

SUPPLEMENTAL INFORMATION

Figure S1. LC-MS and ELSD characterization of an exemplar clinical supply of gentamicin (*sample #3*). (A) The total ion counts from the LC-MS of gentamicin drug product (top trace) and blank injection (bottom trace). Four major peaks presumably representing 4 major gentamicin congeners compose the majority of the overall gentamicin mixture. Many minor peaks with shorter retention times likely compose approximately 10% of the overall mixture (computed based peak integrations; see Table S2). (B) The contents of the gentamicin clinical supply as measured by ELSD mirror the results obtained by LC-MS demonstrating that the complexity of the mixture is not an artifact of the detection method.

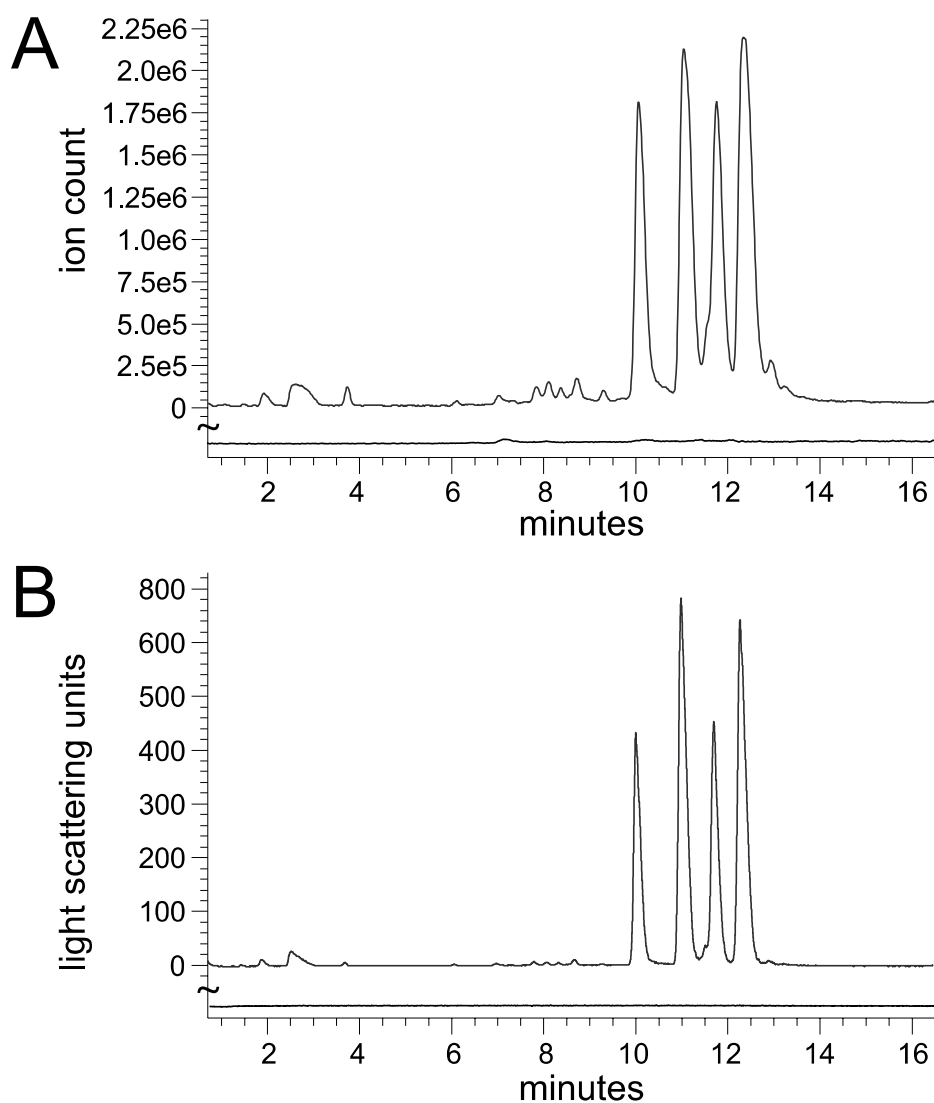


Table S1. Gentamicin clinical supply (*sample #3*) peak integrations and resulting peak area percentages

LC-MS				ELSD			
peak #	retention time (min)	area (TIC×s)	area %	peak #	retention time (min)	area (LSU×s)	area %
1	1.92	9.18e5	0.5	1	1.87	112	0.4
2	2.62	3.70e6	2.2	2	2.52	491	1.8
3	3.73	9.09e5	0.5	3	3.68	36.0	0.1
4	6.11	2.46e5	0.1	4	6.06	21.9	0.1
5	7.03	9.13e5	0.5	5	6.97	30.9	0.1
6	7.84	1.17e6	0.7	6	7.79	60.7	0.2
7	8.11	1.48e6	0.8	7	8.06	49.5	0.2
8	8.37	9.63e5	0.6	8	8.32	55.5	0.2
9	8.72	2.27e6	1.3	9	8.67	95.9	0.4
10	9.30	8.71e5	0.5	10	-	-	-
11	10.1	3.02e7	18.0	11	10.0	4520	17
12	11.0	3.92e7	23.3	12	11.0	8140	30
13	11.8	3.29e7	19.6	13	11.7	5140	19
14	12.3	4.72e7	28.1	14	12.3	8300	31
15	12.9	3.53e6	2.1	15	12.9	108	0.4
16	12.2	1.56e6	0.9	16	-	-	-

