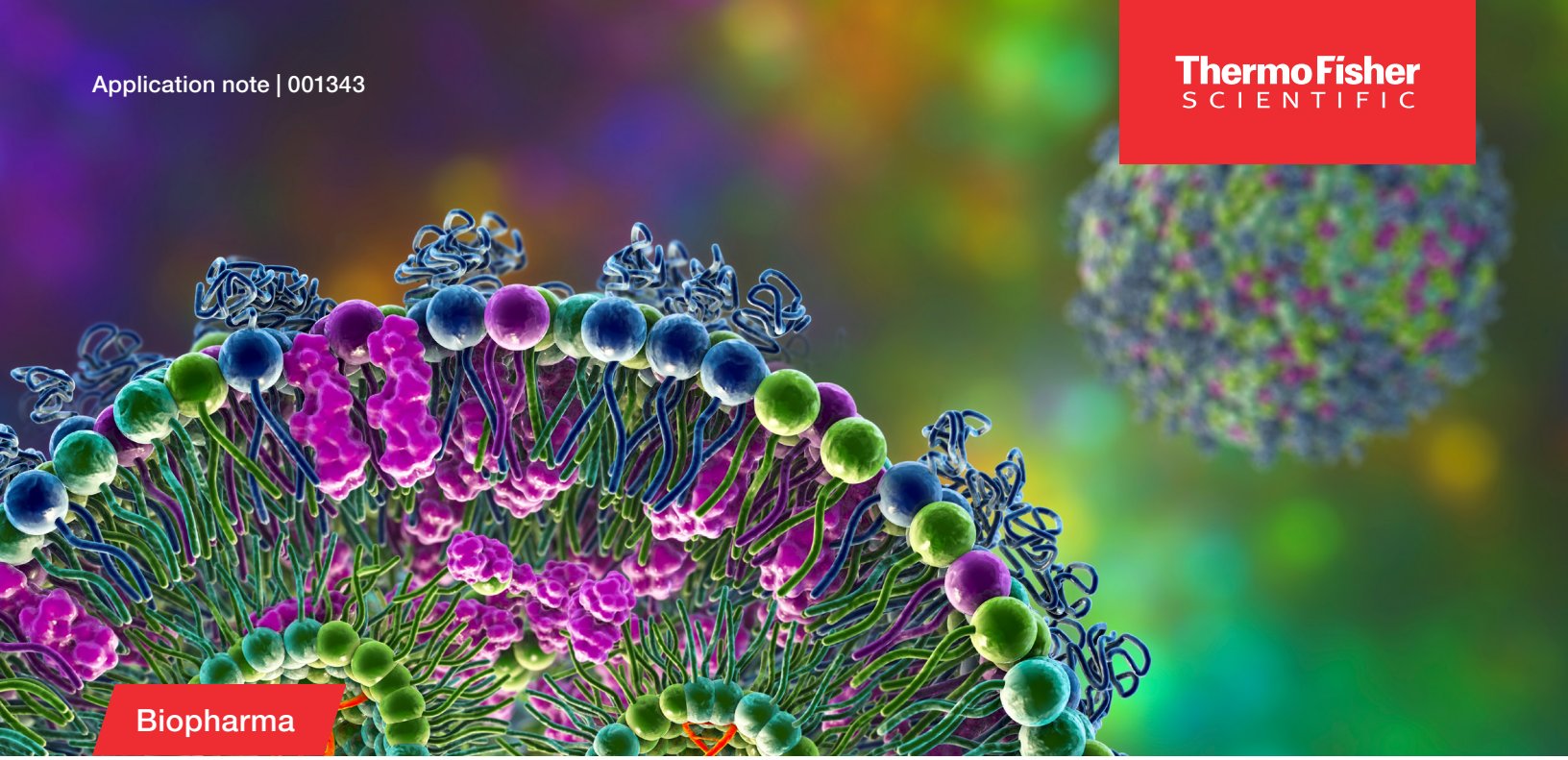
Application note | 001343



Applying UHPLC-HRAM MS technology to characterize and quantify lipid components *in vivo* to support new LNP development

Authors

Reiko Kiyonami,[1] Sinae Lee,[2] Koshi Kunimoto,[2] Min Du,[3] Evgenia Verovskaya,[2] Jason Potter[2]

[1]Thermo Fisher Scientific, San Jose, CA;
[2]Thermo Fisher Scientific, Carlsbad, CA;
[3]Thermo Fisher Scientific, Cambridge, MA

Keywords

Lipid nanoparticle (LNP), ionizable lipids, PEG-lipids, high-resolution accurate mass (HRAM), Orbitrap mass spectrometer, data-dependent MS/MS, targeted MS/MS, simultaneous targeted lipid quantification and lipid metabolite profiling

Application benefits

- A sensitive and robust LC-MS based platform method to support efficient development and preclinical studies of novel lipid nanoparticles (LNPs)
- A single HRAM method for both quantification and characterization of LNP lipid components and their metabolites

Goal

Develop a highly sensitive and selective LC-MS/MS method to simultaneously characterize the metabolites of the LNP lipid components and quantify the LNP components in biological matrix samples

Introduction

LNPs have emerged across the pharmaceutical industry as promising vehicles to deliver a variety of therapeutic agents. Currently in the spotlight as a vital component of the COVID-19 mRNA vaccines, LNPs play a key role in effectively protecting and transporting mRNA to cells.[1] LNP formulations are typically composed of (1) an ionizable or cationic lipid or polymeric material, bearing tertiary or quaternary amines to encapsulate the polyanionic mRNA; (2) a zwitterionic lipid (e.g., 1,2-distearoyl-sn-glycero-3-phosphocholine [DSPC]) that resembles the lipids in the cell membrane; (3) cholesterol to stabilize the lipid bilayer of the LNP; and (4) a polyethylene glycol (PEG)-lipid to lend the nanoparticle a hydrating layer, improve colloidal stability, and reduce protein absorption.[2]

After an LNP product is administered to an animal or human, the ionizable lipid plays a central role in the nucleic acid delivery efficacy. Since ionizable lipids are synthetic components, they should be rapidly degraded into non-toxic metabolites after successful intracellular cargo delivery to avoid immune responses and toxicity mediated by the synthesized lipids. A key research area for new LNP product development is to develop the novel, next-generation ionizable lipids that combine the excellent nucleic acid delivery efficacy with biodegradable functionality leading to rapid elimination *in vivo*.[3–5] To understand the clearance rate and biodistribution of an LNP product, researchers need to rapidly monitor the bio-degradability of the novel ionizable lipids after the LNP administration and identify the metabolites generated through the bio-transformation *in vivo*. Analytical tools that can quantify the ionizable lipids and other synthetic lipids with high sensitivity and simultaneously identify the associated lipid metabolites *in vivo* from various tissue and plasma samples are needed to support new-generation LNP product development and pre-clinical studies.

