Coumermycin A_1—Biopharmaceutical Studies I

HAROLD L. NEWMARK and JULIUS BERGER

Abstract □ Coumermycin A_1, as the free acid or simple salts, is poorly absorbed from the gastrointestinal tract of animals and humans. It is not appreciably degraded in the fluids of the gastrointestinal tract. Mixture of coumermycin with certain additives, such as sugar amines, can significantly enhance oral absorption in dogs, as measured by the increased blood levels achieved. *N*-Methylglucamine, in a 1:4 ratio of antibiotic to *N*-methylglucamine, enhances blood levels 5–15-fold over the antibiotic alone in both dogs and humans.

Keyphrases □ Coumermycin A_1—absorption, oral administration □ Blood levels, coumermycin A_1—formulation effect □ Stability, coumermycin A_1—gastrointestinal tract □ Microbiological analysis—coumermycin A_1

Coumermycin A_1 is a highly potent antibiotic whose antimicrobial spectrum qualitatively resembles that of novobiocin (1–4). Tests *in vitro* and subcutaneously in mice indicated antibacterial activity, particularly against Gram-positive organisms, at concentrations and doses far lower than for novobiocin (*i.e.*, 1/10 to 1/30). However, early trials of the antibiotic orally in humans gave very poor or nondetectable blood levels (5–7). Blood levels far lower than 1 mcg./ml. were achieved even on doses up to 2 g./day, although several forms of the antibiotic were tested, including the water-soluble disodium salt (7), enteric coating (5, 7), and mixing with polyethylene glycol 300 (Carbowax 300) (7). The total amount of antibiotic accounted for in body fluids, calculated at the maximum blood levels achieved, represented less than 0.1% of the total oral drug intake in these clinical studies.

For mice, the toxicity expressed as LD_{50} in mg./kg. for coumermycin has been reported to be p.o., > 4000 (1); i.p., 183 (1); s.c., 380 (1); and i.m., 500 (8). The median curative dose in experimental *Staphylococcus aureus* (Smith) infection in mice has been reported (1, 3) as 0.13 mg./kg. s.c. and 4.3 mg./kg. p.o. Comparable data were reported by others (10). These large differences in toxicity and activity depending on the route of administration strongly suggest poor absorption. The problem of achieving elevated coumermycin blood levels thus can be posed as one of faulty or poor absorption from the gastrointestinal (GI) tract or of instability of the drug on oral administration. Since the preferred route for antibiotic administration is oral, it was considered essential before embarking on clinical trials to attempt to achieve improved blood levels by this route. The dog was chosen as a test animal since it had been found useful in studies of similar problems with novobiocin (9). Considering the *in vitro* antimicrobial activity of coumermycin in the presence of blood serum, a blood level of at least 1 mcg./ml. was somewhat arbitrarily selected as a desirable goal to allow for a reasonable safety factor, pending eventual clinical verification.

MATERIALS AND METHODS

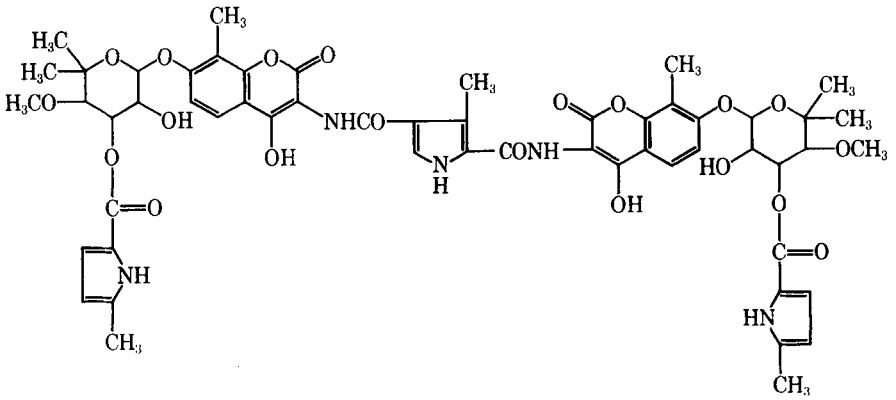
Blood Level Assays—Early studies were performed using an antibiotic bioassay employing serial broth dilutions of 2- or 3-fold, with *Staphylococcus aureus* as the test bacterium. Results of this method had the inherent and substantial potential error of the factor of dilution. During the course of the work, an agar diffusion cup-plate antibiotic bioassay was developed which had a precision of better than ±20% (4). Blood samples (serum) were diluted in 20% dimethylsulfoxide–3% aqueous phosphate buffer solution, pH 6.3, to a concentration which gave inhibition zones against the test organism *S. aureus* within the proper dose–response range (0.5 mcg./ml.). The standard coumermycin monosodium salt was similarly diluted in control blood serum. Quantitative evaluations were obtained as described in (4). Most of the data reported are with the latter method, unless otherwise indicated.

