Supplementary Material for

Development of a Size Exclusion Chromatography Cartridge-Based Analytical Method for Determination of Free Drug in Nano-liposomal Oncology Drug Formulations

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Supplement Data Set #1: Result Summary for Screening of Four Separation Techniques for Liposome Free Drug Analysis

Four separation techniques, UF, SPE cartridge, HPLC-nPEC, and SEC cartridge, were evaluated individually for their feasibility in determining free drug in liposome formulation samples. Passive loading formulations (MR220406 and MR220520) and active loading formulations (09122201 and 10182201F) were used for free drug testing and recovery study. The results are summarized in Table 1.

Table 1. Free drug and recovery study results for liposome samples studied during technique screening.

Technique	Sample [b]	Free drug [c]	Recovery for free drug spiked
UF [a]	MR220406	1.8% to 5.1%	22.9% to 48.3% (spiked at 5%)
SPE cartridge	MR220520	41.4% (n=3)	95.0% (n=3) (spiked at 19%)
	09122201	15.8% (n=2)	N/A
HPLC-nPEC	MR220406	62.8%	N/A
	MR220520	62.0% (n=3)	98.0% (n=3) (spiked at 19%)
	09122201	8.4% (n=3)	99.5% (n=3) (spiked at 10%)
	10182201F	13.8% (n=2)	N/A
SEC cartridge	MR220520	25.9%	78.7% (spiked at 21%)
	10182201F	9.4%	95.9% (spiked at 11%)

a. A series of UF filters with MWCO at 3K, 10K, 30K, 50K, 100K was evaluated.

b. Each sample was diluted prior to the separation: UF (4X dilution); SPE (2X dilution), HPLC-nPEC (10X dilution); SEC (2X and 5X dilution of liposome sample for non-spike and spike sample preparation)

c. Not all samples were available for initial screening of each technique. The number of sample preparations was not controlled when the pre-development screening experiments were performed on different dates.

Poor recoveries (23-48%) for free drug were obtained using UF filters with MWCO from 3K to 100K for separation of a spiked sample of MR220406. It is likely that drug adsorption and clogging of MWCO filter pores by liposomes or lipid-related species happened to the UF filter, generating the significantly underestimated free drug test results (2-5%). On the contrary, extremely high free drug results (62-63%)

were generated from HPLC-nPEC analysis of MR220406 and MR220520 samples that were produced using a similar passive loading procedure. Whether such high free drug results in passive loading liposome samples were real remained a question to answer. Although HPLC-nPEC is the most efficient separation and quantitation technique for on-line liposome free drug analysis with good free drug recovery (See. Fig. 1), high shear force due to instrument and column backpressure would be one of major factors to be concerned with for reliable liposome free drug determination. It was observed in our lab that HPLC-nPEC separation of several active loading formulation samples under high pump pressure (36 bar) generated much higher free drug test results (21-24%) than those (8-9%) determined under normal pressure (20 bar). The difference in free drug test results did exist for active formulations analyzed using SPE, HPLC-nPEC, and SEC techniques, but it was not as significant as those observed in passive loading formulations. Both SPE and SEC cartridge techniques exhibited good potential for reliably analyzing free drug in liposomes.

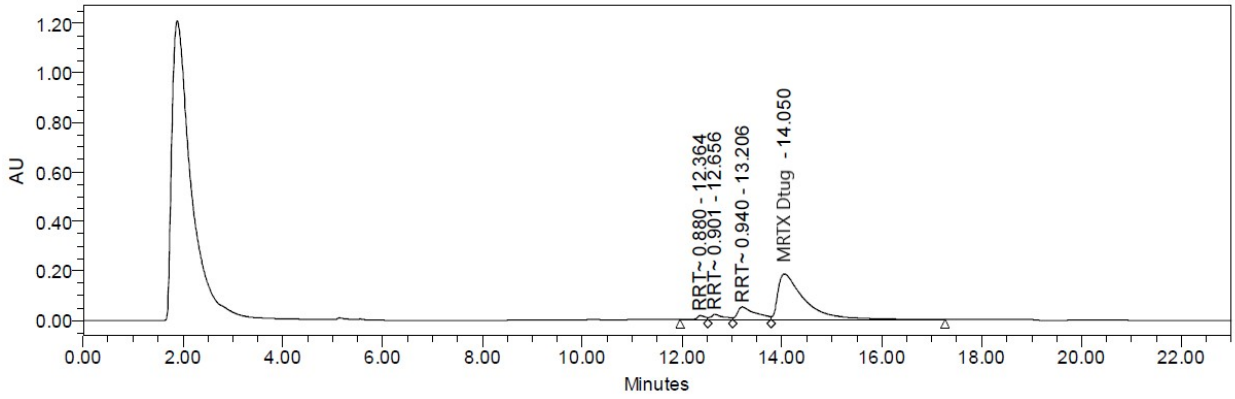


Fig. 1. An example chromatogram of liposome sample solution separated by HPLC-nPEC

Notebook reference: NB0073-011, -017, 034, -037, -038, -047, and -059

Supplement Data Set#2 for SEC Method Development

1) SEC cartridge free drug retention capacity

Table 1. Free drug retention capacity evaluation for a Zebra Spin Desalting SEC cartridge column (7K MWCO_2ML)

API Sol A (178 ug/ml) prepared in 10mM sodium phosphate buffer pH 6.8

Step#	API Study Sol A (mL)	Accumulative Drug Sol Added to SEC (mL)	Conc. of Drug in Study Solution (µg/mL)	Accumulative Drug Loaded to SEC (µg)	Centrifugation [a]	Drug Found (µg/mL) in Elution Sample [b]	Eluted Drug Amount (µg) [c]	%Euted Drug. [d]
1	0.5	0.5	178	89	Yes	0.000	0.00	0.00
2	0.5	1	178	178	Yes	0.000	0.00	0.00
3	0.5	1.5	178	267	Yes	0.000	0.00	0.00
4	0.5	2	178	356	Yes	0.000	0.00	0.00
5	0.5	2.5	178	445	Yes	0.000	0.00	0.00
6	0.5	3	178	534	Yes	2.199	1.10	0.21
7	0.5	3.5	178	623	Yes	5.774	2.89	0.46
8	0.5	4	178	712	Yes	11.123	5.56	0.78
9	0.5	4.5	178	801	Yes	14.386	7.19	0.90
10	0.5	5	178	890	Yes	16.522	8.26	0.93
11	0.5	5.5	178	979	Yes	22.165	11.08	1.13
12	0.5	6	178	1068	Yes	26.094	13.05	1.22
13	0.5	6.5	178	1157	Yes	31.045	15.52	1.34
14	0.5	7	178	1246	Yes	38.622	19.31	1.55
15	0.5	7.5	178	1335	Yes	44.436	22.22	1.66
16	0.5	8	178	1424	Yes	56.462	28.23	1.98
17	0.5	8.5	178	1513	Yes	64.774	32.39	2.14
18	0.5	9	178	1602	Yes	76.053	38.03	2.37
19	0.5	9.5	178	1691	Yes	87.817	43.91	2.60
20	0.5	10	178	1780	Yes	101.160	50.58	2.84

a. Eppendorf 5804R centrifuge (1250RCF/20C/3 min for spinning a test solution at each step)

b. Collected elution sample from each step (0.5 mL) and analyzed by UPLC

c. Eluted Drug Amount = Conc. of Drug (elution sample at each step) X 0.5mL

d. %Eluted Drug = [Eluted Drug Amount (Each Step) / Accumlative Drug (n)] x 100

Notebook reference: NB0212-002

2) SEC cartridge free drug elution (API solution as a study sample)

