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Lyophilization of Sincalide and Correlation of Headspace Relative Humidity with Product Stability

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Abstract ☐ An optimum freeze-drying cycle was developed for use with industrial equipment in the processing in vials of a product degraded by traces of moisture. The relative humidity of the headspace gas of the vials, which contained only 5 μg of active ingredient, was monitored by a GC method and was then correlated with product stability.

Keyphrases ☐ Sincalide—lyophilization, optimum cycle developed, vial headspace relative humidity correlated with product stability ☐ Lyophilization—sincalide, optimum cycle developed, vial headspace relative humidity correlated with product stability ☐ Humidity, relative—in headspace of lyophilized vials of sincalide, correlated with product stability ☐ Stability—sincalide, lyophilized, correlated with headspace vial relative humidity

Lyophilization has become a standard procedure for processing labile ingredients in sterile pharmaceutical products. For substances susceptible to hydrolysis, freeze drying assures the final dried formulation a longer shelf-life at higher storage temperatures than would be possible in solution. The process usually yields a sterile plug of powder in a vial as a final product. When the plug has a small mass, it becomes difficult to determine the moisture.

Ordinarily, the freeze-drying process produces a product containing 1% or less moisture (1); in most cases, this amount is not harmful to the product. However, when small quantities of water have a deleterious effect, the moisture content of the product must be reduced to trace quantities. Consequently, alternative approaches must be devised to determine the moisture content present. This report discusses various procedures for the removal of virtually all water from vials by industrial scale lyophilization and a method for determining the trace amount of water remaining in the sealed vials.

The small quantity of sample and the low moisture levels anticipated made the use of the Karl Fischer method inappropriate for water determination without modification. Based on the work of Litchman and Upton (2), the estimation of in-vial water content through headspace GC was developed. This technique allowed headspace relative humidity to be determined without disturbing the sample environment. The headspace humidity value, supplemented by product stability (bioassay), provided the means for process control.

EXPERIMENTAL

Processing (Lyophilization)—Vials (5 ml, 13-mm neck) were filled with 2.1 (±1.5%) ml of a sterile aqueous solution containing 21.43 mg of sodium chloride/ml for a lyophilization trial. About 9000 filled vials were stoppered with siliconed, notched, sterile butyl rubber closures in the raised position; they were then placed in trays and spread through a 90-tray capacity sterile industrial lyophilizer[1] (Lot A). Ten empty vials similarly prepared were also placed in each tray as controls (Lot B).

In addition, "active" vials were each filled with 2.1 ml of an aqueous solution containing 2.5 μg of sincalide[2] (I)/ml and 21.43 mg of sodium chloride/ml. These active vials were placed with the two other categories in trays in the bottom, middle, and top of the lyophilizer (Lot C). Each of the total of 42 trays was numbered and recorded by shelf location.

Prior to filling of the vials, the eutectic temperature of the active solution was found to be −21.8 ± 0.3° by differential scanning calorimetry. This value compared well with the eutectic reported for sodium chloride, −21.6° (3). To ensure freezing of the liquid beyond the effects that supercooling might induce, the shelves of the lyophilizer were held at −60° for 12 hr.

A vacuum was then drawn. The following drying cycle was immediately begun and continued for 72 consecutive hr: −30° (23 hr), +35° (31 hr), and +50° (8 hr). Transitional periods accounted for 10 hr. During the final hour, the temperature was adjusted to +25°. The shelf temperatures and chamber pressures actually obtained during the lyophilization are given in Table I.

At the conclusion of the cycle, with the temperature at +25°, the evacuated chamber was vented with dry nitrogen. As a flushing operation, the venting was followed by a reevacuation and finally by another venting of the chamber with dry nitrogen. At this point, the vials were closed with the stoppers, inside the lyophilizer, under slight vacuum. After stoppering, the chamber was opened, the trays were removed, and the vials were sealed with crimped aluminum caps. Each tray held ~231 vials, and samples were taken at random for moisture determination.