Administration of Drug to Dogs—For oral studies, dogs fasted 12–18 hr. were administered a single dose of about 5 mg./kg. of coumermycin monosodium salt as a fine powder (average diameter 7 μ) in gelatin capsules. Blood samples were taken at various time intervals as indicated later. It was found that administering an oral dose of water to the dog immediately after the drug dose gave higher and more consistent results. Both males and females were used, since no sex-related difference in response was found.

A parenteral aqueous solution of the monosodium salt of coumermycin A_1 was prepared to contain 20 mg./ml. of coumermycin A_1 monosodium salt (Ro 5-4645/10), 10% *N,N*-dimethyl acetamide, 10% ethanol, 40% propylene glycol, and 1.5% benzyl alcohol in water for injection. This solution was injected intravenously at a drug dose of about 10 mg./kg. Blood samples were taken and the sera assayed as previously indicated.

EXPERIMENTAL

Coumermycin A_1 (formerly also known as sugordomycin D-1a or notomycin), mol. wt. 1110, has the chemical structure (2, 4) shown as I.



I

It is a bishydroxycoumarin with two weakly acidic groups, pKa = 5.8–6.2 depending on solvent. [Kawaguchi *et al.* (1, 2) reported the pKa as 7.76 in 75% dioxane–water and 6.35 in 75% dimethyl formamide–water.] The pyrrole groups have very weak acidic properties, pKa above 11. The drug is sensitive to hydrolysis and rearrangement in strongly alkaline solution. A 1% solution of the soluble disodium salt has a pH ~10. This contrasts with novobiocin, pKa about 3.8, which forms a readily soluble monosodium salt which is only slightly alkaline (9).

Coumermycin free acid has no melting point but decomposes at about 245°, as does the crystalline monosodium salt (8). Coumermycin is soluble in aqueous alkali (*i.e.*, above pH 10), dioxane, dimethylformamide, and dimethylacetamide, and moderately soluble in acetone, methyl isobutyl ketone, methyl ethyl ketone, and ethyl acetate. It is poorly soluble in ethanol, methanol, isopropyl alcohol, chloroform, and benzene, and essentially insoluble in carbon tetrachloride, petroleum ether, and neutral and acidic water. It has a slight solubility in aqueous 6 *N* urea. When dissolved in aqueous alkali and neutralized with dilute acid, precipitation is rapid to form a dense particulate solid. Similarly, dilution of organic solvent solutions (*e.g.*, dimethylacetamide) with water results in rapid precipitation. In dilute aqueous alkali solution, coumermycin has strong surfactant properties. These stability, solubility, and physicochemical properties were taken into consideration in the selection of additives to be tested for enhancement of absorption of the drug.

Stability—To determine the importance of the stability of the antibiotic on blood levels, the effect of several experimental conditions was investigated.

Recovery from Blood In Vivo—The parenteral solution of the drug was injected intravenously into dogs at ~10 mg./kg. Blood samples were taken and the sera were bioassayed. The results were as follows (average of two dogs):

Time after Injection, hr.	Blood Levels (Dilution Assay),[1] mcg./ml.
0	0
0.5	160
1.0	160
2.0	70
4.0	70
24.0	3

Assuming a blood volume about 8% of the dog body weight, the peak level of 160 mcg./ml. in blood is about 1.3 times the administered dose. This is essentially 50–200% recovery of the injected drug, considering the errors of the method used. The results thus indicate that the drug was not rapidly destroyed nor eliminated from the blood.

In the Gastrointestinal Tract—In Vitro—An aqueous solution of the freshly prepared disodium salt of coumermycin was added to freshly prepared gastric juice USP to give a concentration of 20 mcg./ml. Full antibiotic activity (dilution bioassay) was maintained when incubated for 4 and 7 hr., respectively, at 37°.

In Vivo—The pyloric sphincter of a dog was tied off surgically. A dose of 500 mg. of coumermycin monosodium salt was administered orally. Blood samples were taken periodically from the gastroepiploic vein leading from the stomach, as well as samples of the gastric contents by aspiration into a syringe with saline. The bioassay results indicated essentially 100% retention of activity in the gastric contents for 4 hr., while the blood samples (1, 2, and 4 hr.) showed <1 mcg./ml.

These tests indicated that coumermycin is relatively stable in the GI tract, certainly in the stomach, and is not destroyed rapidly by the low pH (about 1.5) or enzymes of gastric juice *in vitro* or *in vivo*. It also appears stable to intestinal juice (pH about 8) *in vitro*.