SEC cartridge elution procedure:

Stage	Eluent Step	Centrifuge Conditions
1) Conditioning	1) Internal storage solution (1.5 mL)	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
2) Liposome Elution	3) 250 µL study API solution	1250 rcf, 20°C, 3 min
	4) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
3) Free Drug Elution	5) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	6) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 × 3 min
	7) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 × 3 min
	8) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 × 3 min
	9) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 × 3 min

An individual elution sample from a conditioned SEC cartridge was collected in a 15-mL centrifuge tube at each step from sample-loading/BF wash step to 5-step organic media wash. For eluted free drug analysis by UPLC, diluted each collected elution sample with 17 mM HEPES BF pH6.8 according to the following scheme:

Elution sample collected from	Final dilution volume
Sample loading / BF wash	No dilution
Organic wash#1	5mL
Organic wash#2	5mL
Organic wash#3	3mL
Organic wash#4	3mL
Organic wash#5	3mL

Notebook reference: NB0073-100

3) SEC cartridge conditioning study

3-conseutive extraction / separation of 0.25 mL of formulation sample solution (1:1) and subsequent eluted liposome samples for free drug analysis using SEC cartridges conditioned with following agents:

- 10mM sodium phosphate buffer pH 6.8
- sodium phosphate buffer and human albumin solution 10% (HAS)
- sodium phosphate buffer and formulation placebo

General SEC cartridge elution procedure:

Stage	Eluent Step	Centrifuge Conditions
1) Conditioning	1) Internal storage solution (1.5 mL)	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 0.5 mL conditioning agent (n = 3)*	1250 rcf, 20°C, 3 min
2) Liposome Elution	1) 250 µL sample solution**	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
3) Free Drug Elution	1) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	2) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	3) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	4) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 9 min

*Described in the tables below for cartridge conditioning study.

**For the initial SEC extraction (1st SEC): 0.25 mL of each diluted formulation sample solution (1:1 (v/v) dilution with 10mM sodium phosphate buffer pH 6.8); For the 2nd extraction: approximate 0.25 mL of liposome elution sample collected from the 1st SEC extraction; For the 3rd extraction: approximate 0.25 mL of liposome elution sample collected from the 2nd SEC extraction. Each of the three consecutive extractions was performed using a freshly conditioned SEC cartridge under the same conditions listed above.

SEC cartridges conditioned with **10mM sodium phosphate buffer pH 6.8:**

Stage	Eluent Step*	Centrifuge Conditions
1) Conditioning	1) Internal storage solution (1.5 mL)	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min

*See general elution procedure for liposomal drug and free drug elution.

SEC cartridges conditioned with **10mM sodium phosphate buffer pH 6.8 + HAS:**

Stage	Eluent Step*	Centrifuge Conditions
1) Conditioning	1) Internal storage solution (1.5 mL)	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 0.5 mL HAS	1250 rcf, 20°C, 3 min
	4) 0.5 mL HAS	1250 rcf, 20°C, 3 min
	5) 0.5 mL HAS	1250 rcf, 20°C, 3 min

*See general elution procedure for liposomal drug and free drug elution.

SEC cartridges conditioned with **10mM sodium phosphate buffer pH 6.8 + Placebo:**

Stage	Eluent Step*	Centrifuge Conditions
1) Conditioning	1) Internal storage solution (1.5 mL)	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
	4) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
	5) 0.5 mL Placebo	1250 rcf, 20°C, 3 min

*See general elution procedure for liposomal drug and free drug elution.

For free drug analysis by UPLC, each of three free drug elution samples for a formulation sample through three consecutive extractions was diluted to volume (10-mL) in a 15-mL centrifuge tube with 17 mM HEPES buffer pH 6.8 as per method.

Notebook reference: NB0073-093

4) Buffer wash steps for liposomal drug elution

SEC cartridge elution procedure:

Stage	Eluent Step	Centrifuge Conditions
1) Conditioning	1) Internal storage solution (1.5 mL)	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
	4) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
	5) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
2) Liposomal Drug Elution [b]	1) 250 µL sample solution (1:1) [a]	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	4) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	5) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
3) Free Drug Elution [c]	1) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	2) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	3) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 6 min
	4) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 9 min

a. Sample solution (1:1) was prepared from the equal volume mixing of a formulation sample with10 mM sodium phosphate BF pH 6.8 (1:1 (v/v)).
b. An individual liposome elution sample was collected in a 15-mL centrifuge tube at each step during the sample loading and four buffer wash steps. For encapsulated drug analysis by UPLC, added 6.9 mL of 9/1 IPA/18mM HCl (v/v) to each centrifuge tube, diluted to volume (10-mL) with 17mM HEPES BF pH 6.8, then incubated at 60°C for 20min to disintegrate the liposomes and dissolve the released drug.
c. All elution samples from 4-step organic media wash were collected in a 15-mL centrifuge tube. For free drug analysis by UPLC, diluted free drug elution sample in the centrifuge tube to volume (10-mL) with 17mM HEPES BF pH 6.8.

Notebook reference: NB0224-001

5) Organic media wash steps for liposome free drug elution

SEC cartridge elution procedure:

Stage	Eluent Step	Centrifuge Conditions
1) Conditioning	1) Internal storage solution (1.5 mL)	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
	4) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
	5) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
2) Liposomal Drug Elution [b]	1) 250 µL sample solution (1:1) [a]	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
3) Free Drug Elution [c]	1) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	2) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	3) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 6 min
	4) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 9 min

a. Sample solution (1:1) was prepared from the equal volume mixing of a formulation sample with10 mM sodium phosphate BF pH 6.8 (1:1 (v/v)).
b. All elution samples from the sample loading and 2-step buffer wash were collected in a 15-mL centrifuge tube for encapsulated drug analysis. For encapsulated drug analysis by UPLC, added 6.9 mL of 9/1 IPA/18mM HCl (v/v) into the centrifuge tube, diluted to volume (10-mL) with 17mM HEPES BF pH 6.8, then incubated each tube at 60°C for 20min to disintegrate the liposomes and dissolve the released drug.
c. An individual elution sample was collected in a 15-mL centrifuge tube at each step during the 4-step organic media wash. For free drug analysis by UPLC, diluted each collected free drug elution sample in the centrifuge tube with 17mM HEPES BF pH 6.8) according to the following scheme:

Free drug elution sample from	Final dilution volume
Organic wash#1	5mL
Organic wash#2	5mL
Organic wash#3	3mL
Organic wash#4	3mL

Notebook reference: NB0224-003

6) Sample size and dilution effects

SEC cartridge elution procedure:

Stage	Eluent Step	Centrifuge Conditions
1) Conditioning	1) Internal storage solution (1.5 mL)	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
	4) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
	5) 0.5 mL Placebo	1250 rcf, 20°C, 3 min
2) Liposomal Drug Elution	1) **X mL** sample preparation [a]	1250 rcf, 20°C, 3 min
	2) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
	3) 1.0 mL 10mM sodium phosphate BF pH 6.8	1250 rcf, 20°C, 3 min
3) Free Drug Elution	1) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	2) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 3 min
	3) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 6 min
	4) 1.5 mL 9/1 EtOH / 18mM HCl	1250 rcf, 20°C, 9 min

a. X= 0.25 mL and 0.5 mL of sample solution (1:1) and 0.25mL of non-diluted sample

Collected elution samples were prepared as per method (described in this paper) for UPLC determination of encapsulated drug and free drug in each study sample.

