Relationship between Acute Toxicity in Mice and Polymorphic Forms of Polyene Antibiotics

G. GHIELMETTI, T. BRUZZESE ˣ, C. BIANCHI, and F. RECUSANI

Abstract □ Marked differences in LD_{50} values were found between various batches of polyene antibiotics (mepartricin and nystatin) after intraperitoneal administration in suspension to mice. Such differences do not seem attributable to the particle size but to different molecular spatial arrangements of the products, which modify their solubility and biological availability. In the samples obtained by chemical treatments of some batches of the polyene substances, it has been possible to change the toxicity drastically and then to bring it back to its original value.

Keyphrases □ Toxicity—relationship to polymorphic forms of polyene antibiotics mepartricin and nystatin, mice □ Polymorphic forms—polyene antibiotics mepartricin and nystatin, relationship to toxicity in mice □ Antibiotics, polyene—mepartricin and nystatin, polymorphic forms related to toxicity, mice □ Mepartricin—polymorphic forms related to toxicity, mice □ Nystatin—polymorphic forms related to toxicity, mice

In continuing chemical and biological studies of mepartricin[1], a semisynthetic polyene antibiotic with high antifungal and antiprotozoal activities (1, 2), it was observed that various batches differed considerably in acute toxicity after intraperitoneal administration (LD_{50} ip) in suspension to mice. These differences did not seem to be justified on the basis of the physicochemical and microbiological analyses, since all samples showed properties within well-defined limits of potency, and on the basis of TLC, NMR, IR, and UV evidence.

The toxicity values of many natural polyenes reported in the literature also show marked discrepancies. For example, the LD_{50} of intraperitoneal nystatin was found to be 8–14 (3), 20–26 (4), or about 200 (5) mg/kg according to different investigations. And the LD_{50} of intraperitoneal amphotericin B under the same experimental conditions seemed to be <99 (6), 280 (7), or even >1200 (8) mg/kg.

Bennet *et al.* (9) attempted to explain the difference in toxicity of various samples of amphotericin B by attributing the variability to the different particle sizes. But preliminary tests with mepartricin showed that this explanation does not apply here because no correlation between the LD_{50} values (intraperitoneal) and the size of the particles was seen. Therefore, it was postulated that occasional modifications of the isolation or purification methods of mepartricin, nystatin, or other polyenes might have given rise to products of different solubility, bioavailability, and toxicity as a result of polymorphic modifications, as reported for other classes of drugs (10).

To support this hypothesis, a few batches of mepartricin and nystatin were selected. A sample from each batch (Sample 1, Table I) was treated twice with organic solvents, the first treatment modifying the crystallographic forms (Sample 2) and the second returning it to the original form (Sample 3). All samples were sieved to keep the particle size uniform, and they were analyzed to control eventual differences in purity. Their solubility and LD_{50} values after intraperitoneal administration in suspension to mice were determined. In some samples, the acute toxicity was determined also after suitable solubilization to obtain values independent of the crystalline structure.

[1] Previously referred to in the literature as partricin methyl ester or SPA-S-160.

EXPERIMENTAL

Materials and Methods—Mepartricin was prepared according to a reported method (11). On the basis of preliminary acute toxicity tests in mice by intraperitoneal administration in suspension, four batches of product with very different LD_{50} values were chosen (Table I). Nystatin was a commercial product, and a batch of high[2] toxicity and one of low[3] toxicity were selected. The original Samples 1 of the two polyenes were treated with organic solvents by one of four methods, A–D, to give Samples 2 and then Samples 3. After treatment, they were passed through a stainless steel sieve of 4450 mesh/cm^2 (sieve opening 0.090 mm)[4]. Particle sizes ranging between 4 and 12 μm were obtained for all samples. Analytical controls by elemental analysis, determination of NMR, IR, and UV spectra, TLC, and testing for antifungal activity were also performed. The modifications of solubility and toxicity were then measured.

TLC—TLC was performed on silica gel[5] with a solvent system of butanol–ethanol–acetone–25% ammonium hydroxide (2:5:1:3). The spots were detected by exposure to UV light and had R_f values of about 0.8 for mepartricin and 0.43 for nystatin.

Antifungal Activity—Antifungal activity was determined by a turbidimetric method (12), suitably modified, that compared the growth inhibition of *Saccharomyces cerevisiae*[6] (ATCC 9763) under the influence of the test samples and known quantities of standard mepartricin and nystatin.

