Evaluating stability and cytotoxicity of human monoclonal antibody in cultured rat hepatocytes with a surrogate peptide approach

Nadya Galeva, Reed Murbach, Krystal Gilligan, Kevin Westland, Seema Muranjan and Joanna E. Barbara
XenoTech LLC, Kansas City, KS, USA

INTRODUCTION

Cultured hepatocytes are an established test system for evaluating the inductive effects of new chemical entities (NCEs). Supportive assays are performed to evaluate the cytotoxicity and stability of these compounds in the test system. The release of LDH, a measure of membrane integrity, is a simple, economical evaluation of cytotoxicity. The direct quantitative measurement of small molecule NCEs by LC-MS/MS from media aliquots taken over the time course of treatment is a simple evaluation of compound stability in the test system. Analytically, however, direct quantitative measurement of a monoclonal antibody (mAb)-based drug is more difficult due to greater structural complexity of protein biopharmaceuticals in comparison to small molecule drugs. Our approach to mAb LC-MS/MS quantitation for predicting drug stability and toxicity in a traditional test system in vitro involved incubations of rat liver cells (cultured primary hepatocytes) with human SiLu™Lite mAb, followed by trypsin digestion using Waters ProteinWorks™ eXpress Direct Digest Kit and quantitation of five tryptic mAb surrogate peptides.

METHODS

Intact monoclonal antibody (mAb) standards

Intact SiLu™Lite mAb standard and SiLu™ mAb labeled standard were purchased from Sigma-Aldrich. ProteinWorks™ eXpress Direct Digest Kit was purchased from Waters Corporation. Figure 1 and Table 1 detail relevant information about the mAb standards used.

Figure 1. Human SiLu™Lite MAb sequence

>light chain human SILu™ mAb
QSALTQPRSVSGSPGQSVTISCTGTSSDIGGYNFVSWYQQHPGKAPKLMIYDATK**RPSGVPDR**FSGSKSGNTASLTISGLQAEDEADYYCCSYAGDYTPG
VVFGGGTK**LTVLGQPK**AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNK**YAASSYLSLTPEQWK**SHRSYSCQ
VTHEGSTVEKTVAPTECS

>heavy chain human SILu™ mAb
EVQLVESGGGLVQPGGSLRLSCVASGFTLNNYDMHWVRQGIGKGLEWVSKIGTAGDRYYAGSVKGRFTISRENAKDSLYLQMNSLRVGDAAVYYCARGAG
RWAPLGAFDIWGQGTMVTVSSASTK**GPSVFPLAPSSK**STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ
TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNK**ALPAPIEK**TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

conserved regions: in blue
variable regions: in red

Table 1. SiLuLite™ mAb and SiLu™ mAb data

	Human SiLuLite™ mAb	Human SiLu™ mAb (stable isotope-labeled mAb standard)
Intact protein formula:	$C_{6374}H_{9864}N_{1708}O_{1992}S_{46}$	$C_{6002}H_{9864}N_{1548}O_{2008}S_{52}[^{13}C_{372}][^{15}N_{160}]$
Average molecular weight:	143,767.8 g/mol	144,744.4 g/mol
Concentration of stock solution:	5,000 µg/mL	1,000 µg/mL

Treatment of cultured rat hepatocytes

Cell culture media containing human SiLu™Lite mAb (0.007, 0.07, 0.7 and 1.4 µM) was applied to cultured rat hepatocytes on the first day of treatment and the media was replaced after 24 hours. The culture multiwell plates were incubated at 37°C, and the incubation atmosphere was a 95:5 mixture of air and CO^2, 95% relative humidity. Approximately 24 h following the final treatment, cultures were visualized with a Nikon TMS Microscope (Nikon Corporation) and representative hepatocytes from each treatment group were photographed with a PAXcam5 (MIS Inc.) digital camera to document morphological integrity. Following the second treatment, equal volumes of incubation medium were collected from triplicate 0, 0.25, 3, and 24 hour samples, flash frozen (-80°C) and stored for subsequent analysis.

Cytotoxicity assessment

The leakage of L-lactate dehydrogenase (LDH) from damaged cultured hepatocytes was determined with a commercial kit. Medium samples were collected from the vehicle and negative control (cell culture medium), positive control, and SiLu™Lite mAb treatment groups immediately prior to treatment on day 2 and approximately 24 h following day 2 treatment. At each collection time point, three vehicle control treatment wells were treated with a 1% Triton X-100 solution and incubated for a period of 30 to 120 min for complete hepatocyte lysis and release of LDH to calibrate LDH release results. Collected medium and samples were stored at 2 to 8 °C until analysis. Aliquots of each incubation medium sample and the lysed controls were transferred to a 96 well plate. The LDH working solution was prepared and added to the plate wells to begin the reactions. Absorbance (λ = 490 nm) was measured for 20 min at 1 min intervals using a Synergy HT Multi-Detection Microplate (BioTek Instruments, Inc.) microtiter plate reader.

