the sulfonamide molecule and the configurational interpretation of the entropy of solution.

In summary, the entropy of solution appears to be a function that becomes very instructive in cases of dilute solutions of pharmaceutically useful solutes dissolved in hydrogen-bonding solvents; furthermore, studies at various temperatures yielded suggestions concerning dissolution behavior that could not be found from single-temperature studies. Although the entropy of solution does not directly allow predictions of solubility, its application is in keeping with Lindstrom's (14) statement that: "it would be of immeasurable aid if explanations of observed solubility phenomena were possible in terms of purely basic theoretical concepts."

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DRUG STANDARDS

Assay of Cyclophosphamide

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Abstract □ A GLC procedure was developed for the assay of cyclophosphamide raw materials and cyclophosphamide in formulations. Results obtained by this technique are comparable to those obtained by IR spectroscopy. The GLC procedure offers increased accuracy, reproducibility, and precision. The method is more specific and less time consuming than the IR procedure.

Keyphrases □ Cyclophosphamide—GLC analysis in raw materials and formulations □ GLC—analysis, cyclophosphamide

Cyclophosphamide[1] is a cytotoxic agent related to the nitrogen mustards and valuable in the palliative therapy of certain malignant neoplasms. Cyclophosphamide was first synthesized by Arnold and Boureaux (1) in 1957. Since that time, there have been several attempts to develop methods of assay for this compound. The published methods of analysis are:

A. Analysis based on nitrogen, phosphorus, or chloride content (2).

B. Colorimetric analysis, based on the intensity of a cobalt thiocyanate–cyclophosphamide complex (3) or by means of 4-(*p*-nitrobenzyl)pyridine after hydrolysis (4).

C. Titrimetric analysis, after precipitation of the digested material by quinoline and citric-molybdic acid solution (5).

D. IR spectroscopy (6).

These methods all have in common the disadvantage that they are not specific for the intact cyclophosphamide molecule. Procedures A–C utilize hydrolysis of the molecule before quantitation. Procedures A and C call for digestion with sulfuric and/or nitric acids. Procedure B utilizes an acid hydrolysis before color development.

Of the methods listed, Procedure D has the greatest degree of specificity. The basis for this procedure is the characteristic stretching frequency of the phosphorus–oxygen bond at 9.5 μ. Quantitation is effected by relating the intensity of this absorption band to an internal standard at 4.9 μ. However, the only degradation dis-

[1] Marketed as Cytoxan, Mead Johnson Laboratories.

Table I—GLC Operating Conditions

Column	1.22 m. (4 ft.) × 3 mm. i.d.	3.8% UCW98 on Diatoport S
Temperature	Injection port	240°
	Detector	250°
	Column	195°
Flow rates	Hydrogen	45 ml./min.
	Air	350 ml./min.
	Helium	55 ml./min.

cernible by this procedure is that in which the heterocyclic ring is altered or opened. Any alteration of the chloroethyl groups would not necessarily be detected by this procedure.

A GLC assay for cyclophosphamide raw material and for cyclophosphamide in products was recently developed in these laboratories. Results of assays using this procedure are presented in the *Discussion* section.

The GLC assay procedure is preferable to the IR procedure for two major reasons:

1. The techniques inherent in the IR procedure (*i.e.*, extraction, addition of internal standard solution, evaporation, pellet preparation, recording of partial spectrum, manual measurement of intensities of respective stretching frequency, and calculation) do not allow for a rapid analysis. This is in contrast with a procedure wherein extraction is made with an internal standard solution and injected directly into the gas chromatograph. Peak areas are determined electronically, giving a much simpler calculation.

2. The precision of both techniques is greatly affected by most of the distinct steps in the analysis scheme. Each additional sample manipulation introduces yet another source of error.

Additionally, quantitation by the IR technique is accomplished with reference to a four-point standard curve. This curve is observed to be variable from day to day and is subject to errors inherent in connecting the four points with a "best fit" line. A GLC assay utilizes a single-point standard and is very reproducible from analysis to analysis.

EXPERIMENTAL

Equipment—GLC determinations were done using a gas chromatograph[2] with both on-column injection and glass columns. Detection was effected by flame ionization. The conditions are listed in Table I.

Integration of areas was accomplished by use of an electronic integrator[3].

Mass spectra were obtained using a mass spectrometer[4] and also a GLC-mass spectrometer system[5] (injection temperature, 200°; column, 190°; helium flow rate, 40 ml./min.; detection by measuring the ionization current).

Reagents—*Internal Standard Solution*—Methyl stearate (1.0 mg./ml.) in chloroform was used.

Sodium Hydroxide Solution—Sodium hydroxide (10%) (reagent grade) in water was used.

