A Simple LC-HRMS-Based Disulfide Mapping Assay Applied to Antibody-Drug Conjugates

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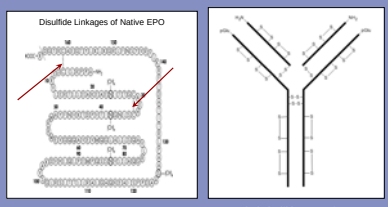
OVERVIEW

Purpose: To develop a simple three-step reaction LC-HRMS-based disulfide site mapping method for common biopharmaceuticals.
Methods: Controlled reduction, cyanylation, and cleavage of modified cysteine residues involved in disulfide linkages monitored by UPLC/LTQ-Orbitrap MS. Technique dependent on molecule class.
Results: The method was able to produce and identify all distinguishable modified peptides of the native structure of Recombinant Human Insulin (rH Insulin). The method was also able to detect unique peptides representative of two possible scrambled products of rH Insulin. Application for Conjugates !

INTRODUCTION

Disulfide bond formation in antibody-drug conjugates (ADC) is an important linkage and post-translational modification essential for the stabilization, activity and structure. In ADC synthesis, disulfide scrambling can be clinical concern since a mis-folded protein or one with abnormal linkages may not have the correct structure or drug-antibody stoichiometry to function properly.

- As mAb models for the study, three proteins with different disulfide mapping complexity defined by the number of disulfide linkages, cysteine residues proximity, and protein size were evaluated: Ribonuclease A and erythropoetin (easy), insulin (intermediate). In this example, Insulin serves as an IgG.
- RNase A was fully characterized by JT Watson and colleagues. Results were published in various journals.
- Erythropoietin has four Cysteine residues all involved in two disulfide linkages. EPO is easy to characterize using in-solution tryptic digestion in its non-reduced form.
- IgG1 mAb (shown below) typically have two hinge disulfide bonds linking the two heavy chains.



Ng T et al. Postgrad Med J 2003;79:367-376

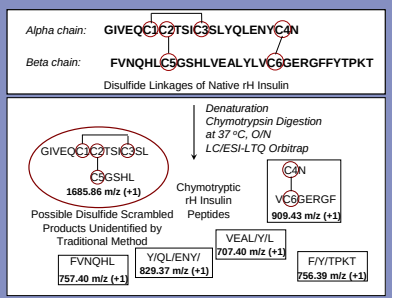
Typical Disulfide Pattern for IgG1 Monoclonal Antibody

- Human insulin, a common biopharmaceutical, has 6 cysteine residues all involved in three disulfide linkages (one intra-chain, and two inter-chain linkages). Three adjacent cysteine residues are present in the alpha-chain. Insulin is more challenging to characterize since no endoproteinase can digest it to produce peptides with one disulfide linkage.

Alpha chain: GIVEQC1C2TSIC3SLYQLENYC4N

Beta chain: FVNQHLC5GSHLVEALYLVC6GERGFFYTPKT

Disulfide Linkages of Native rH Insulin



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METHODOLOGY and RESULTS

Methodology

Recombinant Human Insulin (Sigma), expressed in yeast -- Denatured using GdnHCl in Citrate Buffer, pH 3.0. Minimal to no disulfide scrambling is expected at this acidic pH.

Thermo ESI-LTQ-Oribitrap XL with Accela 1250 UPLC System

Waters Acquity UPLC BEH130 C18 1.7 um 2.1 x 150 mm Column

Reduction at pH 3.0 produces a mixture of isomers of partially reduced insulin protein.

Controlled Reduction at pH 3.0 (TCEP, 1-15 molar equivalent of total Cys; RT or 37 ºC; 10-15 mins)

The addition of 1:5 TCEP:CDAP ratio modifies the free sulfhydryls .

Isomer A | Isomer B | Isomer C

Cyanylation at pH 3.0 (CDAP, 20-75 molar equivalent of total Cys; RT or 37ºC; 15 mins)

Partially Reduced and Cyanylated Products (Table 1) Measurements using LC-HR MS

Cleavage (Methylamine, 2M, 10 mins, 0-2 ºC) Add. TCEP Reduction

All modified proteins are cleaved at the N-terminal side of the cyanylated Cysteine residues.

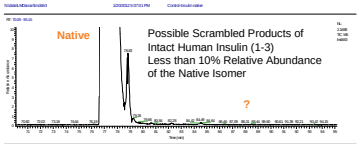
Fragments (Table 2) Measurements using LC-HR MS

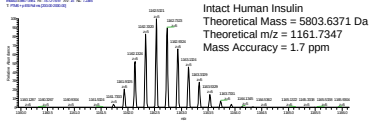
Parameter	Specification
LC Mobile Phase	A – 0.1% FA in Water B – 0.1% FA in ACN
Temperature	40°C (column), 5°C (auto sampler)
Flow Rate	0.2 mL/minute
Injection Vol.	10 µL
Divert Valve	From 0 to 5 minutes, divert to waste
Wavelength	214 nm
LC Gradient	Gradient 1 - Control 0-5 mins, 24 %B; 5-45 mins, 24 to 40 %B Gradient 2 – Cleavage Products 0-5 mins, 0 %B; 5-105 mins, 0 to 40 %B

