High-Performance Liquid Chromatographic Analysis of Cyclophosphamide

TERRY T. KENSLER ˣ, ROBERT J. BEHME, and DANA BROOKE

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Abstract □ A reversed-phase high-performance liquid chromatographic technique is described for the analysis of cyclophosphamide in the presence of its hydrolysis products. The drug was quantified using a UV detector at a low wavelength. A single band was observed for the intact drug, which was well separated from its hydrolysis product(s). Quantification was obtained with adequate precision by the use of an injector loop or a suitable internal standard (hydrocortisone). The technique requires no extraction of the drug from aqueous solution or derivatization for analysis. The method was applied to partially hydrolyzed and to known solutions of cyclophosphamide. With suitable modification, the method may be useful for analysis of dosage forms but probably lacks the sensitivity necessary for analysis of the drug in biological samples.

Keyphrases □ Cyclophosphamide—high-performance liquid chromatographic analysis in prepared samples □ High-performance liquid chromatography—analysis, cyclophosphamide in prepared samples □ Antineoplastics—cyclophosphamide, high-performance liquid chromatographic analysis in prepared samples

Cyclophosphamide[1] is widely used in cancer treatment. A stability-indicating method for analyzing the drug directly in aqueous solution was needed. GLC (1) and other analytical methods (2–6) are available. The GLC method of Boughton *et al.* (1) was adopted, essentially unchanged, as the official USP assay (7).

The GLC method was shown to be more acceptable than the other methods, which lacked specificity, reproducibility, accuracy, or convenience. However, in its application to tablets, the GLC method requires a great deal of sample handling (*e.g.*, as extractions) for both standards and samples. No derivatization is required, but the drug decomposes on the column to give several peaks. A GLC method reported by Pantarotto *et al.* (8) gives a single peak but requires derivatization of the drug before injection. A GLC method has also been reported for the analysis of cyclophosphamide recovered from sheep tissues (9).

This paper reports a new analytical method that takes advantage of the inherent convenience, specificity, precision, and accuracy of reversed-phase high-performance liquid chromatography (HPLC). Reversed-phase HPLC allows direct analysis of aqueous solutions of the water-soluble drug without extraction into an organic liquid. An analytical method that requires no sample extraction and minimizes the number of manipulations is obviously desirable.

Presumably, HPLC has not been used to analyze cyclophosphamide because fixed wavelength UV detectors operating at 254 nm cannot detect the drug. Cyclophosphamide has a low molar absorptivity even at its λ_{max}, which occurs at a low wavelength. However, with modern variable wavelength detectors that can monitor effectively in the 190–200-nm region, this limitation can be overcome.

[1] Cytoxan, Mead Johnson Laboratories. Cyclophosphamide and cyclophosphamide monohydrate are often used interchangably; in this report, both refer to the monohydrate.

EXPERIMENTAL

Reagents and Solvents—Cyclophosphamide and hydrocortisone were drawn from laboratory stock. Chemicals used for buffers were reagent grade.

House-distilled water was redistilled in a glass apparatus[2]. Acetonitrile[3], UV grade, was used as received.

Apparatus—For the kinetic study, a reciprocating pump[4] was used. Samples were injected by the use of a 25-μl loop[5]. The detector[6] was set at 200 nm. The chromatograms were recorded on a strip-chart recorder[7], and the band areas were integrated with an electronic integrator[8].

The chromatogram of cyclophosphamide plus hydrocortisone was obtained with a different pumping system[9], injector[10], detector[11], and recorder–integrator[12].

A pH meter[13] and two UV–visible spectrophotometers[14] also were used.

Columns—Bonded reversed-phase[15] HPLC columns were obtained commercially.

Samples—A stock solution for the kinetic study was prepared by adding 28 g of cyclophosphamide to 700 ml of 0.1 *M* (pH 7.0) phosphate buffer. The resulting solution was filtered through filter paper and divided into four stoppered flasks. The flasks were placed in three constant-temperature ovens (40, 50, and 60°), and the temperature of the fourth flask was brought rapidly to 80° by immersing it in boiling water. When the temperature had reached 80°, the flask was transferred to an 80° water bath[16].

