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High-Performance Liquid Chromatographic Determination of Vincristine Sulfate in Preformulation Studies

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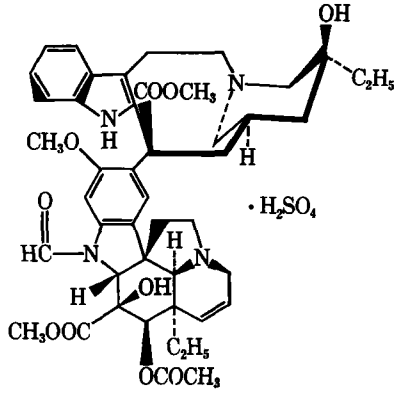
Abstract □ A fast and simple procedure was developed for the quantitative determination of vincristine sulfate for use in preformulation studies. The procedure involves the use of high-performance liquid chromatography with a reverse-phase column and a mobile phase containing the sodium salt of 1-pentanesulfonic acid for ion-pairing. The procedure has been shown to be specific for vincristine sulfate in the presence of forced degradation products of this substance, vinblastine (a structurally similar *Vinca* alkaloid), and several possible formula excipients. The procedure is linear from 10–200% of the normal injection concentration, and has an assay precision (relative 2σ) of ±1.6%. Recovery of known samples averaged 99.7%.

Keyphrases □ Vincristine sulfate—high-performance liquid chromatography, preformulation studies, degradation □ High-performance liquid chromatography—vincristine sulfate, degradation products □ Preformulation studies—vincristine sulfate, degradation products, high-performance liquid chromatography

Vincristine sulfate (I), an antineoplastic agent originally obtained from extracts of *Vinca rosea*, is structurally related to vinblastine. An analytical procedure was required for the determination of vincristine sulfate in samples resulting from preformulation studies. The procedure had to be capable of accurate and precise quantitation, specific in the presence of a number of possible excipients, and stability indicating. Because a large number of samples were to be examined, it was also necessary that the procedure be rapid and simple.

A number of assay procedures have been reported for vincristine sulfate (1); however, some are not stability indicating (direct spectrophotometric analysis), others are time consuming and complex (colorimetric analysis), and some are not sufficiently accurate and precise (TLC analysis). Several high-performance liquid chromatographic (HPLC) methods have been reported. One procedure (2) requires a 40-min gradient with a vincristine retention time of ~25 min. This was deemed too lengthy for the proposed purpose.

Another procedure (3) required the use of ammonium carbonate in the mobile phase. Since the column life might be shortened appreciably by the presence of ammonium carbonate[1], this procedure was not used. This paper reports the development of a simple and rapid HPLC procedure for the determination of vincristine sulfate stability.



EXPERIMENTAL

Reagent and Chemicals—Acetonitrile and methanol were HPLC grade[2], and were used without further purification. Water was distilled and filtered[3] prior to use. Vincristine sulfate was used as received[4]. All other reagents were ACS grade or better and used without further purification.

Equipment—A liquid chromatograph[5] was connected to an injection valve[6], a variable-wavelength detector[7], a recorder[8], and an integrator[9].

A column[10] consisting of a monomolecular layer of a phenylorganosilicone permanently bonded to polar, porous silica particles was used.

The mobile phase consisted of 30% acetonitrile and 70% aqueous solution which contained 0.02 *M* ammonium acetate and 0.005 *M* of the sodium salt of 1-pentanesulfonic acid adjusted to pH 2.0 with 10% v/v nitric acid[11]. The flow rate was 2.5 ml/min (pressure was ~2000 psi). The detector sensitivity was 0.1 AUFS at 254 nm. Chart speed was 60 cm/hr.

Internal Standard Preparation—Propyl *p*-hydroxybenzoate (~30 mg) was accurately weighed and transferred to a 100-ml volumetric flask.