An HPLC MS-MS/MS approach provides excellent analytical solutions for addressing the LNP lipid analytical needs by enabling confident identification of lipid components and associated metabolites using direct accurate mass measurement at both precursor ion and fragment ion levels. It also enables targeted lipid quantification with high sensitivity and selectivity using a targeted MS/MS approach. An HPLC-MS/MS method has been used successfully to study chemical structure and pharmacokinetics of lead lipids in the LNP development of Moderna's Covid-19 vaccine.[6] To take advantage of the HPLC MS-MS/MS approach, we developed an innovative LC MS-MS/MS (LC ddMS^2-tMS^2) method that uses two alternative instrument experiments, a data-dependent MS/MS experiment followed by the targeted MS/MS experiment for simultaneous LNP lipid metabolite characterization and targeted lipid quantification.[7] In this work, we applied the developed LC ddMS^2-tMS^2 method to the lipid analysis of tissues and plasma samples from mice that were administered SM-102 based LNP encapsulated with firefly luciferase mRNA (fLuc mRNA). A Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system was used for all experiments. The LC MS-MS/MS method allowed rapid ionizable lipid SM-102 quantification and lipid metabolite characterization simultaneously in a single 24 min LC/MS run. The analytical results are reported here.

Experimental

LNP formulation preparation

Lipid components including SM-102 (a synthetic ionizable amino lipid that forms part of the LNP for the Moderna COVID-19 vaccine,[8] Figure 1A), DSPC, cholesterol, and DMG PEG2000 were purchased from Cayman Chemical (35425). fLuc mRNA was purchased from TriLink Biotechnologies (L-7602). The lipids were dissolved in 1 mL of ethanol at molar ratios of 50:10:38.5:1.5 (SM102:DSPC:cholesterol:PEG lipid). The fLuc mRNA (0.62 mg) was added to 3.0 mL acidification buffer of 25 mM sodium

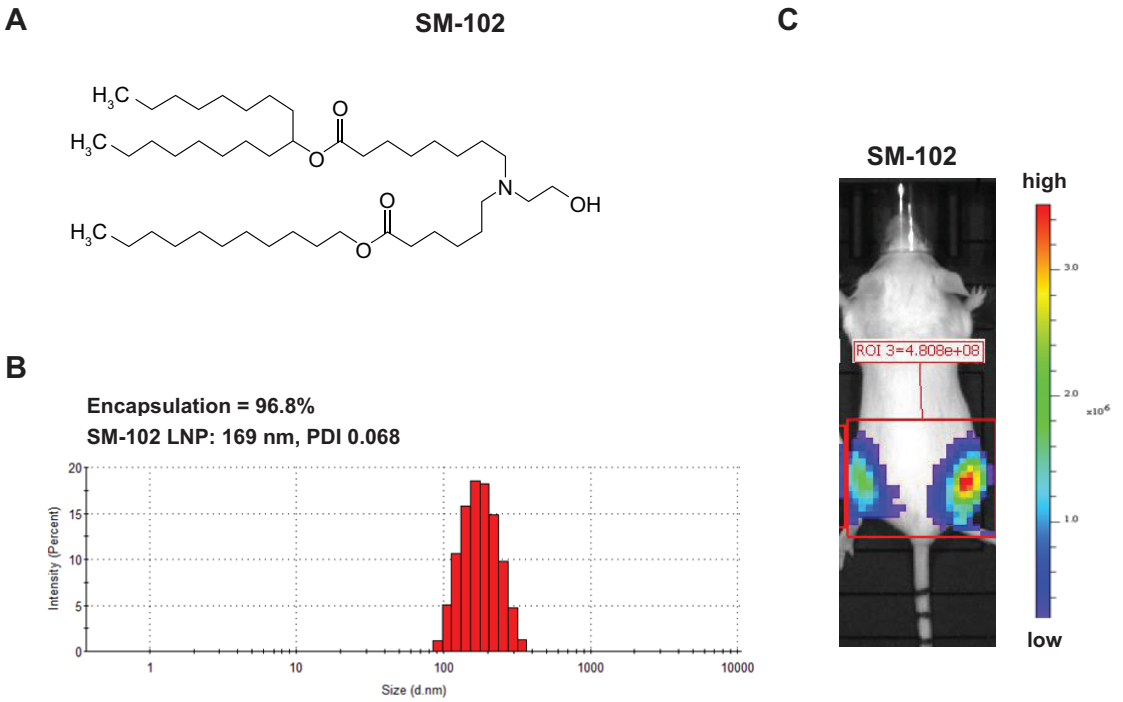


Figure 1. LNP formulation and administration. (A) The structure of lipid SM-102. (B) Dynamic light scattering analysis of LNP size, size distribution, and mRNA encapsulation. (C) *In vivo* bioluminescence images following intramuscular (IM) injection of fLuc mRNA-LNP.

acetate (pH 5.0). The ethanolic lipid mixture was mixed with the aqueous acidic fLuc mRNA solution using a microfluidic mixer (NanoAssemblr™ Benchtop, Precision Nanosystems). Then, the formulation was dialyzed using Thermo Scientific™ Slide-A-Lyzer™ dialysis cassettes against PBS (pH 7.4) for 18 h. The prepared LNP was found to be 169 nm in size and PDI 0.068 using dynamic light scattering (DLS) (Zetasizer™ Nano, Malvern Panalytical). The mRNA encapsulation efficiency was determined to be 96.8% using the Invitrogen™ Quant-it™ RiboGreen RNA Assay (Figure 1B).

