Study Title:

Analysis of Rodent Plasma Samples for GB67B, GB97 and GB594

Ricerca Project Number: **024347**

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Document Number: 024347-1

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Introduction

Objectives

The objectives of this bioanalytical study were to analyze mouse plasma from mouse PK studies for three compounds (GB67B, GB97, and GB594) and to analyze rat plasma from a rat toxicology study for GB67B conducted outside Ricerca.

Methods

Extraction Method

GB67B

1. Prepare a 1 mg/ml of test article in MeOH.
2. Serial diluted test articles from 1 mg/mL in MeOH to obtain 1000, 800, 400, 100, 50, 10, 5 and 2.5, µg/mL stock.
3. Spiked 10 µL of the each stock in 490 µL of mouse or rat plasma to make standard mouse and rat plasma curves at 20000, 16000, 8000, 2000, 1000, 200, 100 and 50 ng/mL.
4. Prepare an internal standard (IS) (GB67B-d9) stock at 0.5 µg/mL in ACN.
5. Transfer 40 µL of the standards and samples to new tubes and quench by 250 µL of IS in ACN, vortex, and spin at 14000 rpm for 10 min at 5 °C.
6. The supernatant were transferred in low-volume plastic HPLC vials for LC/MS/MS.

GB97

1. Prepare a 271 µg/ml of test article in MeOH.
2. Spiked 271 µg/mL of the stock solution in mouse plasma and make a serial dilution from mouse plasma to obtain a standard mouse plasma curve at 20000, 16000, 8000, 4000, 2000, 800, 400 and 200 ng/mL.
3. Prepare an internal standard (IS) (GB97-d9) stock at 2.5 µg/mL in CAN.
4. Transfer 50 µL of the standards and samples to new tubes and quench by 250 µL of IS in ACN, vortex, and spin at 14000 rpm for 10 min at 5 °C.
5. The supernatant were transferred in low-volume plastic HPLC vials for LC/MS/MS.

GB594

1. Prepare a 400 µg/ml of test article in MeOH.
2. Spiked 400 µg/mL of the stock solution in mouse plasma and make a serial dilution from mouse plasma to obtain a standard mouse plasma curve at 20000, 16000, 8000, 2000, 1000, 200, 100 and 50 ng/mL
3. Prepare an internal standard (IS) (GB594-d9) stock at 2.5 µg/mL in ACN
4. Transfer 50 µL of the standards and samples to new tubes and quench by 250 µL of IS in ACN, vortex, and spin at 14000 rpm for 10 min at 5 °C
5. The supernatant were transferred in low-volume plastic HPLC vials for LC/MS/MS.

Bioanalytical Method

Test Article: GB67B

Mass spectrometer:	PE Sciex API 4000 LC-MS/MS System
HPLC Column:	Phenomenex Synergi MAX-RP 4μ 150 x 2.0 mm with Security Guard
Buffers:	A = 1% formic acid in 5 mM ammonium acetate B = 1% formic acid in methanol
Isocratic:	Time (min) %B
	0 68
	3.0 Stop
	Note: The column flow before 1.3 minutes was diverted to waste.
Flow Rate:	0.5 mL/min
Injection Volume:	4 µL
Ionization:	Positive
MRM:	209.1 → 181.2 (GB67B) 218.1 → 190.2 (GB67B-d9, IS)

Test Article:GB97

Mass spectrometer:	PE Sciex API 4000 LC-MS/MS System
HPLC Column:	Phenomenex Synergi MAX-RP 4μ 150 x 2.0 mm with Security Guard
Buffers:	A = 1% formic acid in 5 mM ammonium acetate B = 1% formic acid in methanol
Isocratic:	Time (min) %B
	0 80
	3.0 Stop
	Note: The column flow before 1.0 minute was diverted to waste.
Flow Rate:	0.5 mL/min
Injection Volume:	6 µL
Ionization:	Positive
MRM:	242.4 → 167.1 (GB97) 251.4 → 176.1 (GB97-d9, IS)

Test Article: GB594

Mass spectrometer:	PE Sciex API 4000 LC-MS/MS System
HPLC Column:	Phenomenex Synergi POLAR-RP 4μ 50 x 2.0 mm with Security Guard
Buffers:	A = 1% formic acid in 5 mM ammonium acetate B = 1% formic acid in methanol
Gradient:	Time (min) %B
	0 10
	0.2 10
	2.0 95
	2.8 95
	2.9 10
	5.6 Stop
	Note: The column flow before 1.5 minutes was diverted to waste.
Injection Volume:	5 µL
Ionization:	Positive
MRM:	260.1 → 57.1 (GB594) 269.1 → 66.1 (GB594-d9, IS)

Results

Analytical Results of Mouse Plasma

Analysis result for GB-67B

Std Curve weighted 1/x*x
Intercept = 0.018
Slope = 0.00161
Correlation Coeff = 0.9981

Group	Dose/Route	GB-67B/MPK-08-1	Mouse Number	Dosing Time	Schedule Sample Time	Concentration (ng/mL)
1 (8h)	30mg/kg i.p	1	1	800	1600	No Peak
		2	2	802	1602	No Peak
		3	3	804	1604	No Peak
		4	4	806	1606	No Peak
2 (6h)	30mg/kg i.p	5	1	810	1410	No Peak
		6	2	812	1412	No Peak
		7	3	814	1414	No Peak
		8	4	816	1416	No Peak
3 (4h)	30mg/kg	9	1	820	1220	No Peak
		10	2	822	1222	No Peak
		11	3	824	1224	No Peak
		12	4	826	1226	No Peak
4 (2h)	30mg/kg .i.p	13	1	830	1030	No Peak
		14	2	832	1032	No Peak
		15	3	834	1034	No Peak
		16	4	836	1036	No Peak
5 (1h)	30mg/kg	17	1	840	940	No Peak
		18	2	842	942	No Peak
		19	3	844	944	No Peak
		20	4	846	946	No Peak
6 (30m)	30mg/kg i.p	21	1	1020	1050	No Peak
		22	2	1022	1052	BLQ
		23	3	1024	1054	No Peak
		24	4	1026	1056	No Peak
7 (15m)	30mg/kg	25	1	1100	1115	No Peak
		26	2	1102	1117	No Peak
		27	3	1104	1119	No Peak
		28	4	1106	1121	No Peak
8 (5m)	30mg/kg i.p	29	1	1140	1145	No Peak
		30	2	1142	1147	No Peak
		31	3	1150	1155	No Peak
		32	4	1152	1157	No Peak
Vehicle Control	i.p	33	1	1200	1230	No Peak

BLQ: blow limit of quantitation

Analysis result for GB-97

Std Curve weighted 1/x*x
Intercept = -0.0177
Slope = 0.00104
Correlation Coeff = 0.9978

Group	Dose/Route	GB-97/-MPK-08-1	Animal	Collection Time (nominal)	Collection Time (actual)	Concentration (ng/mL)
G1	30mg/kg/i.p.	1	1	1600	NA	No Peak
		2	2	1602	NA	No Peak
		3	3	1604	NA	No Peak
		4	4	1606	NA	No Peak
G2	30mg/kg/i.p.	5	1	1410	NA	No Peak
		6	2	1412	NA	No Peak
		7	3	1414	NA	No Peak
		8	4	1416	NA	No Peak
G3	30mg/kg/i.p.	9	1	1220	NA	No Peak
		10	2	1222	NA	No Peak
		11	3	1224	NA	No Peak
		12	4	1226	NA	No Peak
G4	30mg/kg/i.p.	13	1	1030	NA	No Peak
		14	2	1032	NA	No Peak
		15	3	1034	NA	No Peak
		16	4	1036	NA	No Peak
G5	30mg/kg/i.p.	17	1	940	NA	No Peak
		18	2	942	NA	No Peak
		19	3	944	NA	No Peak
		20	4	946	NA	No Peak
G6	30mg/kg/i.p.	21	1	1050	NA	330
		22	2	1052	NA	214
		23	3	1054	NA	253
		24	4	1056	NA	182
G7	30mg/kg/i.p.	25	1	1115	NA	1559
		26	2	1117	NA	176
		27	3	1119	NA	802
		28	4	1121	NA	1448
G8	30mg/kg/i.p.	29	1	1145	NA	2813
		30	2	1147	NA	1373
		31	3	1155	NA	1742
		32	4	1157		305
Vehicle Control	i.p.	33	1	1230	NA	No Peak