Table 1. Free drug test results for MRTX liposome samples analyzed using SEC Cartridge Technique (Sample size & dilution effec

Each cartridge conditioned with 3×0.5 mL of correspondong Placebo (**Option#1 for free drug calculation**)

Sample Lot#	SPL Prep. Inf.	Prep#	MRTX Drug		
			Free Drug (mg/mL)	Encap. Drug (mg/mL)	% Free Drug [a]
MR220520	0.25 ML SPL (1:1)	**P1**	0.241	1.383	17.5
	0.25 ML SPL (1:1)	**P2**	0.244	1.365	17.8
		Mean (n=2)	**0.243**	**1.374**	**17.6**
MR220520	0.5 ML SPL (1:1)	**P1**	0.163	1.432	11.4
	0.5 ML SPL (1:1)	**P2**	0.158	1.421	11.1
		Mean (n=2)	**0.161**	**1.427**	**11.3**
MR220520	0.25 ML SPL (NDil)	**P1**	0.170	1.385	12.2
	0.25 ML SPL (NDil)	**P2**	0.166	1.408	11.9
		Mean (n=2)	**0.168**	**1.397**	**12.0**
MTI106-A1	0.25 ML SPL (1:1)	**P1**	0.129	2.007	6.4
	0.25 ML SPL (1:1)	**P2**	0.124	1.995	6.2
		Mean (n=2)	**0.127**	**2.001**	**6.3**
MTI106-A1	0.5 ML SPL (1:1)	**P1**	0.106	2.043	5.2
	0.5 ML SPL (1:1)	**P2**	0.107	2.027	5.3
		Mean (n=2)	**0.107**	**2.035**	**5.2**
MTI106-A1	0.25 ML SPL (NDil)	**P1**	0.115	2.006	5.7
	0.25 ML SPL (NDil)	**P2**	0.115	2.041	5.7
		Mean (n=2)	**0.115**	**2.024**	**5.7**

a. % Free drug = [(Free drug conc. / (Free drug conc. + Encap. drug conc.)] × 100 (**Option#1 calculation**)

BF = 10 mM Na-phosphate buffer pH 6.8 as a Diluent

Notebook reference: NB0224-010

Table 2. Free drug test results for MRTX liposome samples analyzed using SEC Cartridge Technique (Sample size & dilution effec

Each cartridge conditioned with 3×0.5 mL of correspondong Placebo (**Option#2 for free drug calculation**)

Sample Lot#	SPL Prep. Inf.	Prep#	MRTX Drug		
			Free Drug (mg/mL)	Total Drug (mg/mL)	% Free Drug [a]
MR220520	0.25 ML SPL (1:1)	**P1**	0.241	1.647	15.0
	0.25 ML SPL (1:1)	**P2**	0.244	1.575	15.1
		Mean (n=2)	**0.243**	**1.611**	**15.1**
MR220520	0.5 ML SPL (1:1)	**P1**	0.163	1.647	10.1
	0.5 ML SPL (1:1)	**P2**	0.158	1.575	9.8
		Mean (n=2)	**0.161**	**1.611**	**10.0**
MR220520	0.25 ML SPL (NDil)	**P1**	0.170	1.647	10.6
	0.25 ML SPL (NDil)	**P2**	0.166	1.575	10.3
		Mean (n=2)	**0.168**	**1.611**	**10.4**
MTI106-A1	0.25 ML SPL (1:1)	**P1**	0.129	2.187	5.9
	0.25 ML SPL (1:1)	**P2**	0.124	2.186	5.7
		Mean (n=2)	**0.127**	**2.187**	**5.8**
MTI106-A1	0.5 ML SPL (1:1)	**P1**	0.106	2.187	4.8
	0.5 ML SPL (1:1)	**P2**	0.107	2.186	4.9
		Mean (n=2)	**0.107**	**2.187**	**4.9**
MTI106-A1	0.25 ML SPL (NDil)	**P1**	0.115	2.187	5.3
	0.25 ML SPL (NDil)	**P2**	0.115	2.186	5.3
		Mean (n=2)	**0.115**	**2.187**	**5.3**

a. % Free drug = [(Free drug conc. / Avg. toal drug result] ×100 (**Option#2 calculation**)

BF = 10 mM Na-phosphate buffer pH 6.8 as a Diluent

Notebook reference: NB0224-010

Table 3. Concentration data for free drug, encapsulated drug, and total drug in each sample

Sample Lot#	Prep#	MRTX Drug (mg/mL)			%Recovery [a]
		Total Drug	Conc. of Free Drug	Conc. of Encap. Drug	
MR220520 (0.25mL SPL (1:1))	**P1**	1.647	0.241	1.383	100.8
	P2	1.575	0.244	1.365	99.9
	Mean (n=2)	**1.611**	**0.243**	**1.374**	**100.3**
MR220520 (0.5mL SPL (1:1))	**P1**	1.647	0.163	1.432	99.0
	P2	1.575	0.158	1.421	98.0
	Mean (n=2)	**1.611**	**0.161**	**1.427**	**98.5**
MR220520 (0.25mL SPL (NDil))	**P1**	1.647	0.170	1.385	96.5
	P2	1.575	0.166	1.408	97.7
	Mean (n=2)	**1.611**	**0.168**	**1.397**	**97.1**
MTI106-A1 (0.25mL SPL (1:1))	**P1**	2.187	0.129	2.007	97.7
	P2	2.186	0.124	1.995	96.9
	Mean (n=2)	**2.187**	**0.127**	**2.001**	**97.3**
MTI106-A1 (0.5mL SPL (1:1))	**P1**	2.187	0.106	2.043	98.3
	P2	2.186	0.107	2.027	97.6
	Mean (n=2)	**2.187**	**0.107**	**2.035**	**97.9**
MTI106-A1 (0.25mL SPL (NDil))	**P1**	2.187	0.115	2.006	97.0
	P2	2.186	0.115	2.041	98.6
	Mean (n=2)	**2.187**	**0.115**	**2.024**	**97.8**