LNP administration to mice

Female BALB/CJ mice were purchased from the Jackson Laboratory. Mice were acclimated for 1 week before the initiation of a study. Mice were injected intramuscularly into quadriceps muscles with 100 µL containing 5 µg of firefly luciferase mRNA formulated in the LNPs (50 µL per thigh). *In vivo* delivery activity was confirmed 4 hours after delivery by intraperitoneal injection of RediJect™ D-luciferin substrate (Perkin Elmer) and signal quantification using IVIS™ Lumina III analyzer (Perkin Elmer). The *in vivo* bioluminescence images following IM injection of the fLuc mRNA-encapsulated LNP are shown in Figure 1C.

Sample collection

At 1, 2, 4, 8, and 24 h post-injection, two mice were sacrificed and the plasma, spleen, liver, and site of injection muscle were harvested. For liver, the sample was isolated using a biopsy puncher, and for muscle, by excision of the injection point.

Additional liver tissue samples were collected from three control mice without LNP injection and used as the control matrix for generating the SM-102 quantification calibration curve.

All tissue samples were weighed and homogenized using a Fisherbrand™ Bead Mill 24 Homogenizer and 1.4 mm ceramic bead pre-filled 2 mL tubes (Fisher Scientific) following the addition of 19 equivalents (w/v) of water.[8]

Lipid extraction

For plasma samples, 40 µL each were used. The lipids were extracted by adding a mixture of solvent of metahonol/choloroform/water (1/2/0.8). The extracted lipids of each plasma sample were reconstituted in 200 µL of IPA/methanol (50:50) for LC MS-MS/MS analysis.

For tissue samples, the lipids were extracted by adding a mixture of methanol/chloroform (1:2). A variable volume of the mixture of methanol/chloroform was added to each tissue sample depending on the sample's water volume after homogenization to make the proportion of metanol/chloroform/water be close to 1/2/0.8. The extracted lipids of each tissue sample were reconstituted in IPA/methanol (50:50) to make the final concentration of each tissue sample equal to 0.5 mg tissue/µL.

For SM-102 calibration curve generation, SM-102 standard was spiked into the liver control matrix at the concentration levels of 1, 10, 100, 1,000, and 10,000 pg/µL.

Chromatography

For all experiments, chromatographic separations were carried out using a Thermo Scientific™ Accucore™ C30 column (2.1 × 150 mm, 2.6 µm) on the Vanquish Horizon UHPLC system, consisting of the following modules:

- Thermo Scientific™ System Base Vanquish™ Horizon/Flex (P/N VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump H (**P/N VH-P10-A**)
- Thermo Scientific™ Vanquish™ Split Sampler FT (**P/N VH-A10-A**)
- Thermo Scientific™ Vanquish™ Column Compartment (**P/N VH-C10-A**)

Solvent A: 60% ACN/40% H_2O containing 10 mM ammonium formate and 0.1% difluoro acetic acid

Solvent B: 90% IPA/10% ACN containing 10 mM ammonium formate and 0.1% difluoro acetic acid

Chromatographic separation details are shown in Table 1. The column temperature was set to 50 °C with Still Air mode. For the spiked-in SM 102 standard analysis, 1 µL of each known concentration sample was injected and analyzed in triplicate. For the lipid analysis of plasma and tissue samples, 2 µL were injected per sample.

Table 1. UHPLC gradient condition

Time	Flow (mL/min)	%B
0	0.35	30
2	0.35	43
2.1	0.35	55
12	0.35	65
15	0.35	85
16	0.35	100
18	0.35	100
18.1	0.35	30
24	0.35	30

Mass spectrometry

The Orbitrap Exploris 480 mass spectrometer was used for MS data collection. For simultaneous unknown lipid metabolite characterization and targeted SM-102 quantification in a single LC-MS run, a data-dependent MS/MS experiment, followed by a targeted MS/MS experiment, was carried out. Table 2 shows the detail settings for the two alternative experiments. Figure 2 shows the actual method editor images for the MS setups.

Data processing

The lipid metabolite characterization and relative quantification were carried out using Thermo Scientific™ Compound Discoverer™ 3.3 software. The SM-102 quantification was carried out using Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System (CDS) software.

Table 2. MS instrument set up

MS source setting	Value	Experiment #1: full MS/dd-MS/MS setting	Value	Experiment #2: targeted MS/MS setting	Value
Sheath gas	35	**General**		**tMS/MS**	
Aux gas	6	Application mode	Small molecule	Resolution	30,000 at *m/z* 200
Sweep gas	1	Pressure mode	Standard	Isolation window (*m/z*)	2
Spray voltage (+V)	3,400	RF lens (%)	50	AGC target value (%)	100
Capillary temp. (°C)	300	**Full MS**		Max inject time (ms)	200
Vaporizer temp. (°C)	350	Scan range (*m/z*)	300–1400	Fixed first mass (*m/z*)	60
		Resolution	60,000 at *m/z* 200	Collision energy mode	Fixed
		AGC target value (%)	300	HCD collision energy (%)	45
		Max inject time (ms)	100		
		dd-MS/MS	**(cycle time 0.6 s)**	**Targeted precursor mass list**	
		Resolution	30,000 at *m/z* 200	*m/z* 710.6642	RT window: 7.5–9.5 min
		Isolation window (*m/z*)	1.5		
		AGC target value (%)	100		
		Max inject time (ms)	100		
		Fixed first mass (*m/z*)	60		
		Targeted mass exclusion	On		
		Collision energy mode	Fixed		
		HCD collision energy (%)	15, 30		