Sample Preparation—*Raw Material*—Place about 200 mg., accurately weighed, of the raw material in a 125-ml. separator containing 50.0 ml. of internal standard solution and 5 ml. of water.

Tablets[6]—Determine the average weight of 10 tablets. Grind to a fine powder. Place an accurately weighed portion of the powder, equivalent to about 200 mg. of cyclophosphamide monohydrate, in a 125-ml. separator containing 50.0 ml. of internal standard solution and 5 ml. of water.

Vials for Injection—Uncap the vial and weigh. Remove the sample and rinse the vial with water and acetone. After air drying, reweigh the vial to obtain the sample weight.

For 100-mg. vials, transfer the contents with 3 × 1.5-ml. portions of water and 3 × 1.5-ml. portions of $CHCl_3$ to a 125-ml. separator, containing 25.0 ml. of internal standard solution.

For 200-mg. vials, transfer the contents with 3 × 1.5-ml. portions of water and 3 × 1.5-ml. portions of $CHCl_3$ to a 125-ml. separator containing 50.0 ml. of internal standard solution.

For 500-mg. vials, dissolve the contents in 25.0 ml. of water (some stirring may be necessary). Pipet 10.0 ml. of the solution into a 125-ml. separator, containing 50.0 ml. of internal standard solution.

Standard—Place about 200 mg., accurately weighed, of cyclophosphamide monohydrate reference standard in a 125-ml. separator, containing 50.0 ml. of internal standard solution and 5 ml. of water.

[2] Hewlett-Packard model 402.
[3] Hewlett-Packard model 3370A.
[4] CEC 21-104.
[5] LKB-9000.
[6] 53.5 mg. of cyclophosphamide monohydrate per dosage unit.

Table II—Reproducibility, Precision, and Accuracy Studies[a]

	Assay, mg. Cyclophosphamide Monohydrate/Tablet		
	Accuracy	Reproducibility	Precision
Theory	53.5	53.5	53.5
	53.3	53.3	54.6
	54.3	54.6	54.7
	53.9	55.2	54.0
	52.8	55.7	54.9
	54.8	54.7	54.7
	53.8	54.9	54.8
	54.5	55.5	54.2
	54.0	55.0	54.6
	54.1	54.3	54.4
	53.5	54.8	54.3
Mean ± *SD*	53.9 ± 0.6	54.8 ± 0.7	54.5 ± 0.3

[a] See text for details of studies.

