High-Performance Liquid Chromatographic Assay of Cyclophosphamide in Raw Material and Parenteral Dosage Forms

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Abstract □ A simple, specific, high-performance liquid chromatographic method is described for the assay of cyclophosphamide as the raw material and in parenteral dosage forms. The assay involves dilution, addition of an internal standard (methyl 4-aminobenzoate), injection onto a reversed-phase C_{18} column, and quantitation with a UV detector at 200 nm. Sensitivity is ~40 μg/ml. Decomposition products elute at the void volume and do not interfere.

Keyphrases □ Cyclophosphamide—analysis, high-performance liquid chromatography, raw material, parenteral dosage forms □ Antineoplastic agents—cyclophosphamide, high-performance liquid chromatographic analysis, raw material, parenteral dosage forms □ High-performance liquid chromatography—analysis, cyclophosphamide, raw material, parenteral dosage forms

Cyclophosphamide[1], *N,N*-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-oxide (I), is a commonly used antineoplastic drug. Initially, colorimetric (1), titrimetric (2, 3), and IR (4) assays were utilized for I dosage forms, but these methods lacked specificity.

BACKGROUND

A GLC assay for I dosage forms was developed (5) and later adapted by the USP (6). This method was used subsequently to study drug stability in parenteral solutions (7). However, during GLC, the underivatized I partially decomposed on the column, an occurrence reported by others (8, 9). These decomposition peaks were summed with the cyclophosphamide peak in the assay.

The major GLC decomposition product, a dehydrohalogenated compound, also has been proposed as an intermediate in the aqueous hydrolysis of I (10, 11). Therefore, in the analysis of I in aqueous solutions, the USP method might not differentiate the dehydrohalogenated product formed by aqueous hydrolysis from that formed by GLC degradation. Thus, the value of this method is limited, especially in the analysis of parenteral solutions.

More recently, I has been assayed in biological fluids by methods that are sensitive and specific but time consuming. In several methods, the drug is incompletely but quantitatively extracted, and the subsequently produced trifluoroacetyl derivative is analyzed by a gas–liquid chromatograph equipped with either a flame-ionization or an electron-capture detector (12) or by a chemical-ionization mass spectrometer (8, 13). A TLC procedure (14) suffers from the additional problems inherent in that technique.

Compound I also has been assayed in biological samples using a gas–liquid chromatograph equipped with a nitrogen–phosphorus selective detector (15). Again, an extraction was necessary; but even though the drug was underivatized, no mention was made of the on-column decomposition products.

Recently, the Food and Drug Administration proposed the compatibility testing of small-volume parenteral drugs, including I, in large-volume parenteral solutions packaged in plastic containers (16). This notice[2] requires rapid and specific assay methods.

The goal of this study was to develop an assay for I in intravenous solutions that is stability indicating and sufficiently brief to allow the analysis of 40 samples/8 hr. The reported method meets this goal but is not sensitive enough for biological samples. It involves the addition of an internal standard, methyl 4-aminobenzoate (II), to an aliquot of the intravenous solution admixture and subsequent analysis by a high-performance liquid chromatograph equipped with a variable UV detector set at 200 nm. This technique was also used to assay the raw material and cyclophosphamide for injection.

EXPERIMENTAL

Equipment—The microprocessor-controlled high-performance liquid chromatograph was equipped with a variable-volume injector[3], a variable-wavelength detector[4], and a 250-mm × 3.2-mm i.d. reversed-phase C_{18} column[5]. The UV spectrum of I in a 45% methanol solution was obtained[6] using 1-cm quartz cells.

Compounds and Reagents—Methanol[7] was filtered through a 1-μm filter[8]. Distilled water was passed through a deionization apparatus[9] and a 0.8-μm filter[10]. Compounds I[11] and II[12] and intravenous solutions[13] were used as received.

Internal Standard Stock Solution—A solution of II, 100 mg/500 ml of water, was prepared accurately with a sonicator.

