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Antitumor Agents XXX: Evaluation of α-Methylene-γ-lactone-Containing Agents for Inhibition of Tumor Growth, Respiration, and Nucleic Acid Synthesis

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Abstract □ Evidence is presented that a number of sesquiterpene lactones isolated from plants and synthesized pyrimidines containing the α-methylene-γ-lactone moiety are potent inhibitors of Walker 256 carcinosarcoma and Ehrlich ascites tumor growth and marginal inhibitors of P-388 lymphocytic leukemia and Lewis lung tumor growth. *In vitro* aerobic basal respiration and oxidative phosphorylation processes of Ehrlich ascites cells were inhibited by these agents as well as deoxyribonucleic acid polymerase and thymidylate synthetase enzymatic activities. These studies indicate that the α-methylene-γ-lactone moiety, the β-unsubstituted cyclopentenone ring, and the α-epoxycyclopentanone system are the essential moieties for inhibition of these biochemical parameters.

Keyphrases □ α-Methylene-γ-lactone-containing agents—evaluated for effect on tumor growth, respiration, and nucleic acid synthesis □ Antineoplastic activity—various α-methylene-γ-lactone-containing agents evaluated □ Enzyme activity, tumor—effect of various α-methylene-γ-lactone-containing agents evaluated □ Nucleic acid synthesis, tumor—effect of various α-methylene-γ-lactone-containing agents evaluated □ Structure–activity relationships—various α-methylene-γ-lactone-containing agents evaluated for effect on tumor growth, respiration, and nucleic acid synthesis

Recently (1–13), some sesquiterpene lactones and related analogs were isolated or synthesized. A few of these compounds were shown to be potent antitumor agents of Walker 256 ascites carcinosarcoma (Sprague–Dawley rats at 2.5 mg/kg/day) and Ehrlich ascites tumor (CF_1 male mice at 33.3 mg/kg/day) and marginally active against P-388 lymphocytic leukemia (DBA/2 mice at 25 mg/kg/day) (5, 6). Helenalin, tenulin, and eupahyssopin were

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shown to be effective inhibitors of deoxyribonucleic acid and protein synthesis, deoxyribonucleic acid polymerase enzymatic activity (5, 6), and basal and adenosine diphosphate stimulation respiration (14) of Ehrlich ascites cells.

A positive correlation between aerobic respiration inhibition and antitumor–antimitotic activity was observed for a number of nonrelated compounds, *e.g.*, a podophyllotoxin derivative[1], ellipticine (15), 4,6-diaminotriazines, methotrexate, naphthoquinone derivatives, tritylthioalanine, carminomycin, piperazinedione (14), 5-fluorinated pyrimidine-6-carboxaldehydes (16), benzamalecene, and triparanol (17). This paper reports a comparison of the effects of sesquiterpene lactones and related analogs on tumor growth, nucleic acid synthesis, and aerobic respiration.

EXPERIMENTAL

***In Vivo* Tumor Screens**—In the Ehrlich ascites screen, 10^6 cells were implanted intraperitoneally on Day 0. Compounds were suspended in 0.05% polysorbate 80 and homogenized to obtain a fine suspension. Each compound was injected intraperitoneally daily (1 mg/day) at 33.3 mg/kg/day into CF_1 male mice (~30 g). On the 7th day, the mice were sacrificed, and the total volume of ascites fluid and packed cell volume (ascites-crit) was determined to calculate the percent inhibition (18). Mercaptopurine was used as a positive standard.

In the Walker 256 ascites carcinosarcoma screen, 10^6 tumor cells were implanted intraperitoneally into Sprague–Dawley male rats (80 ± 10 g). In the P-388 lymphocytic leukemia screen, 10^6 cells were implanted intraperitoneally into DBA/2 male mice (~20 g) on Day 0. In the Lewis lung carcinoma, 2×10^6 cells were implanted intramuscularly in a hindleg of $C_{57}BL/6$ male mice (~25 g). Test compounds were injected intraperitoneally daily at 2.5 mg/kg/day for rats and 25 mg/kg/day for mice. The treated/control (T/C) values for the average days survived were calculated according to National Institutes of Health protocols (19).

