents—potential, acetamido and trifluoroacetamido analogues of *N*-acetyl-D-mannosamine, tested against Friend leukemia cells

2 - Acetamido - 1,3,4,6 - tetra - *O* - acetyl - 2 - deoxy - β-D-mannopyranose (I), the peracetylated analogue of *N*-acetyl-D-mannosamine, a metabolic precursor in the biosynthetic pathway for sialic acid (1) (Fig. 1), and the corresponding trifluoroacetamido analogue (V) were recently reported to be inhibitors of the growth of Friend erythroleukemia cells in culture (2). Both analogues were equipotent inhibitors of the incorporation of [^{3}H]*N*-acetyl-D-mannosamine into the glycoprotein-bound sialic acid of Friend erythroleukemia cells (2); however, different enzymatic sites appeared to be involved. Compound I caused an accumulation of radioactivity from [^{3}H]*N*-acetyl-D-mannosamine in *N*-acetylneuraminic acid and a decrease in cytidine monophosphate-*N*-acetylneuraminic acid in the ethanol-soluble metabolites of cells, while V caused an accumulation of [^{3}H]cytidine monophosphate-*N*-acetylneuraminic acid. In addition, both I and V produced an increase in the amount of neuraminidase-hydrolyzable sialic acid-like material on the surface of treated cells, presumably as a result of their metabolic utilization and incorporation into cellular macromolecules (3).

Since intracellular deacetylation must precede metabolism of these analogues along the sialic acid biosynthetic pathway, we presumed that I would ultimately give the noncytotoxic metabolite, *N*-acetyl-D-mannosamine; it was therefore surprising to find that I was relatively active as an inhibitor of cellular replication. Consideration of the hydroxyl groups which are required for the conversion of *N*-acetyl-D-mannosamine to *N*-acetylneuraminic acid-9-phosphate (1) (Fig. 1), reveals that such metabolic conversion only necessitates the removal of acetyl groups from the 1, 3, and 6 positions of I, thereby suggesting that the 4-*O*-acetyl group remains intact and is responsible for the

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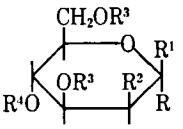
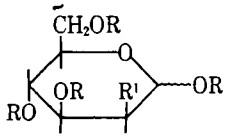


I: R = H, R^1 = $OCOCH_3$, R^2 = $NHCOCH_3$, R^3 = R^4 = $COCH_3$
II: R = $OCOCH_3$, R^1 = H, R^2 = $NHCOCH_3$, R^3 = $COCH_3$, R^4 = H
III: R = $OCOCH_3$, R^1 = H, R^2 = $NHCOCH_3$, R^3 = $COCH_3$, R^4 = SO_2CH_3
VI: R = $OCOCH_3$, R^1 = H, R^2 = CF_3CONH, R^3 = $COCH_3$, R^4 = H
VII: R = $OCOCH_3$, R^1 = H, R^2 = CF_3CONH, R^3 = $COCH_3$, R^4 = SO_2CH_3



IV: R = H, R^1 = CF_3CONH
V: R = $COCH_3$, R^1 = CF_3CONH

biological and biochemical effects of this agent in erythroleukemia cells. In an effort to gain evidence to support these conclusions, the preparation and evaluation of the cytotoxicity in Friend erythroleukemia cells of 2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-mannopyranose (II), 1,3,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-mannopyranose (VI), and their corresponding 4-*O*-mesylates (III and VII) was undertaken.

BACKGROUND

The carbohydrates present on mammalian cell surfaces exist as components of glycolipid, glycoprotein, and glycosaminoglycan macromolecules, which collectively participate in functional properties such as adhesion, cellular interactions, and immunological phenomena (4). In recent years, studies directed at the surface properties of neoplastic cells have highlighted many structural and functional differences between tumor cells and their normal counterparts (5). Although no universal property characteristic of malignancy has resulted from such studies, they have stimulated drug design targeted at the neoplastic cell membrane (6). Of the various cell-surface components, sialic acid (7) is of special interest, since it appears to be involved in fundamental properties of neoplastic cells such as the capacity to metastasize (8–10).