Calibration—Calibration solutions were prepared in water to contain concentrations of I and II that were similar to the concentrations in the appropriate sample solutions. The calibration solution for the raw material and for cyclophosphamide for injection contained 4.4 mg of I/ml and 0.04 mg of II/ml. Fifty-microliter aliquots were injected in triplicate to calibrate the microprocessor.

The calibration solution for the intravenous solution admixture contained 0.44 mg of I/ml and 0.004 mg of II/ml. Aliquots (200 μl) were injected in triplicate to calibrate the microprocessor.

Sample Preparation—*Raw Material*—About 110 mg of the raw material, accurately weighed, and 5.0 ml of the internal standard stock solution were diluted to 25.0 ml with water. A 50-μl aliquot was injected into the appropriately calibrated chromatograph.

Cyclophosphamide for Injection (500 mg)—Water, 25.0 ml, was added to dissolve the powder. Exactly 10 ml of this solution and 10.0 ml of the internal standard stock solution were diluted to 50.0 ml with water. A 50-μl aliquot was injected into the appropriately calibrated chromatograph.

Intravenous Solution Admixture (500 mg of I/Liter)—Exactly 20 ml of the sample intravenous solution admixture and 3.0 ml of a 0.15 dilution

Table I—Cyclophosphamide for Injection, 500-mg Vial

Actual[a] Amount, mg	*n*	Mean Experimental Amount, mg	Mean Percent Recovery	*CV*, %
441.9	5	437.1	98.9	0.2
496.0	5	497.5	100.3	0.4
540.7	5	545.0	100.8	0.8

[a] Amounts expressed on an anhydrous basis. Quantities represent 90, 100, and 110% of label.

[1] CAS Reg. No. 6055-19-2.

[2] Codified as 21 CFR 310.509, effective Feb. 13, 1979.

[3] Model 1084A, Hewlett-Packard, Avondale, Pa.

[4] SF770 Spectroflow, Schoeffel, Westwood, N.J.

[5] Lichrosorb C_{18}, 10 μm, Altex, Berkeley, Calif.

[6] Acta MIV, Beckman, Fullerton, Calif.

[7] HPLC grade, Fisher, Fair Lawn, N.J.

[8] FALP 04700, Millipore, Bedford, Mass.

[9] Nanopure, Barnstead, Boston, Mass.

[10] AAWP04700, Millipore, Bedford, Mass.

[11] The authors are grateful to the Mead Johnson Research Center for the gift of cyclophosphamide monohydrate, which was used in sample preparations. USP reference standard cyclophosphamide monohydrate was used for standard preparations.

[12] Eastman, Rochester, N.Y.

[13] McGaw Laboratories, Irvine, Calif.

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Table II—Cyclophosphamide Intravenous Solution Admixtures

Intravenous Solution	n	Concentration[a], mg/liter	Mean Experimental Concentration, mg/liter	Mean Percent Recovery	CV, %
5% Dextrose injection USP	5	475.8	485.2	102.0	0.7
Sodium chloride injection USP	5	474.9	479.2	100.9	1.0
Lactated Ringer's injection USP	5	474.9	482.8	101.7	1.6
5% Dextrose in 0.9% sodium chloride USP	5	474.9	482.2	101.5	1.2
5% Dextrose in lactated Ringer's injection	4	480.5	488.5	101.7	1.4

[a] Concentrations expressed on an anhydrous basis.

of the internal standard stock solution were mixed, and a 200-μl aliquot was injected into the appropriately calibrated chromatograph.

High-Performance Liquid Chromatographic (HPLC) Conditions—The methanol and water were pumped from separate pumps to achieve a mobile phase of 45% (v/v) methanol at a flow rate of 1.0 ml/min. The oven temperature was maintained at 30°. The detector was set at 200 nm; the detector and microprocessor sensitivity was 0.1 au/cm.