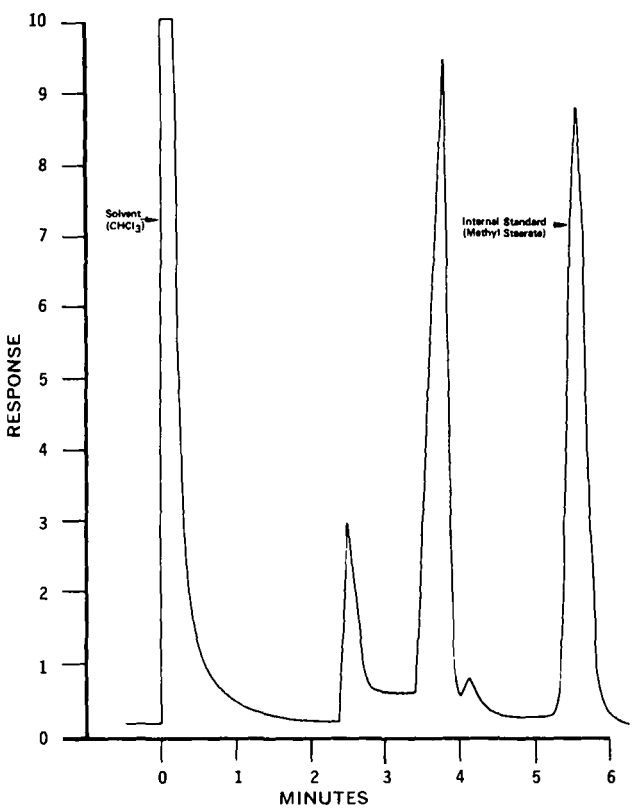


Figure 1—*Chromatogram of cyclophosphamide.*

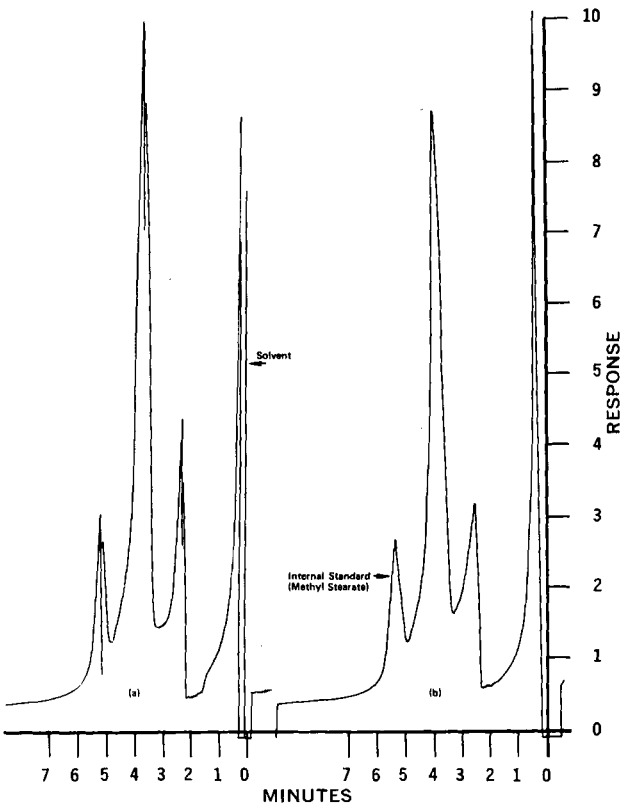


Figure 2—*Chromatogram of cyclophosphamide from a GLC–mass spectrometer system.*

Procedure—Shake each separator 5–10 sec. and let stand for at least 5 min. Shake vigorously for 60 sec. and allow layers to separate. Add 1–2 ml. of sodium hydroxide solution and shake vigorously for 60 sec. Allow the layers to separate and then drain the lower chloroform layer through a pledget of cotton into a screw-cap tube.

Inject 2-μl. aliquots of the standard until reproducibility is obtained on at least three consecutive injections. Average the three values. Inject the duplicate 2-μl. aliquots of the sample dilutions, again using the average values.

Calculation—Arbitrarily select some common value for the area of the internal standard, such as 15,000. The corrected area for the cyclophosphamide monohydrate (use the sum of the three peaks) =

$$\frac{15{,}000}{\text{area of the internal standard}} \times \text{area of cyclophosphamide monohydrate}$$

Table III—Cyclophosphamide Monohydrate Raw Material

Sample	Assay, % Cyclophosphamide Monohydrate: GLC	IR	Theory
1	98.7	102.7	100.0
2	101.1	104.6	
3	101.5	99.6	
4	99.2	102.1	
5	97.7	100.0	
6	97.3	104.0	
7	97.3	98.6	
8	99.7	101.2	
9	100.1	99.0	
10	99.4	99.5	
11	102.1	98.4	
12	100.1	97.4	
13	97.8	96.7	
14	100.0	96.5	
15	100.1	98.5	

Table IV—Cyclophosphamide for Injection, 100 mg.

Sample	Assay, % Cyclophosphamide Monohydrate: GLC	IR	Theory
1	68.2, 66.4	72.5	70.38
2	68.0, 67.5	77.7	
3	66.7, 68.4	73.0	
4	67.1, 65.8	68.7	
5	70.6, 70.0	67.7, 64.1	
6	70.7, 72.0	67.9	

Let:

A = average corrected area for cyclophosphamide monohydrate in the sample

B = average corrected area for cyclophosphamide monohydrate in the standard

Then:

$$C = \frac{B \times 200}{\text{mg. standard used}} = \text{average corrected area for cyclophosphamide monohydrate in 200.0 mg. reference standard}$$

Raw Material—

$$\%\ \text{cyclophosphamide monohydrate} = \frac{A}{C} \times \frac{20{,}000}{\text{sample weight (mg.)}} \quad \text{(Eq. 1)}$$

Tablets—

$$\text{mg. cyclophosphamide monohydrate/tablet} = \frac{A}{C} \times \frac{\text{average tablet weight (mg.)}}{\text{sample weight (mg.)}} \times 200 \quad \text{(Eq. 2)}$$

Vials for Injection—

100-mg. vials:

$$\text{mg. cyclophosphamide monohydrate/vial} = \frac{A}{C} \times 100 \quad \text{(Eq. 3)}$$

200-mg. vials:

$$\text{mg. cyclophosphamide monohydrate/vial} = \frac{A}{C} \times 200 \quad \text{(Eq. 4)}$$

500-mg. vials:

$$\text{mg. cyclophosphamide monohydrate/vial} = \frac{A}{C} \times 500 \quad \text{(Eq. 5)}$$

For all vials for injection:

$$\%\ \text{cyclophosphamide monohydrate} = \frac{\text{mg. cyclophosphamide monohydrate/vial}}{\text{vial contents (mg.)}} \times 100 \quad \text{(Eq. 6)}$$

Table V—Cyclophosphamide for Injection, 200 mg.