Solubility—It was hypothesized that a system that would solubilize coumermycin at the pH range of the GI tract in aqueous solution would permit more efficient absorption. Several different approaches were made.

[1] It should be pointed out again that the serial broth dilution method, employed in this early exploratory experiment, is subject to a potential twofold error and is probably most likely the reason that the 1- and 4-hr. blood values did not "appear" to decline from the preceding values.

Table I—Coumermycin Blood Levels in Dogs after Single 5-mg./kg. Oral Dose (Antibiotic Content) in Different Formulations

Coumermycin Preparation[a]	Dosage Form	Number of Animals	Average Blood Levels, mcg./ml., Hours after Administration 2	4
Monosodium salt (micronized)	Capsule	6	0.4	0.4
Disodium salt	Capsule	2	1.1	0.8
Dicholine salt	Capsule	2	0.3	0.4
Di NMG salt	Capsule	3	0.3	0.2
Monosodium salt with NMG (1:1)	Capsule	2	1.1	1.3
Monosodium salt with NMG (1:4)	Capsule	3	2.1	3.1
Monosodium salt with NMG (1:10)	Capsule	3	7.5	4.0
Free acid + NMG (1:4)	Capsule	7	1.9	1.2
Monosodium salt with glucosamine (1:4)	Capsule	2	2.9	2.3
Monosodium salt with *N*-acetylglucosamine (1:4)	Capsule	2	1.1	0.8
Monosodium salt with *N*-acetylgalactosamine (1:4)	Capsule	2	2.3	1.6
Monosodium salt, Emulphor, DMA (1:2.5:2.5)[b]	Water-dispersible solution	3	1.5	4
Monosodium salt, poloxalene, DMA (1:2.5:2.5)[b]	Water-dispersible solution[c]	2	2	2
Monosodium salt, 0.5% in 70% DMSO[b]	Solution	2	3.8	6.3
Monosodium salt, urea (1:5)[b]	Capsule	2	1.8	0.8
Monosodium salt, urea, DMA (1:5:4)[b]	Capsule	4	3.8	1.4

[a] Numbers in parentheses are the ratio of the weights of the components used in the test preparation in order of listing. [b] Assays performed by serial dilution method. [c] Administered at 50 mg./kg.

Soluble Salts or Ion-Pairs—A series of disubstituted salts or ion-pairs of coumermycin was prepared and their aqueous solutions neutralized by dilute acid to pH 7. Most of these cases resulted in rapid precipitation of free coumermycin or its monosubstituted salt in the form of dense particles. These included magnesium, sodium, calcium (insoluble), aluminum (insoluble), diethanolamine, triethanolamine, lysine, glycine, trishydroxymethyl aminomethane (THAM), proline, leucine, and choline.

Sugar Amines—When aqueous solutions of coumermycin in dilute alkali are neutralized in the presence of sugar amines, a gel is formed rather than dense particles. This is analogous to the "gel-like" precipitate of amorphous novobiocin formed on careful acidification of its sodium salt solution, as reported previously (8). In particular, *N*-methylglucamine (NMG) aqueous solutions with coumermycin produce aqueous gels on neutralization with acid. A 2.5% solution of coumermycin with 10% NMG (pH about 11) will form an opalescent, solid gel on neutralization by dilute HCl or dilute acetic acid. These gels do not appear to have appreciable crystalline coumermycin. Similar behavior can be observed with glucosamine, *N*-acetylglucosamine, and *N*-acetylgalactosamine. A large molar excess of the sugar amine appears to be required.

Complexing Agents—Coumermycin contains two hydroxycoumarin groups that are potentially capable of forming soluble complexes with polyoxyethylene groups. Indeed, Kawaguchi *et al.* (7) claimed some slight success in using polyethylene glycol 300 (Carbowax 300) to enhance blood levels. It was found that solutions of coumermycin monosodium salt in dimethylacetamide with a series of substances containing polyethylene glycol units, including some nonionic surfactants, did form micellar, slightly opalescent dispersions on dilution with water. These include

Table II—Coumermycin Blood Levels[a] in Humans after Single 250-mg. Oral Dose

Preparation	Blood Levels, mcg./ml., Hours after Dose 0	2	4	8	24
Monosodium salt (micronized)	<0.01	0.01 (0.01–0.1)	0.14 (0.09–0.6)	0.34 (0.15–0.52)	0.16 (0.05–0.44)
Monosodium salt plus NMG (1:4)	<0.01	0.94 (0.25–1.5)	2.4 (1.3–2.8)	2.5 (1.3–4.0)	0.71 (0.4–1.2)

[a] Values are average of six patients in each group; the range is given in parentheses.

polysorbate 80, an emulsifying agent (Emulphor EL-620), a propylene glycol derivative (Pluronic F-68), polyethylene glycol 300, polyethylene glycol 4000, and polyethylene glycol monoricinoleate.