The isolation or synthetic methods for the sesquiterpene lactones or related analogs were reported previously and are referred to in Table I under the column noted as "Source of Material."

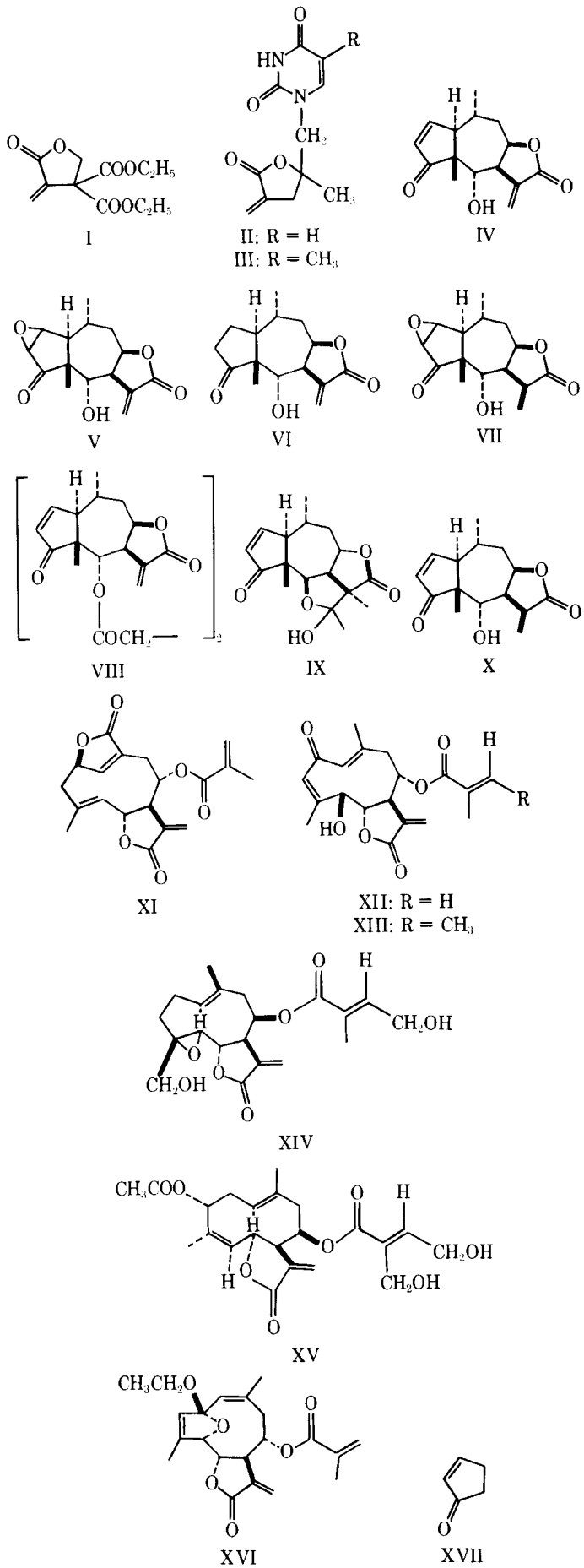
***In Vitro* Tumor Respiration Studies**—Cells for oxidative phosphorylation studies were collected on the 7th day for Ehrlich ascites tumors and the 10th day for Walker 256 and P-388 tumors. Oxygen consumption was measured with a Clark electrode connected to a Gilson oxygraph. The reaction vessel typically contained 55 μmoles of sucrose, 22 μmoles of monobasic potassium phosphate, 22 μmoles of potassium chloride, 90 μmoles of succinate or 60 μmoles of α-ketoglutarate as the substrate, 2 μmoles of adenosine triphosphate, and 0.75 μmole of the test compound in a total volume of 1.8 ml. After the basal metabolic (state 4) level was obtained, 0.257 μmole of adenosine diphosphate was added to obtain the adenosine diphosphate-stimulated respiration rate (state 3) (20).