a. %Recovery for Total Drug = ((Free Drug + Encap. Drug)/Avg. Total Drug] × 100

Notebook reference: NB0224-010

Supplement Data Set #3 for Method Qualification

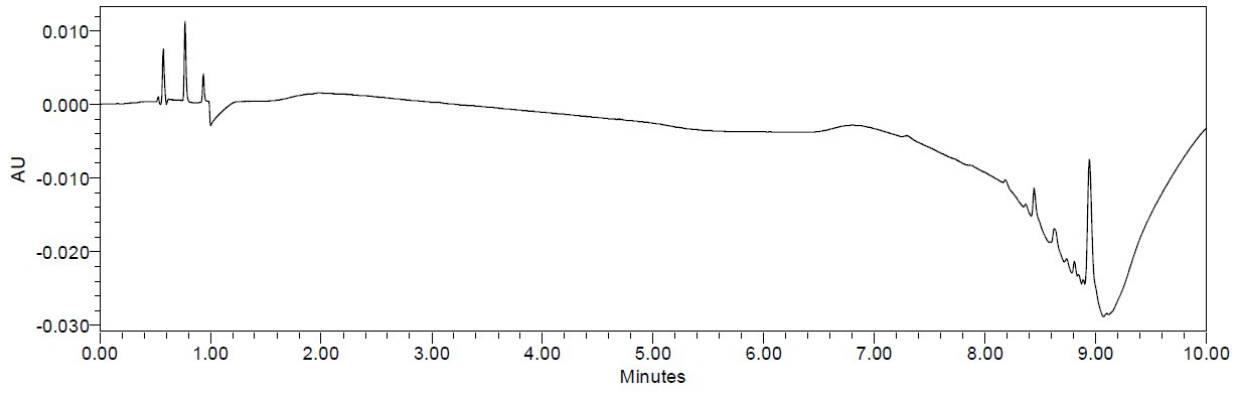
1) Specificity / selectivity study

Table 1. UPLC analysis of specificity / selectivity study solutions @ 240 nm.

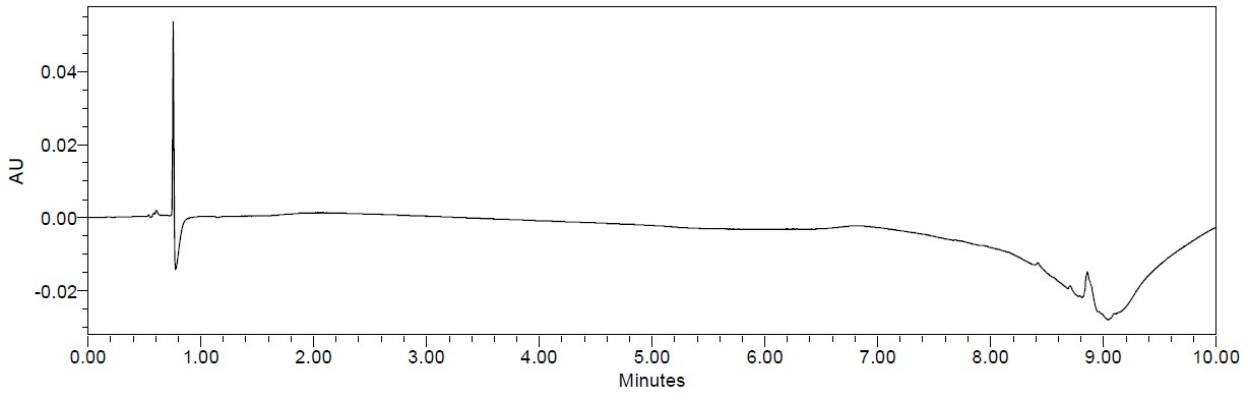
Solution	Information	Interference
1) L-1 standard solution	The lowest calibration solution (about 1.0 µg/mL), drug peak tR at 5.1 min	N/A
2) 40/60 EtOH/H20	Diluent for calibration standard preparations	Not detected
3) 10 mM Sodium phosphate buffer pH 6.8	SEC cartridge conditioning agent and diluent for sample preparations	Not detected
4) 9/1 EtOH/18mM HCl	Media for SEC free drug elution	Not detected
5) 9/1 IPA / 18mM HCl	Diluent for encapsulated drug and total drug sample preparations	Not detected
6) 17 mM HEPES buffer pH 6.8	Diluent for liposomal drug and free drug elution sample preparation	Not detected
7) 25 mM Ammonium acetate buffer pH 4.5	Diluent for total drug sample preparation	Not detected
8) Incubated media blank	Mixture of 9/1 IPA / 18mM HCl and HEPES buffer used for encapsulated drug analysis	Not detected
9) Placebo SEC free drug elution sample	Free drug detection	Not detected
10) Incubated placebo SEC liposome elution sample	Encapsulated drug detection	Not detected
11) Placebo sample solution	Total drug detection	Not detected

