Ambiguities in IR and X-Ray Characterization of Amphotericin B

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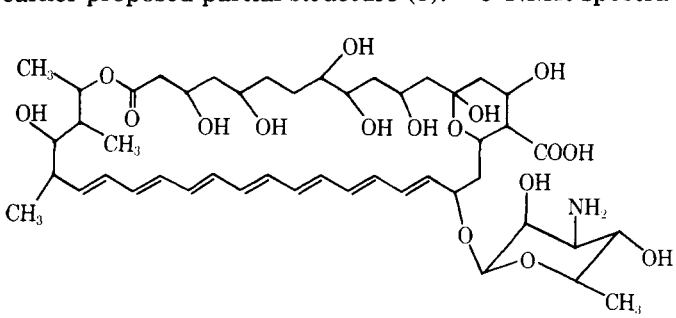
Abstract □ Two distinct IR spectra of amphotericin B have been reported. These differences can be obtained from the same sample by surprisingly small changes in the method of sample preparation. Type I spectra (hand-ground samples) are characterized by a sharp C=O stretching band at 1692 cm^{-1}, and Type II spectra (vibrator-ground samples) are characterized by a broad C=O stretching band near 1710 cm^{-1}. X-ray powder diffraction demonstrates that vibrator grinding promotes a transition from a crystalline to an amorphous phase. The two phases are not bioequivalent. Differential thermal analysis reveals a transition near 157°, and samples heated to 158° give only Type II IR spectra. However, a marked color change accompanies such heating (*i.e.*, structural changes affecting the chromophore have been thermally induced), while X-ray spectra show an increase of only about 30% in amorphous content. Furthermore, hand-ground samples heated to 120° still display only Type I IR spectra. Thus, the vibrator-induced transition is not solely a static thermal effect. Many observed spectral lines can be assigned to specific functional groups.

Keyphrases □ Amphotericin B—different IR spectra attributed to method of sample preparation □ IR spectroscopy—amphotericin B, different spectra attributed to method of sample preparation □ Antifungal agents, topical—amphotericin B, different IR spectra attributed to method of sample preparation

IR absorption spectroscopy is an essential technique in constructing the analytical profile of a drug. Its use depends on comparison of sample spectra to reliable standard spectra. It is disconcerting, therefore, that conflicting IR spectra of amphotericin B have been reported (1, 2). Evidence given in this paper demonstrates that both spectra can be obtained from the same sample at "room temperature," depending on the mode of preparation. Complete IR spectra of both forms are presented and analyzed, and they are supplemented by data obtained by other techniques. Thermally induced changes in amphotericin B (near 157°) also are discussed.

Amphotericin B ($C_{47}H_{73}NO_{17}$) is a mycosamine-containing, macrocyclic, polyene antibiotic, widely used as a fungicide. A proposed structure (I) is based on the X-ray work of Ganis *et al.* (3). This structure is consistent with mass spectroscopic measurements (4) but not with an earlier proposed partial structure (5). ^{13}C-NMR spectra confirm the presence of the hemiketal ring in dimethyl sulfoxide solution (6). Amphotericin B is soluble in dimethyl sulfoxide and, to a lesser extent, in methanol but is virtually insoluble in carbon tetrachloride, chloroform, and neutral water (7, 8).



I

EXPERIMENTAL

Samples of the Food and Drug Administration amphotericin B[1] working standard powder[2] were used without further purification. Samples were ground either by hand or in a vibrator[3] and then made into potassium bromide disks or a liquid paraffin mull[4].

Spectra were taken using several different IR spectrometers[5]. Some disks were made from 1 mg of amphotericin and 200 mg of potassium bromide, hand ground in a 65-mm (o.d.) agate mortar with an agate pestle for 2 min. The disks were machine pressed under vacuum, using a 13-mm die for 2 min at 9080 kg (20,000 lb). Other samples were ground in a vibrator[3] for 2 min in a metal capsule with one steel ball, rather than hand ground, before being pressed into disks.

Mulls were made in the mortar by adding a few drops of mineral oil to fresh amphotericin and hand grinding for 2 min. Mulls were squeezed between sodium chloride plates for observation. Saturated dimethyl sulfoxide solutions (approximately 40 mg/ml) were made from unground samples.