Samples were taken from all solutions at appropriate intervals. The sample size was large enough (~12–25 ml) to allow quantitative dilution of an accurately measured aliquot at a later time. All samples were chilled in an ice-water bath immediately after they were withdrawn and then were stored in a refrigerator until analysis. All samples were analyzed on 1 day and compared to an external standard (curve) prepared on that day. The standard solutions were prepared by dissolving weighed amounts (51.9–221.8 mg) of cyclophosphamide in water and diluting to 50.0 ml.

Other drug solutions were prepared by dissolving weighed amounts of cyclophosphamide in water. The solution containing both hydrocortisone and cyclophosphamide was prepared by dissolving the two drugs in acetonitrile and then diluting that solution quantitatively with water.

Analysis—Accurately measured aliquots of the samples from the kinetic study were diluted quantitatively with water, and a 25-μl portion of each was injected onto the column using a 25-μl injector loop. No internal standard was used. A 25-μl portion of each standard was also analyzed using the same chromatographic conditions.

The chromatographic conditions were: column, 30 cm × 3.9 mm i.d.; flow rate, 1.5 ml/hr; mobile phase, 30% acetonitrile–70% water; detector wavelength, 200 nm; detector scale, 0–1.0; and recorder, 0.64 aufs. The same chromatographic conditions were used to check reproducibility of the band areas at 200 nm. The wavelength was changed to 190 and 254 nm for the analysis of certain samples.

The solution containing cyclophosphamide and hydrocortisone was

[2] Corning water distillation apparatus model AG-2.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] Constametric II G, Laboratory Data Control.
[5] Valco Instruments Co., Houston, Tex.
[6] Vari-Chrom, Varian Instruments.
[7] Varian model 9176.
[8] Minigrator, Spectra-Physics.
[9] Varian model 8500 dual pump.
[10] Rheodyne model 70-10 100-μl loop.
[11] Variscan, model 635, Varian Instruments.
[12] Hewlett-Packard model 3380A.
[13] Corning model 12.
[14] Acta III, Beckman Instruments; Cary 17, Varian Instruments.
[15] μBondapak C_{18}, Waters Associates.
[16] Haake F.J.

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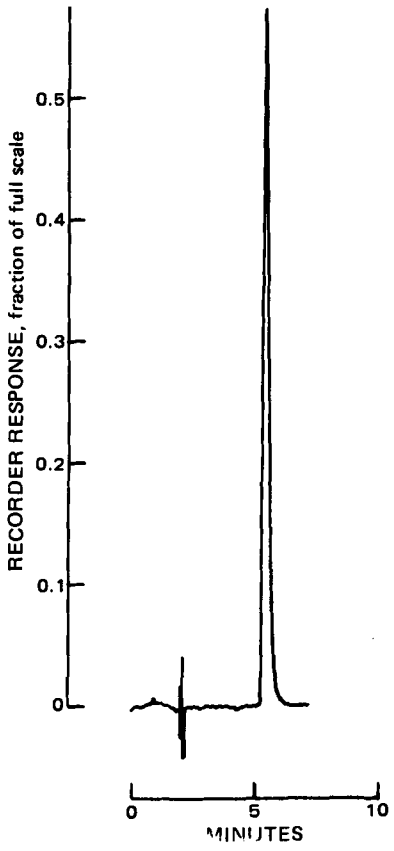

Figure 1—*High-performance liquid chromatogram of cyclophosphamide. Operating conditions were: flow rate, 1.5 ml/min; detector, 200 nm; scale, 0.64 aufs; and sample size, 39 μg (25 μl of 1.56 μg/μl).*