[1] μ-Bondapak and μ-Porasil Liquid Chromatography Columns Care and Use Manual, Waters Associates, Milford, Mass.
[2] Burdick and Jackson Laboratories, Inc., Muskegon, Mich.
[3] Type HA, 0.45μ filter, Millipore Corp., Bedford, Mass.
[4] Gedeon Nichter Ltd., Budapest, Hungary.
[5] Model 5000 Varian Associates, Walnut Creek, Calif.
[6] Valco injection valve with pneumatic actuator, Valco Instruments, Houston, Tx.
[7] Model UV-50 Varichrom, Varian Associates, Walnut Creek, Calif.
[8] Model 9176, Varian Associates, Walnut Creek, Calif.
[9] Model CDS-111L, Varian Associates, Walnut Creek, Calif.
[10] μ-Bondapak Phenyl, Catalog No. 17198 (30 cm × 3.9-mm i.d.), Waters Associates, Milford, Mass.
[11] Beckman Model Zeromatic II, Beckman Instruments, Fullerton, Calif.

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Table I—Forced Degraded Specificity

Product	Amount Degraded, %	295 nm/ 254 nm	Error[a], %	275 nm/ 254 nm	Error[a], %
Intact	—	0.974	—	0.776	—
Acid degradation	65	0.963	−1.1	0.755	−2.7
Base degradation	81	0.964	−1.0	0.770	−0.8
Aqueous degradation	24	0.933	−4.2	0.783	+0.9
Dry powder degradation	27	0.984	+1.0	0.801	+3.2
Light degradation	28	0.969	−0.5	0.801	+3.2

[a] $\text{Error} = \frac{\text{test ratio} - \text{intact ratio}}{\text{intact ratio}} \times 100\%$.

The material was dissolved in 25 ml of methanol and diluted to volume with water.

Standard and Sample Preparation—Approximately 25 mg of vincristine sulfate standard or sample was accurately weighed (or an accurate volume of a sample solution containing ~25 mg of vincristine sulfate was pipeted) and transferred to a 100-ml volumetric flask. Exactly 10 ml of internal standard solution was added and the resulting solution was diluted to volume with water.

Assay Method—A 20-μl aliquot of the standard and of each sample was injected under the chromatographic conditions described. Standards were injected at the beginning, the middle, and the end of each run. Each sample was injected once. Samples and standards were not allowed to remain in solution more than 8 hr and were protected from light. Samples were calculated using a factor derived from the average of all standards injected. A typical chromatogram is shown in Fig. 1.

Calculations—Results were calculated as vincristine sulfate.

$$\text{Standard Factor} = \frac{\text{Standard Wt (mg)} \times \text{\% Purity} \times \text{Internal Standard Area}}{\text{Standard Area} \times \text{Internal Standard Wt (mg)} \times 100\%}$$

$$\text{Vincristine Sulfate (mg)} = \frac{\text{Standard Factor} \times \text{Sample Area} \times \text{Internal Standard Wt (mg)}}{\text{Internal Standard Area}}$$

$$\text{Vincristine Sulfate (mg/ml)} = \frac{\text{Standard Factor} \times \text{Sample Area} \times \text{Internal Standard Wt (mg)}}{\text{Internal Standard Area} \times \text{Volume Sample Taken (ml)}}$$

Degradation Procedure—Vincristine sulfate was degraded under the following conditions to test the procedure for specificity in the presence of vincristine degradation products:

1. A weighed portion of dry material was held at 85° for 80 hr in an open vial.
2. A portion of dry material was placed in an open quartz vessel and subjected to 2437-Å light[12] (~16,000 μW/cm²) for 80 hr.
3. An aqueous solution of the material at a concentration of 0.25 mg/ml was titrated to pH 2.0 with 2 *N* HCl and held for 2 hr at 85°.
4. An aqueous solution of the material at a concentration of 0.25 mg/ml was titrated to pH 11.5 with 2 *N* NaOH and held at room temperature (25°) for 1.5 hr. This solution was neutralized before injection.
5. An aqueous solution of the material at a concentration of 0.25 mg/ml was held unchanged (pH 7.5) at 85° for 4 hr.

In each case, degradation was allowed to proceed until ~25–75% of the vincristine sulfate had been degraded. Thus, some intact vincristine sulfate remained at the end of each time interval. Dry materials were dissolved in water at a concentration of 0.25 mg/ml. Separate injections were made of each of the degradation solutions with detection at 254 nm (vincristine sulfate maximum at 255 nm), 296 nm (maximum), and 275 nm (minimum) (1). Area ratios were then calculated for the peak assumed to be pure vincristine sulfate and these were compared with area ratios obtained from a freshly prepared solution of pure vincristine sulfate at the same wavelengths.