The test strain was cultivated for 18 hr at 37° in a broth containing 1% peptone[7], 0.5% yeast extract[8], and 2% glucose[9]. Then 1.7 ml of suspension was inoculated in 100 ml of a broth of the same composition.

The mepartricin standard was dissolved in dimethyl sulfoxide (1000 μg/ml) and diluted with sterile distilled water. Then 1 ml was added to the inoculated culture medium (9 ml) to obtain the final concentrations of 0.15, 0.20, 0.25, 0.30, and 0.35 μg of polyene compound in 10 ml of broth. After 5 hr of incubation at 37°, the absorbance was determined[10] at 580 nm. Values were plotted on the arithmetic scale of semilogarithmic paper with concentrations on the logarithmic scale, giving a five-point standard response line.

The test samples of mepartricin were worked up in the same way to give the midconcentration of 0.25 μg/10 ml. Then the absorbance was determined, and the potency was calculated from the standard response line.

[2] Batch 7208/P 0866, C.F.M., Milan, Italy.
[3] Batch 134, Gianni, Milan, Italy.
[4] No. 35, UNI Italian series, corresponding to 170 mesh, ASTM series.
[5] Kieselgel F_{254}, Merck, Darmstadt, Germany.
[6] Istituto Sieroterapico Milanese, Milan, Italy.
[7] Baltimore Biological Laboratory, Baltimore, Md.
[8] Difco, Detroit, Mich.
[9] Merck, Darmstadt, Germany.
[10] Spectronic 20, Bausch & Lomb, Rochester, N.Y.

Table I—Solubility of Samples of Mepartricin and Nystatin and Acute Toxicity in Mice following Administration of Aqueous Suspensions and Solutions

Compound (Batch Number)	Sample (Preparation Method)[a]	Microbiol Activity, units/mg[b]	Solubility, units/ml[c]	Acute Toxicity in Mice, LD_{50}, mg/kg (Confidence Limits, $p = 0.05$)		
				Intraperitoneal Suspension	Intraperitoneal Solution	Intravenous Solution
Mepartricin (P.149)	1 (B)	2590	12.196	19.5 (18.1–21.0)	—	—
	2 (A)	2525	3.507	126.3 (91.7–174.0)	—	—
	3 (B)	2632	10.085	22.7 (20.2–25.6)	—	—
Mepartricin (LS/11)	1 (A)	2375	3.539	77.7 (70.3–85.8)	—	—
	2 (B)	2352	12.617	15.4 (14.3–16.8)	—	—
	3 (A)	2357	3.905	100.4 (77.1–133.4)	—	—
Mepartricin (P.166)	1 (A)	2802	4.405	216.1 (144.9–322.3)	13.6 (12.2–14.5)	5.3 (4.4–6.4)
	2 (B)	2803	14.476	19.2 (16.7–22.1)	11.1 (10.5–12.3)	4.9 (4.2–5.7)
	3 (A)	2761	3.809	194.3 (163.8–230.4)	12.2 (11.1–13.3)	5.4 (4.9–6.0)
Mepartricin (P.167)	1 (B)	2455	12.326	14.1 (10.5–18.7)	12.5 (11.4–13.6)	5.3 (4.4–6.3)
	2 (A)	3020	4.798	114.2 (103.0–126.5)	11.6 (10.3–12.9)	4.3 (3.8–4.8)
	3 (B)	3010	14.700	8.8 (8.3–9.3)	11.3 (10.4–12.2)	4.7 (3.9–5.7)
Nystatin (7208/P 0866)	1[d]	4575	8.835	88.5 (77.1–101.5)	5.7 (4.8–6.8)	5.7 (4.7–6.9)
	2 (C)	5245	4.605	519.4 (458.4–577.2)	6.1 (5.5–6.9)	5.3 (4.4–6.4)
	3 (D)	4856	8.210	89.5 (81.4–97.1)	6.9 (5.8–8.2)	5.1 (4.0–6.6)
Nystatin (134)	1[d]	5120	3.925	200.9 (144.7–278.9)	5.5 (4.9–6.3)	6.6 (6.0–7.3)
	2 (D)	4142	8.120	23.2 (20.3–26.8)	5.1 (4.1–6.2)	5.8 (4.3–7.9)
	3 (C)	4905	4.127	635.4 (415.4–971.9)	4.4 (3.8–5.1)	8.3 (7.2–9.5)

[a] Sample 1 represents the original batch. Samples 2 and 3 were obtained in order by subsequent treatments. [b] Against the respective standard samples of mepartricin and nystatin. Note that the first polyene has a minimum inhibitory concentration against *Saccharomyces cerevisiae* and against *Candida albicans* below 0.1 unit/ml, while nystatin has minimum inhibitory concentration values of about 5 units/ml; thus 1 unit of mepartricin is much more potent than 1 unit of nystatin. [c] In aqueous solution of sodium lauryl sulfate at the concentration of 1% for mepartricin and at the concentration of 0.1% for nystatin. [d] Commercial sample.