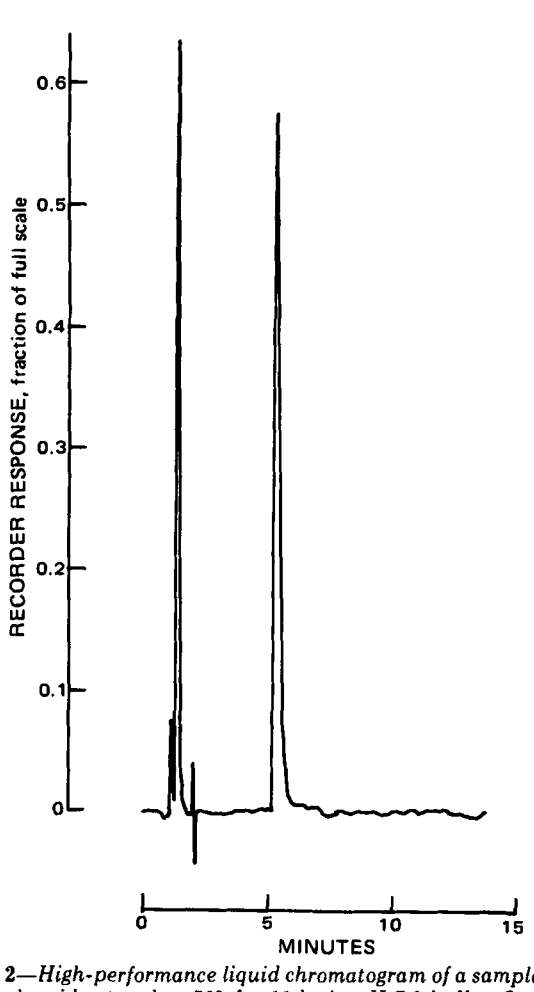

Figure 2—*High-performance liquid chromatogram of a sample of cyclophosphamide stored at 50° for 11 hr in pH 7.0 buffer. Operating conditions were: flow rate, 1.5 ml/min; detector, 200 nm; scale, 0.64 aufs; and sample size, 25 μl of a 1:20 dilution of the reaction mixture that originally contained 40 mg of cyclophosphamide/ml.*

analyzed without further dilution using a 100-μl injector loop. The chromatographic conditions were: column, 30 cm × 3.9 mm i.d.; flow rate, 1.5 ml/hr; mobile phase, 23% acetonitrile–77% 0.1 *M* (pH 7.0) phosphate buffer; detector wavelength, 211 nm; detector scale, 0–0.5; and recorder, 0.4 aufs.

RESULTS AND DISCUSSION

A high-performance liquid chromatogram of a freshly prepared aqueous solution of cyclophosphamide appears in Fig. 1. The drug was eluted in a single band at 325 sec. The fluctuations in the baseline at about 2 min were due to injection anomalies and were not compound related. The single peak illustrated in Fig. 1 gave approximately 60% of a full-scale deflection (0.64 aufs) and was due to about 39 μg of the drug. This result is in contrast with the USP GLC method (7) in which decomposition on the column gives multiple peaks.

The UV absorbance spectrum of cyclophosphamide, in water and in a solvent (25% acetonitrile–75% water) approximating the HPLC mobile phase, was recorded. A local maximum occurred in both solvents at or below 190 nm, but even at this wavelength the drug had a low molar absorptivity (ϵ = 1020 in water). At 200 nm, ϵ equaled 550. The compound had no absorbance at 254 nm, where fixed wavelength detectors operate at maximum sensitivity.

The chromatogram depicted in Fig. 1 was recorded using a variable wavelength detector[6] set at 200 nm. The choice of this wavelength allowed quantification of the chromatographic band due to intact drug, which would not have been observed at 254 nm. The greatest sensitivity was obtained using the lowest wavelength compatible with the detector, flowcell, and solvent used. The chromatograms from the brief kinetic study to be described also were recorded at 200 nm. However, it was possible to operate the same detector at wavelengths as low as 190 nm if the column was equilibrated thoroughly with the eluant (water and UV grade acetonitrile). The cyclophosphamide band area (from an 86-μg sample) was 1.8 times greater at 190 nm than at 200 nm, as expected from the UV spectrum. The ability to monitor at wavelengths as low as 190 nm is not critical for the accurate measurement of cyclophosphamide concentrations, however.