Sample	Assay, % Cyclophosphamide Monohydrate: GLC	IR	Theory
1	70.5, 74.2	73.3	70.38
2	70.1, 71.6	68.5	
3	71.0, 69.1	70.0	
4	72.4, 72.0	71.0	
5	72.9, 73.4	71.7	
6	71.5, 70.9	72.1	
7	71.5, 72.8	71.2	
8	70.3	63.0	
9	71.0	69.9	
10	70.1	62.5	

Table VI—Cyclophosphamide for Injection, 500 mg.

Sample	Assay, % Cyclophosphamide Monohydrate: GLC	IR	Theory
1	68.5, 67.4	72.7	70.38
2	68.5, 69.0	68.2	
3	68.6, 67.4	67.1	
4	71.1	62.7	
5	77.2	71.0	

DISCUSSION

Chromatography of cyclophosphamide under the conditions listed in Table I yielded a chromatogram with three peaks (Fig. 1). These three peaks constituted approximately 9, 90, and 1% of the total area, respectively. Observation of variability of these areas from injection to injection seemed to indicate that the more reproducible, total area should be used in subsequent calculations.

The accuracy of the procedure was determined by injection of solutions of known concentration and comparison of calculated with actual values. Reproducibility was determined by grinding a large number of cyclophosphamide tablets and taking 10 weighed portions through the assay procedure. Precision of the method was determined by injection of one solution 10 times. These data are listed in Table II.

Assay values of cyclophosphamide raw material and cyclophosphamide in cyclophosphamide formulations were determined gas chromatographically and compared with results obtained by the usual IR procedure (6). These comparisons are listed in Tables III–VII for cyclophosphamide raw materials and cyclophosphamide formulations. The results obtained are comparable to the IR procedure.

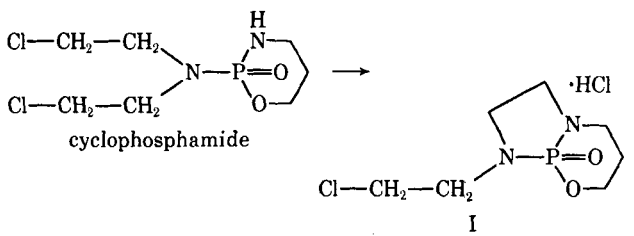
To ascertain the specificity of the GLC method, it was necessary to determine the identity of the species causing the three peaks in the chromatogram. The two major peaks (Fig. 2*b*) arising from cyclophosphamide were identified by mass spectrometry as dehydrohalogenated cyclophosphamide (the 9% peak) and intact cyclophosphamide (the 90% peak). The first peak had a molecular ion of 224 mass units (one chlorine). The second peak had a molecular ion of 260 mass units (two chlorines) and the same fragmentation pattern as intact cyclophosphamide. Thus, the first peak appears to be cyclophosphamide with a loss of 36 mass units. These 36 mass units are due to hydrogen chloride, which would be eluted from the column before the sample peaks and would not be discernible in the mass spectrum.

A possible structure for the dehydrohalogenated compound is shown in Scheme I. Structure I could be formed by an intramolecular alkylation, corresponding to a structure proposed by Friedman *et al.* (7) in the aqueous hydrolysis of cyclophosphamide.

The identity of the minor peak (1%) has not been determined.

It is postulated that multiple peaks arise from a partial thermal degradation (loss of HCl) on the GLC column. The degree of degradation is apparently related to the polarity of the liquid phase used on the packing material. With a relatively nonpolar liquid phase (UCW98) (8), the amount of dehydrohalogenation observed was approximately 9%; with a much more polar liquid phase (HiEFF8BP), the amount of dehydrohalogenation increased to 80–85%. Another indication that this phenomenon is an on-column occurrence is that a cyclophosphamide sample, whose purity and identity had been verified by elemental and spectroscopic analyses, also gave rise to three peaks in the chromatogram.

It would thus seem that quantitation based on total area of the three observed peaks would offer a valid representation of the amount of intact cyclophosphamide present in the sample.



Scheme I

Table VII—Cyclophosphamide Tablet

Sample	Assay, mg. Cyclophosphamide Monohydrate/Tablet: GLC	IR	Theory
1	51.8	53.7	53.5
2	52.1	54.5	
3	52.2	56.3	
4	52.5	53.8	
5	53.8	55.7	
6	54.4	54.1	
7	52.8	55.0	
8	52.8	58.3	
9	53.9	56.8	
10	53.6	56.2	
11	53.0	58.4	
12	53.3	54.5	
13	53.0	57.1	

CONCLUSION

The GLC procedure for cyclophosphamide determination in raw materials and formulations yields results comparable to those obtained by IR spectroscopy. The GLC procedure is less time consuming and offers increased accuracy, reproducibility, and precision. The specificity of this procedure is greater than any developed to date.

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