Chromatographic Separation of the C(1), C(1a), and C(2) Components of Gentamicin and the Assessment of Their Individual Binding to Serum Proteins

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Received December 21, 1983, from the **Department of Pathology and Laboratory Medicine, Division of Biology and Medicine, Roger Williams General Hospital and Brown University, Providence, RI 02908* and the *‡Department of Chemistry, Providence College, Providence, RI 02918.* Accepted for publication March 9, 1984.

Abstract □ [^{3}H]gentamicin and [^{14}C]gentamicin samples were purified by Sephadex column chromatography and separated by an HPLC technique into the three major, medicinally active gentamicin components. These separated components were used in equilibrium dialysis studies to determine their percent binding to serum proteins. The bindings of the components were inversely related to concentrations of ionized calcium and magnesium. When dialyzed against a buffer containing physiological concentrations of the divalent cations, the binding of the C(1) component was 2.2 ± 1.0%, the binding of the C(1a) component was 1.2 ± 1.9%, and the binding of the C(2) component was 5.0 ± 2.0%. The percent bindings are not identical and, due to their low values, probably have negligible clinical significance. The radioactive composition and purity of the ^{3}H- and ^{14}C-labeled gentamicin samples differed and these may be important factors in the variance of reported gentamicin bindings.

Keyphrases □ Gentamicin—component separation serum protein binding, radiolabels, HPLC □ Serum protein binding—radiolabeled gentamicin, component separation, HPLC

Since its discovery in 1963, the use of the broad spectrum antibiotic gentamicin has found acceptance in the treatment of gram negative infections (1). Because nephrotoxicity and ototoxicity are associated with gentamicin administration, its use has been somewhat limited (2). In evaluating the pharmacokinetics and renal cortical uptake of gentamicin, its binding to serum proteins was investigated. However, reported gentamicin binding varies from 0 to 30% (3–8). Parameters which influence the binding of gentamicin to serum proteins and which must be controlled in these binding studies are the levels of ionized calcium and magnesium (6, 7) and the use of serum rather than heparinized plasma (6). However, the chemical purity of the radioactively labeled gentamicin used in some studies was not well defined. To further examine gentamicin binding to serum proteins, our study was designed to separate both ^{3}H- and ^{14}C-labeled gentamicin samples into the three major, medicinally active components [C(1), C(1a), and C(2)] and to determine the percent binding of these components to the proteins of a serum pool prepared from hospital patient samples.

EXPERIMENTAL SECTION

Gentamicin sulfate[1] and [^{14}C]gentamicin sulfate[2] (120 mg, specific activity 830 μCi/mg) were generously donated. Unlabeled gentamicin (100 mg) was tritium labeled[3] by the high-pressure gas phase technique (crude product specific activity 0.37 mCi/mg). The scintillation cocktail was commercially obtained[4]. Unless otherwise specified, all other chemicals were reagent grade. The serum used in the experiments was prepared by pooling the excess of >2000 specimens from adult hospital patients. The pool was divided into 5-mL aliquots, which were kept at −5°C until use, to a maximum of 6 months.

[1] Lot No. 1045-268-3194; Schering Corp.
[2] Lot No. 5883-118; Schering Corp.
[3] New England Nuclear, Boston, Mass.
[4] Aquasol; New England Nuclear.

Purification of Radioactive Gentamicin with Thin-Layer Chromatographic Detection—Radioactive gentamicin was partially purified with Sephadex column chromatography using a modification of the method of Mahon *et al.* (9). Aliquots (1 mL) were collected into test tubes. One microliter from each aliquot was placed into scintillation vials containing 10 mL of the scintillation fluid. The vials were counted in a liquid scintillation counter[5]. The fractions showing the highest level of radioactivity were saved and the others were discarded. The fractions containing gentamicin were identified by TLC. Aliquots (100 μL) were spotted on a silica gel TLC plate[6]. A 1-mg/mL nonradioactive gentamicin solution (25 μL) in water was used as a marker on either side of the plate. The solvent system was chloroform–17% NH_4OH–methanol–H_2O (1:2:4:1, by volume). One percent ninhydrin in ethanol was used as the visualization agent. This system separates gentamicin into three bands which correspond to the three major components of the active drug.