The detector was set at 254 nm for the same 86-μg sample to test the hypothesis that no HPLC absorbance band should be seen. As expected, no band was seen even with the sensitivity increased 100-fold to 0.0064 aufs. A sample 10 times as large (*i.e.*, 855 μg) also gave no chromatographic band (due to absorbance) at this increased sensitivity. However, this concentrated sample did give a deflection in the baseline (first negative and then positive), which was attributed to a refractive index change in the eluant as the drug passed through the detector. This deflection was not suitable for quantification of samples.

Figure 2 illustrates the chromatogram of a buffered (pH 7.0) aqueous sample of cyclophosphamide in which a portion of the drug had been hydrolyzed. Drug solutions buffered at pH 7.0 and stored at 40, 50, 60, and 80° gave chromatograms that were all qualitatively like the one shown in Fig. 2. All injections were made with the same 25-μl loop without an internal standard. Each chromatogram contained a band due to the remaining intact drug at about 325 sec and only one other band (at about 90 sec) due to the degradation product(s). More than one product can result from cyclophosphamide hydrolysis. The band near 90 sec may have been due to more than one product, but no attempts were made to resolve the possible components.

Plots of the natural log of the area of the band due to intact cyclophosphamide *versus* time, for solutions stored at each of the four temperatures, appear in Fig. 3. The plots are linear, and the rate constants calculated from the slopes of the regression lines agree well with those that can be predicted from the Arrhenius equation and data published previously (10) (Table I). The linearity of the plots and agreement with the predicted values indicate that the HPLC technique can provide specific and reliable stability data for the intact drug in the presence of its hydrolysis products. The activation energy of 26.8 kcal/mole/deg calculated from the HPLC data also is in general agreement with the value (~24.5 kcal/mole/deg) previously determined (4).

The brief kinetic study described here was conducted only to show the

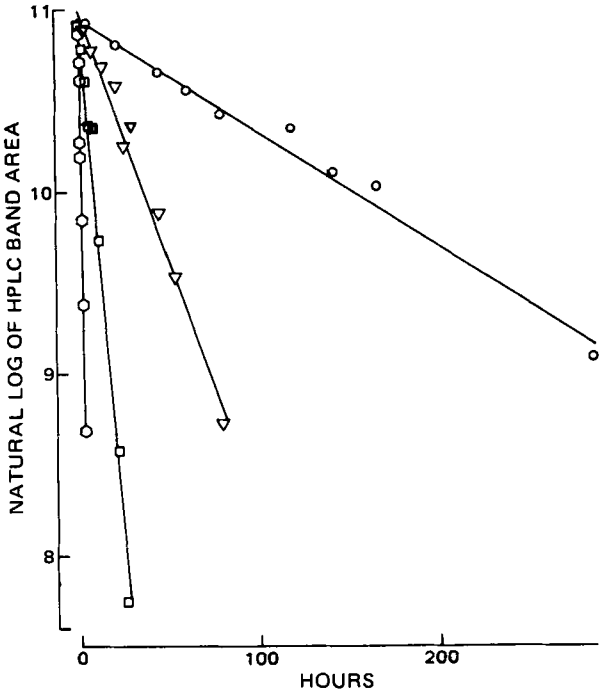

Figure 3—*Semilogarithmic plots of the cyclophosphamide HPLC band area* versus *time for pH 7.0 solutions stored at 40° (○), 50° (▽), 60° (□), and 80° (○).*

possible utility of an HPLC method for analyzing the drug in the presence of its hydrolysis products. No internal standard was used because the precision using an injector loop was considered adequate for the desired results. Of course, each HPLC column and instrument is unique; variations in mobile phase, flow rate, and detector settings for optimum performance are to be expected.

The standard calibration curve used to determine sample concentration was prepared by three or more injections of four standard solutions (1.038–4.436 mg/ml). A least-squares analysis of integrator counts *versus* concentration defined a straight line ($r^2 = 0.9987$) with a slope of 1.305×10^5 counts/(mg/ml), a y-intercept of 1.646×10^4 counts, and a relative standard deviation of the slope of 1.05%. The highest relative standard deviation of the areas (within the group) at any one concentration was 2.1% (at 1.563 mg/ml, three injections). Ten replicate 25-μl injections of a 3.42-mg/ml solution with no internal standard gave band areas with a relative standard deviation of ±1.5%.