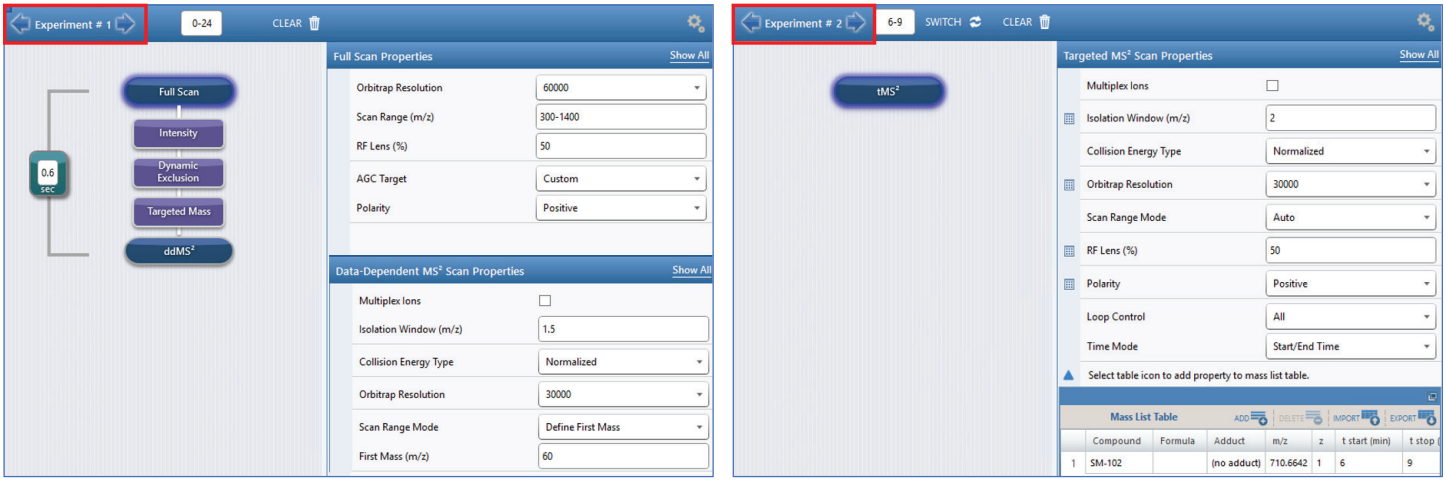


Figure 2. Screen captures of the MS method editor for two alternative experiments

Results and discussion

Quantification of SM-102

Calibration curve generation of SM-102

The SM-102 standard was spiked in the control liver lipid extract matrix at different concentration levels (1 pg/µL – 10,000 pg/µL) for calibration curve generation. 1 µL of each sample was injected in triplicate. Figure 3A shows the base peak chromatogram from one of the control liver lipid extracs (SM-102: 10 pg/µL). Many lipid components across different lipid classes were detected from the control liver sample (data not shown), and the normalized intensity signal of the spiked-in SM-102 (10 pg on column) was less than 0.2% compared to the co-eluting lipid species (Figure 3B). Even at such low concentration range, high-quality MS/MS data was acquired for the spiked-in SM-102 (Figure 3C) with excellent mass accuracy of 2 ppm, enabling unambiguous SM-102 identity confirmation. The most abundant and unique fragment ions of 300.2173, 318.2280, and 472.4003 were selected for SM-102 quantification. Figure 4 shows the SM-102 was clearly detected and quantified at 1 pg on column with less than 5% CV. Figure 5 shows the generated calibration curve of SM-102. Over 4 orders of linear dynamic range were observed.