Calculations—The microprocessor, operating in the internal standardization mode, automatically calculated the results, C_I, in terms of percent purity (raw material), milligrams of I per vial[14] (cyclophosphamide for injection), or milligrams of I per liter[14] (intravenous solution admixture). The general equation applied was:

$$C_I = \frac{R_I}{R_{II}} \frac{A_I}{A_{II}} C_{II} D F \qquad \text{(Eq. 1)}$$

where R_I is the concentration of I (hydrous basis) in the calibration solution divided by the area counts of peak I in the calibration graph; R_{II} is the concentration of II in the calibration solution divided by the area counts of peak II in the calibration graph; A_I is the area counts of peak I in the sample graph; A_{II} is the area counts of peak II in the sample graph; C_{II} is the concentration of II in the sample solution injected into the chromatograph; D is the dilution factor, which is 25 for the raw material, 125 for the vial, and 1.15 for the intravenous solution admixture; and F is the factor that converts to the anhydrous basis (F = 1 for the raw material and 0.936 for the other dosage forms).

Interference Study—To study the potential interference from degradation products, a 1% solution of I in water was heated to 83°. Aliquots were withdrawn at about 15-min intervals up to 4 hr and injected without the addition of an internal standard.

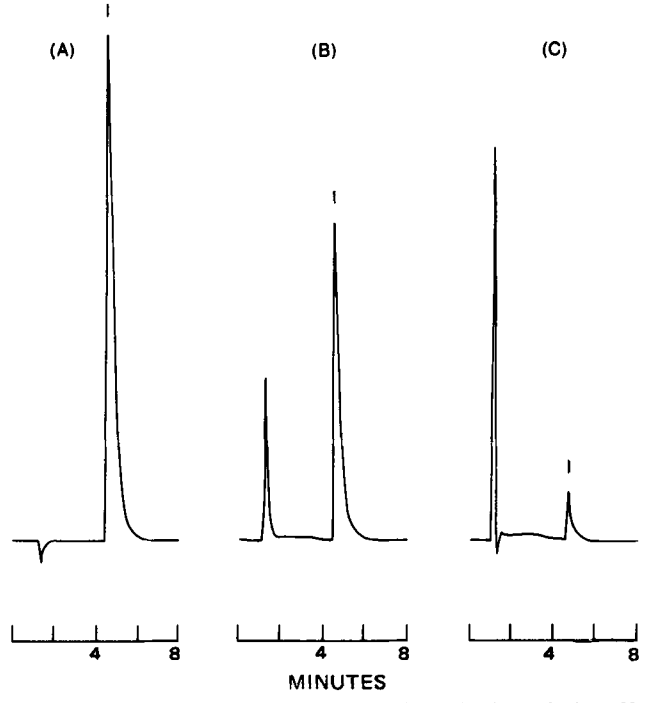

Figure 1—*Chromatograms of cyclophosphamide degradation. Key: A, zero time; B, 2 hr; and C, 3.5 hr.*

[14] Concentration of I expressed on an anhydrous basis.

RESULTS AND DISCUSSION

UV Spectrum—A solution of I in 45% methanol, 7.1 mg/25 ml, had a λ_{max} of 198 nm (ϵ = 464).

Interference Study—Selected chromatograms of the interference study are shown in Fig. 1. Once the temperature of 83° was reached, peak I decreased logarithmically with time, indicating first-order kinetics as previously reported (7). The peak at the void volume, representing the decomposition product(s), continually increased. No peaks eluted at the retention time of the internal standard. Therefore, it was concluded that decomposition products do not interfere.

The UV spectrum of the final reaction mixture exhibited a λ_{max} of 198 nm, the same maximum as for I.

Assays—The retention times of I and II were ~5.1 and ~3.3 min, respectively.

Two lots of raw material were assayed in quintuplicate against a USP reference standard. Lot 1 had a purity of 99.4% with a coefficient of variation (CV) of 0.7%; Lot 2 had a purity of 98.6% with a CV of 0.5% (Fig. 2A).

Simulated vials of cyclophosphamide for injection containing 90, 100, and 110% of the label amount of I were prepared by mixing I of known purity with sodium chloride. Each sample was assayed five times. HPLC recoveries of 98.9–100.8% and CV's of less than 1% were achieved (Table I). The chromatogram (Fig. 2B) is similar to that of the raw material. Separations were baseline. The calculated performance parameters for peak I were: N = 1850, k' = 2.6, and the asymmetry factor (17) at 10% of peak height = 2.8. The resolution was 4.6.