Notebook reference: NB0224-021 and -034

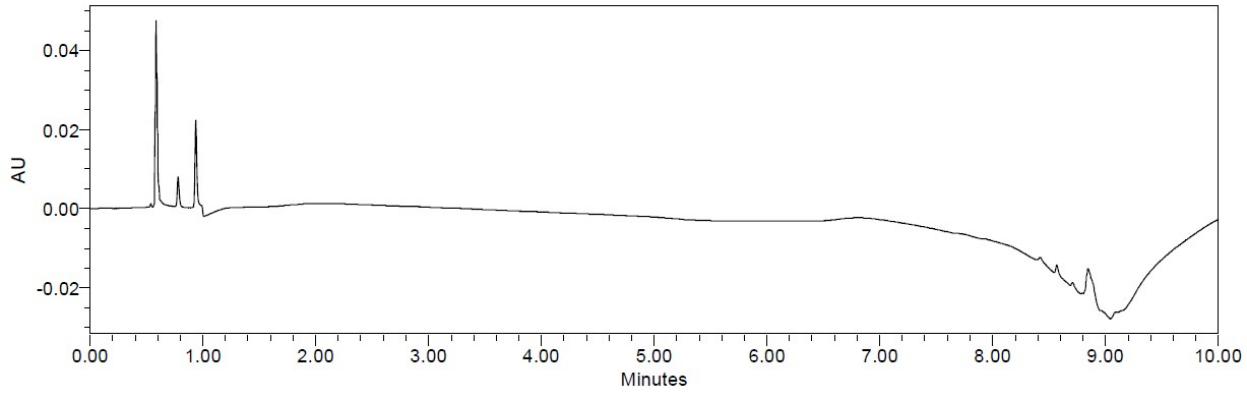
Incubated media blank



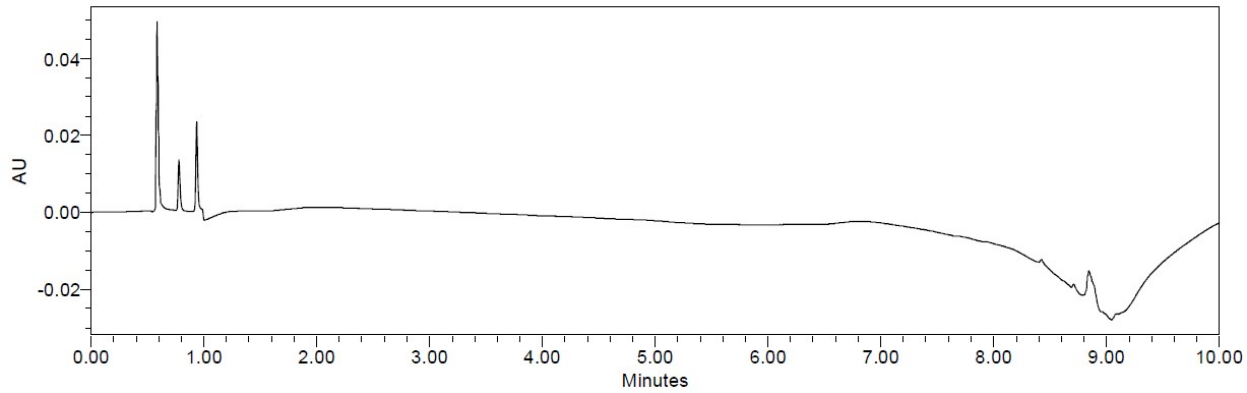
Placebo SEC free drug elution sample



Incubated placebo SEC liposome elution sample (for encapsulated drug detection)



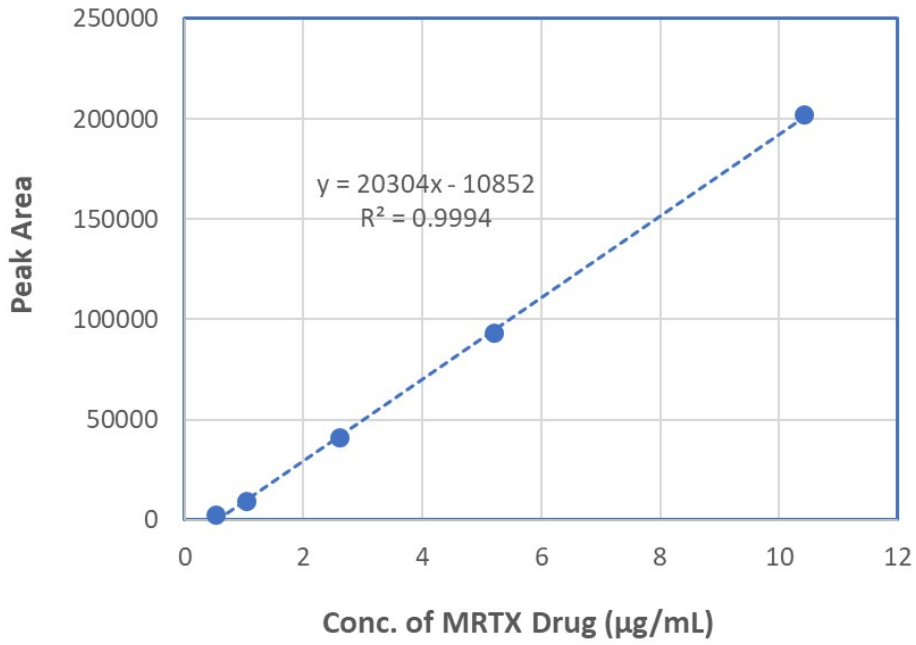
Placebo sample solution (2µL) (for total drug detection)



2) Linearity and LOQ/LOD

5-Level calibration data and plot (2µL injection @240 nm):

Sol	Theore. Conc. (µg/mL)	Peak Area	Calc. Conc.(µg/mL) [a]	Recovery (%) [b]
L-0.5/LOQ	0.52132	2641	0.66455	127.5
L-1	1.04264	9282	0.99163	95.1
L-2	2.60661	40815	2.54467	97.6
L-3	5.21322	93214	5.12539	98.3
L-4	10.42644	202003	10.48340	100.5
a. Calculate drug concentration from calibration equation according to peak area response				
b. %Recovery = (Calc. Conc. / Theore. Conc.) × 100				



If Target drug level is set at about 5 µg/mL (L-3), % Y-Intercept = (10852 / 93214) × 100 = 11.7%

Since the recovery at L-0.5/LOQ is 127.5%, beyond 80-120% (usually set for LOQ), calibration range would be better to set within L1 to L-4 for quantitation of drug in samples.

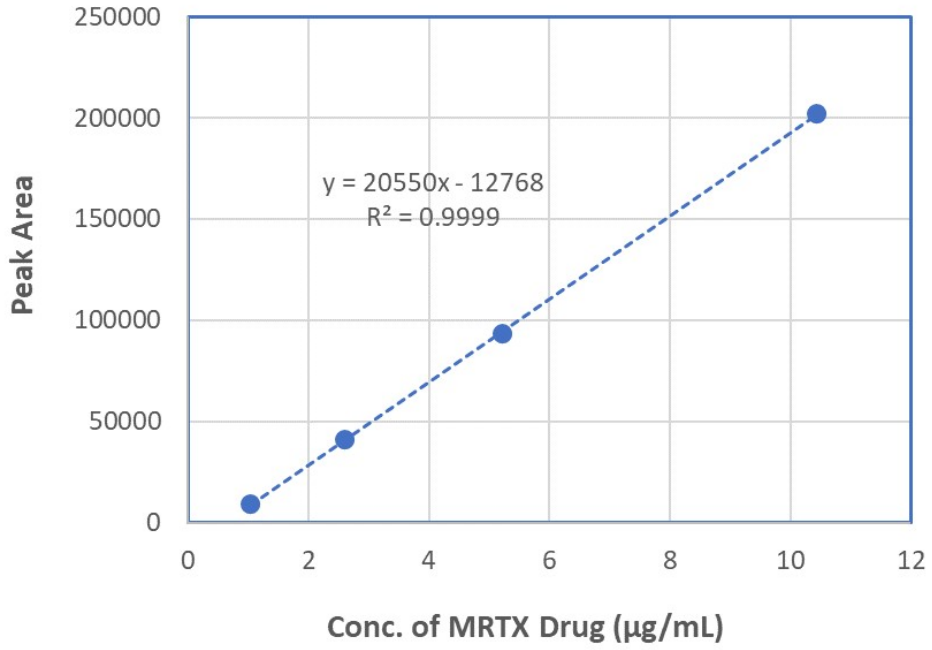
Notebook reference: NB0224-025

4-Level calibration data and plot (2µL injection @240 nm):