In optimizing the freeze-drying procedure, other cycles were employed. Lots D and E were lyophilized (after freezing) in laboratory equipment[3] for 42.5 hr: −28° (1.5 hr), slow transition (24 hr), and +30° (17 hr). Lot F was lyophilized in laboratory equipment[3] for 72 hr: −30° (24 hr), slow transition (24 hr), +22° (21 hr), and transition to +35° (3 hr). Lot G was lyophilized in the industrial equipment[1] for 72 hr: transition from −45 to −27° (4 hr), −27° (20 hr), slow transition from −27 to +25° (12 hr), +25° (34 hr), and transition to +35° (2 hr). Lot H-1 was lyophilized in laboratory equipment[3] for 72 hr, approximating the cycle of Lot F.

Humidity Determination—The headspace humidity determination was based on the GC estimation of water in a headspace gas sample. The

[1] Model 179FXS350, Hull Corp., Hatboro, Pa.
[2] L-Aspartyl-L-tyrosyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartylphenyl-L-alaninamide (hydrogen sulfate). The trade name for this gallbladder contractile agent is Kinevac (Squibb).
[3] Model 2004-LLX3, F. J. Stokes Machine Co., Philadelphia, Pa.

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Table I—Details of the Lyophilization Cycle for Production of Vials with Low Relative Humidity (Lots A, B, and C)

Hours	Shelf Temperature	Average Product Temperature	Chamber Pressure, μ	Remarks
0	−55°	−54°	—	Product had been frozen overnight; vacuum in chamber begun
1.5	−26°	−41°	140	
2.5	−25°	−39°	140	
5	−25°	−37°	140	
6	−25°	−37°	140	
9	−25°	−35°	140	
11	−25°	−34°	140	
13	−25°	−32°	140	
24	−25°	−27°	140	Low heat applied to shelves
25	−16°	−23°	—	
26	−4°	−13°	—	
27	2°	−5°	—	
28	9°	−1°	—	
29	33°	15°	140	
30	35°	29°	132	
31	35°	30°	120	
32	36°	33°	110	
33	34°	32°	109	
34	35°	32°	105	
35	34°	32°	100	
36	34°	32°	100	
47	34°	32°	95	
60	34°	33°	95	High heat applied to shelves
61	45°	37°	95	
62	48°	45°	95	
70	52°	47°	96	Shelf cooling applied
72	27°	32°	94	Vacuum discontinued
73	27°	31°	—	Stoppers set into vials

water was quantitated against that found under identical conditions in the headspace of salt solutions prepared to yield a progression of humidities specifically for calibration.

Preparation and Use of Calibration Standards—Four or more saturated aqueous salt solutions were prepared in individual 500-ml septum closure bottles (Table II). Each contained ~100 ml of solution and excess salt. The bottles were sealed at atmospheric pressure and allowed to equilibrate at room temperature for at least 48 hr before use.

The humidity standards were retained equilibrated indefinitely. The creation of a small negative pressure within the bottles following sample withdrawals, however, necessitated readjustment to atmospheric pressure. The momentary insertion of a hypodermic needle into the septum closure of each bottle following a day's use accomplished this readjustment. Reequilibration occurred overnight.

GC—A gas chromatograph[4] equipped with a glass column using on-column injection, a thermal conductivity detector, and a 1-mv recorder[5] constituted the system.

A 1-m glass column, 6 mm i.d., was packed with 50–80-mesh Poropak Q[6]. It was conditioned prior to use according to the manufacturer's instructions. Daily reconditioning for 0.5 hr was generally not necessary but was advisable.

Operating conditions were: column temperature, ~100°[7]; detector temperature, 200°; detector current, >200 mamp; carrier gas, helium, 45–50 ml/min; and gas sample, 0.5 ml.

Sampling—The headspace gas sampling of both calibration standards and samples was accomplished with a gas-tight syringe. For this purpose, a plastic 1-ml tuberculin syringe[8] equipped with a 2.54-cm 26-gauge needle was utilized. The standard gas-tight syringes[9] were unsatisfactory. Excessive deadspace and, more important, the reversible adsorption of moisture by the glass barrel led to cross-contamination and significant analytical bias.

The sampling procedure consisted of two steps. First, the syringe was thoroughly flushed with either dry helium or nitrogen to clear it of atmospheric moisture. Upon expulsion of the drying gas, the syringe needle was plunged through the septum of the vessel to be sampled. Second, the syringe was pumped slowly four or five times, filled to ~0.6 ml, and withdrawn from the sample container. The volume was adjusted to exactly 0.5 ml prior to injection into the chromatograph.