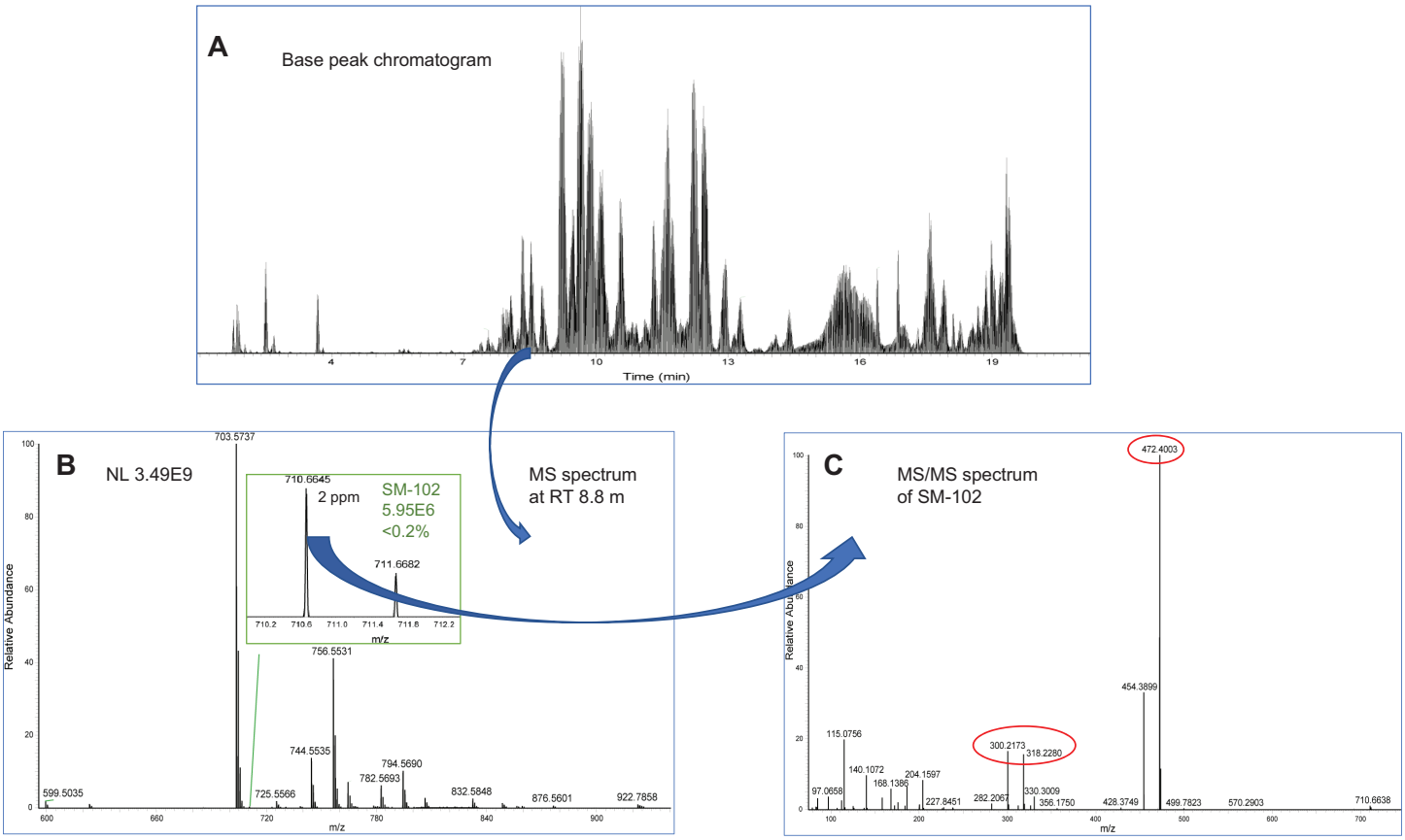


Figure 3. SM-102 quantification using targeted MS/MS. (A) The base peak chromatogram of control liver lipid extract with spiked-in SM-102 standard at 10 pg/µL. (B) Full scan MS spectrum observed at the retention time of 8.8 min where the SM-102 was eluted with other co-eluting lipid species. The relative intensity signal ratio of SM-102 (shown in the insert) vs. the strongest lipid species (precursor ion: *m/z* 703.5737) was less than 0.2%. (C) MS/MS spectrum of the SM-102 observed with the targeted MS/MS experiment of *m/z* 710.6642. The fragment ions highlighted with red circles were used for SM-102 quantification.

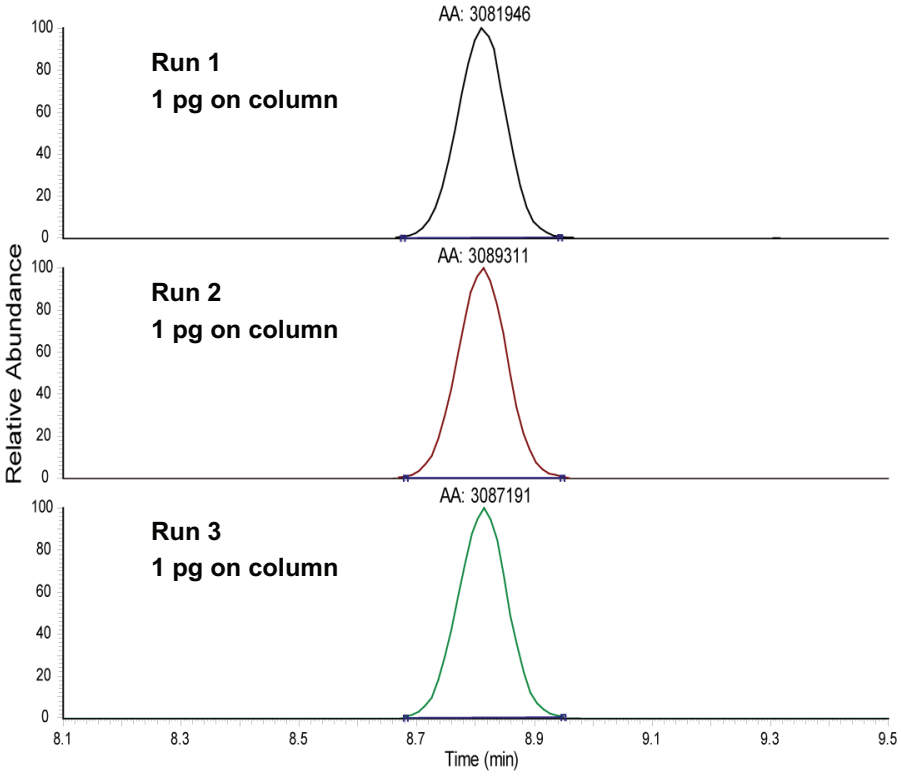


Figure 4. Extracted fragment ion chromatogram (300.2173 + 318.2280 + 472.4003) of the SM-102 from the triplicate analysis of the control liver lipid extract with spiked-in SM-102 standard at 1 pg/µL. The integrated peak area of each run was shown on the top of the peak. The observed CV% across three replicate runs was less than 5%.

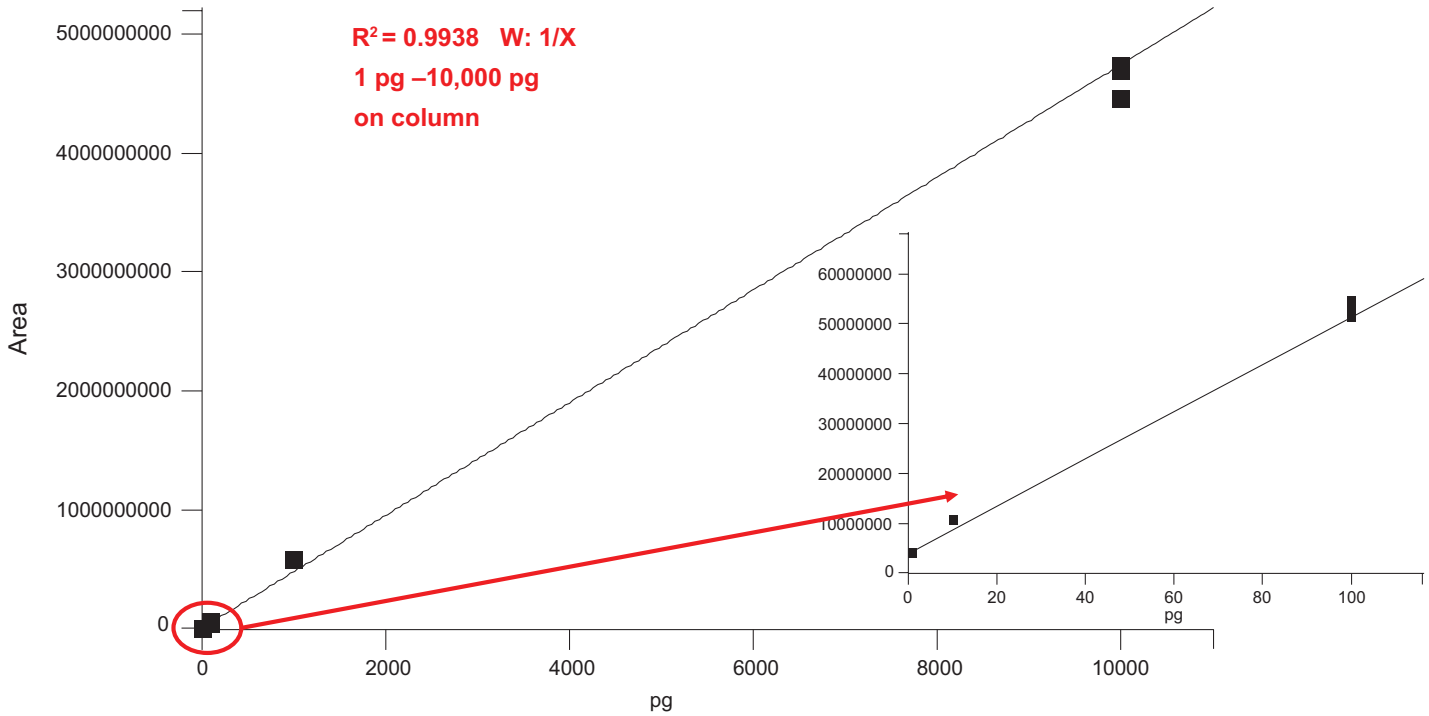


Figure 5. Calibration curve of SM-102 spiked-in the control liver lipid extract matrix