RESULTS AND DISCUSSION

The method originally used an eluant consisting of 50% methyl alcohol and 50% 0.01 *M* ammonium acetate adjusted to pH 4.0, and an octadecylsilane reverse-phase column[13]. However, peak shape was rather poor. It was found that the peak shape could be improved by lowering the pH to 2.0 with 10% v/v nitric acid. Preliminary examination of this method with a partially degraded vincristine sulfate solution indicated a lack of resolution between vincristine sulfate and an unknown degradation product. Addition of a 0.005 *M* solution of the sodium salt of heptanesulfonic acid and increasing the ammonium acetate concentration to 0.02 *M* improved the resolution, but not sufficiently. Switching from methyl alcohol to acetonitrile (which required an adjustment in the aqueous–organic ratio from 50:50 to 70:30 for adequate retention) greatly increased resolution. The resolution between vincristine sulfate and the unknown degradation product at this point was ~1.0. Although this may have been adequate, it was found that by changing from an octadecylsilane to a phenyl column, baseline separation could be achieved between these two compounds.

Table II—Relative Retention Times of Some Compounds of Interest

Compound	Relative Retention[a]
Vincristine sulfate	1.00
Vinblastine sulfate	1.57
Major acid degradation product	1.32
Major base degradation product	0.40, 0.51
Light degradation product	1.34
Benzyl alcohol	0.27
Parabens	
Methyl	0.40
Ethyl	0.55
Propyl	0.78
Butyl	1.20

[a] $\text{Relative retention} = \frac{\text{retention time of compound (min)}}{\text{retention time of vincristine sulfate (min)}}$

In the subsequent use of this system, it was found that minor changes in the acetonitrile concentration of the eluant produced major changes in the retention time of vincristine sulfate. To reduce this tendency, and also to shorten the retention time and further improve peak shape, the ion-pairing reagent was changed from a 0.005 *M* solution of the sodium salt of heptanesulfonic acid to a 0.005 *M* solution of the sodium salt of pentanesulfonic acid. This produced the system described above (*Experimental*), which was tested for specificity, linearity, precision, and accuracy.

Specificity—The forced degradation products of vincristine sulfate, which were produced as described above, were examined using the HPLC procedure to ensure that none would interfere with intact vincristine. The results (Table I) indicate that the forced degradation products do not interfere with the vincristine sulfate. A few of the results have 3–4% errors. If the three wavelengths had been monitored simultaneously for each injection, this might have been significant. However, each of these ratios is the result of two separate injections each of which has a precision of ~ ±2%.

Vincristine sulfate is isolated from extracts of the periwinkle plant; thus, isolated precursors probably are nonexistent. However, other *Vinca* alkaloids are possible interferences. One of these, vinblastine sulfate, was found to have a retention time of 12.1 min. Vinblastine sulfate differs structurally from vincristine sulfate only by the presence of a methyl group instead of an aldehyde moiety at the anilino-nitrogen in the vindoline portion of the molecule (1).

A number of possible formulation excipients were examined for interference with vincristine sulfate in this system:

1. Buffers: citrate–citric acid, acetate–acetic acid, and phosphate buffers at 0.1 *M* and 0.2 *M* did not interfere.
2. Sugars: lactose and mannitol at 1 mg/ml showed no interference.
3. Sodium chloride: sodium chloride did not interfere.
4. Benzyl alcohol: benzyl alcohol had a retention time of 2.2 min.
5. Parabens: none of the parabens (methyl, ethyl, propyl, or butyl) interfered with vincristine sulfate using this system. Table II shows the relative retention times of some compounds of interest using this system.

Linearity—Vincristine sulfate showed a linear response from 10–200% of the normal injection concentration. The linear correlation coefficient was found to be +0.99990, and the intercept was −0.06% of the response of the normal injection concentration. Thus single-point standardization was deemed to be adequate.

[12] Rayonet Photochemical Reactor, The South New England Ultraviolet Co., Middletown, Conn.

[13] Internal communication from J. T. Woolever, Analytical Research and Development Department, Bristol Laboratories, Syracuse, NY 13201.