In thermal studies, unground samples were heated in glass test tubes immersed in an oil bath (158°) prior to processing. X-ray powder diffraction spectra were taken with a wide angle X-ray diffractometer[6] with a theta compensating slit and focusing monochromator. Differential thermal analysis and thermal gravimetric analysis data also were obtained[7]. Such heat-treated samples were made into disks and mulls for IR measurements.

DISCUSSION

IR Spectra at Room Temperature—IR spectra of amphotericin B powder at room temperature seem to represent a mixture of two distinct forms, Types I and II. Type I predominates in hand-ground samples (Fig. 1A), whereas Type II usually predominates in vibrator-ground samples (Fig. 1B). Some vibrator-ground samples display a more equal mixture of forms (Fig. 1C). Since mulls are usually hand ground and samples for disks are often vibrated along with potassium bromide, this effect can cause considerable confusion. However, when prepared under identical conditions (*i.e.*, hand ground or vibrator ground), potassium bromide disks and liquid paraffin mulls give the same spectra.

Type I spectra are characterized by a sharp C=O stretching band at 1692 cm^{-1} (with a high frequency shoulder), a 1556-cm^{-1} C=C stretching band, and considerably more detail in the CH band and stretching regions (*e.g.*, near 850, 1380, and 2860 cm^{-1}). Type II spectra typically display broader, less resolved bands, including a broad C=O stretching band (width of approximately 50 cm^{-1} rather than approximately 10 cm^{-1})

[1] Lot Ampho B-2, E. R. Squibb & Sons, New Brunswick, N.J.
[2] Supplied by the National Center for Antibiotics Analysis.
[3] WIG-L-BUG, Crescent Dental Manufacturing Co., Chicago, Ill.
[4] Nujol.
[5] Models 521, 567, and 621, Perkin-Elmer Corp., Norwalk, Conn.
[6] Generator XRG-3000, goniometer 15U10021, and electronic panel 3005/220, Phillips Electronics Instruments, Mount Vernon, N.Y.
[7] Model 990 thermal analyzer with a model 950 thermogravimetric attachment, E. I. du Pont de Nemours & Co., Wilmington, Del.

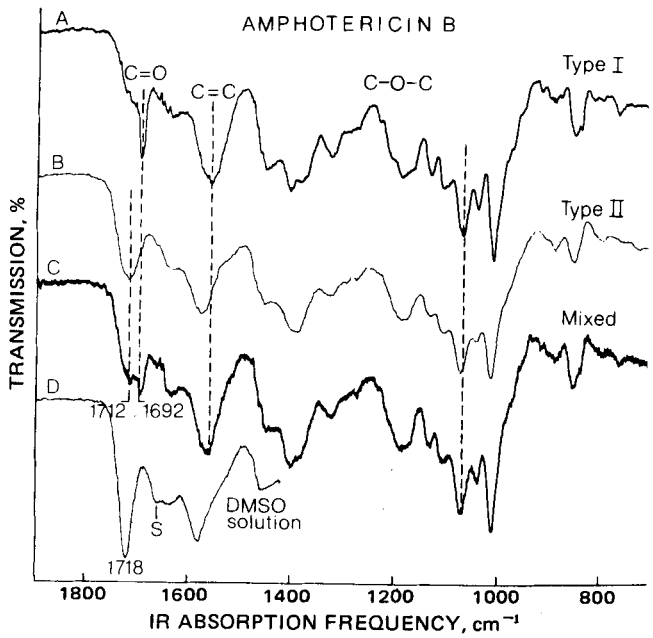


Figure 1—*IR spectra of amphotericin B powder, showing differences between hand-ground (A) and vibrator-ground (B) samples, some vibrator-ground samples displaying mixed forms (C), and samples in dimethyl sulfoxide (D).*

at 1712 cm^{-1}, and a 1566-cm^{-1} C=C stretching band. Other differences are found near 900, 1233, 2930, and 2970 cm^{-1}.

Spectra of amphotericin B in dimethyl sulfoxide (Fig. 1D) have C=O and C=C stretching bands near 1718 and 1580 cm^{-1}, respectively, and tend to resemble Type II. The observed absorption frequencies are listed in Table I. Many peaks can be tentatively assigned to specific structural features (9–11), using such model compounds as glucosamine.