Enzyme Studies—*In vitro* DNA polymerase activity of 7-day Ehrlich ascites cells was determined on isolated nuclei from the ascites cells prepared by a literature method (21). The incubation method was that described by Sawada *et al.* (22) except that 2-^{14}C-deoxythymidine triphosphate (1–45 Ci/mmole) was used with 0.75 μmole of test compound in a total volume of 0.35 ml. The insoluble nucleic acids were precipitated with 1 *M* perchloric acid containing 1 mmole of tetrasodium pyrophosphate monohydrate and were collected on glass fiber paper[2] by vacuum suction (22).

In vitro thymidylate synthetase activity was determined by the method of Kampf *et al.* (23), utilizing a postmitochondrial supernate (9000×*g*) from 7-day Ehrlich ascites cells, 0.75 μmole of the test compound, and 5 μCi of ^{3}H-5′-deoxyuridine monophosphate (11 Ci/mmole) in a total volume of 0.1 ml. Preliminary studies indicated that 0.75 μmole *in vitro* resulted in the same magnitude of inhibition of deoxyribonucleic acid polymerase activity as previously demonstrated *in vivo* (5).

RESULTS

All compounds tested in the Ehrlich ascites tumor screen caused at



[1] VM-26.
[2] Whatman GF/F.

Table I—Antitumor Activity of Sesquiterpene Lactones and Related Analogs in Rodents

	Compound (n = 6)[a]	*In Vivo* Antitumor Activity: Ehrlich Ascites, % Inhibition	Walker 256 Carcinosarcoma, T/C	Lewis Lung, T/C	P-388 Lymphocytic Leukemia, T/C	Percent Control *In Vitro* Ehrlich Ascites: DNA Polymerase Activity	Thymidylate Synthetase Activity	Source of Material (Reference)
I	α-Methylene-β,β-di(ethoxycarbonyl)-γ-butyrolactone	98	140	—	—	44 ± 11[b]	—	1
II	Uracil lactone	100	131	—	115	29 ± 4[b]	—	2
III	Thymine lactone	99	127	—	127	38 ± 11[b]	79 ± 7[b]	2
IV	Helenalin	99	316	142	127	35 ± 2	49 ± 8[b]	3
V	2,3-Epoxyhelenalin	99	145	—	117	44 ± 4[b]	70 ± 14[b]	4
VI	2,3-Dihydrohelenalin	64	134	—	110	64 ± 19[c]	83 ± 3[b]	4
VII	11,13-Dihydro-2,3-epoxyhelenalin	89	264	—	134	77 ± 11[c]	79 ± 9[b]	4
VIII	Dimeric helenalin[d]	100	187	142	168	39 ± 2[b]	56 ± 2[b]	—
IX	Tenulin	97	266	126	131	83 ± 9[c]	56 ± 11[b]	5
X	Plenolin	100	207	—	138	74 ± 7[b]	73 ± 13[c]	7
XI	Deoxyelephantopin	99	226	—	123	67 ± 7[b]	73 ± 19[e]	8
XII	Molephantin	75	149	80	118	55 ± 6[b]	81 ± 18[f]	9
XIII	Molephantinin	88	397	123	146	37 ± 5[b]	77 ± 9	10
XIV	Eupahyssopin	93	330	132	147	34 ± 5[b]	63 ± 7[b]	11
XV	Eupaformosanin	—	471	—	147	72 ± 10[b]	69 ± 4[b]	13
XVI	Phantomolin	87	378	123	113	38 ± 4[b]	72 ± 14[c]	12
XVII	Cyclopentenone	75	188	—	—	43 ± 4[b]	65 ± 8[b]	—
XVIII	Mercaptopurine	99	—	—	—	—	—	—
XIX	Fluorouracil	—	—	—	186	—	39 ± 7[b]	—
XX	Melphalan	96	317	—	168	—	—	—
XXI	Cyclophosphamide	—	—	140	—	—	—	—
XXII	Iodoacetate	—	—	—	—	41 ± 4[b]	—	—
	0.05% polysorbate 80	0	100	100	100	100 ± 5[g]	100 ± 3[h]	—