Sol	Theore. Conc. (µg/mL)	Peak Area	Calc. Conc.(µg/mL) [a]	Recovery (%) [b]
L-1	1.04264	9282	1.07299	102.9
L-2	2.60661	40815	2.60745	100.0
L-3	5.21322	93214	5.15727	98.9
L-4	10.42644	202003	10.45114	100.2

a. Calculate drug concentration from calibration equation according to peak area response

b. %Recovery = (Calc. Conc. / Theore. Conc.) × 100

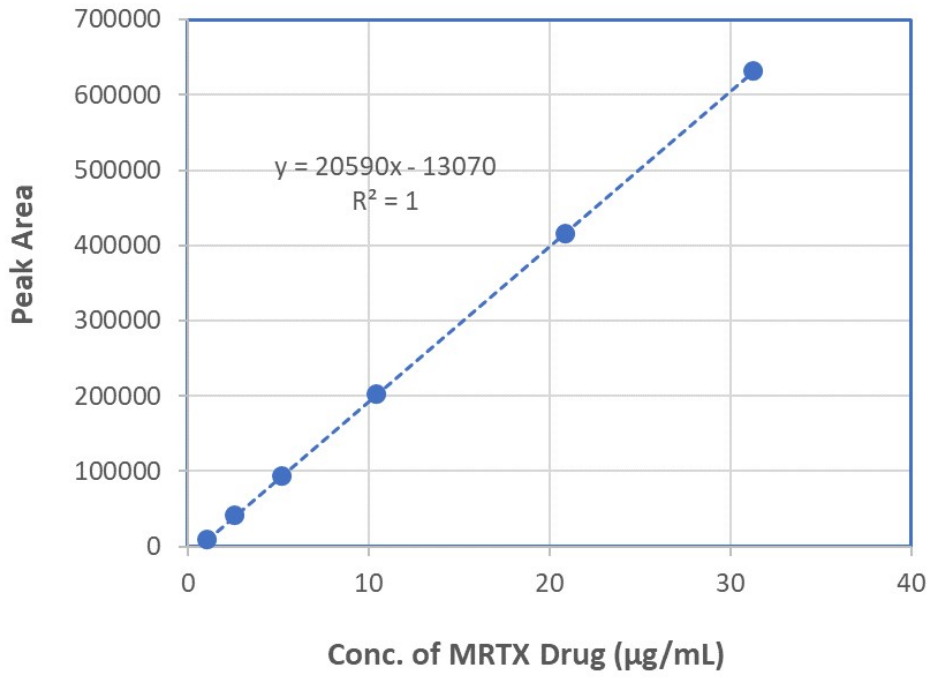


If Target drug level is set at about 5 µg/mL (L-3), % Y-Intercept = (12768 / 93214) × 100 = 13.7%

Notebook reference: NB0224-025

6-Level calibration data and plot (2µL injection @240 nm):

Sol	Theore. Conc. (µg/mL)	Peak Area	Calc. Conc.(µg/mL) [a]	Recovery (%) [b]
L-1	1.04264	9282	1.08558	104.1
L-2	2.60661	40815	2.61705	100.4
L-3	5.21322	93214	5.16192	99.0
L-4	10.42644	202003	10.44551	100.2
L-5	20.85288	414978	20.78912	99.7
L-6	31.27932	631835	31.32127	100.1
a. Calculate drug concentration from calibration equation according to peak area response				
b. %Recovery = (Calc. Conc. / Theore. Conc.) × 100				

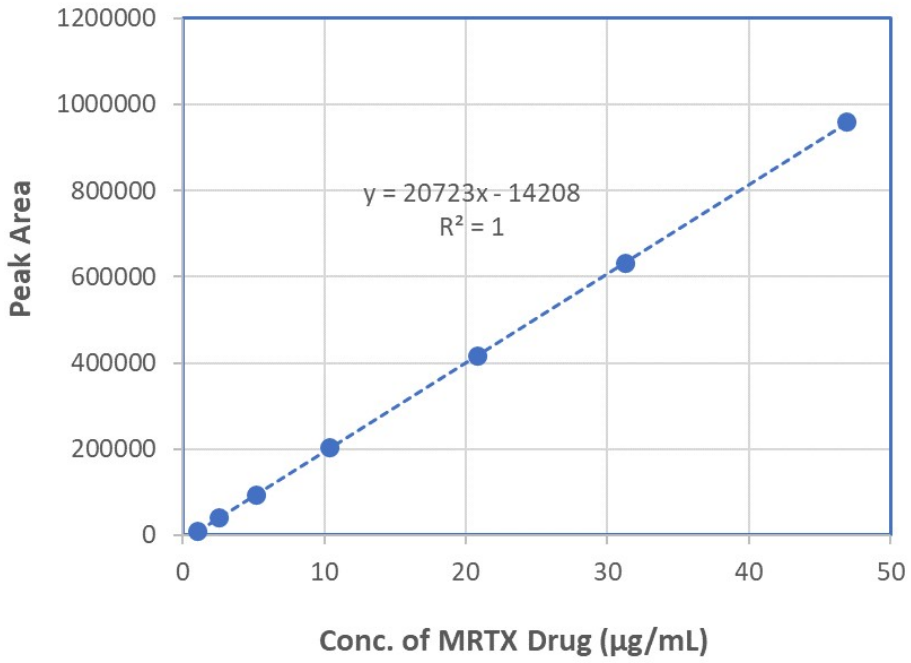


If Target drug level is set at about 5 µg/mL (L-3), % Y-Intercept = (13070 / 93214) × 100 = 14.0%

Notebook reference: NB0224-025

7-Level calibration data and plot (2µL injection @240 nm):

Sol	Theore. Conc. (µg/mL)	Peak Area	Calc. Conc.(µg/mL) [a]	Recovery (%) [b]
L-1	1.04264	9282	1.13352	108.7
L-2	2.60661	40815	2.65517	101.9
L-3	5.21322	93214	5.18371	99.4
L-4	10.42644	202003	10.43338	100.1
L-5	20.85288	414978	20.71061	99.3
L-6	31.27932	631835	31.17517	99.7
L-7	46.91898	960788	47.04898	100.3
a. Calculate drug concentration from calibration equation according to peak area response				
b. %Recovery = (Calc. Conc. / Theore. Conc.) × 100				



If Target drug level is set at about 5 µg/mL (L-3), % Y-Intercept = (14208 / 93214) × 100 = 15.2%

Notebook reference: NB0224-025

Verification injection results for LOQ and LOD determination (2µL injection @240 nm):

Injection No.	LOD Test Sol (0.177 µg/mL)		LOQ Test Sol (0.521 µg/mL)	
	Peak Area	S/N	Peak Area	S/N
1	721	4.8	2568	11.6
2	629	4.1	2534	18.6
3	448	3.2	2591	17.5
4	397	2.4	2597	17.3
5	433	2.5	2538	20.6
6	453	3.0	2559	19.8
Mean (n=6)	513	3.3	2564	17.6
%RSD (n=6)	25.3	N/A	1.0	N/A

Notebook reference: NB0224-025

3) Method accuracy / recovery

Table 1. Accuracy/Recovery Study Result Summary for Free drug in Spiked Liposome Samples Analyzed using SEC Cartridge Technique

0.25 mL of liposome study sample (1:1 dilution); each cartridge conditioned with 3×0.5 mL of Placebo (NB0100-21-Pacebo)