The same procedure was followed with the standard and test samples of nystatin, using concentrations 50-fold higher.

Solubility Tests—Since the two polyenes were too sparingly soluble in distilled water and formed cloudy solutions, which could not be clarified by filtering or centrifuging, the tests were performed in a 1% solution of sodium lauryl sulfate for mepartricin and in a 0.1% solution of sodium lauryl sulfate for nystatin. Accurate and reproducible results for the various samples were obtained.

Suspensions containing 1% of the polyene in these solvents were prepared and stored in darkness at 4° with slow agitation; samples were taken after 1, 8, 24, and 48 hr. Every sample was carefully filtered on a porous membrane, and the solution was immediately subjected to microbiological assay. The results were constant in time and were expressed as the mean of the values for different samples.

Chemistry—*Method A*—Mepartricin (5 g) was treated with 150 ml of methylene chloride–methanol (9:1) at room temperature. The solution was evaporated to dryness at 35–40° under reduced pressure. The oily residue solidified on standing, forming a compact yellow solid, which was pulverized through a screen and dried *in vacuo* at room temperature until complete elimination of the solvent. The product was obtained in the form of layered plates with polygonal surfaces and regular contours.

Method B—Mepartricin (5 g) was treated with 100 ml of acetone–water (8:2) and heated to incipient boiling. The solution was rapidly cooled to room temperature, and 800 ml of ether was added with agitation. The precipitate was collected by filtration and dried *in vacuo* at room temperature, giving almost quantitative yields of yellow rounded granules with porous surfaces and irregular contours.

Method C—Nystatin (5 g) was treated with 50 ml of water saturated with methyl ethyl ketone and heated carefully to 60° with agitation. A partial solution was obtained from which the product rapidly began to separate by crystallization.

After a few minutes, heating was stopped. The mixture was slowly cooled to 10° in 1 hr with agitation. The precipitate was isolated by filtration, washed with acetone, and dried *in vacuo* at room temperature. Yellow needle crystals were obtained.

Method D—Nystatin (5 g) was dissolved in 200 ml of chloroform–methanol–ammonium hydroxide (7:2.5:0.5). The solution was evaporated to dryness *in vacuo* at a temperature below 25° to minimize the degradation of the product; this procedure was followed by trituration through a screen and drying *in vacuo* at room temperature. The product was obtained as yellow rounded granules with porous surfaces.

Pharmacology—The acute toxicity was determined in male and female Swiss mice, 18–22 g, by intraperitoneal administration of mepartricin or nystatin suspensions and, for a few samples, by intraperitoneal and intravenous administrations of solutions. Not less than eight groups of 10 mice each were used in each test. The mortality recorded in 7 days of observation served for calculating the LD_{50} by the Thompson (13) method of moving averages as described by Weil (14).

The compounds, sieved as already described, were suspended in carboxymethylcellulose (0.5% w/v), polysorbate 80 (0.05% w/v), and benzyl alcohol (0.5% v/v) and injected in the constant volume of 0.2 ml/10 g. When solutions were used, the compounds were dissolved in dimethyl sulfoxide with a sodium lauryl sulfate addition (62.5 mg of active agent in 0.45 ml of dimethyl sulfoxide and 125 mg of sodium lauryl sulfate in 25 ml of water). Stock solutions were prepared immediately before use, diluted with water as need-

ed, and injected intraperitoneally and intravenously in the constant volume of 0.2 ml/10 g.

In each test, the interval between doses was calculated with the geometric factor of 1.26 so that the logarithms of successive dosage levels differed by a constant (0.1).

The method of Thompson (13) and Weil (14) for calculating LD_{50} values was preferred to the usual method of Litchfield and Wilcoxon (15) for its simplicity, provided that the logarithm of successive dosage levels differed by a constant and that the number of animals used was the same in each group. The results obtained with the two methods are quite similar.

RESULTS AND DISCUSSION

The batches of mepartricin and nystatin, the samples obtained by the sequence of treatments, and the data of microbiological activity, solubility, and toxicity are described in Table I. These data indicate that, by varying the treatment method, it is possible to obtain samples of higher or lower solubility and, consequently, of very different acute toxicity after intraperitoneal administration in suspension.