Elevated levels of sialyltransferase activity (11, 12) and cell surface-bound sialic acid (13, 14) have been reported in some transformed cell lines in culture. Although such changes have not been observed uniformly, elevated levels of sialic acid and sialyltransferase activity have been reported in the serum of animals (15–17) and humans (16, 18–20) bearing malignant neoplasms, and a variety of human lung carcinomas also have been shown to contain higher levels of protein-bound sialic acid than corresponding normal tissue (21). Since drugs that are capable of altering the synthesis, and thereby the content, of cell surface sialic acid-containing macromolecules may have utility as therapeutic agents, analogues of *N*-acetyl-D-mannosamine (a biosynthetic precursor of sialic acid) have been prepared.

EXPERIMENTAL[1]

Methods—All evaporations were performed under reduced pressure, and melting points[2] were uncorrected. Column chromatography[3] and TLC[4] were performed on silica gel, the latter being developed by heating after the application of an ethanol–5% sulfuric acid spray. ^{1}H-NMR spectra[5] were run in deuterochloroform. Petroleum ether refers to a fraction having a bp of 35–60°.

2-Acetamido-1,3,6-tri-O-acetyl-2-deoxy-α-D-mannopyranose (II)—Aliquots of acetyl chloride were added to a solution of *N*-acetyl-β-D-mannosamine·H_2O (1 g, 4.5 mmoles) in pyridine (15 ml) at room temperature. The reaction was monitored by TLC (chloroform–methanol, 9:1, v/v) until optimal conversion to an anomeric mixture of tri-*O*-acetyl derivatives was obtained. Toluene was added and the mixture was evaporated under reduced pressure. The residue was chromatographed on silica gel (chloroform–methanol, 97.5:2.5) to give a mixture of tri-*O*-acetyl derivatives (0.51 g, 33%) which showed as three major spots on TLC. This material was crystallized from acetone–petroleum ether to give II (0.19 g, 12%), mp 216–217° (dec), $[\alpha]_D^{23}$ − 63° (*c* 1, methanol); ^{1}H-NMR[6]: δ 5.84 (d, 1, $J_{1,2}$ 1.5 Hz, H-1), 5.76 (d, 1, $J_{2,NH}$ 9.1 Hz, NH), 4.90 (dd, 1, $J_{2,3}$ 4.0, $J_{3,4}$ 9.5 Hz, H-3), 4.73 (m, 1, H-2), 4.52 (dd, 1, $J_{5,6}$ 4.1, $J_{6,6'}$ −12.2 Hz, H-6), 4.30 (dd, 1, $J_{5,6'}$ 1.6 Hz, H-6'), 3.76–3.63 (m, 2, H-4, H-5), 2.80 (d, 1, $J_{4,OH}$ 3.7 Hz, OH), 2.14 (3, $NHCOCH_3$), 2.10, 2.08, 2.07 (9H, $OCOCH_3$).

Anal.—Calc. for $C_{14}H_{21}NO_9$: C, 48.41; H, 6.05; N, 4.03. Found: C, 48.29; H, 6.22; N, 4.25.

2-Acetamido-1,3,6-tri-O-acetyl-2-deoxy-4-O-mesyl-α-D-mannopyranose (III)—Methanesulfonyl chloride (0.05 g, 0.44 mmole) was added to a solution of II (0.1 g, 0.29 mmole) in pyridine (5 ml) cooled to −10°. The mixture was allowed to stand at room temperature overnight, after which time TLC (chloroform–methanol, 9:1, v/v) indicated a new product. Toluene was added and the mixture was then evaporated under reduced pressure. The residue was chromatographed on silica gel to afford the 4-mesylate III, (chloroform–methanol, 49:1) as a syrup (0.1 g, 82%), $[\alpha]_D^{23}$ − 18.9° (c 1, chloroform); ^{1}H-NMR[6]: δ 5.86 (d, 1, $J_{1,2}$ 1.8 Hz, H-1), 5.81 (d, 1, $J_{2,NH}$ 8.8 Hz, NH), 5.14 (dd, 1, $J_{2,3}$ 4.1, $J_{3,4}$ 9.9 Hz, H-3), 4.82–4.75 (m, 2, H-2, H-4), 4.45 (dd, 1, $J_{5,6}$ 4.4, $J_{6,6'}$ −12.4 Hz, H-6), 4.25 (dd, 1, $J_{5,6'}$ 2.2 Hz, H-6'), 3.86 (m, 1, $J_{4,5}$ 9.5 Hz, H-5), 3.07 (OSO_2CH_3), 2.11, 2.10, 2.05 (12, $NHCOCH_3$, $OCOCH_3$).

