

Technical Report

LCMS Bioanalysis of Antibody Drugs Using Fab-selective Proteolysis “nSMOL Method” — Selection of Signature Peptide —

Noriko Iwamoto¹, Takashi Shimada¹

Abstract:

nSMOL (nano-surface and molecular-orientation limited proteolysis) is Shimadzu's completely new, proprietary, and innovative technique for selective proteolysis of Fab region of monoclonal antibodies. nSMOL allows analytical method development of antibody drugs independent of a variety of antibody drugs. Fab-derived peptide fragments produced by nSMOL can be precisely quantified by multiple reaction monitoring (MRM) using the Shimadzu LCMS-8050/8060 triple quadrupole liquid chromatograph mass spectrometer (TQ-LCMS). This report describes a selection protocol of signature peptides suitable for pharmacokinetic studies.

Keywords: nano-surface and molecular-orientation limited proteolysis, antibody drug, bioanalysis, LC/MS/MS

1. Introduction

Pharmacokinetic information provides some of the most fundamental indicators. The effective drug discovery is supported by the overall pharmacokinetic profile such as for drug efficacy and toxicity.

The current method used for measuring drug concentration in blood is enzyme-linked immunosorbent assay (ELISA). However, there are critical issues with ELISA, including influences from cross-reaction and inhibitory materials. In contrast, by MS, analysis is performed based on the structural information; thus, the aforementioned issues can potentially be resolved.

The LCMS analysis of high-molecular-weight proteins, such as antibodies, is normally performed after fragmentation of the protein into smaller peptides using a protease, such as trypsin or lysyl endopeptidase. However, this process also generates a large number of peptides including the signature peptides. These peptides increase the background noise and ionization suppression, and become a major cause of instability in the LCMS system. nSMOL can decrease these issues by selective proteolysis on the analytical target region of the antibody Fab. Therefore, the use of this approach can improve the reproducibility and robustness of the analytical system with maintaining antibody specificity.

A peptide for quantitation (signature peptide) is selected from the tryptic peptides containing a complementarity-determining region (CDR), which defines the specificity of the antibody. However, it is not possible that the CDR-containing peptide does not have the same amino acid sequence as that in the endogenous IgGs. At this point, it must be confirmed that there is no competition with the signature peptide in the biological matrix.

2. Structure and Specificity of Antibodies

Antibodies are large biological molecules with a heterotetrameric protein containing two heavy and light chains. Antibody structures are extraordinarily well conserved, and antibodies have a flexible hinge region between the rigid Fc and the variable Fab. A variable region, Fv, is positioned at the top of the Fab. The antibody specificity in this region is defined as amino acid substitution by somatic mutation. Portions of the Fv with a particularly high frequency mutation are called CDRs, which play a major role in antigen binding (see Fig. 1).

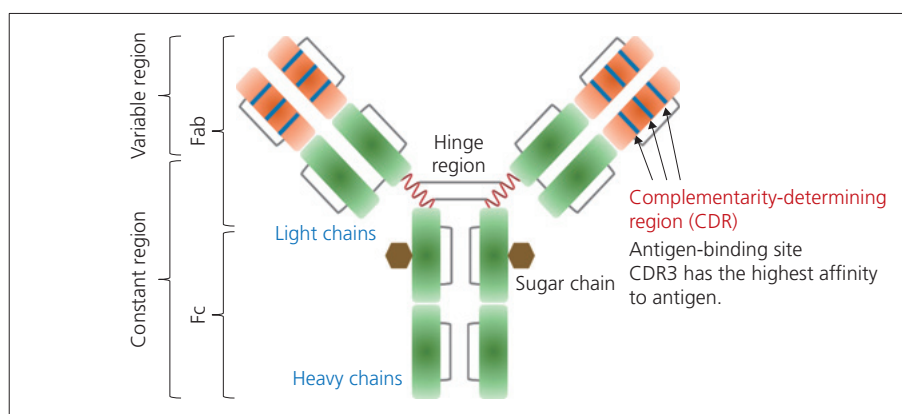


Fig. 1 Basic Antibody Structure

3. Frequency of Amino Acid Substitution in the Fv

The Fv contains three regions of high-frequency amino acid substitution, namely CDR1, CDR2, and CDR3 (see Fig. 2). Almost all amino acid residues in these regions can undergo substitution; therefore, an extremely large number of possible sequences can be generated. Consequently, predicting amino acid sequences is difficult. Therefore, nSMOL bioanalysis requires the setting and selection of signature peptides based on actual analysis data.