Sample Name	Prep#	MRTX Drug			
		Free Drug (mg/mL)	Rec. Free Drug Drug (mg/mL) [a]	Spiking Free Drug (mg/mL)	Recovery (%) [b]
Non-spiked Sample (MTI106-A1)	**1**	0.1517	N/A	N/A	N/A
	2	0.1431	N/A	N/A	N/A
	3	0.1441	N/A	N/A	N/A
	Mean (n=3)	**0.1463**	N/A	N/A	N/A
	%RSD (n=3)	**3.2**	N/A	N/A	N/A
Spike Sample A (4.5% Spike level)	**1**	0.2230	0.0767	0.0999	76.8
	2	0.2278	0.0815	0.0999	81.6
	3	0.2319	0.0856	0.0999	85.7
				Mean (n=3)	**81.3**
				%RSD (n=3)	**5.5**
Spike Sample B (8.9% Spike Level)	**1**	0.3131	0.1668	0.1997	83.5
	2	0.3180	0.1717	0.1997	86.0
	3	0.3070	0.1607	0.1997	80.5
				Mean (n=3)	**83.3**
				%RSD (n=3)	**3.3**
Spike Sample C (17.9% Spike Level)	**1**	0.5141	0.3678	0.3995	92.1
	2	0.4934	0.3471	0.3995	86.9
	3	0.4980	0.3517	0.3995	88.0
				Mean (n=3)	**89.0**
				%RSD	**3.1**
				Ooverall Mean (n=9)	**84.6**
				%RSD (n=9)	**5.4**

a. Conc. of Recovered Free Drug = Conc. of Free Drug (spiked sample) - Mean Conc. of Free Drug (non-spiked sample)

b. Recovery for Free Drug (%) = (Conc. of Recovered Free Drug /Conc. of Spiking Free Drug) × 100

Spike Level (%) = (Conc. of Spiking Free Drug / Total Drug in Non-Spiked Sample (2.2332 mg/mL)) × 100

Note: An eppendorf research plus 30-300 µL pipet (not a positive displacement) was used for sample transfer during the study.

Notebook reference: NB0224-021

The results of accuracy/recovery study performed at CDMO lab during method evaluation:

With Placebo Conditioning

MTI108-A	Replicate#	Measured Free, µg/mL	DF	Free drug, µg/mL [a]		
Unspiked	1	2.5037	80	200.30	N/A	
	2	2.5950	80	207.60		
	3	2.5502	80	204.02		
	Avg.	**2.55**	N/A	**203.97**		
	RSD (%)	**1.8**		**1.8**		
Spiked with 10% API	**Replicate#**	**Measured Free, µg/mL**	**Recovered Spiked API, µg/mL [b]**	**DF**	**Recovered Spiked API as Free drug, µg/mL [c]**	**%Recovery [d]**
	1	7.0182	4.4686	40	178.74	89.4
	2	6.9834	4.4338	40	177.35	88.7
	3	6.8935	4.3439	40	173.75	86.9
	Avg.	**6.97**	**4.42**	N/A	**176.62**	**88.3**
	RSD (%)	**0.9**	**1.5**		**1.5**	**1.5**

With Placebo Conditioning

MTI108-B	Replicate#	Measured Free, µg/mL	DF	Free drug, µg/mL [a]		
Unspiked	1	2.4964	80	199.71	N/A	
	2	2.5238	80	201.90		
	3	2.5492	80	203.94		
	Avg.	**2.52**	N/A	**201.85**		
	RSD (%)	**1.0**		**1.0**		
Spiked with 10% API	**Replicate#**	**Measured Free, µg/mL**	**Recovered Spiked API, µg/mL [b]**	**DF**	**Recovered Spiked API as Free drug, µg/mL [c]**	**%Recovery [d]**
	1	7.3209	4.7978	40	191.91	96.0
	2	7.3173	4.7942	40	191.77	95.9
	3	7.4021	4.8790	40	195.16	97.6
	Avg.	**7.35**	**4.82**	N/A	**192.95**	**96.5**
	RSD (%)	**0.7**	**1.0**		**1.0**	**1.0**

a. Free Drug in formulation sample = Measured Free (Unspiked sample sol) × Sample dilution factor (DF)

b. Recovered spiked API (spiked sample solution) = Meas. Free (Spiked sample sol) - Avg. Meas. Free (Unspiked sample sol)

c. Recovered Spiked API as Free Drug = Recovered Spiked API (Spike sample sol) × Dilution factor (DF)

d. %Recovery = (Recovered Spiked API as Free Drug / Theoret. Spiking API Conc. (200µg/mL)) ×100

Note: A positive displacement pipet was used to load each study sample solution to a SEC cartridge.

4) Method precision

Table 1. Six Replicate Determinations of Free Drug in MTI106-A1 (non-spike sample) for Method Precision Study (SEC Cartridge Techniqu

0.25 mL of diluted liposome sample solution (1:1); each cartridge conditioned with 3x0.5 mL of Placebo (NB0100-21-Placebo)

(Option#1 calculation for both free drug and encap. drug**)**

Sample Lot#	Prep#	MRTX Drug			
		Free Drug (mg/mL)	Encap. Drug (mg/mL)	Free Drug (%) [a]	Encap. Drug (%) [b]
MTI106-A1	1	0.1488	2.1024	6.6	93.4
	2	0.1429	2.1226	6.3	93.7
	3	0.1382	2.0729	6.3	93.7
	4	0.1403	2.0670	6.4	93.6
	5	0.1342	2.1031	6.0	94.0
	6	0.1338	2.0627	6.1	93.9
Mean		**0.1397**	**2.0993**	**6.3**	**93.7**
SD		**0.0057**	**N/A**	**0.2**	**0.2**
%RSD		**4.1**	**N/A**	**3.4**	**0.2**

a. % Free drug = (Free drug conc. / (Encap. drug conc. + Free drug conc.)) ×100 (**Option#1 calculation**)

b. % Encap. drug = (Encap. drug conc. / (Encap. drug conc. + Free drug conc.)) × 100 = 100 - %Free drug (**Option#1 calculation**)

Table 2. Six Replicate Determinations of Free Drug in MTI106-A1 (non-spike sample) for Method Precision Study (SEC Cartridge Techniqu

0.25 mL of diluted liposome sample solution (1:1); each cartridge conditioned with 3x0.5 mL of Placebo (NB0100-21-Placebo)

(**Option#2 calculation** for both free drug and encap. drug)

Sample Lot#	Prep#	MRTX Drug			
		Free Drug (mg/mL)	Total Drug (mg/mL)	Free Drug (%) [a]	Encap. Drug (%) [b]
MTI106-**A1**	1	0.1488	2.2448	6.6	93.4
	2	0.1429	2.2494	6.3	93.7
	3	0.1382	2.2578	6.1	93.9
	4	0.1403	N/A	6.2	93.8
	5	0.1342	N/A	6.0	94.0
	6	0.1338	N/A	5.9	94.1
Mean		**0.1397**	**2.2507**	**6.2**	**93.8**
SD		**0.0057**	**N/A**	**0.3**	**0.3**
%RSD		**4.1**	**N/A**	**4.1**	**0.3**

a. % Free drug = (Free drug conc. / Avg. toal drug conc.) x 100 (**Option#2 calculation**)

b. % Encap. drug = 100 - %Free drug (**Option#2 calculation**)

Table 3. Concentration data for free drug, encapsulated drug, and total drug in each sample

