Recovery of Tissue Bound Polymyxin B and Colistimethate[1] (35667)

Calvin M. Kunin and Ann Bugg

Department of Medicine, University of Wisconsin School of Medicine, and Veterans Administration Hospital, Madison, Wisconsin 53705

Previous studies in this laboratory have demonstrated that the antibacterial activity of the polymyxin antibiotics (polymyxin B and colistin) is inhibited by ordinary bacteriologic agar, dextran sulfate, and homogenates of a wide variety of tissues (1, 2). Colistimethate, the methane sulfonate derivative of colistin, is only slightly inhibited by these substances. The tissue factor responsible for inactivation of the polymyxin antibiotics has been shown to be acid phospholipids present in chloroform–methanol extracts. Liver and kidney are most active, followed by lung, heart, skeletal muscle, and brain. It was postulated that this effect could best be explained by formation of complexes between the highly positively charged antibiotics, containing multiple free amino groups, and negatively charged acid phospholipids. This notion was supported by demonstration of precipitin bands formed in gel diffusion tests between polymyxin B and inositol phosphatide (3). It was not possible, however, to determine from these experiments whether the drugs were permanently altered by tissue or bound in a repository form.

This report describes a method which liberates antibacterially active polymyxin B and colistimethate or free colistin from tissue binding sites. The method is based on ability to separate acid phospholipids from tissue by chloroform–methanol extraction, the relative resistance of the polymyxin antibiotics to heat and acid and availability of an inhibitor-free, cup-plate agarose diffusion assay method.

The marked difference between binding by tissues of polymyxin B and colistimethate are confirmed. The method should be suitable for use in studies of the distribution and persistence of the drugs in the body.

Materials and Methods. Tissues were obtained from New Zealand white rabbits sacrificed by exsanguination. Homogenates (25% wet wt) were prepared in a Sorvall Omni-Mixer (Ivan Sorvall) cooled in ice water, and stored at −20° until used. Antibiotics were polymyxin B (Aerosporin, Burroughs Wellcome) and sodium colistimethate (Colymycin, Warner-Chilcott) obtained from the hospital pharmacy. These were prepared as 100-μg/ml solutions in water and stored at −20° until used. Assays were performed by the cup-plate method using *Bordetella bronchiseptica* ATCC 4617 seeded in plastic petri dishes containing 10 ml of 2% agarose medium (Sea-Kem, Marine Colloids) and medium 199, adjusted to pH 8.0. All extracts and standard curves were performed at pH 8.0. The minimum sensitivity of this assay was 2.5 μg/ml for polymyxin B and 0.62 μg/ml for colistimethate.

Tissues extracts were prepared by adding 2 vol of chloroform–methanol (2:1) to tissue homogenates followed by blending for 1 min in the homogenizer. The mixture was then centrifuged at 10,000 rpm for 10 min in a Sorvall refrigerated centrifuge (Ivan Sorvall). Examination of the tube revealed an upper aqueous methanol, a middle tissue and a bottom chloroform layer. The upper and lower layers were removed and the tissue was subjected to two further extractions with chloroform–methanol. The chloroform extracts were pooled and dried in 100-mm glass petri dishes at 60°. The methanol layers were pooled and treated in the same manner. After a series of studies it was found that the antibiotics were most effectively released from the dried chloroform layer by addition

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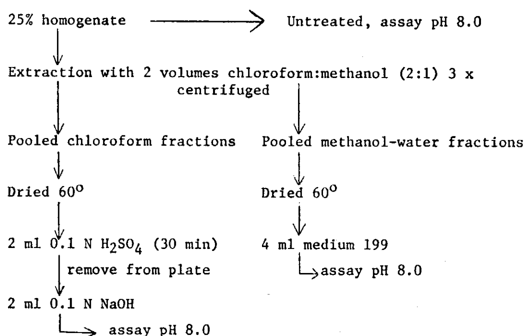
of 2 ml of 0.1 H_2SO_4 to the plates and incubation for 30 min at ca. 25°. Fluid was then *removed* from the plates, neutralized with 2 ml of 0.1 *N* NaOH, and adjusted to pH 8.0 prior to assay. The dried methanol–water layer was taken up in 4 ml of medium 199 (pH 8.0) and assayed directly. A summary of this method is presented in Table I.