4. Amino Acid Sequence Alignment Using ClustalW

An example is shown the prediction of candidate signature peptides in four chimeric antibodies (Rituximab, Brentuximab vedotin, Cetuximab, and Infliximab) by ClustalW alignment (Fig. 3, black: common residues, gray: similar residues, white: unique residues).

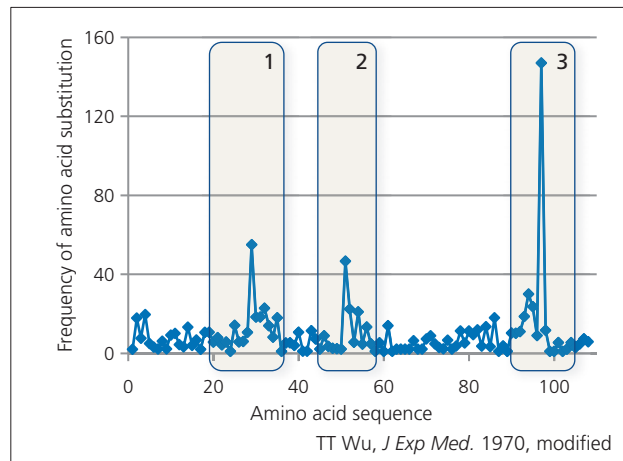


Fig. 2 Frequency of Amino Acid Substitution in Fv

Amino acid sequence alignment of heavy chains																																																														
RTX H	1	QVQLTQSPG	GAELV	PKGASV	KMSCK	ASGYTF	TSY	NMH	WV	QTP	PGR	GLEW	IG	ATYP	PGN	--	GD	58																																												
BRX H	1	QIQIQQS	GPPEV	VKFGAS	VKISCK	ASGYTF	FTD	YI	TWV	KQK	PGQ	GLEW	IGW	ATYP	PGS	--	GN	58																																												
CTX H	1	QVQLTQ	QS	GPGLV	QPSQ	LSITCT	VSGF	SL	TNY	GVH	WV	QSP	PK	GLEW	LGV	ATWS	---	GN	57																																											
IFX H	1	EVKLE	EESGG	GLVQ	PGGSM	KLSCV	ASGFI	F	SNH	WMN	WV	RQSP	PK	GLEW	V	AER	RSK	SIN	SA	60																																										
RTX H	59	S	YNOK	F	KGKAT	L	TADK	SS	STAYM	QL	SSL	T	SE	D	SAV	Y	C	A	R	S	T	Y	Y	G	G	D	W	F	N	W	G	A	G	T	V	T	118																									
BRX H	59	K	YNEK	F	KGKAT	L	TVD	T	SS	TAF	M	QL	SSL	T	SE	D	TAV	Y	C	A	N	---	Y	G	-	N	Y	W	F	A	Y	W	G	Q	T	Q	V	T	114																							
CTX H	58	D	YNT	P	F	T	S	R	L	S	I	N	K	D	N	S	K	S	O	V	F	F	M	N	S	L	Q	S	N	D	T	A	I	Y	C	A	R	A	L	T	Y	Y	-	D	Y	E	F	A	Y	W	G	Q	T	L	V	T	116					
IFX H	61	H	Y	A	B	S	V	K	G	R	F	T	I	S	R	D	S	K	S	A	V	Y	L	Q	M	T	D	L	R	T	E	D	T	G	V	Y	C	S	R	N	-	Y	G	-	-	S	T	Y	D	Y	W	G	Q	T	L	T	117					
RTX H	119	V	S	A	A	S	T	K	G	P	S	V	F	P	L	A	P	S	S	K	S	T	S	G	G	T	A	A	L	G	C	L	V	K	D	Y	F	P	E	P	V	T	V	S	W	N	S	G	A	L	T	S	G	V	H	T	F	P	A	V	L	178