Anal.—Calc. for $C_{15}H_{23}NO_{11}S$: C, 42.35; H, 5.41; N, 3.29; S, 7.53. Found: C, 42.10; H, 5.68; N, 3.02; S, 7.74.

2-Deoxy-2-trifluoroacetamido-D-mannopyranose (IV)—A solution of methanol (5 ml) and ethyl trifluoroacetate (1 ml) was added to a mixture of D-mannosamine hydrochloride[7] (0.5 g, 2.3 mmoles) and sodium carbonate (0.25 g). The reaction was shown by TLC (ethyl acetate–methanol, 4:1, v/v) to be essentially complete after 2–3 hr. The mixture was evaporated with silica gel and applied to a dry-packed column of silica gel. Elution with chloroform–methanol (9:1, v/v) afforded IV (0.44 g, 69%) as an immobile syrup, $[\alpha]_D^{23}$ + 19° (c 1, methanol).

Anal.—Calc. for $C_8H_{12}F_3NO_6$: C, 34.91; H, 4.36; N, 5.09; F, 18.54. Found: C, 35.13; H, 4.67; N, 4.89; F, 18.16.

1,3,6-Tri-O-acetyl-2-deoxy-2-trifluoroacetamido-α-D-mannopyranose (VI)—Acetyl chloride was added to a solution of IV (2.83 g, 7.1 mmoles) in pyridine (180 ml) at room temperature. The reaction was conducted and processed as described for the preparation of II. Chromatography of the mixture of products on silica gel (chloroform), afforded the anomeric mixture of tri-*O*-acetyl derivatives (0.88 g, 21.2%) from which VI (0.22 g, 5.3%) was obtained on crystallization from ether, mp

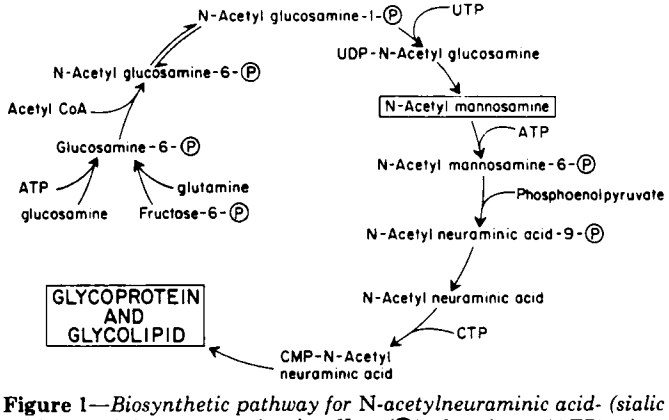


Figure 1—*Biosynthetic pathway for* N-*acetylneuraminic acid- (sialic acid) containing macromolecules. Key: (Ⓟ) phosphate; (ATP) adenosine triphosphate; (UTP) uridine triphosphate; (UDP) uridine diphosphate; (CTP) cytidine triphosphate; (CMP) cytidine monophosphate.*

[1] Elemental analyses and optical rotations were performed by Baron Consulting Co., Orange, Conn.
[2] Thomas-Hoover capillary melting point apparatus.
[3] Silica gel 60 (70–230 mesh), EM Laboratories, Inc., Elmsford, NY 10523.
[4] Silica gel GF, 250-μm layer prescored 10 × 20-cm plates, Analtech, Inc., Newark, DE 19711.
[5] 270-MHz Bruker HX-270 spectrometer.
[6] The proton at C-6 giving a higher field signal is designated H-6'.
[7] Sigma Chemical Co., St. Louis, MO 63178.