A complete chromatographic run required about 2 min. All GC analyses were in triplicate from each sample, and the average peak height of the three samplings was used in computation. Typical chromatographic curves are displayed in Fig. 1, representing 11 and 75% relative humidities (RH).

Calibration and Quantitation—Headspace gas samples of the calibration standards were taken and chromatographed as specified. A response plot (peak height *versus* relative humidity) was constructed. A typical calibration curve gave a slope of 1.20, an intercept of −0.7, and a correlation coefficient of 0.999. The small variable intercept found may be indicative of constant atmospheric moisture contamination during sampling or simply the experimental error of the method.

Quantitation was achieved by reading the relative humidities of the unknown directly from a calibration curve constructed prior to or immediately following the samples. Because of the sensitivity of the water peak height to changes in experimental conditions, it was imperative that the conditions remain constant throughout the entire calibration–sample sequence.

Precision and Accuracy—The precision of standard or sample replicates varied with relative humidity. At 11% RH, it was within ±4%; at 30% RH, it was ±3%; and at 50% RH, it was ±1%. Precision did not improve greatly at relative humidities above 50%.

The accuracy of the measurements is demonstrated in Table III

Table II—Saturated Salt Solution: Humidity Relationships at 25° (4)

Aqueous Saturated Salt Solution	Headspace Relative Humidity, %
Lithium chloride	11
Calcium chloride hexahydrate	29
Magnesium nitrate hexahydrate	52
Sodium chloride	75

Table III—Atmospheric Relative Humidity Determinations

Percent by GC	Percent by Sling Psychrometry
36	40
44	40
59	62

[4] Model K-2, Burrell Corp., Pittsburgh, Pa.
[5] Brown Instruments, Division of Minneapolis Honeywell Corp., Philadelphia, Pa.
[6] Waters Associates, Framingham, Mass.
[7] The temperature was adjusted so that the retention time to the front of the water peak was 0.6 min.
[8] Stylex tuberculin syringe, no needle, Z020, Pharmaseal Laboratories, Glendale, Calif.
[9] Hamilton.

Table IV—Moisture Content of Formulated Sincalide as a Function of Headspace Relative Humidity

Humidistat Relative Humidity, %	Headspace Relative Humidity (GC), %	Powdered Sample Moisture (TGA), %
0	6	0
11	16	0
23	26	0
33	35	0.01
52	53	0.05
67	69	0.08
79	84	33.8

through a comparison of atmospheric relative humidity measured simultaneously by GC and sling psychrometry.

Equilibrium Moisture Content: Sodium Chloride—Duplicate 1-g samples of lyophilized sodium chloride were weighed accurately and placed in desiccators. Each desiccator contained the appropriate saturated salt solution to maintain the atmosphere within the desiccator at the desired relative humidity. After 25 days at 22°, the samples were again weighed and the percent change in weight from the initial was calculated. The values obtained for the duplicate samples within a desiccator were averaged.

Equilibrium Moisture Content: Sincalide—An electrobalance[10] was used to determine the change in weight of a sample under various humidity conditions. The sample weighing pan was hung from the balance beam into a glass cylinder with two air entry ports and one air exit port. The tare weighing pan was hung in a closed glass cylinder.

To maintain the humidity of the weighing chamber, a compressed air stream was split in two. One stream was passed through a tower containing an indicating desiccant[11] to ensure that the humidity was less than 1%. The second stream was passed through a diffusion tube into a tower containing distilled water. The stream exiting this tower was composed of high relative humidity air. The two flows were then recombined in a desiccator into one stream.

A hygrometer sensing probe[12] was placed in the desiccator for measurement of the relative humidity of the air before it entered the weighing chamber. The humidity of the air was regulated by varying the ratio of the air going through each tower. The air was then passed through a flowmeter and again split into two streams. The two streams were fed through the entry ports into the weighing chamber above and below the sample. The air then passed out through the exit port to a second hygrometer[13] where the humidity was again measured.

A sample of I (approximately 4.5 mg) was placed on the sample pan. Anhydrous air was then passed over the sample for 48 hr to ensure equilibration of the sample with the atmosphere. The sample weight was recorded after equilibration and after each subsequent reequilibration. Low humidity air was then passed into the weighing chamber and equilibrated with the sample. After equilibrium was established, the air entering the weighing chamber was increased to a new relative humidity. The anhydrous weight of the sample was redetermined after equilibration at each relative humidity.