BRX H	115	V	S	A	A	S	T	K	G	P	S	V	F	P	L	A	P	S	S	K	S	T	S	G	G	T	A	A	L	G	C	L	V	K	D	Y	F	P	E	P	V	T	V	S	W	N	S	G	A	L	T	S	G	V	H	T	F	P	A	V	L	174
CTX H	117	V	S	A	A	S	T	K	G	P	S	V	F	P	L	A	P	S	S	K	S	T	S	G	G	T	A	A	L	G	C	L	V	K	D	Y	F	P	E	P	V	T	V	S	W	N	S	G	A	L	T	S	G	V	H	T	F	P	A	V	L	176
IFX H	118	V	S	S	A	S	T	K	G	P	S	V	F	P	L	A	P	S	S	K	S	T	S	G	G	T	A	A	L	G	C	L	V	K	D	Y	F	P	E	P	V	T	V	S	W	N	S	G	A	L	T	S	G	V	H	T	F	P	A	V	L	177
RTX H	179	Q	S	S	G	L	Y	S	L	S	S	V	V	T	V	P	S	S	L	G	T	Q	T	Y	I	C	N	V	N	H	K	P	S	N	T	K	V	D	K	K	V	E	P	K	S	---	C	D	K	T	H	T	C	P	P	C	P	A	235			
BRX H	175	Q	S	S	G	L	Y	S	L	S	S	V	V	T	V	P	S	S	L	G	T	Q	T	Y	I	C	N	V	N	H	K	P	S	N	T	K	V	D	K	K	V	E	P	K	S	---	C	D	K	T	H	T	C	P	P	C	P	A	231			
CTX H	177	Q	S	S	G	L	Y	S	L	S	S	V	V	T	V	P	S	S	L	G	T	Q	T	Y	I	C	N	V	N	H	K	P	S	N	T	K	V	D	K	R	V	E	P	K	S	P	K	S	C	D	K	T	H	T	C	P	C	P	A	236		
IFX H	178	Q	S	S	G	L	Y	S	L	S	S	V	V	T	V	P	S	S	L	G	T	Q	T	Y	I	C	N	V	N	H	K	P	S	N	T	K	V	D	K	K	V	E	P	K	S	---	C	D	K	T	H	T	C	P	C	P	A	234				
RTX H	236	P	E	L	L	G	G	S	V	F	L	F	P	P	K	P	K	D	T	L	M	I	S	R	T	P	E	V	T	C	V	V	D	V	S	H	E	D	P	E	V	K	F	N	W	Y	V	D	G	V	E	V	H	N	A	K	T	K	P	295		
BRX H	232	P	E	L	L	G	G	S	V	F	L	F	P	P	K	P	K	D	T	L	M	I	S	R	T	P	E	V	T	C	V	V	D	V	S	H	E	D	P	E	V	K	F	N	W	Y	V	D	G	V	E	V	H	N	A	K	T	K	P	291		
CTX H	237	P	E	L	L	G	G	S	V	F	L	F	P	P	K	P	K	D	T	L	M	I	S	R	T	P	E	V	T	C	V	V	D	V	S	H	E	D	P	E	V	K	F	N	W	Y	V	D	G	V	E	V	H	N	A	K	T	K	P	296		
IFX H	235	P	E	L	L	G	G	S	V	F	L	F	P	P	K	P	K	D	T	L	M	I	S	R	T	P	E	V	T	C	V	V	D	V	S	H	E	D	P	E	V	K	F	N	W	Y	V	D	G	V	E	V	H	N	A	K	T	K	P	294		
RTX H	296	R	E	E	Q	N	S	T	Y	R	V	S	V	L	T	V	L	H	Q	D	W	L	N	G	K	E	Y	K	C	K	V	S	N	K	A	L	P	A	P	I	E	K	T	I	S	K	A	K	G	O	P	R	E	P	O	V	Y	T	L	355		
BRX H	292	R	E	E	Q	N	S	T	Y	R	V	S	V	L	T	V	L	H	Q	D	W	L	N	G	K	E	Y	K	C	K	V	S	N	K	A	L	P	A	P	I	E	K	T	I	S	K	A	K	G	O	P	R	E	P	O	V	Y	T	L	351		