Analysis result for GB594

Std Curve weighted 1/x*x
Intercept = -0.0223
Slope = 0.00186
Correlation Coeff = 0.9939

Group	Dose/Route	GB-594/MPK-08-1	Animal	Collection Time (nominal)	Concentration (ng/mL)
G1	30mg/kg/i.p.	1	1	1600	No Peak
		2	2	1602	No Peak
		3	3	1604	No Peak
		4	4	1606	No Peak
G2	30mg/kg/i.p.	5	3	1414	No Peak
		6	4	1416	No Peak
G3	30mg/kg/i.p.	7	1	1220	No Peak
		8	2	1222	No Peak
		9	3	1224	No Peak
		10	4	1226	No Peak
G4	30mg/kg/i.p.	11	1	1030	No Peak
		12	2	1032	No Peak
		13	3	1034	No Peak
		14	4	1036	No Peak
G5	30mg/kg/i.p.	15	1	940	110
		16	2	942	118
		17	3	944	72.9
		18	4	946	BLQ
G6	30mg/kg/i.p.	19	1	1050	463
		20	2	1052	326
		21	3	1054	619
		22	4	1056	251
G7	30mg/kg/i.p.	23	1	1115	2567
		24	2	1117	1555
		25	3	1119	2023
		26	4	1121	1080
G8	30mg/kg/i.p.	27	1	1145	1501
		28	2	1147	1003
		29	3	1155	1106
		30	4	1157	2189
Vehicle Control	i.p.	31	1	1230	No Peak

BLQ: blow limit of quantitation

Analytical Results of Rat Plasma

Std Curve weighted 1/x*x
Intercept = -0.00614
Slope = 0.00183
Correlation Coeff = 0.9992

DAY 1 (05-Feb-2009) RAT PLASMA SAMPLES (5017-5039)

GB-67B/ 044RE27.003	Animal	Sex	Group	Dose (mg/kg/day)	Predose Sample	Time of Dose	1 hour Postdose	Differ-ence	4 hours Postdose	Differ-ence	Conc. (ng/mL)
	5017	M	5	6							
0001					7:09	8:00					No Peak
0002							9:00	1:00			No Peak
0003									12:00	4:00	No Peak
	5019	M	5	6							
0004					7:11	8:03					No Peak
0005							9:03	1:00			No Peak
0006									12:03	4:00	No Peak
	5021	F	5	6							
0007					7:13	8:06					No Peak
0008							9:06	1:00			No Peak
0009									12:06	4:00	No Peak
	5023	F	5	6							
0010					7:15	8:09					No Peak
0011							9:09	1:00			No Peak
0012									12:09	4:00	No Peak
	5025	M	6	18							
0013					7:17	8:12					No Peak
0014							9:12	1:00			No Peak
0015									12:12	4:00	No Peak
	5027	M	6	18							
0016					7:19	8:15					No Peak
0017							9:15	1:00			No Peak
0018									12:15	4:00	No Peak
	5029	F	6	18							
0019					7:22	8:18					No Peak
0020							9:18	1:00			No Peak
0021									12:18	4:00	No Peak
	5031	F	6	18							
0022					7:24	8:21					No Peak
0023							9:21	1:00			No Peak
0024									12:21	4:00	No Peak
	5033	M	7	36							
0025					7:26	8:24					No Peak
0026							9:24	1:00			No Peak
0027									12:24	4:00	No Peak
	5035	M	7	36							
0028					7:28	8:27					No Peak
0029							9:27	1:00			No Peak
0030									12:27	4:00	No Peak
	5037	F	7	36							
0031					7:30	8:30					No Peak
0032							9:30	1:00			No Peak
0033									12:30	4:00	No Peak
	5039	F	7	36							
0034					7:32	8:33					No Peak
0035							9:33	1:00			No Peak
0036									12:33	4:00	No Peak

DAY 1 (05-Feb-2009) RAT PLASMA SAMPLES (2018-5040A)

GB-67B/ 044RE27.002	Animal	Sex	Group	Dose (mg/kg/day)	Time of Dose	30 min Posdose	Differ-ence	2 hours Postdose	Differ-ence	Conc. (ng/mL)
	5018	M	5	6						
0037					8:01	8:31	0:30			No Peak
0038								10:01	2:00	No Peak
	5020	M	5	6						
0039					8:04	8:34	0:30			No Peak
0040								10:04	2:00	No Peak
	5022	F	5	6						
0041					8:07	8:37	0:30			No Peak
0042								10:07	2:00	No Peak
	5024	F	5	6						
0043					8:10	8:40	0:30			No Peak
0044								10:10	2:00	No Peak
	5026	M	6	18						
0045					8:13	8:43	0:30			No Peak
0046								10:13	2:00	No Peak
	5028	M	6	18						
0047					8:16	8:46	0:30			No Peak
0048								10:16	2:00	No Peak
	5030	F	6	18						
0049					8:19	8:49	0:30			No Peak
0050								10:19	2:00	No Peak
	5032	F	6	18						
0051					8:22	8:52	0:30			No Peak
0052								10:22	2:00	No Peak
	5034	M	7	36						
0053					8:25	8:55	0:30			No Peak
0054								10:25	2:00	No Peak
	5036	M	7	36						
0055					8:28	8:58	0:30			No Peak
0056								10:28	2:00	No Peak
	5038	F	7	36						
0057					8:31	9:01	0:30			No Peak
0058								10:31	2:00	No Peak
	5040	F	7	36						
0059					8:34	9:04	0:30			No Peak
0060								10:34	2:00	No Peak

DAY 1 (5 Feb 2009) RAT PLASMA SAMPLES (5018-5040B)

GB-67B/044RE27.003	Animal	Sex	Group	Dose (mg/kgday)	Time of Dose	8 hours Postdose	Difference	Conc. (ng/mL)
0061	5018	M	5	6	8:01	16:01	8:00	No Peak
0062	5020	M	5	6	8:04	16:04	8:00	No Peak
0063	5022	F	5	6	8:07	16:07	8:00	No Peak
0064	5024	F	5	6	8:10	16:10	8:00	No Peak
0065	5026	M	6	18	8:13	16:13	8:00	No Peak
0066	5028	M	6	18	8:16	16:16	8:00	No Peak
0067	5030	F	6	18	8:19	16:19	8:00	No Peak
0068	5032	F	6	18	8:22	16:22	8:00	No Peak
0069	5034	M	7	36	8:25	16:25	8:00	No Peak
0070	5036	M	7	36	8:28	16:28	8:00	No Peak
0071	5038	F	7	36	8:31	16:31	8:00	No Peak
0072	5040	F	7	36	8:34	16:34	8:00	No Peak

DAY 7 (11 Feb 2009) RAT PLASMA SAMPLES (5017-5039)

Std Curve weighted 1/x*x
Intercept = 0.00133
Slope = 0.00155
Correlation Coeff = 0.9993