This procedure was repeated for the system without any sample. In the range of 0–60% RH, the blank weight varied up to a maximum of 6.8 μg or approximately 0.2% of the sample weight.

RESULTS AND DISCUSSION

The degradation of I was first studied under conditions of controlled relative humidity. Degradation in excess of 40% after storage of I at 46 and 76% RH was found. Only minor changes were detected at 0% RH[14].

With the role of moisture in I degradation established, the total volatiles in lyophilized plugs of formulated I were determined by thermal gravimetric analysis. The formulated plugs contained sodium chloride, 45 mg, and I, 5.25 μg. per 5-ml vial. The moisture values found in the plugs by thermal gravimetric analysis ranged from 0 to 33.8% (*i.e.*, to 15 mg) (Table IV), but the method was not sensitive enough to differentiate among plugs of the formulation when the moisture present was at the desired very low magnitudes. For example, the moisture present in the plug at 67% RH was only 0.08% or 36 μg of moisture.

Table V—Equilibrium Moisture Contents of Bulk Sincalide (Nonformulated)

Relative Humidity, %	Moisture, %
5	1.6
12	2.2
14	2.8
20	3.6
30	4.2
42	4.8
49	5.2

Table VI—Equilibrium Moisture Contents of Lyophilized Sodium Chloride

Relative Humidity, %	Increase in Weight from Initial, %
0	0.037
11	0.067
23	0.083
33	0.060
42.5	0.022
67	0.068
79	200.0

Theimer and Pavelek (5) used the Karl Fischer method to determine the moisture content in small plugs of lyophilized mannitol and acetylcholine chloride in vials. This work was done on vials containing 0.50–1.63 mg of water each, a much greater amount than is present in the lyophilized formulation of I. Since the precision of the reported data was already inadequate to achieve the accuracy required here and since the precision probably would deteriorate at lower levels, this line of investigation was also not pursued.

The relative humidity of the headspace gas of the vials containing the formulated plug was then explored as an indicator of moisture conditions within a vial. It was assumed that a stable product containing I could be obtained if the relative humidity of the headspace gas was maintained at a low level. Determination of the equilibrium moisture content of I alone was proportional to the relative humidity. The equilibrium moisture content of the sodium chloride in the formulation was essentially independent of the relative humidity at relative humidities of 67% or less. The equilibrium moisture contents of I and sodium chloride at various relative humidities are shown in Tables V and VI, respectively.

The headspace moisture is in equilibrium with the moisture in the plug in a vial. Because of the hygroscopic nature of I compared to the other ingredient, sodium chloride, a relatively larger percent of the moisture in a plug is associated with I. This condition causes I to become vulnerable to degradation by hydrolysis even at exceptionally low levels of moisture in the vials. The level of moisture that may be tolerated in the vials, as shown by the maintenance of the potency of I, was determined by a number of optimizing trials. The trials provided vials containing the formulated product with headspace relative humidities of a few percent to as high as 88%. For example, the relative humidity of the headspace gas in the plant lyophilized vials at the conclusion of the 72-hr cycle described in Table I was determined for the three categories of vials in the chamber. Results are given in Table VII.

As shown, the values for these plant-processed vials were at 5% RH or below. Since the values found for the "active" vials (Lot C) and the pla-

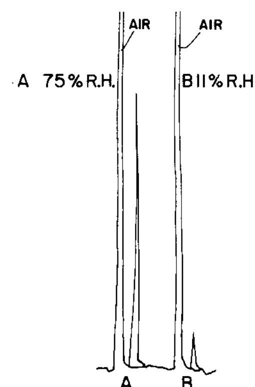

Figure 1—*Typical chromatographic curves.*

[10] Model RG, Cahn Division, Ventron Instruments Corp., Paramount, Calif.
[11] Drierite, Hammond Drierite Co., Zenia, Ohio.
[12] Electric hygrometer, Hygrodynamics, Inc., Silver Spring, Md.
[13] Hygroline, Beckman Instruments, Irvine, Calif.
[14] O. Kocy, Squibb Institute for Medical Research, unpublished data.