Admixtures were prepared by adding simulated vials of cyclophosphamide for injection to five different intravenous solutions at known concentrations of ~0.5 mg of I/ml. Each admixture was assayed four or five times. Mean recoveries ranged from 100.9 to 102.0% with CV's of

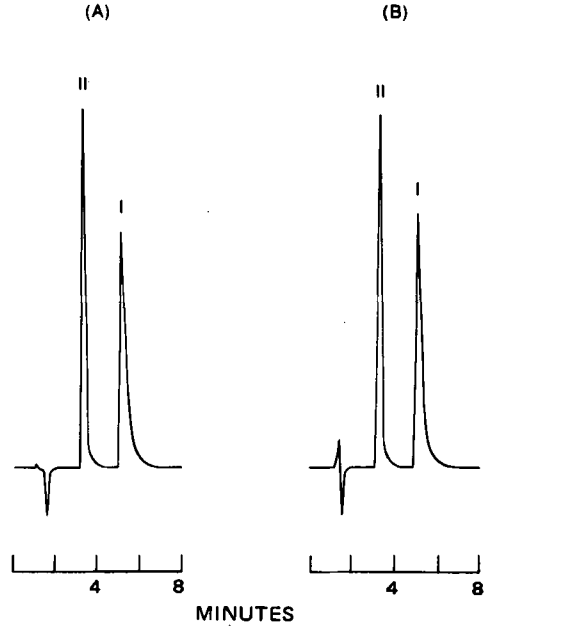

Figure 2—*Chromatograms of cyclophosphamide. Key: A, raw material; and B, cyclophosphamide for injection.*

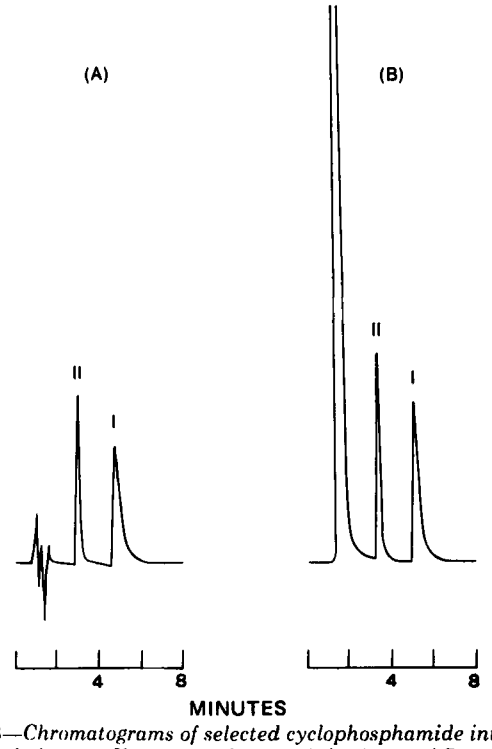

Figure 3—*Chromatograms of selected cyclophosphamide intravenous solution admixtures. Key: A, 5% dextrose injection; and B, 5% dextrose in lactated Ringer's injection.*

0.7–1.6% (Table II). In all admixtures, baseline separations were achieved. Chromatograms of selected admixtures are illustrated in Fig. 3. Peaks at the void volume are intravenous solution components and decomposition product(s), if present. The negative peaks in Fig. 3A represent the water used as the sample diluent. The minimum concentration that can be quantitated is ~0.2 mg/ml, and the detection limit is ~40 μg/ml (three times noise). This sensitivity is sufficient for parenteral solutions.

For all three sample types, blank solutions were prepared containing all appropriate ingredients (without I). In all cases, the chromatograms exhibited no peaks eluting after the peak due to the water used as the sample diluent.

In summary, the reported method is precise, accurate, specific, extremely brief, and applicable to dosage forms of I. It is particularly suited to the analysis of I in large-volume parenteral solutions.

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