GB-67B/ 044RE27.002	Animal	Sex	Group	Dose (mg/kg/day)	Predose Sample	Time of Dose	1 hour Postdose	Differ-ence	4 hours Postdose	Differ-ence	Conc. (ng/mL)
	5017	M	5	6							
0073					7:15	8:00					No Peak
0074							9:00	1:00			No Peak
0075									12:00	4:00	No Peak
	5019	M	5	6							
0076					7:18	8:03					No Peak
0077							9:03	1:00			No Peak
0078									12:03	4:00	No Peak
	5021	F	5	6							
0079					7:19	8:06					No Peak
0080							9:06	1:00			No Peak
0081									12:06	4:00	No Peak
	5023	F	5	6							
0082					7:21	8:09					No Peak
0083							9:09	1:00			No Peak
0084									12:09	4:00	No Peak
	5025	M	6	18							
0085					7:24	8:12					No Peak
0086							9:12	1:00			No Peak
0087									12:12	4:00	No Peak
	5027	M	6	18							
0088					7:27	8:15					No Peak
0089							9:15	1:00			No Peak
0090									12:15	4:00	No Peak
	5029	F	6	18							
0091					7:29	8:18					No Peak
0092							9:18	1:00			No Peak
0093									12:18	4:00	No Peak
	5031	F	6	18							
0094					7:32	8:21					No Peak
0095							9:21	1:00			No Peak
0096									12:21	4:00	No Peak
	5033	M	7	36							
0097					7:34	8:24					No Peak
0098							9:24	1:00			No Peak
0099									12:24	4:00	No Peak
	5035	M	7	36							
0100					7:36	8:27					No Peak
0101							9:27	1:00			No Peak
0102									12:27	4:00	No Peak
	5037	F	7	36							
0103					7:38	8:30					No Peak
0104							9:30	1:00			No Peak
0105									12:30	4:00	No Peak
	5039	F	7	36							
0106					7:40	8:33					No Peak
0107							9:33	1:00			No Peak
0108									12:33	4:00	No Peak

DAY 7 (11 Feb 2009) RAT PLASMA SAMPLES (5018-5040A)

GB-67B/ 044RE27.003	Animal	Sex	Group	Dose (mg/kg/day)	Time of Dose	30 minutes Postdose	Differ-ence	2 hours Postose	Differ-ence	Conc. (ng/mL)
	5018	M	5	6						
0109					8:01	8:31	0:30			No Peak
0110								10:01	2:00	No Peak
	5020	M	5	6						
0111					8:04	8:34	0:30			No Peak
0112								10:04	2:00	No Peak
	5022	F	5	6						
0113					8:07	8:37	0:30			No Peak
0114								10:07	2:00	No Peak
	5024	F	5	6						
0115					8:10	8:40	0:30			No Peak
0116								10:10	2:00	No Peak
	5026	M	6	18						
0117					8:13	8:43	0:30			No Peak
0118								10:13	2:00	No Peak
	5028	M	6	18						
0119					8:16	8:46	0:30			No Peak
0120								10:16	2:00	No Peak
	5030	F	6	18						
0121					8:19	8:49	0:30			No Peak
0122								10:19	2:00	No Peak
	5032	F	6	18						
0123					8:22	8:52	0:30			No Peak
0124								10:22	2:00	No Peak
	5034	M	7	36						
0125					8:25	8:55	0:30			No Peak
0126								10:25	2:00	No Peak
	5036	M	7	36						
0127					8:28	8:58	0:30			No Peak
0128								10:28	2:00	No Peak
	5038	F	7	36						
0129					8:31	9:01	0:30			No Peak
0130								10:31	2:00	No Peak
	5040	F	7	36						
0131					8:34	9:04	0:30			No Peak
0132								10:34	2:00	No Peak

DAY 7 (11 Feb 2009) RAT PLASMA SAMPLES (5018-5040B)

GB-67B/ 044RE27.002	Animal	Sex	Group	Dose (mg/kg/day)	Time of dose	8 hours Postdose	Difference	Conc. (ng/mL)
0133	5018	M	5	6	8:01	16:01	8:00	No Peak
0134	5020	M	5	6	8:04	16:04	8:00	No Peak
0135	5022	F	5	6	8:07	16:07	8:00	No Peak
0136	5024	F	5	6	8:10	16:10	8:00	No Peak
0137	5026	M	6	18	8:13	16:13	8:00	No Peak
0138	5028	M	6	18	8:16	16:16	8:00	No Peak
0139	5030	F	6	18	8:19	16:19	8:00	No Peak
0140	5032	F	6	18	8:22	16:22	8:00	No Peak
0141	5034	M	7	36	8:25	16:25	8:00	No Peak
0142	5036	M	7	36	8:28	16:28	8:00	No Peak
0143	5038	F	7	36	8:31	16:31	8:00	No Peak
0144	5040	F	7	36	8:34	16:34	8:00	No Peak

Figure 1: GB67B-MPK-08-1-027 (upper) and GB67B_d_9 (internal standard, lower)

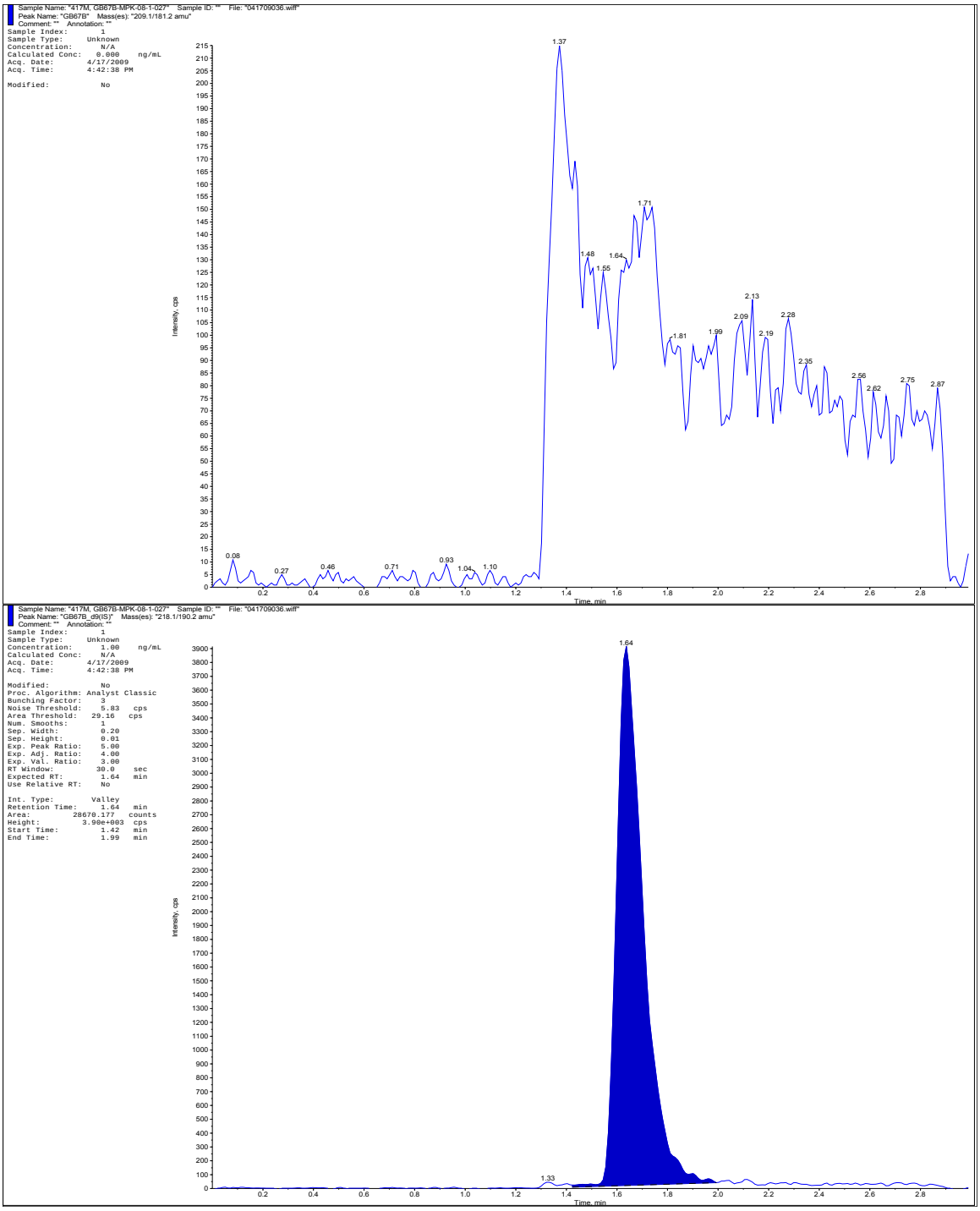

Figure 2: Typical Chromatograms of GB67B in a Mouse Plasma Standard Curve Sample (LLOQ 50 ng/mL) GB67B (upper) and GB67B_d_9 (internal standard, lower)