[a] n = number of animals per group. [b] $p = 0.001$. [c] $p = 0.005$. [d] The synthesis of VIII is unpublished. [e] $p = 0.010$. [f] $p = 0.025$. [g] 47,424 dpm/mg of DNA. [h] 103,328 dpm/mg of protein.

least 60% inhibition of tumor growth. Compounds I–V, VIII–XI, and XIV inhibited Ehrlich ascites tumor growth greater than 90% (Table I). All compounds tested with the Walker 256 ascites carcinosarcoma were active; *i.e.*, the T/C >125. Compounds VII and IX–XI doubled the life expectancy, and IV, XIII, XIV, and XVI tripled the survival time of these animals; XV improved it four times. Of the compounds tested in the Lewis lung, only IV and VIII were active (T/C = 140 required for activity). In the P-388 lymphocytic leukemia screen, III, IV, VII–X, and XIII–XV demonstrated a T/C >125.

The basal respiration (state 4) was significantly reduced by all compounds tested with both succinate (flavin adenine dinucleotide-linked dehydrogenase) (Table II) and α-ketogluturate (nicotinamide adenine dinucleotide-linked dehydrogenase) for Ehrlich, P-388 leukemia, and Walker 256 cells (Table III). The oxidative phosphorylation processes, *i.e.*, coupled adenosine triphosphate synthesis, were all significantly suppressed by all compounds tested for all three types of cells. *In vitro* deoxyribonucleic acid polymerase activity of Ehrlich ascites cells was inhibited drastically (>50%) by I–V, VIII, XI, XIII, XIV, XVI, XVII, and XXII at 0.75 μmole. *In vitro* thymidylate synthetase activity was inhibited significantly by all compounds tested. Compounds IV, VIII, and IX demonstrated at least 40% inhibition, and XIV, XV, XVII, and XIX showed at least 30% inhibition at 0.75 μmole.

The probable (p) significant difference was determined by the Student t test. Data are expressed in Tables I–III as percent control of average mean and standard deviation.

DISCUSSION

In view of the wide diversity of structural components of I–XVII, it can be surmised that an α-methylene-γ-lactone moiety, a β-unsaturated cyclopentenone ring, or an α-epoxycyclopentanone system is one essential structural requirement for significant *in vivo* antitumor activity in Ehrlich and Walker 256 carcinosarcoma tumors. This conclusion is consistent with the previous observation that any one of these three functions is essentially responsible for the *in vitro* cytotoxic activity against H.Ep-2 cells (4, 24–28).

Compounds IX and X, which contain only the cyclopentenone ring of these three functional groups, must also possess some other characteristic in the molecule that adds to the antitumor activity since they were more active against Walker 256 and Ehrlich ascites tumor growth than the cyclopentenone ring alone (XVII). The presence of the allylic ester side chain, *e.g.*, in the germacranolides, molephantinin (XIII), eupahyssopin (XIV), eupaformosanin (XV), and phantomolin (XVI), may enhance antitumor activity, perhaps facilitating the membrane transport of these

Table II—*In Vitro* Effects of Antitumor Agents on Basal and ADP-Stimulated Respiration (Microliters of Oxygen per Hour per Milligram of Protein) Using Sodium Succinate as the Substrate (Percent Control)