While this degree of reproducibility was deemed adequate here, an internal standard may be desirable or necessary for precise kinetic measurements or for accurate day-to-day control of the potency of a drug product. An internal standard would also be useful if the drug were to be analyzed in biological samples. (However, the techniques outlined here probably would not provide the sensitivity, without a drug concentrating step, necessary for the analysis of the small amounts of intact drug in biological samples.) Nevertheless, for the analysis of drug products or relatively concentrated solutions, the sensitivity is adequate, and hydrocortisone is a suitable internal standard.

Table I—Rate Constants for the Hydrolysis of Cyclophosphamide at pH 7.0

T	k[a], min^{-1}	r^2	$k_{expected}$[b,c]
40°	1.04×10^{-4}	0.986	1.48×10^{-4}
50°	4.62×10^{-4}	0.982	5.12×10^{-4}
60°	1.94×10^{-3}	0.995	1.65×10^{-3}
80°	1.37×10^{-2}	0.992	1.40×10^{-2}

[a] Plot of log k *versus* $1/T$ gives a value of $E_a = 26.8$ kcal/mole/deg ($r^2 = 0.994$). [b] These values were calculated from the constants given in Ref. 8 and the Arrhenius equation $k = Ae^{-E_a/RT}$, where $A = 4.22 \times 10^{-13}$ min^{-1} and $E_a = 25.0$ kcal/mole/deg. [c] These values are expected to be nearly independent of pH in the pH 3–10 region, as evidenced by the work of Hirata *et al.* (4).

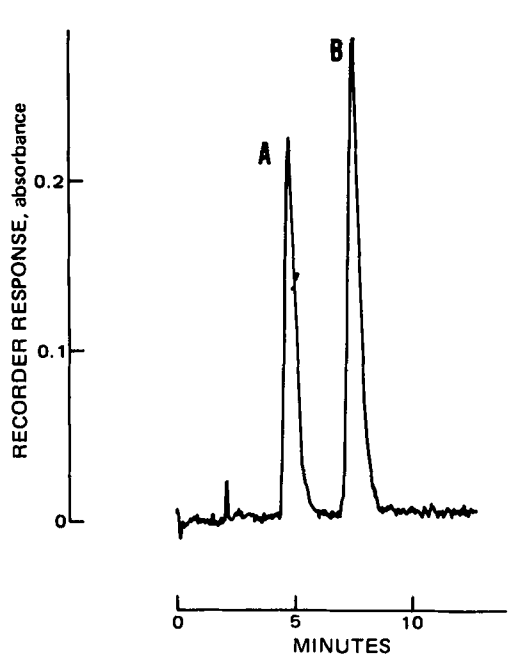

Figure 4—*High-performance liquid chromatogram of cyclophosphamide (A) (4.85 min) and hydrocortisone (B) (7.71 min). Operating conditions were: flow rate, 1.5 ml/min; detector, 211 nm; scale, 0.4 aufs; and sample size, 100 μl of a solution containing 12.5 mg of cyclophosphamide/ml and 0.2 mg of hydrocortisone/ml.*

A chromatogram of a solution containing hydrocortisone and cyclophosphamide is shown in Fig. 4. Since hydrocortisone elutes after the drug, it does not overlap with either the drug or hydrolysis products. Its use in biological samples would require further experimentation. The detector was set at 211 nm for this chromatogram because this detector[11] would not operate below that wavelength with the indicated mobile phase. For this reason, the sensitivity was lower than before, as expected.

Detectors other than UV types (*e.g.*, refractive index) may also be useful for the analysis of cyclophosphamide in certain instances. Although a degree of specificity might be lost in going from a UV to a refractive index detector, advantage might be gained in detecting hydrolysis products or impurities that do not absorb UV light. The data indicate that reversed-phase HPLC is ideal for the analysis of cyclophosphamide when the appropriate detector is employed. Specific methods for the analysis of the drug in solid or liquid dosage forms should easily be developed based on the chromatographic system given.

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