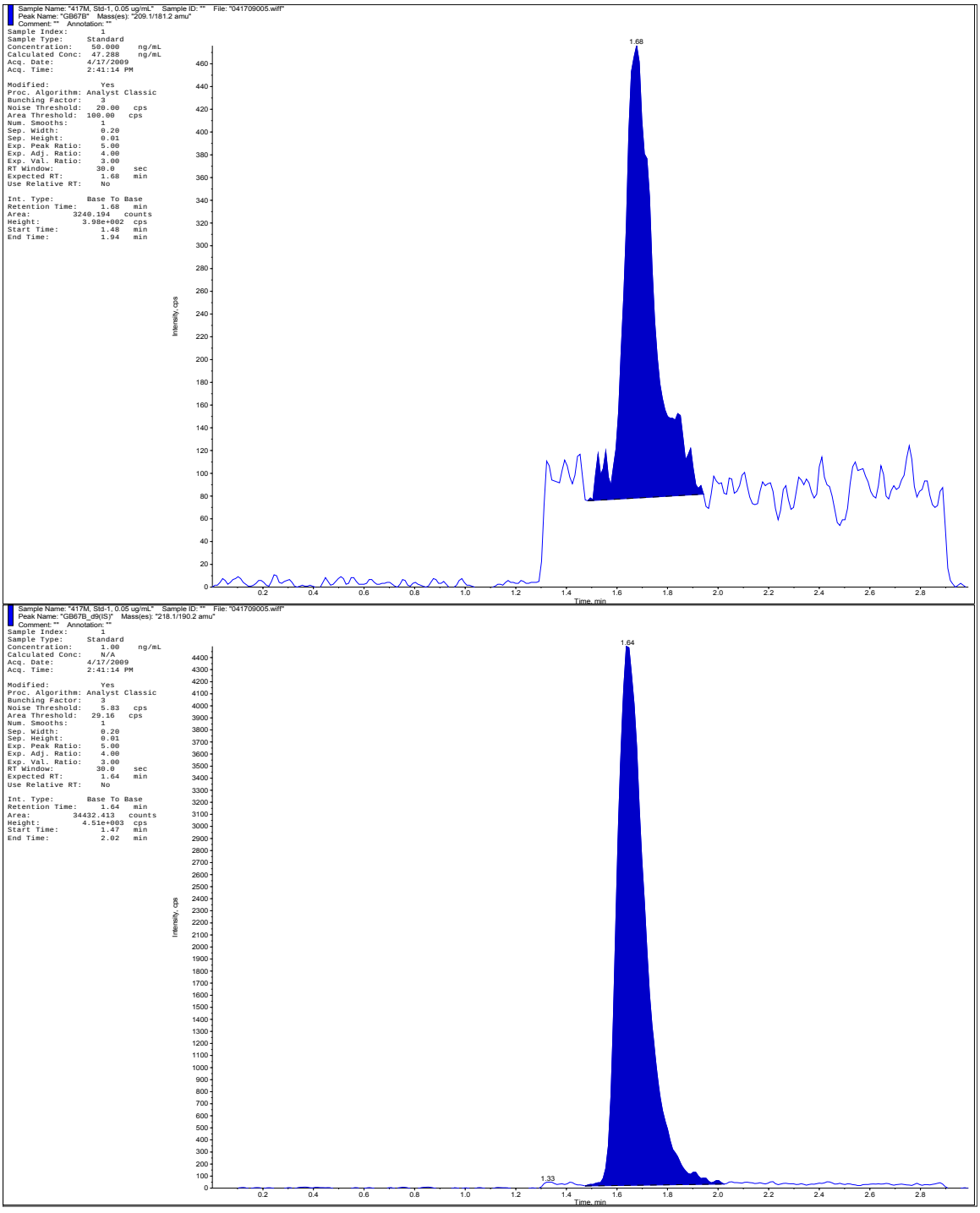

Figure 3: Typical Chromatograms of GB67B in a Mouse Plasma Standard Curve Sample (ULOQ 20 µg/mL) GB67B (upper) and GB67B_d_9 (internal standard, lower)

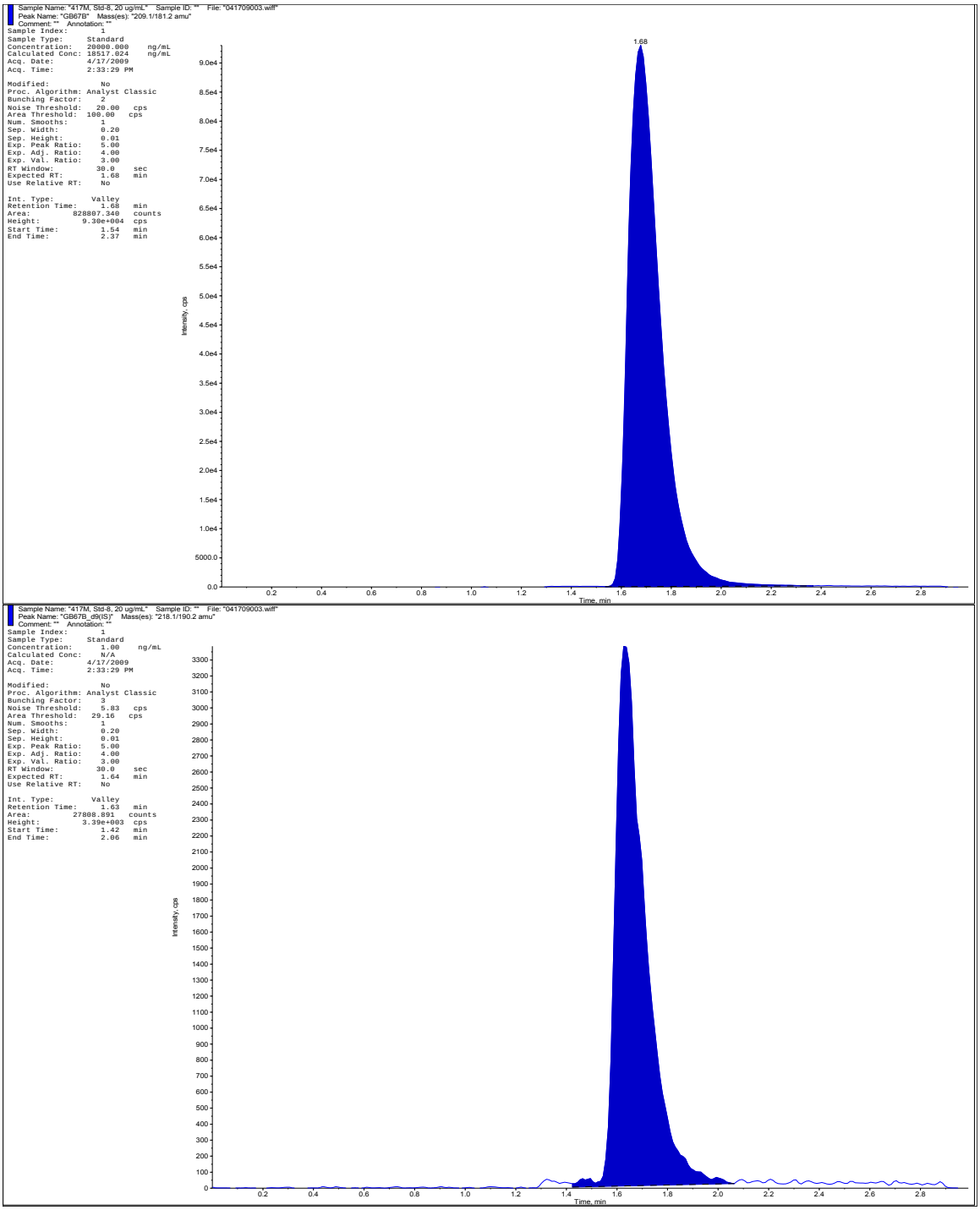

Figure 4: GB97-MPK-08-1-0029 (upper) and GB97_d_9 (internal standard, lower)