Compound (n = 6)	Walker 256 State 4	Walker 256 State 3	P-388 Leukemia State 4	P-388 Leukemia State 3	Ehrlich Ascites State 4	Ehrlich Ascites State 3
I	47 ± 5[a]	50 ± 4[a]	77 ± 7[a]	70 ± 13[b]	55 ± 8[a]	49 ± 3[a]
II	—	—	—	—	—	—
III	—	—	—	—	74 ± 9[a]	81 ± 7[c]
IV	52 ± 9[a]	73 ± 9[a]	63 ± 14[a]	53 ± 20[a]	72 ± 8[a]	60 ± 8[a]
V	60 ± 9[a]	72 ± 5[a]	69 ± 8[a]	62 ± 17[a]	66 ± 8[a]	77 ± 11[c]
VI	36 ± 10[a]	42 ± 4[a]	83 ± 9	62 ± 13	49 ± 7[a]	59 ± 5[a]
VII	75 ± 14[c]	80 ± 7[c]	87 ± 2[b]	76 ± 3[a]	78 ± 8[a]	84 ± 4[c]
VIII	75 ± 7[a]	80 ± 9[c]	62 ± 13[a]	52 ± 15[a]	59 ± 7[a]	64 ± 6[a]
IX	36 ± 8[a]	70 ± 7[a]	51 ± 9[a]	65 ± 22[c]	52 ± 6[a]	64 ± 5[a]
X	62 ± 7[a]	75 ± 5[a]	62 ± 19[c]	58 ± 16[a]	65 ± 10[a]	52 ± 14[a]
XI	65 ± 6[a]	69 ± 6[a]	73 ± 5[a]	70 ± 8[a]	69 ± 12[a]	75 ± 3[a]
XII	48 ± 5[a]	50 ± 7[a]	69 ± 7[a]	69 ± 6[a]	68 ± 5[a]	55 ± 5[a]
XIII	64 ± 7[a]	82 ± 6[c]	67 ± 17[c]	66 ± 16[c]	64 ± 9[a]	75 ± 8[a]
XIV	—	—	—	—	82 ± 11[c]	87 ± 5[c]
XV	—	—	—	—	69 ± 12[a]	69 ± 9[a]
XVII	—	—	—	—	78 ± 4[c]	74 ± 3[a]
0.05% polysorbate 80	100 ± 5	100 ± 8	100 ± 8	100 ± 11	100 ± 3	100 ± 4
Control	4.494	5.506	4.196	4.532	5.506	6.487

[a] $p = 0.001$. [b] $p = 0.010$. [c] $p = 0.005$.

Table III—*In Vitro* Effects of Antitumor Agents on Basal and ADP-Stimulated Respiration (Microliters of Oxygen per Hour per Milligram of Protein) Using α-Ketoglutarate as Substrate (Percent Control)

Compound (n = 6)	Walker 256		P-388 Leukemia		Ehrlich Ascites	
	State 4	State 3	State 4	State 3	State 4	State 3
I	35 ± 9[a]	43 ± 11[a]	68 ± 8[a]	53 ± 6[a]	46 ± 11[a]	42 ± 12[a]
II	62 ± 4[a]	73 ± 3[a]	55 ± 6[a]	69 ± 3[a]	—	—
III	63 ± 10[a]	71 ± 14[b]	39 ± 9[a]	57 ± 17[a]	—	—
IV	49 ± 21[a]	58 ± 12[a]	70 ± 9[a]	70 ± 6[a]	53 ± 9[a]	66 ± 10[a]
V	52 ± 17[a]	54 ± 17[a]	64 ± 10[a]	95 ± 2	51 ± 9[a]	56 ± 7[a]
VI	49 ± 16[a]	57 ± 17[a]	74 ± 13[b]	85 ± 7[c]	56 ± 10[a]	66 ± 10[a]
VII	54 ± 14[a]	70 ± 17[b]	56 ± 8[a]	78 ± 5[a]	70 ± 5[a]	79 ± 9[b]
VIII	54 ± 16[a]	54 ± 15[a]	41 ± 15[a]	65 ± 4[a]	66 ± 9[a]	18 ± 13[b]
IX	47 ± 9[a]	53 ± 15[a]	59 ± 7[a]	66 ± 13[a]	51 ± 5[a]	55 ± 12[a]
X	67 ± 8[a]	63 ± 10[a]	59 ± 4[a]	82 ± 7[b]	60 ± 10[a]	77 ± 5[b]
XI	57 ± 13[a]	67 ± 15[b]	95 ± 3[a]	58 ± 6[a]	59 ± 12[a]	73 ± 11[b]
XII	55 ± 11[a]	55 ± 14[a]	70 ± 6[a]	68 ± 6[a]	56 ± 18[a]	63 ± 9[a]
XIII	60 ± 31[c]	76 ± 12[b]	61 ± 7[a]	73 ± 13[b]	69 ± 8[a]	67 ± 9[a]
XIV	49 ± 14[a]	46 ± 25[a]	68 ± 9[a]	87 ± 10	59 ± 14[a]	53 ± 8[a]
XV	59 ± 15[a]	52 ± 16[a]	65 ± 10[a]	66 ± 10[a]	52 ± 14[a]	53 ± 8[a]
XVII	—	—	—	—	—	—
0.05% polysorbate 80	100 ± 8	100 ± 7	100 ± 5	100 ± 8	100 ± 5	100 ± 11
Control	2.729	2.691	2.029	1.958	2.525	4.049