Analysis of incubation medium samples

The collected incubation samples stored at -80°C were thawed for analysis. Briefly, an aliquot was transferred into ProteinWorks™ (Waters Corporation) tubes containing digestion buffer with internal standard for further sample preparation. Samples were analyzed by LC-MS/MS for quantitation of SiLu™Lite mAb against six levels of calibration standards ranging from 0.0007 to 0.7 µM prepared in duplicate.

Surrogate peptide selection – Analytical Method development for analysis of incubation medium samples

Both light and heavy chain sequences of SiLu™Lite mAb were compared to the database of rat protein sequences using Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/). Regions of SiLu™Lite mAb established as different from proteins endogenous to the test system were used to generate a list of tryptic peptides of convenient length. Peptides specific to the variable or conserved regions of SiLu™Lite mAb light and heavy chains (underlined in Figure 1) were selected for method development and LC-MS/MS quantitation using Skyline and MassLynx® software (Table 2). Figure 2 depicts the workflow used to generate the method for quantitation of the surrogate peptides selected.

Figure 2. Method development workflow with BLAST, Skyline and MassLynx®

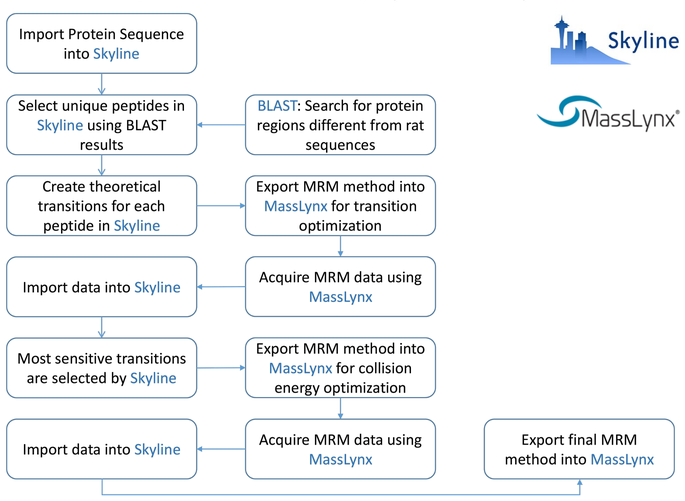


Table 2. Surrogate peptides from SiLu™Lite mAb and SiLu™ mAb (internal standard)

Peptide #	Surrogate peptide sequence	SiLu™Lite mAb (Analyte)			SiLu™ mAb (IS)		
		Elemental composition	MW	MRM transition	IS Elemental composition	IS MW	IS MRM transition
1	RPSGVPDR	$C_{36}H_{62}N_{14}O_{12}$	882.4672	442.24> 709.36	$C_{24}H_{62}N_6O_{12}$ $[^{13}C_{12}][^{15}N_8]$	902.4837	452.26> 719.37
2	LTVLGQPK	$C_{39}H_{70}N_{10}O_{11}$	854.5226	855.53> 542.33	$C_{33}H_{70}N_8O_{11}$ $[^{13}C_6][^{15}N_2]$	862.5368	863.54> 550.34
3	YAASSYLSLTPEQWK	$C_{81}H_{118}N_{18}O_{25}$	1742.8516	872.43> 687.35	$C_{75}H_{118}N_{16}O_{25}$ $[^{13}C_6][^{15}N_2]$	1750.8658	876.44> 695.36
4	GPSVFPLAPSSK	$C_{55}H_{87}N_{13}O_{16}$	1185.6394	593.83> 418.23	$C_{49}H_{87}N_{11}O_{16}$ $[^{13}C_6][^{15}N_2]$	1193.6536	597.83> 426.24
5	ALPAPIEK	$C_{39}H_{67}N_9O_{11}$	837.4960	838.50> 654.38	$C_{33}H_{67}N_7O_{11}$ $[^{13}C_6][^{15}N_2]$	845.5102	846.52> 662.40

Sample and calibration standard preparation for cultured rat hepatocyte incubations

Calibration standards (0.1 – 100 µg/mL; 0.0007-0.7 µM) were prepared by serial dilution in MCM+ culture media. Samples and SiLu™Lite mAb calibration standards were prepared for LC-MS analysis using ProteinWorks™ eXpress Digest Kit (Waters Corporation) with the 3-step protocol. The internal standard concentration was approximately 10 µg/mL. Briefly, 35 µL hepatocyte incubation samples were aliquoted into ProteinWorks tubes containing 135 µL digestion buffer with internal standard for 10 minutes at 80°C, digested with trypsin for 2 hours at 45°C, centrifuged at 920 RPM for 1 min, and incubated for another 15 min with inactivation reagent at 45°C. Where applicable, samples were diluted using appropriate dilution factors into the method range prior to analysis.