Table VII—Relative Humidity of the Headspace Gas in Vials Lyophilized by the Optimum 72-hr Cycle

Lot	Shelf Number in Chamber	Tray Number	Vials Tested per Tray	Relative Humidity in Headspace Gas of Vials[a], %
A	1 (bottom)	1, 4	2	All 3
A	2–11	7, 10, 12, 15, 17, 20, 23, 27, 31, 34, 36, 38	2	All 3
A	12 (top)	42	2	Both 3
B	1	4	3	All 3
B	6	20	4	3, 3, 3, 4
B	12	42	3	4, 4, 3
C	1	4	3	5, 4, 4
C	6	20	4	4, 4, 4, 3
C	12	42	3	4, 3, 4

[a] The nitrogen found was 99.5%/vial.

Table VIII—Comparison of Percent Relative Humidity Found with Potency of Stability Samples in Vials

Months Stored	Sample Lot Number	Storage Temperature	Relative Humidity, % (Number of Replicates in Parentheses)	Sincalide Bioassay, % of Theory Relative to Standard
0	C	—	3–5 (10)	130
9	C	22°	10–17 (10)	105
0	D	—	—	96
12	D	22°	26, 30	98
12	D	40°	25	91
20	D	22°	26–29 (10)	97
20	D	33°	26–29 (10)	91
36	D	22°	26–29 (10)	97
0	E	—	—	121
12	E	22°	22, 34	131
12	E	40°	20	119
20	E	22°	25–27 (10)	119
20	E	33°	26–29 (10)	117
36	E	22°	24–30 (9) 32 (1)	112
0	F	—	—	125
12	F	22°	20–27 (3)	105
12	F	40°	—	98
19	F	22°	20–26 (10)	103
19	F	33°	22–24 (10)	110
36	F	22°	26–30 (10)	98
0	G	—	55–58 (6)	99
6	G	22°	—	81
6	G	40°	—	70
13	G	22°	52–61 (30)	—
16	G	−20°	—	83
16	G	5°	51–59 (10)	84
0	H-1	—	—	106
25	H-1	22°	19–29 (12)	103
3	H-2[a]	22°	46–47 (12)	114
14	H-2[a]	22°	34–35 (4)	94
3	H-3[b]	22°	81–88 (12)	66
14	H-3[b]	22°	54–72 (4)	36

[a] Opened vials of Lot H-1 were subjected to moderate humidity, sealed, stored, and tested at the time period shown. [b] Opened vials of Lot H-1 were subjected to high humidity, sealed, stored, and tested at the time period shown.

cebo vials (Lot A) were virtually no higher than those found for the empty vials (Lot B), drying of the product apparently had reached an end-point and could not be improved by extending the length of the cycle or applying greater heat to the shelves. Although the initial freeze-drying period for the product at −30° might be considered overlong (23 hr), it did allow drying to proceed without collapse of the matrix due to vibration in the lyophilizer and thus afforded a freer flow of vapor from the solids.

Furthermore, with this drying cycle, there was no evidence that the location of the vials in the trays or of the trays on the shelves affected moisture retention. Therefore, the lyophilization cycle was felt to be suitable.

The relative humidity of the headspace gas, reflecting the moisture content of the lyophilized plug within the sealed vials, also correlated well with the stability profile of formulated I.

The bioassay for I consists of determining the potency by giving graded doses of unknown and of reference standard intravenously to anesthetized guinea pigs and statistically comparing the recorded contractile responses of the gallbladder *in situ* (6–8). The special skill and care required by the operator during the *in vivo* assay on the excised animals and the time-consuming nature of the operation made it necessary to use the bioassay as sparingly as possible. In addition, variables inherent in such a bioassay required many replicates to determine accurately the potency of any one sample. Usually, 24 injections had to be made for each bioassay result independent of the administration of the reference standard. Thus, the relative humidity may be a better predictor of stability than any single bioassay value. The substitution may be made in view of the correlation of the relative humidity values and the potency (Table VIII).

Table VIII shows the relationship between the range of the relative humidity values found for the headspace gas of vials and the corresponding bioassays after various periods of stability storage. A minimum potency of 85% of theory by bioassay meets the adopted specification for stability compliance of the product.