Liquid Chromatographic Separation into Gentamicin Components—The HPLC separation of radioactive gentamicin into its three major components was accomplished by a modification of the ion-pair method of Anhalt and Brown (10). This was done with an HPLC unit using a C_{18} column[7] with a mobile phase of 0.2 M Na_2SO_4, 0.02 M sodium pentane sulfonate, and 0.1% (v/v) acetic acid in water–methanol (97:3) at a flow rate of 2 mL/min at ambient temperature. Samples (25 μL) were injected and collected fractions were analyzed both by TLC and liquid scintillation counting.

Dialysis Studies—Equilibrium dialysis was carried out in a multicavity dialysis cell[8] (eight chambers, 1.0-mL capacity each) with cellulose dialysis membranes[9]. Human pooled serum was dialyzed against an equivalent amount of a buffer containing a radioactive gentamicin component (purified by Sephadex and HPLC) and nonradioactive gentamicin at a concentration of 10 μg/mL. Divalent cation concentrations were varied by changing the concentration of these species in the buffer from zero to four times the physiological concentrations. Divalent cation concentrations of the serum samples were varied by adding equivalent amounts of EDTA to the serum to reduce their concentrations to zero or by adding the appropriate amount of these cations to the buffer to bring the concentrations to four times the physiological value. Dialysis membranes were prepared by boiling them in distilled water three times and then soaking them overnight in the dialysis buffer of 0.05 M Tris, 0.1% sodium azide, and 0.15 M NaCl (adjusted to pH 7.4 with 1 M HCl). All dialysis experiments were equilibrated for a 48-h period in a thermally controlled (37°C) shaking waterbath[10]. Control experiments, consisting of buffer with gentamicin against buffer without gentamicin, were also run.

[5] Model 3385, TRI-CARB; Packard.
[6] LK5D, silica gel 8 nm; Whatman, Clifton, N.J.
[7] μ-Bondapak C_{18} column, Model 204; Waters.
[8] Model 348; Bel Art Products, Inc.
[9] Model No. 43020; Roche.
[10] Model 50; GCA/Precision Scientific.

Table I—Percent Radioactivity of Gentamicin Components Separated by Liquid Chromatography

Component	^{3}H-Labeled Sample[a]	^{14}C-Labeled Sample	
		Our Data[a]	Manufacturer's Data
C(1)	21%	58%	54.4%
C(1a)	42%	6%	10.4%
C(2)	37%	36%	35.2%

[a] These percentages correspond to the radioactivity of the HPLC-separated components used in the dialysis studies, not to the overall radioactive composition of the initial sample.

0022-3549/84/1200-1836$01.00/0

Table II—Percent Binding of Gentamicin Components to Serum Proteins[a]

Serum Environment	C(1) Component		C(1a) Component		C(2) Component	
			Absence of Ca^{2+} and Mg^{2+}			
^3H-Label data	8.7 ± 3.9 (5)	$p < 0.01$	2.8 ± 1.1 (4)	$p = 0.02$	5.7 ± 3.3 (5)	$p < 0.01$
^{14}C-Label data	5.8 ± 2.2 (4)	$p < 0.01$	3.4 ± 2.9 (3)	$p = 0.02$	7.2 ± 1.5 (5)	$p < 0.01$
^3H- and ^{14}C-Label data	7.4 ± 2.4 (9)	$p < 0.01$	3.1 ± 1.0 (7)	$p < 0.01$	6.5 ± 1.4 (10)	$p < 0.01$
			Normal Ca^{2+} and Mg^{2+}			
^3H-Label data	2.1 ± 1.5 (10)	$p < 0.01$	1.5 ± 1.8 (12)	$p = 0.05$	6.8 ± 2.2 (10)	$p < 0.01$
^{14}C-Label data	2.3 ± 0.6 (5)	$p = 0.02$	0.2 ± 8.1 (4)	$p = 0.70$	1.4 ± 1.5 (5)	$p = 0.12$
^3H- and ^{14}C-Label data	2.2 ± 1.0 (15)	$p = 0.01$	1.2 ± 1.9 (16)	$p = 0.27$	5.0 ± 2.0 (15)	$p < 0.01$
			Elevated Ca^{2+} and Mg^{2+} (× 4)			
^3H data	3.6 ± 4.2 (4)	$p < 0.01$	2.3 ± 3.0 (4)	$p = 0.14$	5.1 ± 4.6 (5)	$p < 0.01$
^{14}C data	−1.1 ± 1.7 (5)	$p = 0.57$	−2.8 ± 5.3 (4)	$p = 0.11$	0.6 ± 1.2 (5)	$p = 0.12$
^3H and ^{14}C data	1.0 ± 0.8 (9)	$p = 0.46$	−0.3 ± 3.2 (8)	$p = 0.50$	2.2 ± 2.9 (10)	$p = 0.05$
			Controls			
^3H-Label data			0.8 ± 0.6 (34)			
^{14}C-Label data			−0.4 ± 1.1 (21)			
^3H- and ^{14}C-Label data			0.4 ± 0.6 (55)			

[a] All bindings are given as: mean ± 95% confidence limit (number of data points); p values are the probabilities for the t test comparing the percent binding values to the control values. Total gentamicin concentrations in serum at equilibrium were at 5 μg/mL during this study.