[a] $p = 0.001$. [b] $p = 0.005$. [c] $p = 0.010$.

compounds. This possibility is especially obvious with the introduction of the primary alcohol into the side chain (XIV and XV).

Gosalvez *et al.* (29) attempted to correlate antitumor activity with suppression of tumor aerobic respiration in the presence and absence of adenosine diphosphate. With respect to the sesquiterpene lactones or synthetic compounds containing the α-methylene-γ-lactone moiety, there appeared to be a strong positive correlation between antitumor activity and suppression of basal (4) and coupled oxidative phosphorylation processes (3) of Ehrlich and Walker 256 tumor cells with both nicotinamide adenine dinucleotide and flavin adenine nucleotide-linked dehydrogenases. The adenosine triphosphate derived from state 3 respiration is critical in rapidly dividing tumor cells since energy is required for synthetic processes, *e.g.*, nucleic acids, protein, and cellular membranes during G_1 and S phases of the cell cycle.

However, the ability of these compounds to suppress aerobic respiration processes, *i.e.*, states 3 and 4, appeared to exceed their ability to inhibit tumor growth. This observation is confirmed by the fact that these compounds effectively inhibited P-388 leukemic cell respiration but only marginally inhibited P-388 tumor growth. Kupchan *et al.* (30) demonstrated that compounds containing the α-methylene-γ-lactone moiety act as antitumor agents by alkylating sulfhydryl biological nucleophiles by a rapid Michael-type addition. A similar phenomenon was demonstrated *in vitro* with tenulin (IX) and helenalin (IV), indicating that both the α-methylene-γ-lactone and the cyclopentenone moieties act as electrophiles (6). The cytochrome cofactors of the electron transport chain contain iron–sulfur centers or sulfide ions as components of their structure, and a number of Krebs cycle dehydrogenases contain sulfhydryl groups within their structures (31). Theoretically, these compounds can be alkylated by a Michael reaction by these antitumor agents, reducing activity and state 4 respiration.

Compound IV was shown to be an effective inhibitor of nuclei DNA polymerase activity of Ehrlich ascites cells *in vivo* (5). Supposedly, the DNA polymerase alpha and gamma enzymes contain exposed sulfhydryl groups (31) that can be alkylated. From the present study, not only were the compounds containing the α-methylene-γ-lactone moiety inhibitors of deoxyribonucleic acid polymerase activity, but compounds containing only a cyclopentenone ring (IX and X) or an α-epoxycyclopentanone system (VII) were also marginal inhibitors of this enzyme.

Sesquiterpene lactones containing two of these functions were more active than compounds containing only one function in inhibiting deoxyribonucleic acid polymerase activity. Compounds containing the allylic ester side chain (XI–XVI) also demonstrated higher activity, with the exception of XV. Thymidylate synthetase was reported to be a sulfhydryl-bearing enzyme (32). Sesquiterpene lactone and related analogs can significantly inhibit this enzyme but to a slightly less magnitude than the deoxyribonucleic acid polymerase enzymes. All three of these functional moieties appear to be active in the inhibition of thymidylate synthetase activity of tumor cells.

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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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