As the results indicate, the potency was maintained at approximately the initial activity for at least 18 months at 22° in those instances where the headspace relative humidity for the lot was below 30%. However, activity had decreased at higher humidities. Therefore, an upper limit of not more than 30% RH has been specified for the headspace gas of vials for product acceptance.

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GLC–Mass Spectrometry of Several Important Anticancer Drugs I: Pertrimethylsilylation and *O*-Methoxime Formation

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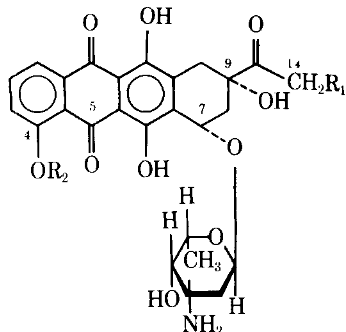
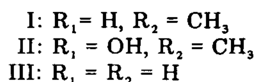
Received July 5, 1977, from the *School of Pharmacy, University of Southern California, Los Angeles, CA 90033.* Accepted for publication January 4, 1978.

Abstract ☐ Procedures are reported for the formation of pertrimethylsilyl and pertrimethylsilyl methoxime derivatives of the aglycones of doxorubicin, daunorubicin, carminomycin, chromomycin A_3, and mithramycin. The mass spectra are consistent with the formation of these derivatives. Fragmentation patterns highly specific for these derivatives are proposed, and the potential application for the identification of metabolites of these compounds is discussed.

Keyphrases ☐ Doxorubicin—GLC–mass spectral analysis, bulk drug ☐ Daunorubicin—GLC–mass spectral analysis, bulk drug ☐ GLC–mass spectrometry—analyses, doxorubicin, daunorubicin, and other antineoplastic agents, bulk drug ☐ Antineoplastic agents, various—GLC–mass spectral analyses in bulk drug

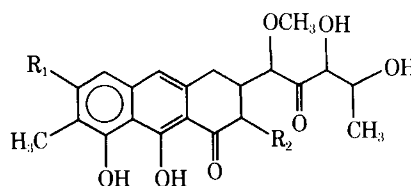
Doxorubicin[1,2] (I), daunorubicin[1,3] (II), carminomycin[1,4] (III), chromomycin A_3[1,5] (IV), and mithramycin[1,6] (V) are naturally occurring antibiotics (1–6) which possess significant antineoplastic activities. The first three are structurally related, and the last two differ only in the sugar moieties (6). Both I and V have been marketed in the United States. Of these five drugs, I is the most important clinically and has proven to be extremely effective against various tumors (7–9).

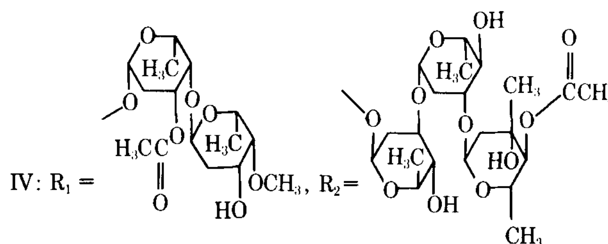


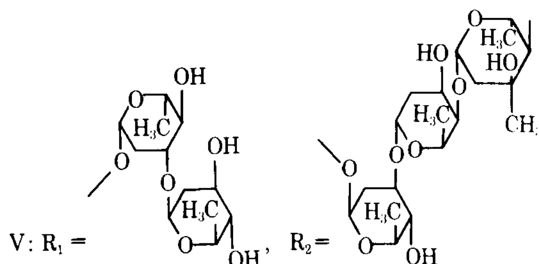


The disposition of I and II in animals and humans has been studied in this and other laboratories (1–14). Of major concern has been the development of sensitive, specific analytical procedures for I in physiological fluids and the identification of its metabolites.

GLC–mass spectrometry is one of the most effective instrumental methods for the separation and identification of compounds extracted from biological fluids. The relative retention times and the supplemental mass fragmentographic data provide for the positive characterization of unknown compounds. No reports have yet been published







[1] Supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.
[2] Adriamycin hydrochloride, NSC-123127, Adria Laboratories.
[3] Daunomycin, rubidomycin, NSC-82151.
[4] NSC-180024.
[5] NSC-58514, Toyomycin, Takeda Chemical Industries, Osaka, Japan.
[6] NSC-143020, Mithracin, Pfizer.

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