Sample Lot#	MRTX Drug (mg/mL)			%Recovery [a]
	Total Drug	Conc. of Free Drug	Conc. of Encap. Drug	
MTI106-A1	2.2448	0.1488	2.1024	100.0
	2.2494	0.1429	2.1226	100.7
	2.2578	0.1382	2.0729	98.2
	N/A	0.1403	2.0670	98.1
	N/A	0.1342	2.1031	99.4
	N/A	0.1338	2.0627	97.6
Mean	**2.2507**	**0.1397**	**2.0885**	**99.0**
SD	**0.0066**	**0.0057**	**0.0243**	**1.2**
%RSD	**0.3**	**4.1**	**1.2**	**1.2**

a. %Recovery for Total Drug = ((Free Drug + Encap. Drug)/Avg. Total Drug] × 100

Notebook reference: NB0224-021

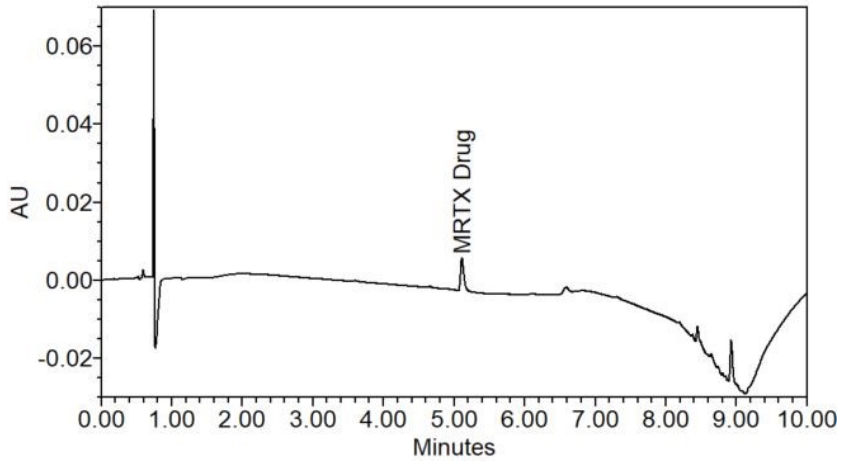


Fig. 1: Chromatogram of free drug sample solution (Prep#1) (Sample dilution factor: 80)

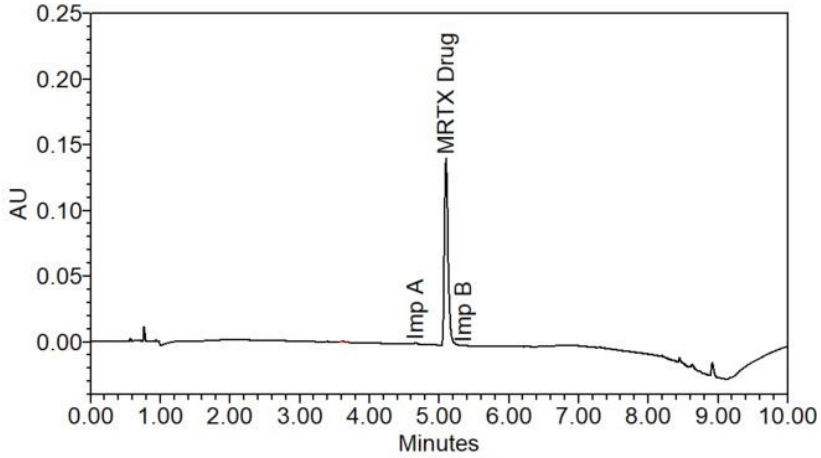


Fig. 2: Chromatogram of encapsulated drug sample solution (Prep#1) (Sample dilution factor: 80)

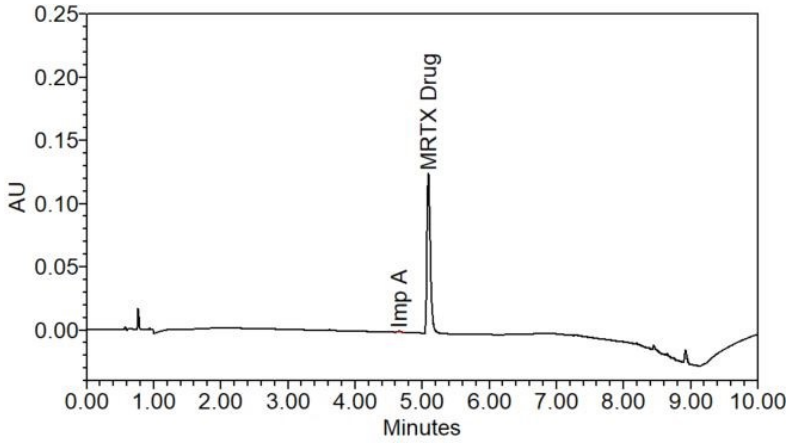


Fig. 3: Chromatogram of total drug sample solution (Prep#1) (Sample dilution factor: 100)

5) Solution stability

Table 1. Standard Solution Stability Test Results (Stored in both flasks@2-8°C and HPLC vials@8°C)

Sol name	Conditions	MRTX Drug		
		Theore. Conc. (µg/mL) (t0)	**Retest (µg/mL) (in 3 days)** [a]	**Recovery (%)** [b]
L-1	Flask @2-8˚C	1.0520	0.9782	**93.0**
	HPLC Vial @8˚C	1.0520	1.0298	**97.9**
L-4	Flask @2-8˚C	10.5204	10.4813	**99.6**
	HPLC Vial@8˚C	10.5204	10.4665	**99.5**
L-7	Flask @2-8˚C	47.3419	46.9221	**99.1**
	HPLC Vial@8˚C	47.3419	47.1736	**99.6**

a. Determined by UPLC using freshly prepared calibration std solutions

b. % Recovery = [Result (t) /Initial result] x 100

Table 2. Sample Solution Stability Test Results (Stored in both centrifuge tubes or flask @2-8˚C and HPLC vials@8˚C)

Sol name	Conditions	MRTX Drug		
		Initial Test (mg/mL) (t0)	**Retest (mg/mL) (in 3 days)**	**Recovery (%)** [a]
FD-106A1_P1 [b]	Tube @2-8˚C	148.8397	148.0192	**99.4**
	HPLC Vial @8˚C	148.8397	153.5660	**103.2**
TD_106A1_P1 [c]	Flask @2-8˚C	2244.8330	2248.7588	**100.2**
	HPLC Vial@8˚C	2244.8330	2241.7595	**99.9**
LD_106A1_P1 [c]	Tube @2-8˚C	2102.4137	2089.4012	**99.4**
	HPLC Vial@8˚C	2102.4137	2097.7905	**99.8**

a. % Recovery = [Result (t) /Initial result] × 100

b. Determined using freshly prepared **L-1 to L-4** as linear calibration standards

c. Determined using freshly prepared **L-1 to L-6** as linear calibration standards

FD = Free drug in MTI106-A1 sample; TD = Total drug in MTI106-A1 sample; LD = Liposomal or encapsulated drug in MTI106-A1 sample

Notebook reference: NB0224-021 and -022