Table 3. List of plasma and tissue samples collected from mice. Two mice were sacrificed per time point.

Sample #	Mouse	Blood/Organ	Sample #	Mouse	Blood/Organ
1	1 h -1	Plasma	21	4 h -2	Plasma
2	1 h -1	Liver	22	4 h -2	Liver
3	1 h -1	Spleen	23	4 h -2	Spleen
4	1 h -1	Muscle	24	4 h -2	Muscle
5	1 h -2	Plasma	25	8 h -1	Plasma
6	1 h -2	Liver	26	8 h -1	Liver
7	1 h -2	Spleen	27	8 h -1	Spleen
8	1 h -2	Muscle	28	8 h -1	Muscle
9	2 h -1	Plasma	29	8 h -2	Plasma
10	2 h -1	Liver	30	8 h -2	Liver
11	2 h -1	Spleen	31	8 h -2	Spleen
12	2 h -1	Muscle	32	8 h -2	Muscle
13	2 h -2	Plasma	33	24 h -1	Plasma
14	2 h -2	Liver	34	24 h -1	Liver
15	2 h -2	Spleen	35	24 h -1	Spleen
16	2 h -2	Muscle	36	24 h -1	Muscle
17	4 h -1	Plasma	37	24 h -2	Plasma
18	4 h -1	Liver	38	24 h -2	Liver
19	4 h -1	Spleen	39	24 h -2	Spleen
20	4 h -1	Muscle	40	24 h -2	Muscle

In vivo SM-102 quantification results

The plasma, spleen, liver, and site of injection muscle of the mice were collected at 1, 2, 4, 8, and 24 h after the SM-102 LNP injection. Two mice were sacrified per time point, yielding total 40 biological samples (Table 3).

The lipids were extracted from plasma and tissue samples using a chloroform and methanol mixture as described in the sample preparation section. The extracted lipid mixture per biological sample was analyzed using the LC MS-MS/MS method described in the experimental section. The SM-102 quantification was performed using the targetd MS/MS data. The absolute amount of SM-102 was calculated using the SM-102 calibration curve generated in the control mice liver matrix.

For SM-102 quantification results, we focus on clearance profiling of tissue samples because only a trace amount of SM-102 was detected from plasma samples collected at the 1 h time point post injection (data not shown) and no SM-102 was detected from other plasma samples.

Table 4 summarizes the SM-102 quantification results of mouse liver samples across different time points. Although some variations in the measured amounts were observed between two biological replicates per time point, it is clear that the amount of SM-102 decreased consistently across the time course (Figure 6). Comparing the average amount of SM-102 at the 1 h time point (3677 ng/g) and 2 h time point (512 ng/g), around 86% of SM-102 was efficiently cleared from liver tissue during the 1 h time period. Table 5 summarizes the SM-102 quantification results of mouse spleen samples across different time points. The clearance efficiency of SM-102 was even greater for spleen tissue. Almost 99% of SM-102 was cleared from spleen tissue at the 2 h time point and only a very trace amount of SM-102 was detected after 2 h post injection (Figure 6). Table 7 summarizes the SM-102 quantification results of muscle samples across different time points. The SM-102 concentation also decreased steadily across the time course (Figure 6). When comparing the concentration of SM-102 among liver, spleen, and muscle tissues, muscle tissue had the highest concentration of SM-102, as expected.

Table 4. SM-102 quantification summary for the mouse liver tissue samples

Sample #	Mouse	Sample weight (g)	Lipid extract in IPA/MeOH (µL), 0.5 mg/µL	2 µL injection, observed amount (pg)
2	1 h -1	0.05	100	3119
6	1 h -2	0.05	100	4235
10	2 h -1	0.06	120	398
14	2 h -2	0.05	100	625
18	4 h -1	0.06	120	228
22	4 h -2	0.04	80	242
26	8 h -1	0.05	100	115
30	8 h -2	0.1	200	83
34	24 h -1	0.05	100	102
38	24 h -2	0.05	100	91

Table 5. SM-102 quantification summary for the spleen tissue samples

Sample #	Mouse	Sample weight (g)	Lipid extract in IPA/MeOH (µL), 0.5 mg/µL	2 µL injection, observed amount (pg)
3	1 h -1	0.04	80	1614
7	1 h -2	0.06	120	927
11	2 h -1	0.06	120	8
15	2 h -2	0.05	80	17
19	4 h -1	0.06	120	6
23	4 h -2	0.06	120	9
27	8 h -1	0.1	200	4
31	8 h -2	0.1	200	3
35	24 h -1	0.1	200	<1
39	24 h -2	0.06	120	<1

Table 6. SM-102 quantification summary for the muscle tissue samples

Sample #	Mouse	Sample weight (g)	Lipid extract in IPA/MeOH (µL), 0.5 mg/µL	2 µL injection, observed amount (pg)
4	1 h -1	0.05	100	12860
8	1 h -2	0.04	80	9360
12	2 h -1	0.04	80	5452
16	2 h -2	0.11	220	7099
20	4 h -1	0.06	120	3513
24	4 h -2	0.06	120	4240
28	8 h -1	0.1	200	2592
32	8 h -2	0.11	220	3194
36	24 h -1	0.05	100	1437
40	24 h -2	0.06	120	2135