Results. Recovery of drugs from tissues. Polymyxin B or colistimethate was added to 4 ml of tissue homogenates (1 g of tissue, wet wt), final concentration 25 μg/ml; incubated for 30 min at ca. 25°; and extracted by the procedure outlined in Table I. Recovery of the drugs from each fraction is shown in Table II. As shown, most of the polymyxin B was recovered in the chloroform layer of the tissues studied. In one instance (brain), the sum of polymyxin B recovered from chloroform and direct assay exceeded 100% (110.5%). This is thought to be due to the intrinsic variation of cup-plate assay methods which have an error of about ±10%.

In contrast to polymyxin B, large amounts of colistimethate were recovered directly from untreated homogenates with a proportionately smaller amount in the chloroform layer. As in the polymyxin B study, total recovery from both assays varied, in this test, probably related to the sensitivity of the assay. Note that about the same amount of drug was recovered in the methanol–water fraction as from untreated material except in the case of brain. This suggests that the methanol–water fraction also contains free drug. The proportion of drug present in the bound form (that is released from the chloroform layer by acid) was much higher for polymyxin B than for colistimethate.

The experiment was repeated with a one-step modification (Table III), in which mixtures of the drugs and tissues were centrifuged at 15,000 rpm for 15 min and the supernatant fraction and sediments were extracted separately. Note that chloroform extractable activity was present in both supernatant fluid and sediment. In this experiment, polymyxin B was not detectable in untreated homogenates or in the methanol–water layer, but colistimethate was present in

TABLE II. Recovery of Polymyxin B and Sodium Colistimethate from 25% Rabbit Tissue Homogenates Incubated with 25 μg/g (expressed as % recovered).

Tissue	Untreated	Methanol–water	Chloroform	Bound[a] (%)
		Polymyxin B		
Liver	5.0	0	94.0	95.0
Kidney	—	—	—	—
Lung	7.5	0	77.5	91.2
Brain	13.0	0	97.5	88.0
		Colistimethate		
Liver	52.5	53.0	67.5	56.3
Kidney	47.5	58.0	27.0	36.2
Lung	43.5	21.6	17.0	28.1
Brain	87.5	8.8	33.0	27.4

[a] Calculated as drug recovered in chloroform extract divided by recovered chloroform extract plus by direct assay × 100.

TABLE III. Localization of Polymyxin B and Sodium Colistimethate in Supernate and Sediment of 25% Aqueous Rabbit Tissue Homogenates Incubated with 25 μg/g (expressed as % recovered).

Tissue		Untreated	Methanol–water	Chloroform	(%) Recovered[a]
		Polymyxin B			
Liver	supernate	0	0	40.0	
	sediment	—	0	40.0	80.0
Kidney	supernate	0	0	25.6	
	sediment	—	0	100.0	125.6
		Colistimethate			
Liver	supernate	57.5	32.0	20.0	
	sediment	—	5.0	15.5	93.0
Kidney	supernate	47.5	31.0	11.6	
	sediment	—	5.2	31.0	90.1

[a] Derived from percentage recovered from direct assay and chloroform extract.

both. Total recovery of drug from different preparations varied from 80.0 to 125.6%.

The effect of lowering the concentration of drug to 10 μg/g added to tissues was studied next (Table IV). In this experiment 50 μg of antibiotic was added to 20 ml of 25% homogenate and the extraction procedure was followed as before. Polymyxin B was found only in the chloroform layer with the highest recoveries from liver and kidney. It is likely that the remainder of drug which was not recovered from other tissues may have been present in the aqueous fractions at concentrations below the sensitivity of the test (2.5 μg/ml). In contrast, except for a small amount in the chloroform extract of lung, colistimethate was found almost entirely in untreated tissue and in small amounts in the methanol–water layer. Failure to account for all of the drug may be related to small amounts bound to the chloroform layer, but at concentrations below the sensitivity of the test (0.63 μg/ml).

Factors which influence recovery of the drugs from tissue. In the course of developing this extraction procedure a number of variables were examined. Direct treatment of tissue homogenates with 0.1 or 1 *N* H_2SO_4 or HCl for 30 min at ca. 25° liberates an antibacterial factor active against not only the assay strain, but also against *Pseudomonas, Staphylococcus, E. coli, Klebsiella and Proteus.* The nature of this substance, which resembles that described by Hirsch and Dubos (4) and by Skarnes and Watson (5), will be the subject of another report. This

TABLE IV. Recovery of Polymyxin B and Colistimethate from 25% Rabbit Tissue Homogenates Incubated with 10 μg/g (expressed as % recovered).