Liquid scintillation counting was done on the serum and buffer portions after minimizing quenching differences through a matrix equalization procedure. In this procedure, pooled serum without gentamicin was dialyzed against buffer without gentamicin to reproduce the slight changes due to the osmotic effect during dialysis. The dialyzed serum without gentamicin was added to an equal volume of radioactive buffer, and the dialyzed buffer without gentamicin was added to an equal volume of radioactive serum before counting on the liquid scintillation counter. The percentage of drug bound to the serum proteins was calculated as published by Olsen (11, 12). Radiochemical analysis of the dialysis membrane indicated that negligible binding-absorption of gentamicin had occurred to this membrane.

RESULTS AND DISCUSSION

TLC analysis of the Sephadex-enriched [^3H]gentamicin showed significant amounts of radioactive material at the origin and in locations other than those attributable to known gentamicin components, demonstrating incomplete purification.

The results of the HPLC separation of Sephadex-treated radioactive gentamicin into its components is presented in Table I. Essentially all of the radioactivity of the [^{14}C]gentamicin can be ascribed to the gentamicin components, whereas significant radioactivity (~25%) of the [^3H]gentamicin was found in HPLC fractions that contained negligible gentamicin, mostly in the fractions eluting early. Some tritiated impurities possibly also bind to serum proteins. This binding of impurities may explain the observation that a tritiated gentamicin sample, purified by Sephadex but not HPLC, gave 15, 10, and 6% binding with zero, normal, and elevated divalent cation levels as compared with 5.1, 3.6, and 3.6% for the combined bindings of the HPLC purified-separated tritiated components.

Multiple equilibrium dialysis experiments were carried out using either [^3H]gentamicin components or [^{14}C]gentamicin components. The data are summarized in Table II. There were also 34 control experiments using [^3H]gentamicin and 21 control experiments using [^{14}C]gentamicin, all run concurrently with one or another serum dialysis experiment. The criterion for assessing the difference in binding from zero of a given gentamicin component in a given environment was the comparison of the average test experiment data with the average control result, using the Student's t test.

The bindings are inversely related to concentrations of ionized calcium and magnesium as reported earlier (6, 7). In the absence of ionized calcium and magnesium in the serum pool, partially separated gentamicin components, C(1), C(1a), and C(2), all showed binding different from zero, at 7.4, 3.1, and 6.5%, respectively. In the presence of physiological concentrations of calcium and magnesium, components C(1) and C(2) showed binding. The averages were 2.2 and 5.0%, respectively. Component C(1a) showed no binding under these conditions. In the presence of calcium and magnesium concentrations which were four times the usual physiological concentrations, none of the components showed binding when average data were used. There were two discrepancies, however, in that the tritium-labeled C(1) and C(2) components showed binding at 3.6 and 5.1%, respectively. This finding is probably due either to incomplete separation of the tritiated gentamicin components from nondrug impurities, or to a large precision error. A three-way analysis of variance using the Fisher F ratio showed that the 4X physiological divalent cation, physiological divalent cation, and no divalent cation binding results were different for the C(1) component ($p < 0.001$), for the C(2) component ($p = 0.030$), but not for the C(1a) component [for which the only binding was in the absence of calcium and magnesium ($p = 0.179$)].

Several fresh serum samples, drawn from volunteers, were used in dialysis experiments and showed degrees of binding comparable to those obtained using pooled serum. The bindings of the individual gentamicin components to serum proteins are different and, due to their low values, this binding effect probably has no clinical significance. This study indicates that, besides the use of serum rather than heparinized plasma and control over the divalent cation concentrations, other significant factors in the variance of reported gentamicin bindings may be the radioactive purity and relative composition of labeled gentamicin mixtures. Thus, these factors must be better controlled or, at least, better documented in protein binding studies.

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