Analytical method

All samples were analyzed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with a Waters Xevo TQ-S tandem quadrupole mass spectrometer, in positive MRM mode with electrospray ionization. Tryptic peptides were separated using a Waters Acquity UPLC®Peptide BEH C18, 300 Å (2.1 x 150 mm, 1.7 µm) column and mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A chromatographic gradient was ramped from 10 to 50% B over 6 minutes at 0.3 mL/min. The capillary voltage applied was 3.0 kV, and the cone voltage was 35 V. The table below shows the LC-MS/MS parameters for the 5 surrogate peptides selected.

Peptide #	Surrogate peptide sequence	MRM transition	Charge	Collision energy (eV)	Retention time (min)
1	RPSGVPDR	442.2>709.4	2+	15	5.52
2	LTVLGQPK	855.5>542.3	1+	31	3.51
3	YAASSYLSLTPEQWK	872.4>687.3	2+	15	4.79
4	GPSVFPLAPSSK	593.8>418.2	2+	21	4.46
5	ALPAPIEK	838.5>654.4	1+	30	3.41

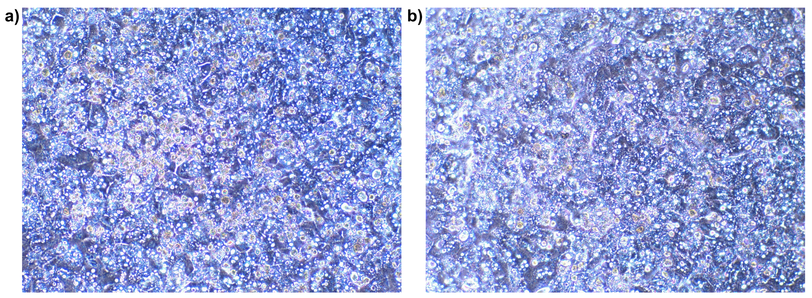
SiLu™Lite mAb was quantified by back calculation of concentrations against duplicate calibration curves using the simplest appropriate weighting and regression algorithm based on analyte/internal standard peak area ratios.

RESULTS

Hepatocyte viability and morphology

During and after the 24 h adaptation period, cell cultures were observed daily by light microscopy and judged to be morphologically normal with confluency adequate for treatment with SiLu™Lite mAb. Within 24 h after the final treatment, hepatocytes were photographed to document their morphological integrity and any overt signs of cytotoxicity of SiLu™Lite mAb. Representative photomicrographs showing cells 24 h post final treatment are shown in Figure 3. Hepatocytes treated with up to 1.4 µM SiLu™Lite mAb exhibited normal hepatocyte morphology.

Figure 3. Hepatocytes at 24 h post final treatment with (a) blank media and (b) treated with 1.4 µM SiLu™Lite



Lactate dehydrogenase release

In cultured rat hepatocytes, when the plasma membrane is damaged, LDH is released into cell culture medium. Treatment of hepatocytes with up to 1.4 µM SiLu™Lite mAb had little or no effect on LDH release in any of the cultures evaluated. No evidence of cytotoxicity was observed.

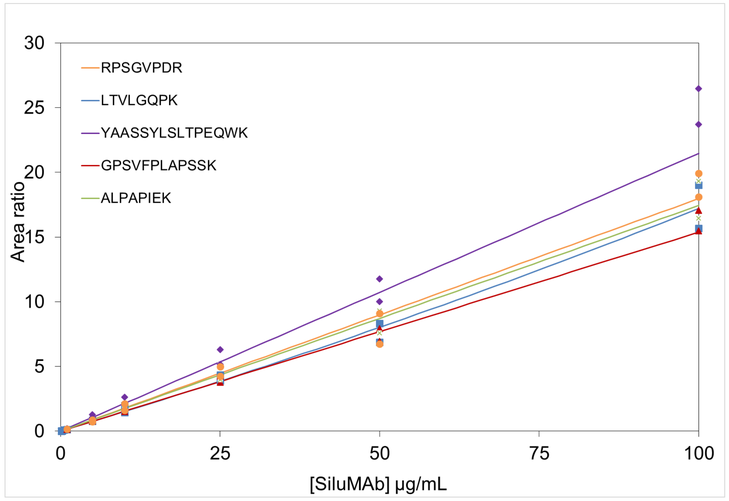
Incubation medium analysis for SiLu™Lite mAb

Table 3 shows the calibration curve parameters for the five surrogate peptides monitored for experiments with cultured rat hepatocytes. The calibration standards for all five peptides had accuracy ranges within ± 25 % of the theoretical concentrations with linear or quadratic 1/x regression ($r^2 \geq 0.99$). The calibration curve plots for the surrogate peptides are shown in Figure 4.