Tissue	Polymyxin B (chloroform extract)[a]			Colistimethate (untreated)[b]		
Expt:	A	B	Mean	A	B	Mean
Liver	92.0	108.0	100.0	68.0	92.0	80.0
Kidney	84.0	96.0	90.0	84.0	72.0	78.0
Lung	43.2	56.0	49.6	40.0	52.0	46.0
Brain	57.6	69.6	63.6	68.0	68.0	68.0
Head	46.4	81.6	64.0	75.0	77.8	76.4
Muscle	60.0	68.0	64.0	84.0	72.0	78.0

[a] No drug detectable by assay of untreated tissue or in the methanol–water fraction.

[b] No drug detectable in the chloroform extract, 8–15% recovered in the methanol–water fraction; 12% recovered in chloroform extract of lung in both experiments A and B.

material appeared to be associated with a dialyzable peptide substance and interfered with the test. For this reason, lipids were first extracted from the homogenates and then treated with acid to liberate the drugs. No antibacterial activity was detected after acid treatment of lipid extracts of tissues which had not been preincubated with the antibiotics.

Treatment of polymyxin B or colistimethate solutions by drying at 60°, followed by incubation with 0.1 *N* H_2SO_4 and neutralization with 0.1 *N* NaOH did not interfere with antibacterial activity.

Various concentrations of H_2SO_4 and HCl, incubation times and heating at 100° were explored. The HCl failed to liberate as much drug as H_2SO_4. Concentrations of H_2SO_4 of 0.1 and 1.0 *N* were equally effective. Prolonged incubation and boiling did not improve recovery. An important feature of the method is the need to separate the acid solution from the dried chloroform layer prior to neutralization with alkali. Recovery is poor if this is not done, presumably because the liberated polymyxin antibiotic is bound once again to the lipids. For example, in early studies in which the acid extract was not separated prior to neutralization, recovery from liver and kidney was less than 50% of the drug that was added.

Discussion. The polymyxin antibiotics are believed to exhibit antibacterial activity by virtue of their ability to bind to bacterial membrane phospholipids (6). Binding to membranes of mammalian cells probably accounts as well for their toxic properties in mammalian tissue. Colistimethate may be less toxic than polymyxin B and more readily excreted in the urine by virtue of the fact that it less readily binds to phospholipids. It has been shown previously that colistin sulfate binds to tissue just as readily as polymyxin B, but that the methane sulfonation derivative, colistimethate, which possesses fewer free amino groups behaves quite differently. The ability to recover most of polymyxin B and colistimethate from tissue extracts indicates that inhibition of antibacterial activity by tissue homogenates is due to binding rather than chemical alteration of the drugs.

Sande and Kaye (7) have suggested that *in vitro* assays of colistimethate may be inaccurate because of "activation" *in vivo* as the sulfomethyl groups are hydrolyzed. The assay procedure used in the current study avoids some of these problems since the agarose–199 medium is free of inhibitors normally present in bacteriologic agar and various growth media. Both colistin and colistimethate diffuse well in agarose. The ability to partition polymyxin B and colistimethate into tissue bound and unbound fractions also provides information concerning the relative abundance of highly charged (bound) and uncharged (free) drug. Adaptation of the extraction procedure to *in vivo* studies, now underway in experimental animals, should help clarify the distribution and persistence of the drugs in various tissues. Preliminary studies clearly indicate sequestration and persistence of both drugs in tissues long after they are no longer detectable in the blood. Experiments to determine the rate of elution of the drugs from tissues *in vivo* are now in progress.

Summary. A method for recovery of tissue bound polymyxin B and colistimethate has been developed. It is based on the finding that these drugs bind strongly to acid phospholipids present in cell membranes. The method employs extraction of lipid fractions by chloroform–methanol followed by release from the binding sites by dilute H_2SO_4. Polymyxin B and colistimethate differ markedly in binding properties probably related to coverage of free amino groups in the latter compound by methane-sulfonation. This method should prove useful in understanding the pharmacologic behavior of these antibiotics when employed in animal models.

This approach may also prove useful in studies of the behavior in the body of other highly positively charged compounds.

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