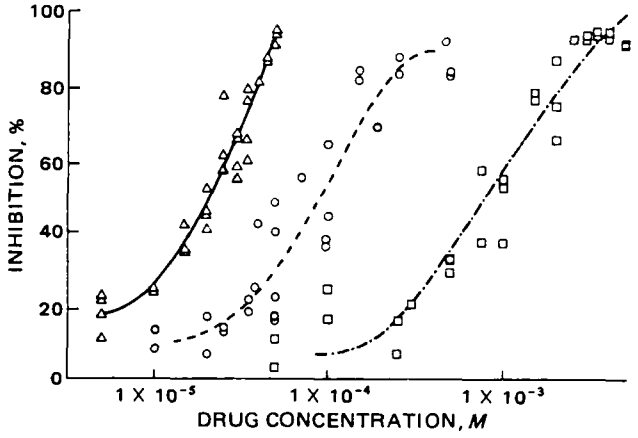


Figure 2—*Effects of* N-*acetyl-*D-*mannosamine analogues on the proliferation of Friend erythroleukemia cells. Key: (---) I, ID_{50} ~9 × 10^{-5} M; (-·-) II, ID_{50} ~8.5 × 10^{-4} M; (—) III, ID_{50} ~2 × 10^{-5} M.

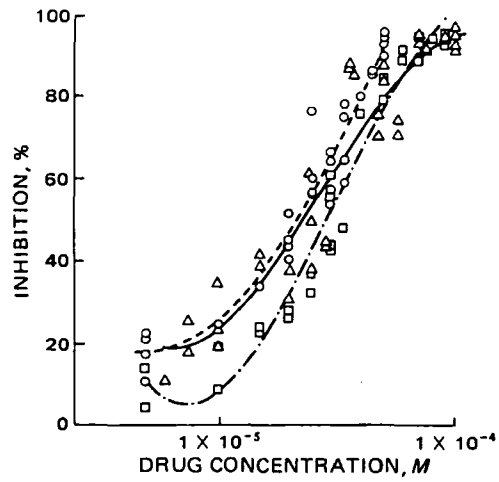


Figure 3—*Effects of* N-*trifluoroacetamido-*D-*mannosamine analogues on the proliferation of Friend erythroleukemia cells. Key: (---) VII, ID_{50} ~2 × 10^{-5} M; (—) V, ID_{50} ~2 × 10^{-5} M; (-·-) VI, ID_{50} ~3 × 10^{-5} M.

148–150°, $[\alpha]_D^{23}$ + 36° (c 1, methanol); ^{1}H-NMR[6]: δ 6.49 (d, 1, $J_{2,NH}$ 8.8 Hz, NH), 6.07 (d, 1, $J_{1,2}$ 1.8 Hz, H-1), 5.24 (dd, 1, $J_{2,3}$ 4.4, $J_{3,4}$ 9.9 Hz, H-3), 4.62 (m, 1, H-2), 4.55 (dd, 1, $J_{5,6}$ 4.0, $J_{6,6}$ −12.4 Hz, H-6), 4.25 (dd, 1, $J_{5,6'}$ 2.4 Hz, H-6'), 3.97 (m, 1, H-5), 3.74 (td, 1, $J_{4,5}$ 9.9 Hz, H-4), 2.86 (d, 1, $J_{4,OH}$ 4.4 Hz, OH), 2.20, 2.14, 2.08 (9, $OCOCH_3$).

Anal.—Calc. for $C_{14}H_{18}F_3NO_9$: C, 41.90; H, 4.49; N, 3.49; F, 14.21. Found: C, 41.78; H, 4.68; N, 3.37; F, 13.92.

*1,3,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-4-*O*-mesyl-α-*D*-mannopyranose (VII)*—Methanesulfonyl chloride (0.22 g) was added to a solution of VI (0.25 g, 0.52 mmole) in pyridine (2.5 ml) cooled to −10°. The mixture was allowed to stand at room temperature overnight, when TLC (chloroform) indicated completion. The mixture was processed as described for III; the product was chromatographed on silica gel (chloroform–petroleum ether 3:1, v/v) to afford VII as a syrup (0.275 g, 93%), $[\alpha]_D^{23}$ + 68.3 (c 1, chloroform); ^{1}H-NMR[6]: δ 6.79 (d, 1, $J_{2,NH}$ 8.8 Hz, NH), 6.12 (d, 1, $J_{1,2}$ 2.2 Hz, H-1), 5.48 (dd, 1, $J_{2,3}$ 4.4, $J_{3,4}$ 10.3 Hz, H-3), 4.88 (t, 1, $J_{4,5}$ 9.9 Hz, H-4), 4.67 (m, 1, H-2), 4.47 (dd, 1, $J_{5,6}$ 4.0, $J_{6,6'}$ −12.4 Hz, H-6), 4.23 (dd, 1, $J_{5,6'}$ 2.2 Hz, H-6'), 4.14 (m, 1, H-5), 3.08 (3H, OSO_2CH_3), 2.18, 2.13, 2.09 (9H, $OCOCH_3$).