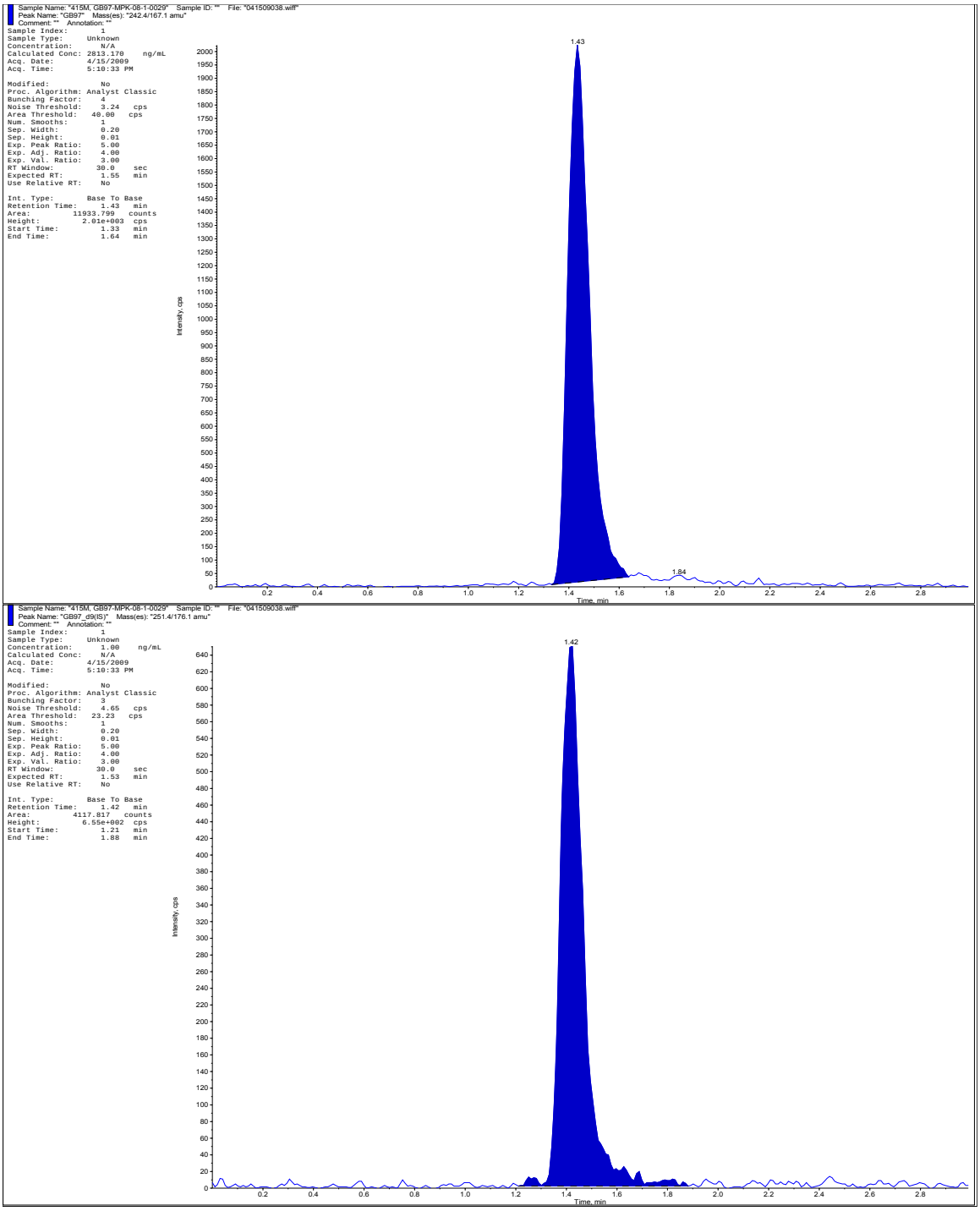

Figure 5: Typical Chromatograms of GB97 in a Mouse Plasma Standard Curve Sample (LLOQ 200 ng/mL) GB97 (upper) and GB97_d_9 (internal standard, lower)

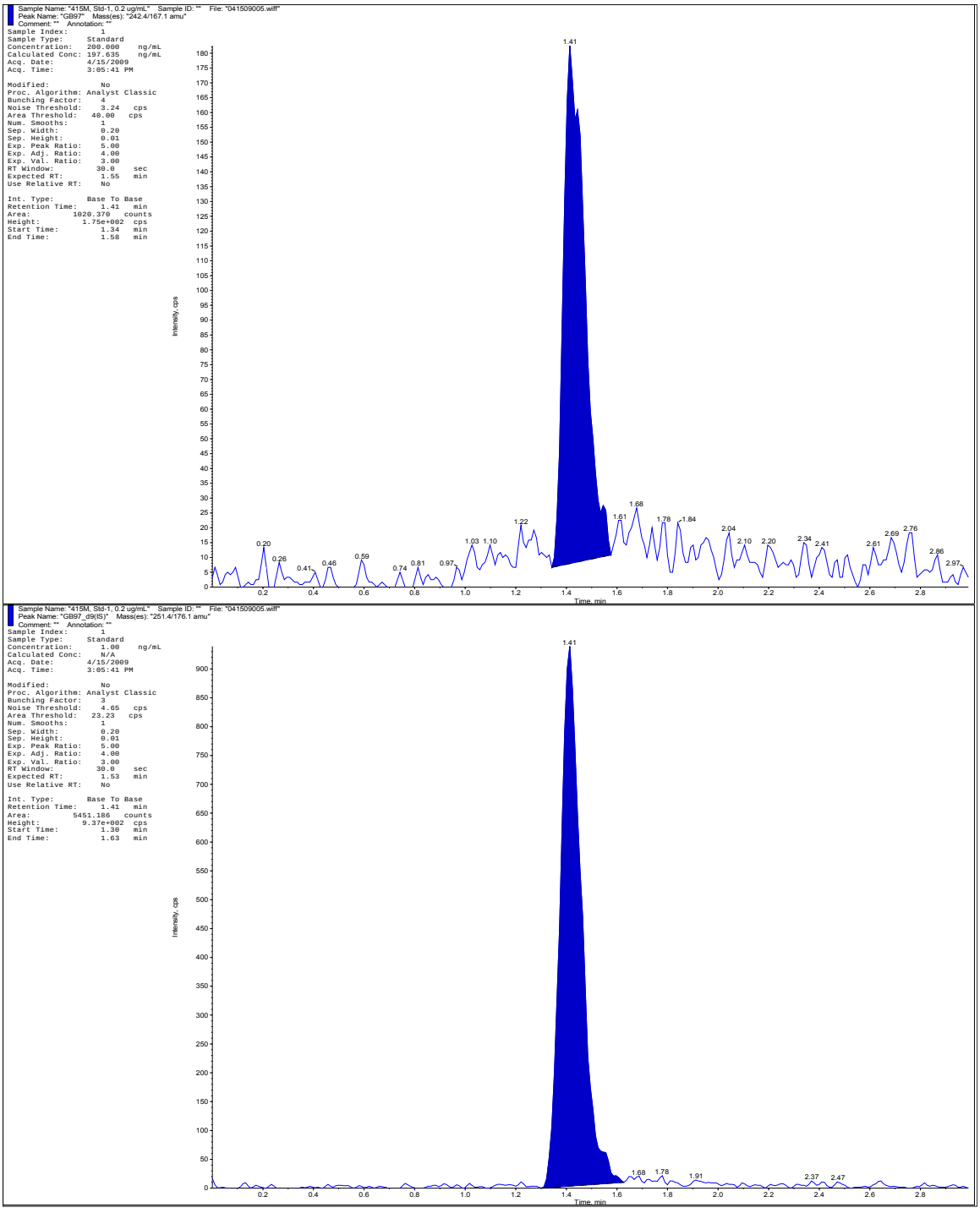

Figure 6: Typical Chromatograms of GB97 in a Mouse Plasma Standard Curve Sample (ULOQ 20 µg/mL) GB97 (upper) and GB97_d_9 (internal standard, lower)