Microscopic examination showed the FDA amphotericin B working standard powder to consist of thin, irregularly shaped fragments, which tended to clump together into approximately 80-μm clusters. The fragments were roughly 5–15 μm long in hand-ground or untreated samples and 1–5 μm long in vibrator-ground samples; they were less than 0.3 μm thick. The irregular, shattered appearance of untreated samples indicates considerable grinding and other comminution as part of the working standard's final processing by the manufacturer. This treatment could account for the broad 1710-cm^{-1} band (typical of Type II grinding) seen as a shoulder in all spectra of hand-ground or even untreated samples, both disks and mulls.

Nonetheless, the observed spectral differences between hand-ground and vibrator-ground samples do not appear to be a size effect (12, 13) since: (*a*) the differences in particle sizes are not very great; (*b*) if a difference does exist, Type II spectra should be of higher resolution since the particle size of Type II is smaller (yet compare, for example, the 800–950-cm^{-1} regions of Figs. 1A and 1B); (*c*) the observed differences

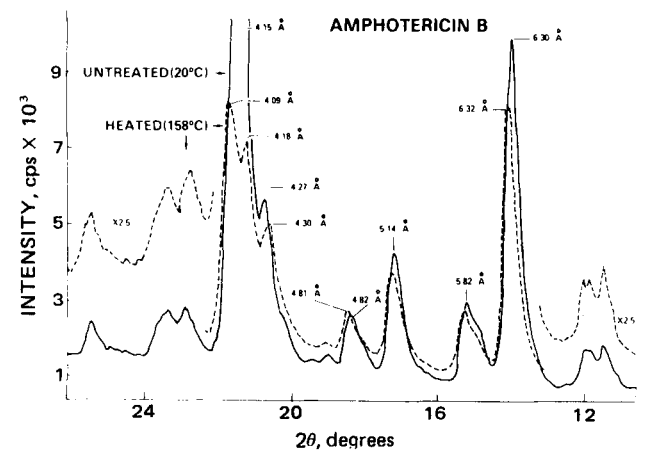


Figure 2—*X-ray diffraction spectrum of unground and unheated amphotericin B powder.*

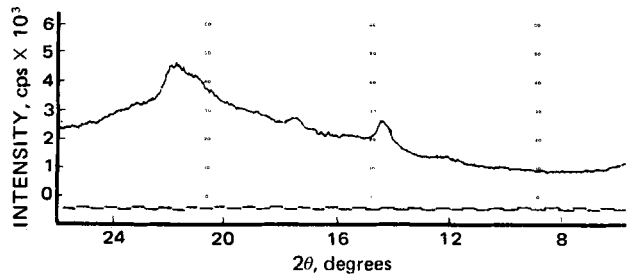


Figure 3—*X-ray diffraction spectrum of vibrator-ground amphotericin B powder.*

involve both frequency shifts and changes in the width of the bands; and (*d*) the powders are somewhat fine for an appreciable Christiansen effect, and no marked asymmetries are seen.

X-Ray Powder Diffraction—The X-ray powder diffraction spectrum of untreated (unground and unheated) amphotericin B (solid line, Fig. 2) demonstrates that the substance has a definite crystal structure. Material heated to 158° produces a pattern with less intense peaks, small shifts in *d*-spacings, and increased background. These changes show that some strain has been introduced in the crystal lattice and that the amorphous fraction of the powder has increased. The diffraction pattern of vibrator-ground powder (ground in 2-mg aliquots, 2 min each; Fig. 3) has only a few broad peaks of very low intensity and much higher background, characteristic of patterns obtained from almost completely amorphous powders.

This result demonstrates that the original crystalline sample has undergone a transition to an amorphous form. There is no change in color, but the transition is associated with a 40% loss in biological activity, as measured by the CFR *Saccharomyces cerevisiae* assay (14). Since the first step of the assay is to dissolve the sample, an accompanying irreversible chemical change is indicated.

The phase transition is apparently the physical basis for the observed changes in the IR spectrum; a similar effect was observed in the Cinchona alkaloids (15). Spectra of amphotericin B heated 15 min at 120° and then ground by hand are still Type I, which suggests that the elevated temperatures in the vibrator capsule during grinding are not themselves sufficient to induce the observed phase transition. Nor is salt complexation responsible, since samples ground with and without potassium bromide have identical IR spectra. However, if mulls are made by adding liquid paraffin directly to the vibrator capsule before grinding, the spectra are of Type I. This result suggests that pressure effects, probably aided by elevated temperatures, may be important.