Anal.—Calc. for $C_{15}H_{20}F_3NO_{11}S$: C, 37.58; H, 4.80; N, 2.92; S, 6.68; F, 11.90. Found: C, 37.79; H, 4.38; N, 3.30; S, 6.38; F, 11.89.

Assay of Cytotoxic Activity in Cell Culture—Friend erythroleukemia cells were grown at 37° in a suspension culture of Dulbecco's modified Eagle's medium containing 15% fetal calf serum and 0.02 m*M* glutamine. Exponentially growing Friend cells were seeded in 25-cm² plastic culture flasks at 1 × 10^5 cells/ml in 5 ml of medium. The test substance at various concentrations in phosphate-buffered saline was added at time zero, and the cells were incubated for 48 hr. The total number of cells in each of duplicate flasks was determined for the three separate experiments with a particle counter[8].

RESULTS AND DISCUSSION

In an effort to determine whether the biological properties of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-mannopyranose (I) were related to the presence of a more stable 4-*O*-acetyl group relative to the other acetyl groups and, in addition, to ascertain whether this substituent is also involved in the cytotoxic action of the trifluoroacetamido analogue (V), 2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-mannopyranose (II), 1,3,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-mannopyranose (VI), and their corresponding 4-*O*-mesylates (III) and (VII) were prepared and evaluated for their cytotoxicity in culture. It was speculated that removal of the 4-*O*-acetyl group from I to give II would result in a significant reduction in the cytotoxic properties in culture, while substitution of the 4-*O*-mesyl group, which is structurally similar and might be expected to be relatively more resistant to enzymatic hydrolysis, would result in greater activity than I.

The synthesis of trifluoroacetamido derivatives has been reported utilizing a variety of reagents, including ethyl trifluoroacetate (22, 23), trifluoroacetyl triflate (24), and trichlorotrifluoroacetone (25). *S*-Ethyl trifluorothioacetate (26) and trifluoroacetic anhydride (27–30) have been used previously in the synthesis of amino sugar nucleosides, in which the trifluoroacetyl moiety served as an *N*-blocking group. Accordingly, treatment of D-mannosamine hydrochloride with ethyl trifluoroacetate in methanol in the presence of sodium carbonate afforded 2-deoxy-2-trifluoroacetamido-D-mannopyranose (IV) as an immobile syrup in 69% yield. Compounds II and VII were prepared by a selective reaction involving treatment of *N*-acetyl-β-D-mannosamine and IV in pyridine with acetyl chloride until an optimum conversion to a triacetylated mixture was apparent. These products were purified by silica gel column chromatography, to afford anomeric mixtures of triacetylated derivatives from which the appropriate α-anomers II and VII were crystallized in 12% and 5.3% yields, respectively.

The structures of the analogues were confirmed by ^{1}H-NMR spectroscopy. The $J_{1,2}$ coupling constants for II and VI were 1.5 and 1.8 Hz, respectively, indicating the α-configuration (31). The signals due to H-4 coincided with H-5 (δ 3.76–3.63) in II and appeared at 3.74 in VI, essentially resonating at a higher field when compared with the other ring protons (as would be expected). Doublets at δ 2.80 in II and δ 2.86 in VI were assigned to the OH groups at H-4, since addition of deuterium oxide caused the signals to collapse while the triplet of doublets due to H-4 in VI reverted to a triplet. Further treatment of II and VII with methanesulfonyl chloride in pyridine afforded mesylates (III) and (VII) as syrupy products. The ^{1}H-NMR spectra of III and VII both showed shifts of their H-4 resonance signals to lower field in comparison with II and VI, respectively, while three proton singlets at δ 3.07 in II and δ 3.08 in VII were assigned to the mesyl groups.