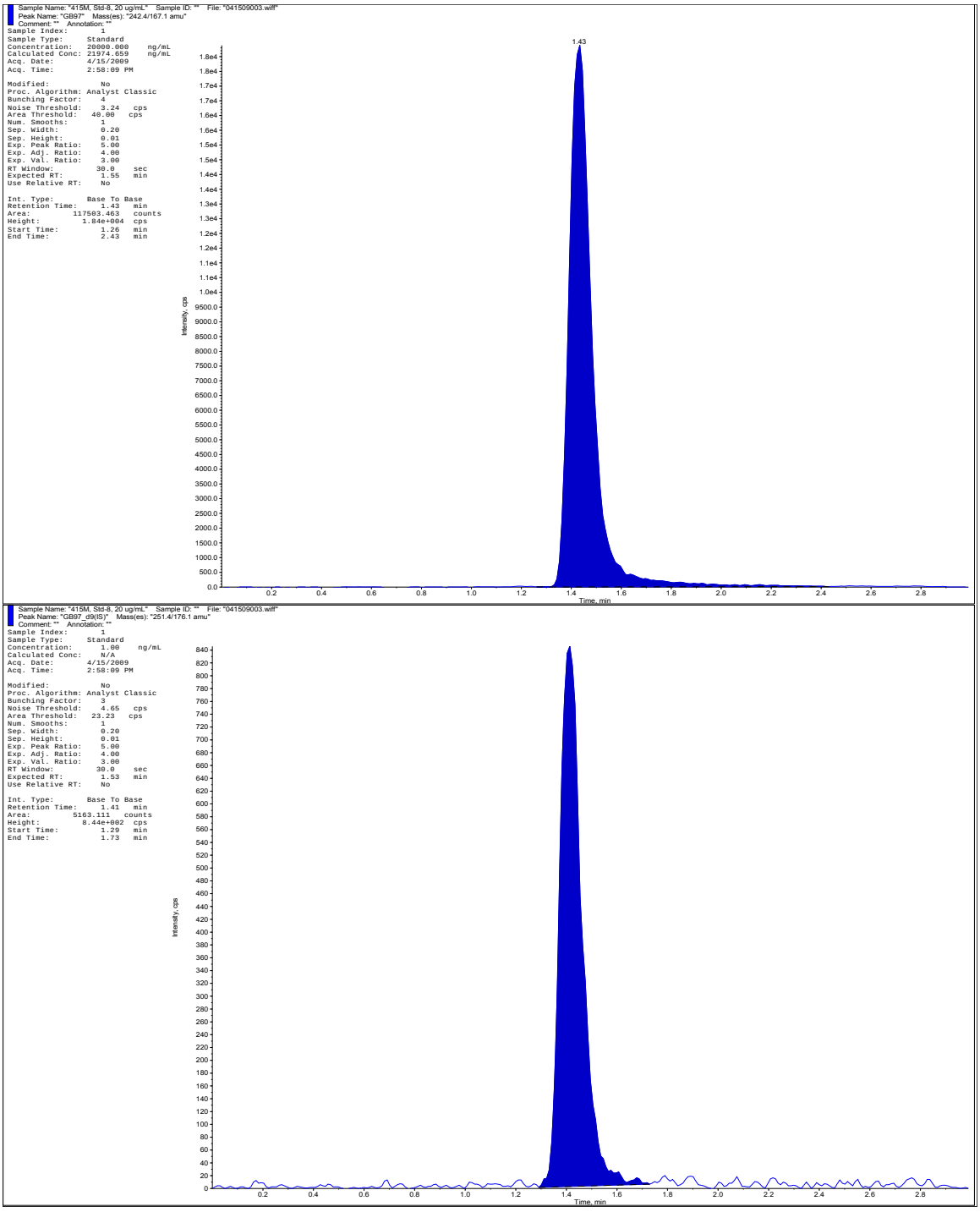

Figure 7: GB594-MPK-08-1-027 (upper) and GB594_d_9 (internal standard, lower)

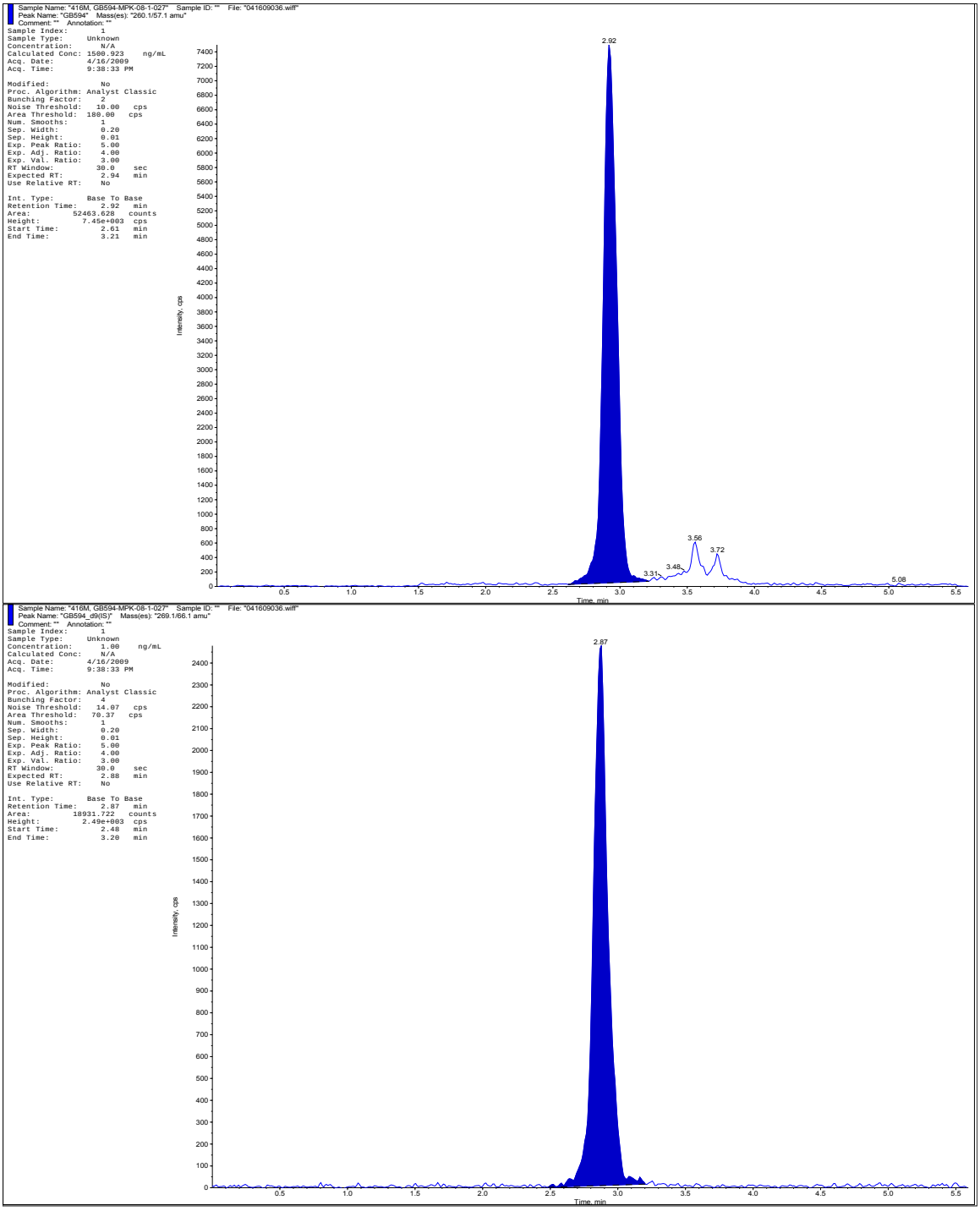

Figure 8: Typical Chromatograms of GB594 in a Mouse Plasma Standard Curve Sample (LLOQ 50 ng/mL) GB594 (upper) and GB594_d_9 (internal standard, lower)