Figure S2. The structure of kanamycin with AAC(6')-Ib informs a model to account for the susceptibility differences between gentamicin C2 and C2a against strains containing AAC6'-Ib. **(A)** 2D structures of gentamicin C1a and kanamycin B illustrating the similarities of the two molecules particularly at the 6' carbon position highlighted in red and orange for gentamicin C1a and kanamycin B, respectively. **(B)** X-ray crystal structure of AAC(6')-Ib from *Salmonella enterica* subsp. *enterica* (PDB code 2QIR) in complex with kanamycin B at 2.40 Angstrom resolution. Kanamycin is shown as sticks colored with green carbon, red oxygen, and blue nitrogen. The enzyme is shown as a cartoon model in magenta with a gray transparent surface illustrating the pocket occupied by kanamycin. The 6' carbon atom is labeled and white hydrogen atoms were modelled on this atom to illustrate where the methyl attachment points would be on gentamicin C2 and C2a. One of the attachment points is near the back wall of the active site pocket while the other position points out toward solvent in this structure.

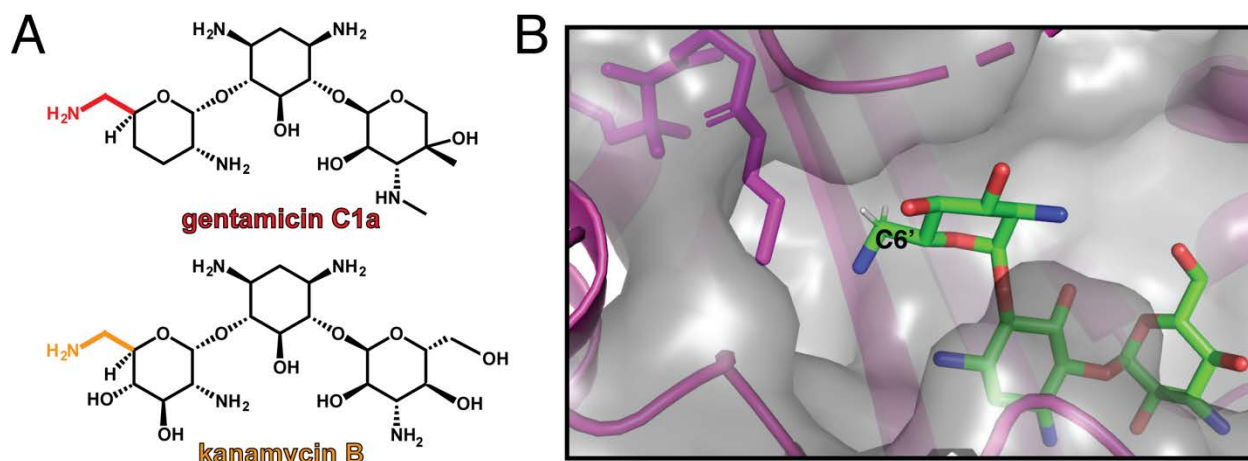


Table S2. Description of gentamicin sources and batch numbers characterized in this manuscript

Clinical Dosing Solution Number	Product Name	Batch
USP ref std.	USP Ref Std 1289003	MOD314
1	GENT-40-10AMP-UK	1802200
2	GENT-240/80-20VIA-UK	17467414
3	GENT-160/2-5VIA-HEX	FN8794
4	GENT-40-5VIA-HEX	GS0977AA
5	GENT-40-10VIA-DE	GF9422AB
6	GENT-80/2-5VIA-HEX	GL8882
7	GENT-80/2-5(4)VIA-DE	GG4567
8	GENT-160/2-5VIA-RAT	T19094A
9	GENT-40-5VIA-RAT	T09675A
10	GENT-80/2-5VIA-RAT	T07104A
11	REFO-120/2-5VIA-DE	229506
12	REFO-40-5VIA-DE	242374
AST Disks	ID	Catalogue no.
1	Hardy 10 µg	Z8541
2	BD BBL 10 µg	231227
3	BD BBL 120 µg	231693
4	Oxoid 10 µg	CT0024B
5	Oxoid 120 µg	CT0794B

Figure S3. The four major gentamicin congeners can be purified from the gentamicin mixture. The laboratory batch of the gentamicin mixture from which the congeners were purified (top black line), purified gentamicin congeners C1a (red), C2 (blue), C2a (green), and C1 (purple) and a water-only sample (bottom black line). Total ion count is depicted on the y-axis.