The broad 1710-cm^{-1} shoulder in Type I spectra could represent the presence of some amorphous material (Type II) in the original standard. Indeed, about 30–40% of the area in the C=O stretch band of Fig. 1A may have such an origin (the absorption efficiencies of the two forms may differ, however).

Thermal Transitions above 157°—Differential thermal analysis of amphotericin B (14) shows a sharp drop beginning near 140°, with a minimum near 157°. Thermal gravimetric analysis reveals no corresponding weight loss, and sample decomposition occurs only above 200°. Amphotericin B heated at 158° for 15 min irreversibly changes color from bright yellow to orange brown. Such samples display a loss of about 30% in antifungal effectiveness (14), a 20–25% decrease in UV absorbance (14), a 25–30% decrease in crystalline peak scattering intensities in powder X-ray diffraction spectra (Fig. 2), and Type II (or mixed type) IR spectra.

Such results suggest that irreversible chemical changes occur near 158°. Heating amphotericin B to 120° for approximately 15 min has little effect on its IR spectrum, although heating for a longer period may affect the 1712- and 1692-cm^{-1} bands. The X-ray results confirm that heating to 158° can partially increase the amorphous fraction of amphotericin B; slight shifts (0.38 Å) in several peaks of the X-ray powder diffraction spectrum (Fig. 2) further suggest some additional strain in the remaining crystalline fraction. However, lack of a complete transition to the amorphous form and the presence of a color change indicate that the effects of static heating cannot totally account for those observed upon grinding with a vibrator. Neither process markedly affects the resonance Raman spectrum[8].

Evidently, sample preparation can be very important in obtaining

[8] I. M. Asher, M. Bunow, and I. Levin, unpublished data.

Table I—IR Spectra of Amphotericin B [a]

Type I	Type II	Dimethyl Sulfoxide	Tentative Assignment
(625)			
664		(661)	OH out-of-plane bend
697			
(732)			
762	(sh)	758	Pyranose ring breathing
795	(792)	791	
812	(804) sh	(s1)	
818			
(837) sh			
851	850	850	CH bend (G)
(878)			
889	888	888	CH bend, CH_3 rock
(898) sh		(900) sh	
916		(915) sh	Pyranose ring vibration
(931) sh			
(953) sh			
(972) sh		(970) sh	
(981) sh	(s1)		CH out-of-plane bend (*trans*-polyene)
1009	1010	1010	
1041	1040	(1036) sh	
1070	1068	1069	
1109	1106	1106 sh	CO asymmetric stretching (COC, OOH)
1132	1130	1132	
1164 sh	(1173)		
1186	(1188)	1183	COC asymmetric stretching (COC=O)
1210 sh			
1233 sh	(1230) sh	(1232) sh	
1272 sh	1269	1273	CH_2 wag, bend (skeletal)
(sh)	(1291)	1292	
1324	1322	1321	
(1338) sh			
(1371) sh			
1381	(1385)	(1380)	CH_3 symmetric bend
1401	(1400)	(1402)	CH in-plane bend (polyene)
1448	1449	1452 sh	CH_2, CH_3 asymmetric bend
1556*	1566*	1565	Polyene C=C stretching
(1628)	1628 sh	S	NH_2 in-plane bend
1692*			
(1710) sh	1712*	1716	
(sh)	2859*	S	CH_2, CH_3 symmetric stretching
2918			CH_2, asymmetric stretching
	2925		
2940			
(2960) sh			CH_3 asymmetric stretching
2978	(2979) sh		
3009	3015		CH stretching (polyene)
(3370)			OH stretching
3390	3390	3350	(Strongly H-bonded)

[a] B = broad; sh = shoulder; sl = slant; S = solvent peaks; () = weak, frequency uncertain; * = frequency characteristic of Type I or II; and + = may arise from slight admixture of Type II.

consistent IR spectra of pharmaceuticals. Ideally, both samples and standards should be prepared by identical techniques at the same time. In general, hand-ground samples seem preferable.

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