CTX H	297	R	E	E	Q	N	S	T	Y	R	V	S	V	L	T	V	L	H	Q	D	W	L	N	G	K	E	Y	K	C	K	V	S	N	K	A	L	P	A	P	I	E	K	T	I	S	K	A	K	G	O	P	R	E	P	O	V	Y	T	L	356		
IFX H	295	R	E	E	Q	N	S	T	Y	R	V	S	V	L	T	V	L	H	Q	D	W	L	N	G	K	E	Y	K	C	K	V	S	N	K	A	L	P	A	P	I	E	K	T	I	S	K	A	K	G	O	P	R	E	P	O	V	Y	T	L	354		
RTX H	356	P	P	S	R	D	E	L	T	K	N	Q	V	S	L	T	C	L	V	K	G	F	Y	P	S	D	I	A	V	E	W	E	S	N	G	O	P	E	N	N	Y	K	T	P	P	V	L	D	S	D	G	S	F	F	L	Y	S	K	L	T	415	
BRX H	352	P	P	S	R	D	E	L	T	K	N	Q	V	S	L	T	C	L	V	K	G	F	Y	P	S	D	I	A	V	E	W	E	S	N	G	O	P	E	N	N	Y	K	T	P	P	V	L	D	S	D	G	S	F	F	L	Y	S	K	L	T	411	
CTX H	357	P	P	S	R	D	E	L	T	K	N	Q	V	S	L	T	C	L	V	K	G	F	Y	P	S	D	I	A	V	E	W	E	S	N	G	O	P	E	N	N	Y	K	T	P	P	V	L	D	S	D	G	S	F	F	L	Y	S	K	L	T	416	
IFX H	355	P	P	S	R	D	E	L	T	K	N	Q	V	S	L	T	C	L	V	K	G	F	Y	P	S	D	I	A	V	E	W	E	S	N	G	O	P	E	N	N	Y	K	T	P	P	V	L	D	S	D	G	S	F	F	L	Y	S	K	L	T	414	
RTX H	416	V	D	K	S	R	W	Q	Q	N	V	F	S	C	S	V	M	H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	L	S	P	G	K	451																									
BRX H	412	V	D	K	S	R	W	Q	Q	N	V	F	S	C	S	V	M	H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	L	S	P	G	K	447																									
CTX H	417	V	D	K	S	R	W	Q	Q	N	V	F	S	C	S	V	M	H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	L	S	P	G	K	452																									
IFX H	415	V	D	K	S	R	W	Q	Q	N	V	F	S	C	S	V	M	H	E	A	L	H	N	H	Y	T	Q	K	S	L	S	L	S	P	G	K	450																									
Amino acid sequence alignment of light chains																																																														
RTX L	1	Q	I	V	L	S	O	S	P	A	I	L	S	A	S	P	G	E	K	V	T	M	T	C	R	A	S	S	S	V	---	S	I	H	W	F	O	O	K	P	G	S	S	P	K	P	W	I	Y	A	T	S	N	L	A	S	55					
BRX L	1	D	I	V	L	S	O	S	P	A	I	L	S	A	S	P	G	E	K	V	T	M	T	C	R	A	S	S	S	V	---	S	I	H	W	F	O	O	K	P	G	S	S	P	K	P	W	I	Y	A	T	S	N	L	A	S	60					
CTX L	1	D	I	L	L	S	O	S	P	A	I	L	S	V	S	P	G	E	R	V	S	F	S	C	R	A	S	O	S	I	G	---	T	I	H	W	Y	O	O	R	T	N	G	S	P	R	L	L	I	K	Y	A	S	E	S	I						