Compounds I–III and V–VII were tested for their ability to inhibit the growth of Friend erythroleukemia cells in culture. The tetraacetate (I) had an ID_{50}[9] of ~9 × 10^{-5} *M*, while that of II and III were ~8.5 × 10^{-4} *M* and 2 × 10^{-5} *M*, respectively (Fig. 2). In contrast, the trifluoroacetamido analogues V, VI, and VII all gave similar ID_{50} values of 2–3 × 10^{-5} *M* (Fig. 3)[10]. The triacetate (II) is, therefore, 10-fold less active than I, while the 4-*O*-mesylate (III) is at least fourfold more active. These findings support the conclusion that the 4-*O*-acetyl and 4-*O*-mesyl groups play an important role in the cytotoxic properties of these compounds. The finding of similar activity for analogues in the trifluoroacetamido series (V–VII) implies that the biological properties of these compounds are due predominantly to the trifluoroacetamido moiety and are independent of substituents at the 4-position.

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[8] Model ZBI particle counter, Coulter Electronics, Hialeah, Fla.

[9] The ID_{50} is the concentration necessary to inhibit cell replication by 50%.

[10] Compound IV was inactive up to a concentration of 10^{-3} *M*.

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High-Performance Liquid Chromatographic Determination of Pralidoxime Chloride and Its Major Decomposition Products in Injectable Solutions

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Abstract □ A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of pralidoxime chloride (I) and its major decomposition products in an injectable formulation is described. I and its decomposition products were detected and quantitated by their UV absorbances at 270 nm, after being separated from related compounds and formulation excipients on a reverse-phase C-18 column using a mobile phase consisting of 52% acetonitrile and 48% of an aqueous solution containing 0.005 *M* phosphoric acid and 0.001 *M* tetraethylammonium chloride. The major decomposition products of I in the injectable formulation were identified by their retention times and stop-flow spectroscopy as 2-carboxy-*N*-methylpyridinium chloride, *N*-methyl-2-pyridone, 2-carbamoyl-*N*-methylpyridinium chloride, 2-hydroxymethyl-*N*-methylpyridinium chloride, and 2-cyano-*N*-methylpyridinium chloride. A substance of unknown identity also was detected in degraded solutions of I. Stop-flow spectroscopy, employing the spectral discrimination technique, showed that the method is specific for I. Recovery of I from a spiked placebo formulation averaged 99.9%. The accuracy of the method was also demonstrated for the decomposition products over a range of concentrations representing 1–50% decomposition. Replicate determinations of I in degraded solutions gave coefficients of variation of 1.0 and 1.5%, while the precision of determining the decomposition products range from 1.3 to 6.5%. Regression lines with correlation coefficients >0.9999 were obtained for I and its decomposition products, and solutions of these compounds were shown to be stable in the mobile phase for several days. Results for I by the HPLC and USP procedures are compared.

Keyphrases □ Pralidoxime chloride—decomposition products in aqueous solutions, concurrent high-performance liquid chromatographic determination □ High-performance liquid chromatography—concurrent determination of pralidoxime chloride and its decomposition products in aqueous solutions, comparison to USP procedure □ Degradation products—of pralidoxime chloride, concurrent high-performance liquid chromatographic determination

Pralidoxime chloride, 2-[(hydroxyimino)methyl]-1-methylpyridinium chloride (I), is a reactivator of organophosphate-inhibited cholinesterase. It has therapeutic value as an antidote to poisoning by organophosphate agricultural chemicals, chemical warfare agents, and drugs acting as cholinesterase inhibitors. Compound I is typically formulated as an aqueous injectable solution which is administered intramuscularly immediately after the onset of anticholinesterase poisoning.

Unfortunately, aqueous solutions of the various salts of pralidoxime have been shown to be unstable. The instability of pralidoxime in both acidic and basic media was first described in a series of reports (1–4) which proposed the existence of several decomposition products of pralidoxime iodide: the iodide salts of the 2-carboxy-, 2-formyl-, 2-carbamoyl-, and 2-cyano-*N*-methylpyridinium ions and *N*-methyl-2-pyridone. In addition, two major decomposition products were detected in solutions of pralidoxime

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