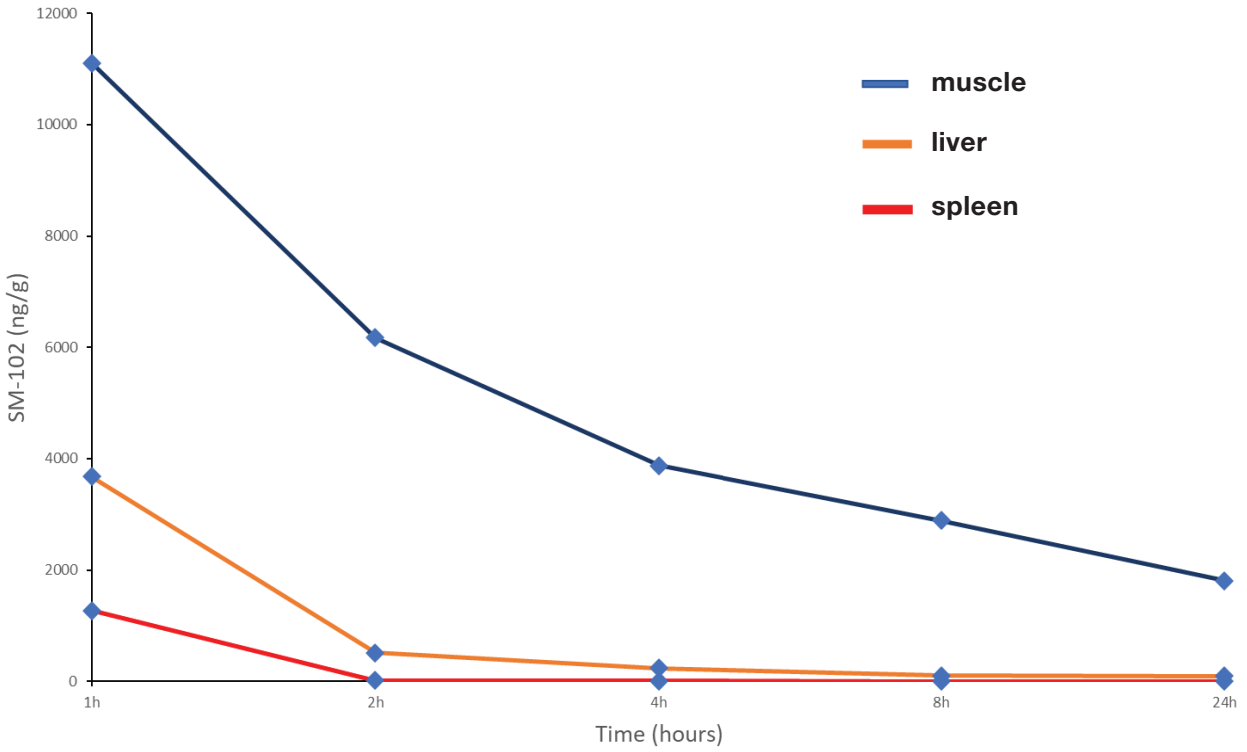


Figure 6. Clearance profile of SM-102 in multiple tissues. The average observed amount of two biological replicates was used for the plot per time point.

In vivo SM-102 metabolite characterization

Since SM-102 is a synthetic amino lipid, it will go through biotransformation *in vivo* like other small molecular drugs. It is important to identify SM-102 metabolites in order to understand the SM-102 clearance pathways involved. Compound Discoverer 3.3 software includes knowledge-based biotransformation reaction rules that can be used to predict expected metabolic transformations of small molecules in both phases 1 and 2. By providing the structure of SM-102, we were able to use the metabolite ID workflow of Compound Discoverer 3.3 software to process the data collected from the data-dependent MS/MS experiment for the SM-102 metabolite identification study (Figure 7). The software detects the potential metabolites from the expected metabolite list, which was generated based on the common reation rules. Subsequently, the FISh Scoring node (FISh = fragment ion search) automatically annotates the MS/MS fragments that match the theoretical fragment ions predicted with the known fragmentation rules based on the parent structure and provides a FISh score. A higher score means more fragment ions can be annotated.