5. Discussion of Alignment Analysis

From the sequence alignment analysis, it can be assumed that areas with a predominance of unique residues (white) are CDRs. The results also demonstrated the presence of unique residues throughout the Fv. The prediction of signature peptides is simplified by amino acid sequences of several representative antibodies according to antibody classification (chimeric, humanized, or fully human antibody).

During quantitative analysis, identified signature peptides are highlighted in ■ for Rituximab, ■ for Brentuximab vedotin, ■ for Cetuximab, and ■ for Infliximab. Some of the signature peptides include N-terminal sequences that may become heterogeneity during post-translational modification or may interfere with another biological matrix during preclinical studies. Therefore, N-terminal sequences are not used during an actual nSMOL bioanalysis.

6. Conclusions

A reliable selection of signature peptides is essential for clinical pharmacokinetic studies.

Fig. 4 shows a procedure from a structural analysis technique by mass spectrometry and a recent information-based approach. Identification of the peptide structure and its actual valence by high-resolution MS is the biggest advantage in the viewpoint of scientific evidence. The information-based approach configures MRM conditions based on sequence information. These two techniques should be used at different times depending on the circumstances.

Reference

- Iwamoto N et al. *Analyst*, DOI:10.1039/c3an02104a
 Iwamoto N et al. *Bioanalysis*, DOI:10.4155/bio-2016-0018
 Iwamoto N et al. *Biol. Pharm. Bull.*, DOI:10.1248/bpb.b16-00230
 Iwamoto N et al. *Clin. Pharm. Biopharm.*, DOI:10.4172/2167-065X.1000164

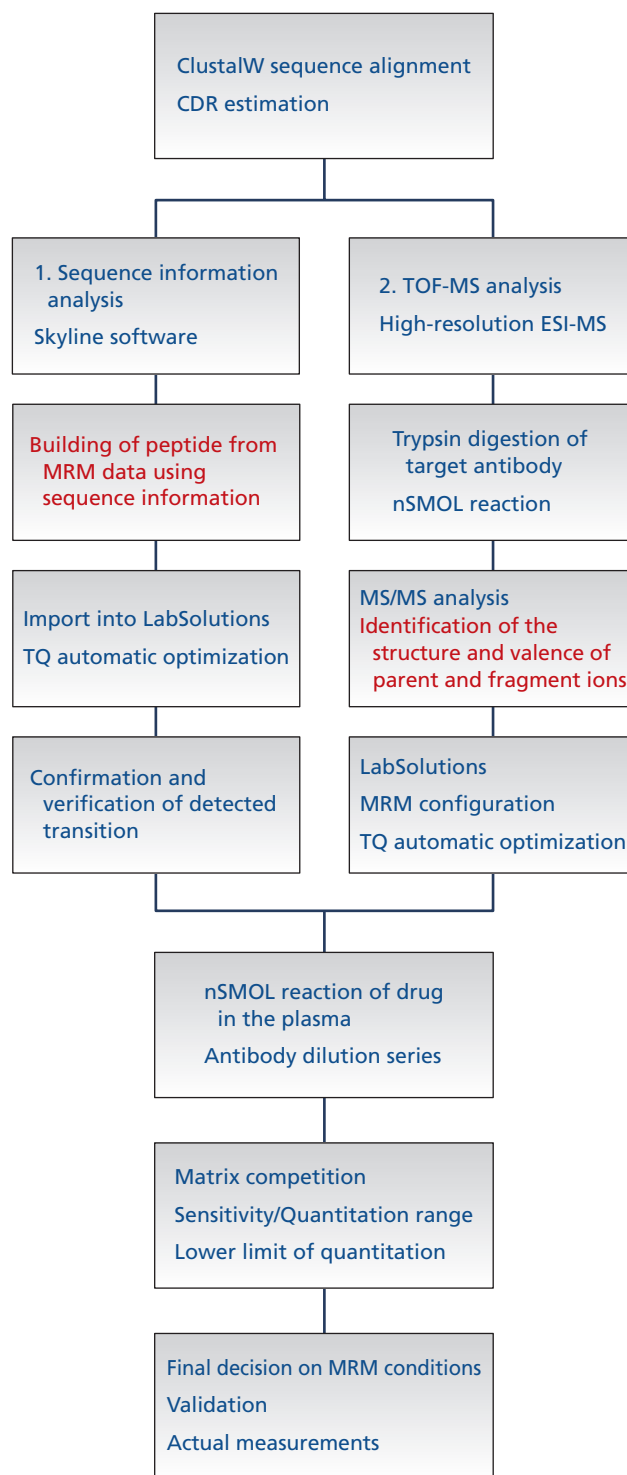


Fig. 4 Signature Peptide Selection Procedure

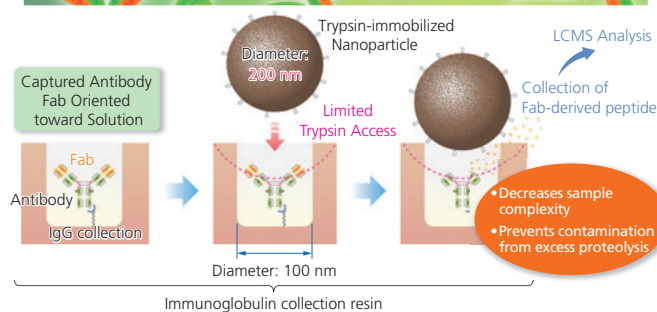
nSMOL Antibody BA Kit

LC/MS/MS Sample Prep Kit
for Quantitative Analysis of Monoclonal Antibodies

nSMOL Technology Increasing Confidence in Therapeutic Monoclonal Antibody Bioanalysis