For example, Sample 1 of mepartricin, Batch P.166, obtained by Method A, showed relatively low solubility and reduced toxicity, with an LD_{50} in mice of 216.1 mg/kg. When treated according to Method B, it became the more soluble and more toxic Sample 2 (LD_{50} ip 19.2 mg/kg). Treating Sample 2 by Method A then provided Sample 3, of relatively low solubility and toxicity (LD_{50} ip 194.3 mg/kg) like Sample 1. This return to the initial characteristics is obviously important in demonstrating that the changes of toxicity are not the result of a variation in the amount of eventual impurities during the treatment, as confirmed by the usual chemical and microbiological controls.

Furthermore, when the toxicity was determined by the same method but in solution, the differences between the treated Samples 2 and 3 and the original Sample 1 proved to be insignificant, with LD_{50} values for mepartricin Batch P.166 between 11.1 and 13.6 mg/kg, thus demonstrating their essential equivalence under identical bioavailability conditions and confirming the hypothesis concerning the influence of the crystal structure.

Analogous results were obtained when the solutions were administered intravenously, the LD_{50}'s being between 4.9 and 5.4 mg/kg. When the data for the more soluble mepartricin samples are compared, the toxicity in suspension proves to be comparable to that of the solutions, demonstrating a very high bioavailability of these materials. In contrast, more sparingly soluble samples exhibit a much lower *in vivo* utilization and toxicity than in solution.

In conclusion, the principal reason for the variability of the acute toxicity test results obtained with different batches of mepartricin and nystatin suspensions, seems to be attributable to differences in crystal structure, *i.e.*, the existence of polymorphic forms. The same might be true for many other polyenes cited in the literature, and this finding would explain the differences in toxicity data found by different laboratories. Consequently, it is imperative to standardize the methods of the final isolation of the products to have constant toxicity.

These results also suggest that, when a drug of inherently low bioavailability is to be used undissolved in various dosage forms, it may be advantageous to conduct pharmacological tests with several batches of different LD_{50} values (intraperitoneal) to choose the one with the best activity–toxicity ratio. Knowledge of the corresponding acute toxicity of the suitable solubilized product may give further indications as to the possibility of increasing the bioavailability of the undissolved product.

REFERENCES

(1) T. Bruzzese and R. Ferrari, Brit. pat. appl. 52,271 (Nov. 3, 1970).

(2) T. Bruzzese, I. Binda, A. Di Nardo, G. Ghielmetti, and M. Riva, *Experientia*, **28**, 1515(1972).

(3) H. Umezawa, "Index of Antibiotics from Actinomycetes," University of Tokyo, Tokyo, Japan, 1967, p. 474.

(4) E. L. Hazen and R. Brown, *Proc. Soc. Exp. Biol. Med.*, **76**, 93(1951).

(5) "The Merck Index," 8th ed., Merck & Co., Rahway, N.J., 1968, p. 753.

(6) D. P. Bonner, W. Mechlinski, and C. P. Schaffner, *J. Antibiot.*, **25**, 261(1972).

(7) B. A. Steinberg, W. P. Jambor, L. O. Suydam, and A. Soriano, *Antibiot. Ann.*, **1955–56**, 574.

(8) T. H. Sternberg, E. T. Wright, and M. Oura, *ibid.*, **1955–56**, 566.

(9) J. E. Bennet, G. J. Hill, II, W. T. Butler, and C. W. Emmons, "Antimicrobial Agents and Chemotherapy 1963," American Society for Microbiology, Ann Arbor, Mich., 1964, p. 745.

(10) J. Haleblian and W. McCrone, *J. Pharm. Sci.*, **58**, 911(1969).

(11) T. Bruzzese, M. Cambieri, and F. Recusani, *ibid.*, **64**, 462(1975).

(12) F. Kavanagh, "Analytical Microbiology," vol. II, Academic, New York, N.Y., 1972, p. 148.

(13) W. Thompson, *Bacteriol. Rev.*, **11**, 115(1947).

(14) C. Weil, *Biometrics*, **8**, 249(1952).

(15) J. T. Litchfield and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96**, 99(1949).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 31, 1974, from the *Research Laboratories, SPA–Società Prodotti Antibiotici S.p.A., Via Biella 8, 20143 Milano, Italy.*

Accepted for publication July 25, 1975.

[x] To whom inquiries should be directed.