Formulations—A summary is presented in Table I of the data obtained for blood levels in dogs administered different formulations of coumermycin designed to give enhanced levels. A review of these results in dogs resulted in the selection of NMG formulation with the monosodium salt of coumermycin for trial in humans, using a 1:4 weight ratio equivalent to about 1:20 molar ratio. Although higher ratios of NMG to coumermycin increased the blood levels of drug in dogs, practical limitations such as capsule size led to the choice of 1:4 for clinical capsules. NMG has the practical advantages of being commercially available, nonhygroscopic, and chemically stable.

Capsules containing 50 mg. of coumermycin (monosodium salt) with 200 mg. of NMG were tested in humans in comparison to capsules of micronized coumermycin monosodium salt alone. The capsules were administered as a single oral dose of 250 mg. of coumermycin to six humans in each group. The results are presented in Table II.

DISCUSSION

The blood level data presented of coumermycin with NMG in dogs and humans clearly show the enhancement of absorption produced by this additive. At a ratio of 1:4 (drug–NMG), both dogs and humans show a 5–15-fold enhancement of oral absorption of coumermycin as measured by blood levels achieved. This effect is produced by simple physical mixture of the two ingredients in capsules. This represents about a 20 molar ratio of NMG to coumermycin. *In vitro*, this mixture dissolves in water to form a solution at pH 10.5–11.0, which in turn gives a gel-like noncrystalline mass on acidification. Presumably, a similar dissolution and then acidification to form a gel occur in the stomach after oral administration. The resultant gel probably has a greatly enhanced surface area to aid in absorption of coumermycin from the GI tract.

The blood levels achieved with the 1:4 mixture in dogs at 5 mg./kg. p.o. are roughly comparable to those achieved with the same mixture at 250 mg./human p.o. (approximately equivalent to 4 mg./kg.). This substantiates the choice of dogs as a test animal for biopharmaceutical studies of coumermycin.

The NMG mixture with coumermycin rapidly achieves blood levels appreciably higher than the 1 mcg./ml. originally desired on single doses of 250 mg./human, and levels above 1 mcg./ml. are maintained for over 12 hr.

SUMMARY

Coumermycin A_1, as the free acid or simple salts, is poorly absorbed from the GI tract of animals and humans. It is not appreciably degraded in the fluids of the GI tract. Mixtures of coumermycin with certain additivies, such as sugar amines, can significantly enhance oral absorption in dogs, as measured by the increased blood levels achieved. NMG, in a 1:4 ratio of antibiotic to NMG, enhances blood levels 5–15-fold over the antibiotic alone in both dogs and humans.

REFERENCES

(1) N. Kawaguchi, H. Tsukiura, M. Okanishi, T. Miyaki, T. Ohmori, K. Fujisawa, and H. Koskiyama, *J. Antibiot. Ser. A*, **18,** 1(1968).

(2) H. Kawaguchi, T. Naito, and H. Tsukiura, *ibid.*, **18,** 11 (1968).

(3) H. Kawaguchi, T. Miyaki, and H. Tsukiura, *ibid.*, **18,** 220 (1968).

(4) J. Berger, A. J. Schocher, A. D. Batcho, B. Pecherer, O. Keller, J. Maricq, A. E. Karr, B. P. Vaterlaus, A. Furlenmeier, and H. Spiegelberg, *Antimicrob. Ag. Chemother.*, **1965,** 778.

(5) W. Abrams, E. Grunberg, and A. J. Schocher, private communications.

(6) S. Nakazawa, H. Oka, H. Sato, N. Cho, H. Sunada, S. Nohara, K. Kunii, and K. Arai, *Chemotherapy*, **18,** 580(1966).

(7) H. Kawaguchi, H. Yeda, and S. Ishiyama, *Int. Congr. Chemother. 5th*, **1,** 1015(1967).

(8) J. D. Mullins and T. J. Macek, *J. Amer. Pharm. Ass., Sci. Ed.*, **49,** 245(1960).

(9) H. Kawaguchi, M. Okanishi, and T. Miyaki, U. S. pat. 3,201,386, Aug. 17, 1965.

(10) E. Grunberg and M. Bennett, *Antimicrob. Ag. Chemother.*, **1965,** 786.

ACKNOWLEDGMENTS AND ADDRESSES

Received October 20, 1969, from *Product Development Department and Chemical Research Department, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication February 18, 1970.

The authors thank Drs. J. Gerke, E. Grunberg, A. J. Schocher, and J. Unowsky for various microbiological assays; Dr. E. Whitman for the tests in humans; and Drs. R. Bagdon, R. Banziger, W. Pool, L. Hines, and S. Rubin for assistance in the design and execution of the animal tests.