nSMOL (nano-surface and molecular orientation limited proteolysis) is Shimadzu's proprietary, innovative technique that enables selective proteolysis of the Fab region of monoclonal antibodies. The nSMOL Antibody BA Kit is a ready-to-use reagent kit for collecting monoclonal antibodies from blood or other biological samples using immunoglobulin collection resin, and then performing selective proteolysis of the Fab region of these antibodies via trypsin-immobilized nanoparticles. Variable region-derived peptides produced by limited proteolysis can then be quantified via MRM measurements utilizing a high-performance LCMS-8050/8060 triple quadrupole liquid chromatograph mass spectrometer.

An unparalleled convenient and rapid workflow provided by the nSMOL Antibody BA Kit dramatically improves the productivity and robustness of LCMS mAb bioanalysis.



Kit Contents

The nSMOL Antibody BA Kit provides prepared reagents and protocols for sample prep via the nSMOL method.

Reagent	Quantity	Capacity	Storage Temperature
Immunoglobulin collection resin	2	1.3 mL/each	4 °C
Wash solution 1 (Binding solution)	1	80 mL	4 °C
Wash solution 2	1	80 mL	4 °C
Reaction solution	1	10 mL	4 °C
Enhanced reaction solution	1	Freeze-dried	4 °C
Reaction stop solution	1	1 mL	4 °C
FG beads Trypsin DART	1	1.1 mL	-20 °C *1

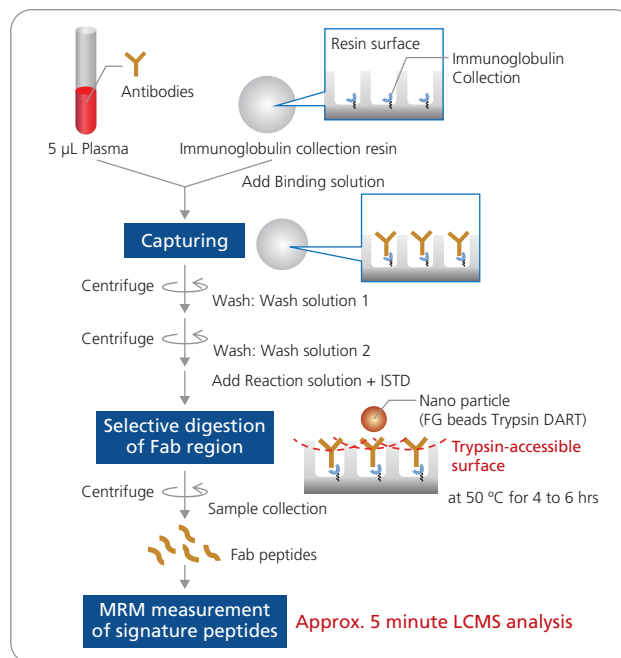
*1: If it is being stored for a month or longer, store it at -80 °C.



Note: The reagent kit is transported at refrigeration temperatures (2 to 8 °C).

Simple Workflow

This kit enables highly reproducible data and avoids the troublesome steps of denaturing, reduction, and alkylation normally associated with protein digestion. There is also no need for solid phase extraction after reaction. After nSMOL preparation, samples can be injected directly onto the LCMS.



First Edition: March, 2017



For Research Use Only. Not for use in diagnostic procedures.

This publication may contain references to products that are not available in your country. Please contact us to check the availability of these products in your country.

The content of this publication shall not be reproduced, altered or sold for any commercial purpose without the written approval of Shimadzu. Company names, products/service names and logos used in this publication are trademarks and trade names of Shimadzu Corporation, its subsidiaries or its affiliates, whether or not they are used with trademark symbol "TM" or "®".

Third-party trademarks and trade names may be used in this publication to refer to either the entities or their products/services, whether or not they are used with trademark symbol "TM" or "®".

Shimadzu disclaims any proprietary interest in trademarks and trade names other than its own.

The information contained herein is provided to you "as is" without warranty of any kind including without limitation warranties as to its accuracy or completeness. Shimadzu does not assume any responsibility or liability for any damage, whether direct or indirect, relating to the use of this publication. This publication is based upon the information available to Shimadzu on or before the date of publication, and subject to change without notice.

Shimadzu Corporation

www.shimadzu.com/an/