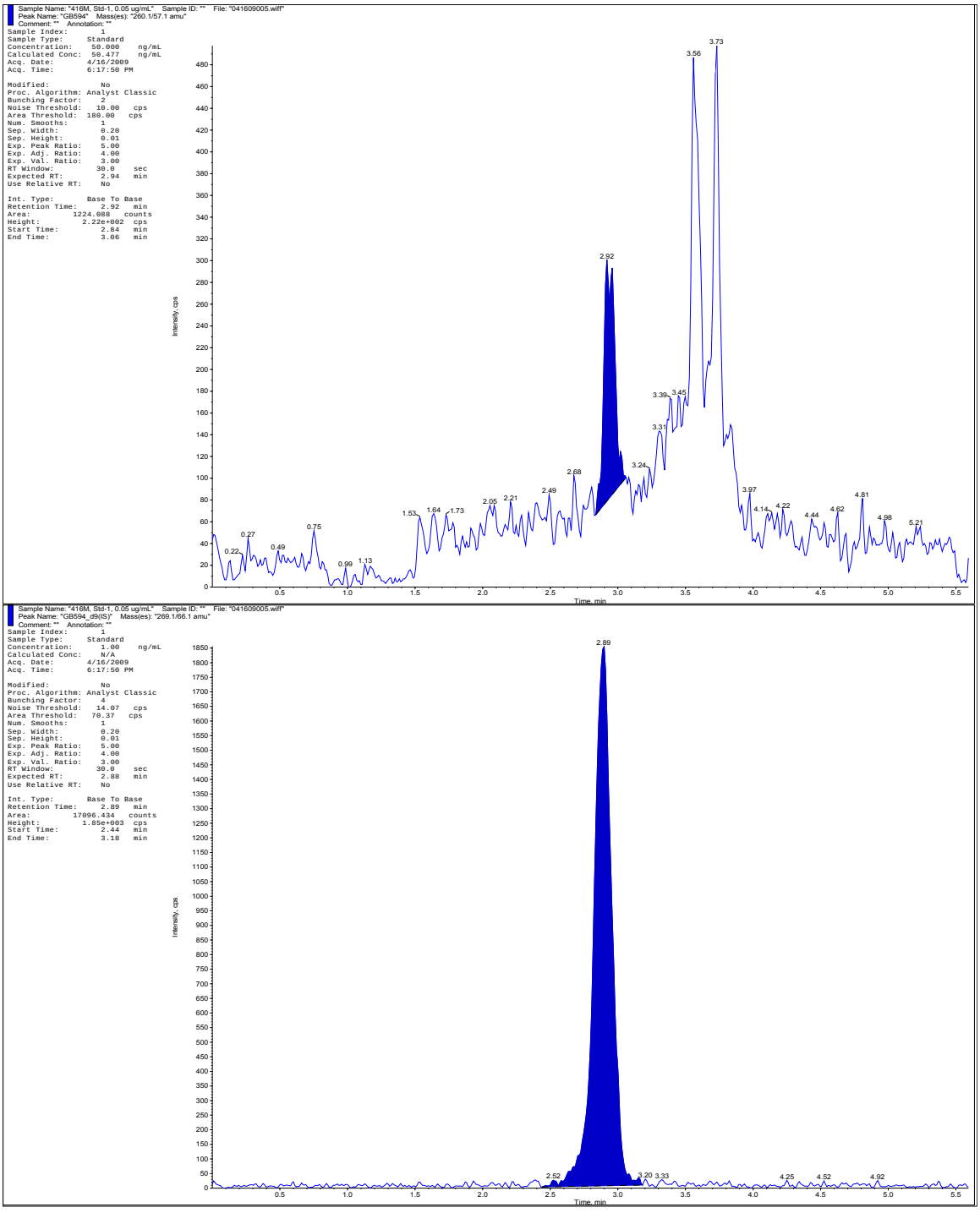

Figure 9: Typical Chromatograms of GB594 in a Mouse Plasma Standard Curve Sample (ULOQ 20 µg/mL) GB594 (upper) and GB594_d_9 (internal standard, lower)

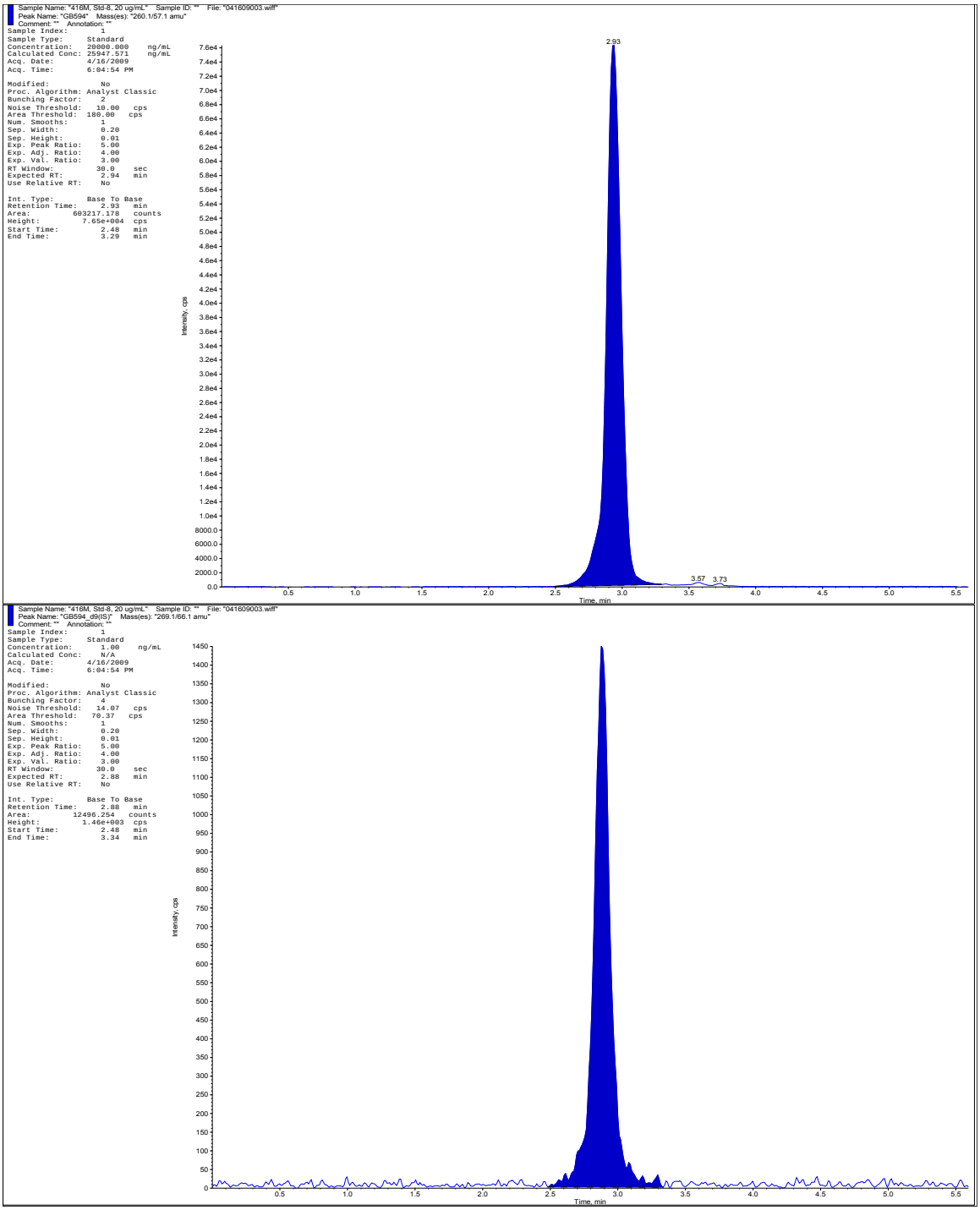

Figure 10: GB67B 5030, day1, 30 min (upper) and GB67B_d_9 (internal standard, lower)