Table 3. Calibration curve (0.1-100 µg/mL; 0.0007-0.7 µM) parameters for surrogate peptides from SiLu™Lite mAb in cultured rat hepatocytes

Peptide #	Surrogate peptide sequence	Regression and weighting algorithm	Regression fit (R^2)	% Accuracy
1	RPSGVPDR	Linear 1/x	0.995	76.4 to 121
2	LTVLGQPK	Quadratic 1/x	0.991	86.4 to 112
3	YAASSYLSLTPEQWK	Linear 1/x	0.991	78.6 to 118
4	GPSVFPLAPSSK	Linear 1/x	0.995	87.5 to 115
5	ALPAPIEK	Linear 1/x	0.990	85.9 to 116

Figure 4. Calibration curve for SiLu™Lite mAb surrogate peptides in MCM+ medium



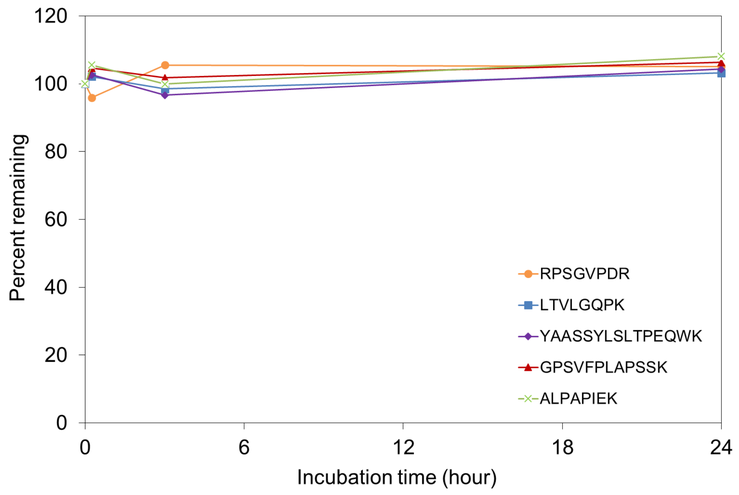
The quantitation data obtained agreed well between the triplicates analyzed for each peptide at designated concentrations and treatment timepoints, showing robustness of the digestion procedure as well as the LC-MS/MS method.

Results from sample analysis of SiLu™Lite mAb in cultured rat hepatocytes over the course of 24 h are shown in Table 4. Stability results for SiLu™Lite mAb (0.07 µM) incubated with cultured rat hepatocytes and monitored by five surrogate peptides are graphically represented in Figure 5. Overall, SiLu™Lite mAb was stable in cultured rat hepatocytes over the incubation time course and no detectable substrate loss was observed.

Table 4. Stability of human SiLu™Lite mAb monitored using surrogate peptides in cultured rat hepatocytes incubated for up to 24 h

SiluTMmAb concentration (µM)	Time (h)	Substrate (surrogate peptide) remaining (%)				
		RPSGVPDR	LTVLGQPK	YAASSYLSLTP EQWK	GPSVFPLAPSSK	ALPAPIEK
0.007	0	100	100	100	100	100
	0.25	108	94.8	107	96.3	97.8
	3	112	99.6	104	100	107
	24	102	107	110	102	110
0.07	0	100	100	100	100	100
	0.25	95.4	106	103	104	104
	3	107	102	96.7	102	99.9
	24	102	105	104	106	107
0.7	0	100	100	100	100	100
	0.25	93.5	99.2	96.4	99.1	103
	3	99.6	101	99.4	102	103
	24	107	102	103	104	109
1.4	0	100	100	100	100	100
	0.25	101	103	98.7	103	103
	3	102	102	103	104	101
	24	111	99.6	102	103	103

Figure 5. Stability of SiLu™Lite mAb (0.07 µM) in cultured rat hepatocytes for up to 24 h



CONCLUSIONS

- Treatment of cultured rat hepatocytes with up to 1.4 µM SiLu™Lite mAb caused no detectable cytotoxicity based on cell morphology or LDH release.
- An LC-MS/MS method for the quantitation of mAb in cultured hepatocytes using five surrogate peptides was successfully developed and applied.
- The calibration curve (0.0007-0.7 µM in MCM+ medium) accuracy for each peptide was within ± 25 % of the theoretical concentrations with linear or quadratic regression ($r^2 \geq 0.99$).
- The quantitation data obtained for all five of the surrogate peptides yielded consistent results, establishing good representation of the intact mAb.
- SiLu™Lite mAb was stable over the relevant incubation time course in suspended cultured hepatocytes.

ACKNOWLEDGEMENTS

We greatly appreciate the collaboration and support from Waters Corporation-Mary Lame, Steven Calciano, Sukhdev Bangar, Scott Toerber

XENOTECH
A BioIVT Company