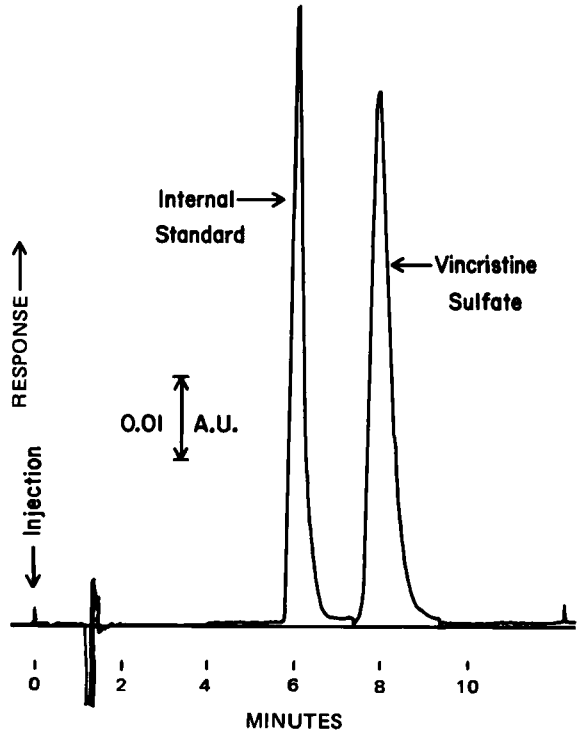

Figure 1—*Typical vincristine sulfate chromatogram with retention times.*

Chromatographic Precision—The chromatographic precision was determined by making six injections from a single freshly prepared solution of vincristine sulfate in water (0.25 mg/ml) on each of 3 days. The precision was calculated each day as two standard deviation units (95% confidence interval) relative to the mean expressed as a percentage. The chromatographic precision was taken to be the root mean square of the three relative 2σs, and was found to be ±2.3%.

Accuracy and Assay Precision—An estimate of the procedural accuracy was obtained by assaying six replicate weighings of vincristine sulfate *versus* a seventh weighing used as a standard. The reproducibility of the recoveries was used as an estimate of assay precision. Table III lists the results.

Stability in Diluent—The six solutions used for accuracy determination were held for 24 hr in the dark at room temperature (25°). They were then reassayed *versus* a fresh standard solution as an estimate of stability in diluent. As shown in Table IV, the average loss over 24 hr was ~5%. Therefore automated runs are feasible as long as vincristine sulfate is not held in solution for >8 hr and is protected from light.

In the routine use of this procedure, several precautions need to be taken. The mobile phase should be prepared with care, since small changes in the acetonitrile percentage produce large retention-time changes. However, as the column ages, small changes in the acetonitrile concentration may be necessary to maintain vincristine–internal standard resolution. Prior to beginning a run, the column should be conditioned with eluant for at least 30 min and several standard injections should be made until retention times are constant (usually ~3–5 injections). After use, the column should be rinsed with 30–50 ml of 1:1 v/v acetonitrile–water. Vincristine sulfate is extremely toxic and possibly carcinogenic; therefore, it should be handled with caution. Protective apparel should be worn, and all solutions (including spent HPLC eluant) should be disposed of properly.

Table III—Accuracy and Assay Precision

Sample	Calc. Wt, mg	Actual Wt, mg	Recovery[a], %
1	24.71	25.09	98.5
2	26.36	26.49	99.5
3	25.26	25.03	100.9
4	24.40	24.44	99.8
5	25.85	25.85	100.0
6	25.17	25.29	99.5

[a] Average is 99.7% with a relative 2σ value of ±1.57%.

Table IV—Stability in Diluent

Sample	Calc. Wt, mg	Actual Wt, mg	Recovery[a], %
1	23.88	25.09	95.2
2	24.81	26.49	93.6
3	24.26	25.03	96.9
4	23.28	24.44	95.2
5	24.81	25.85	96.0
6	23.89	25.29	94.5

[a] Average is 95.2%.

As a measure of system suitability, typical chromatographic parameters are shown below:

	Vincristine Sulfate	Propylparaben
Efficiency (plates/column)	1700	3500
HEPT[14]	0.018	0.008
Asymmetry	1.2	1.05
k'	7	5

The minimum acceptable values for vincristine sulfate are an efficiency of 1000 plates/column and a k' value of 6–10. In addition, there should be baseline separation between vincristine sulfate and propylparaben, the internal standard.

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[14] Height equivalent of a theoretical plate.