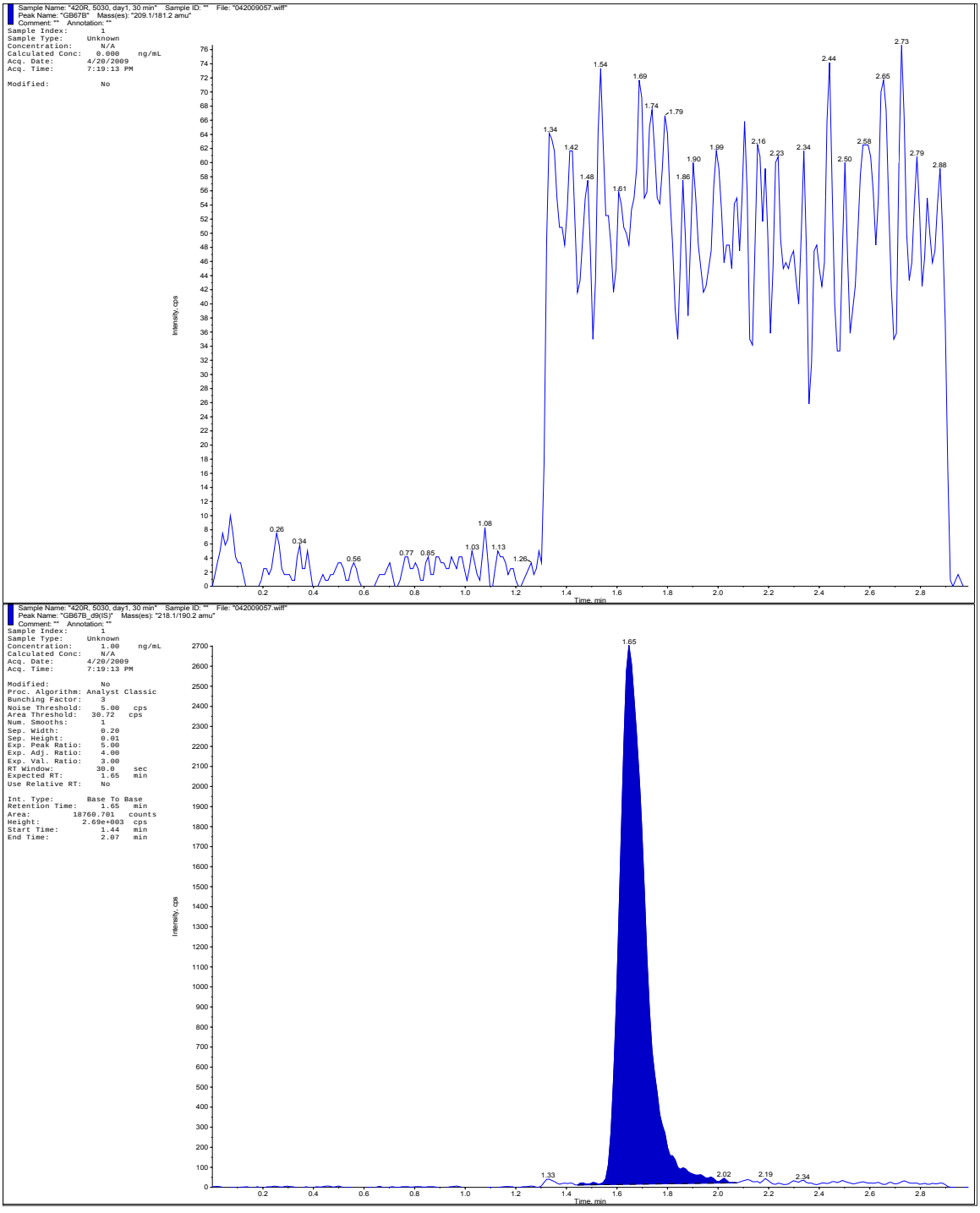

Figure 11: Typical Chromatograms of GB67B in a Rat Plasma Standard Curve Sample (LLOQ 50 ng/mL) GB67B (upper) and GB67B_d_9 (internal standard, lower)

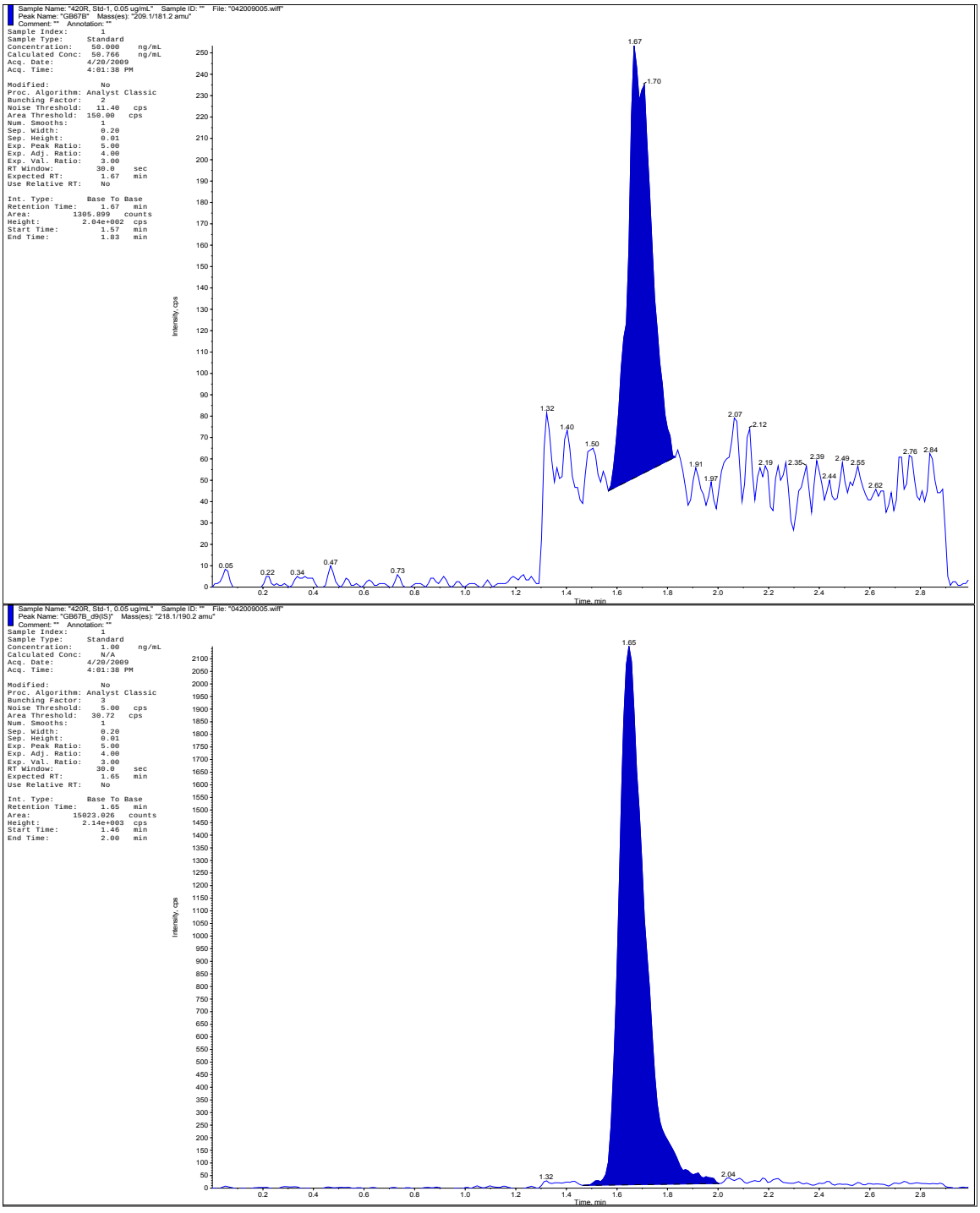

Figure 12: Typical Chromatograms of GB67B in a Rat Plasma Standard Curve Sample (ULOQ 20 µg/mL) GB67B (upper) and GB67B_d_9 (internal standard, lower)