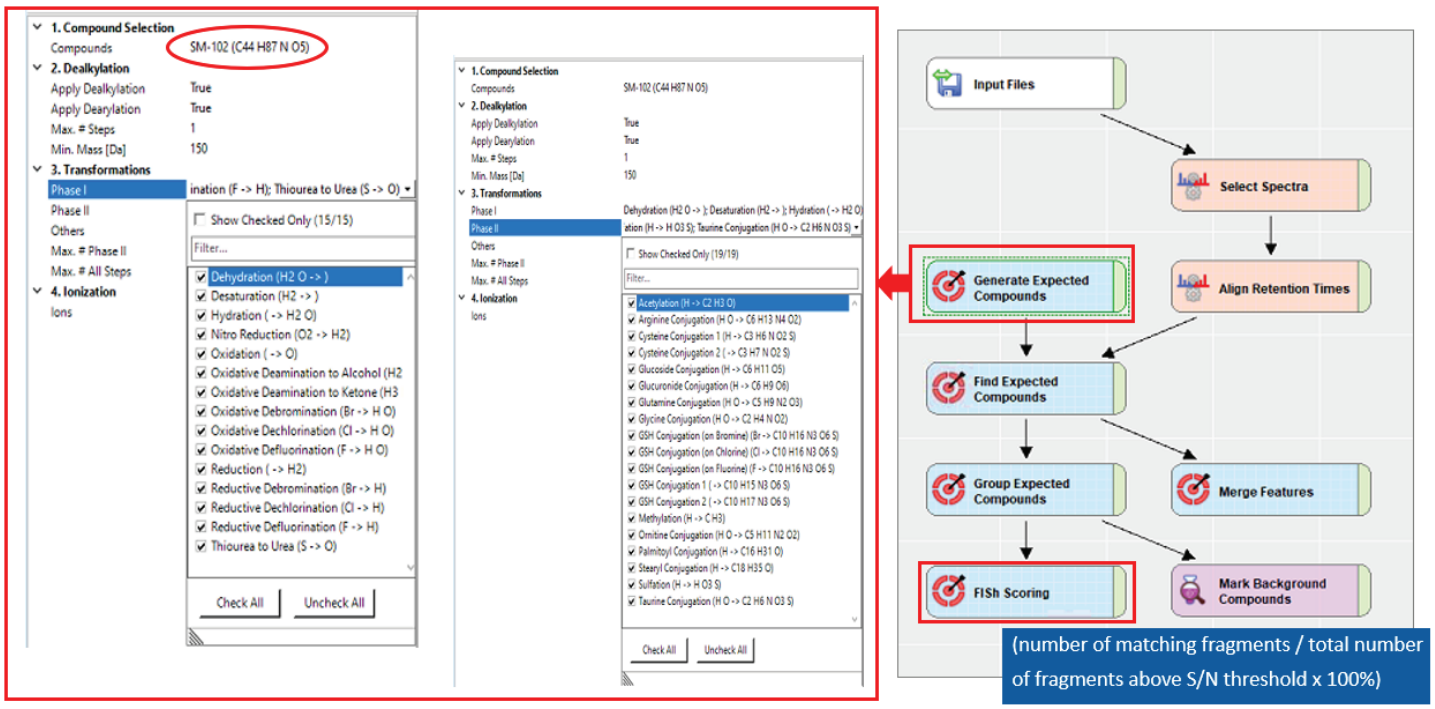


Figure 7. Compound Discoverer software predefined processing workflow: MetID with Stats Expected

Figure 8 shows one example of potential SM-102 metabolite identification in liver using the metabolite ID workflow. The relative concentration of the identified potential SM-102 metabolite decreased over the time course, showing it was cleared from the liver tissue over time. The measured molecule weight error for the potential metabolite was less than 1 ppm. The structure of the potential metabolite was proposed by the annotation of the fragment ions using the FISh Scoring node.

Formula	Parent Compound	Dealkylated	Transformations	Composition Change	Annot. ΔMass [ppm]	Calc. MW	m/z	RT [min]	FISh Coverage	Area (Max.)	MS2	Reference Ion
C42 H79 N O3	SM-102	X	Dehydration, Desaturation	-(C2 H8 O2)	0.79	645.60651	646.61378	16.816	60.00	100584341	■	[M+H]+1

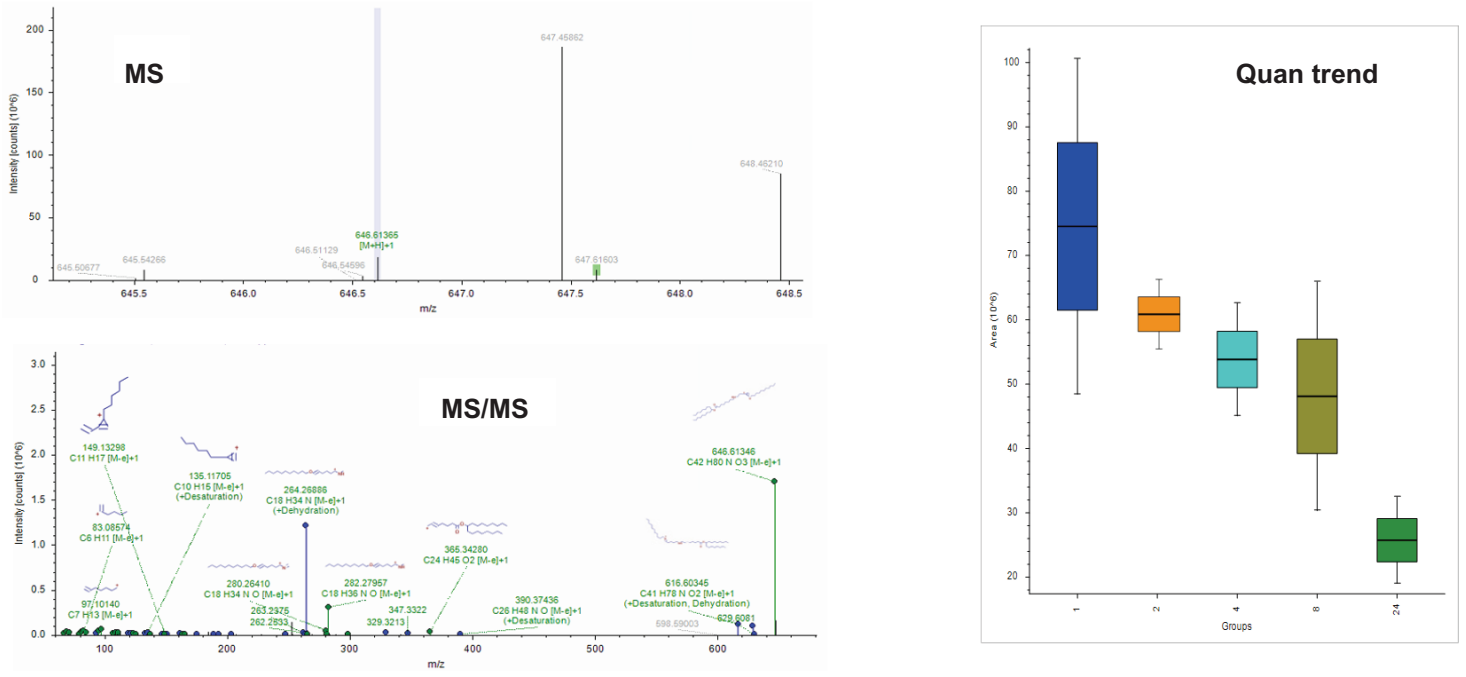


Figure 8. Potential metabolite identification from mouse liver tissue samples using Compound Discoverer 3.3 software

Conclusion

We have successfully applied our developed LC ddMS^2-tMS^2 method to understand the pharmacokinetics and metabolism of SM-102 in mice that were injected fLuc mRNA – encapsulated LNPs with IM.

Within a single LC-MS/MS method,

- the targeted HRAM MS/MS experiment ensured sensitive quantification of SM-102 with high selectivity from complex mouse tissues and plasma samples, establishing SM-102 clearance profiles after LNP injection.
- the data-dependent experiment, combined with streamlined data processing of Compound Discoverer software, allowed confident identification and relative quantification of potential SM-102 metabolites.

The developed LC ddMS^2-tMS^2 method provides a useful analytical tool to support the new generation of LNP development and preclinical study.

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