Parameter	Specification
Ion Polarity	Positive
Duration	50 min; 110 min
Lock Mass List	391.2843 413.2662
Scan event 1	FTMS, resolution 60000 Mass Range: 150-2000 Da

All mass measurements and assignments were performed within 5 ppm mass accuracy when compared to its respective theoretical mass in Tables 1 and 2. All TIC abundances of reduction and cyanylation products were confirmed by UV absorbance at 214 nm.
$CDAP_1$ = 1-cyano-4-dimethylamino-pyridinium tetrafluorate.

Recombinant Human Insulin





Scrambled Product 1:
GIVEQC1C2TSIC3SLYQLENYC4N
FVNQHLC5GSHLVEALYLVC6GERGFFYTPKT
itz-C1C2TSIC3SLYQLENYC4N-OH
m/z = 941.37 (+2)

Scrambled Product 2:
GIVEQC1C2TSIC3SLYQLENYC4N
FVNQHLC5GSHLVEALYLVC6GERGFFYTPKT
GIVEQC1C2TSI-NHCH$_3$
m/z = 533.26 (+2)

Two possible disulfide scrambled products based on proximity of linkages with representative peptides not found in the native secondary structure

Controlled Reduction and Cyanylation Products

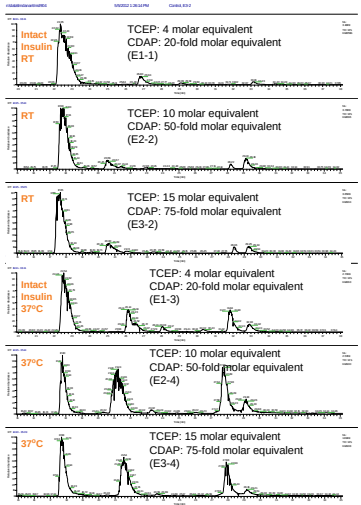
Objective: Formation of a Mixture of Insulin Protein Isomers with 1 and 2 Disulfide Linkages Reduced and Cyanylated

Experimental Design:
- **Total Number of Cysteines in Protein**
- **$[TCEP]_{min}$ = 4 molar equivalent for every Cys**
- **$[CDAP]_{min}$ = 20 molar equivalent for every Cys; [CDAP] should be minimum 5x of [TCEP]**
- **Both reactions performed at pH 3.**

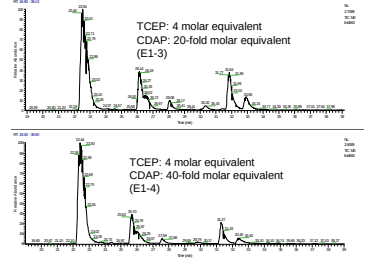
Table 1. Mass-to-charge Values of Insulin Partial Reduction and Cyanylation Products

		Mass-to-Charge Values, m/z						
	M, Da	[M+H]+	[M+2H]2+	[M+3H]3+	[M+4H]4+	[M+5H]5+	[M+6H]6+	[M+7H]7+
Intact Non-Reduced rH Insulin	5803.6371	5804.6444	2902.8259	1935.5530	1451.9166	1161.7347	968.2802	830.0983
Controlled Reduction Products								
Two Cys – Intact rH Insulin	5805.6517	5806.6590	2903.8332	1936.2245	1452.4202	1162.1376	968.6159	830.3861
Four Cys – intact rH Insulin	5807.6663	5808.6736	2904.8405	1936.8961	1452.9239	1162.5406	968.9517	830.6739
Two Cys - A-Chain (1DB intrachain)	2379.9842	2380.9915	1190.9994	794.3354	596.0034	477.0041	397.6713	341.0050
Two Cys - B-Chain (fully reduced)	3427.6845	3428.6918	1714.8496	1143.5688	857.9284	686.5442	572.2881	490.6765
Four Cys - A-Chain (fully reduced)	2382.0000	2383.0073	1192.0073	795.0073	596.5073	477.4073	398.0073	341.2930
Cyanylation Products								
Two Cys – intact rH Insulin	5855.6719	5856.6792	2928.8433	1952.8979	1464.9253	1172.1417	976.9526	837.5319
Four Cys – intact rH Insulin	5907.7067	5908.7140	2954.8607	1970.2426	1477.9545	1182.5486	985.6351	844.9654
Two Cys - A-Chain (1DB intrachain)	2430.0044	2431.0117	1216.0095	811.0088	608.5084	487.0082	406.0080	348.1508
Two Cys - B-Chain (fully reduced)	3477.7047	3478.7120	1739.8597	1160.2422	870.4305	696.5482	580.6248	497.8223
Four Cys - A-Chain (fully reduced)	2482.0404	2483.0477	1242.0275	828.3541	621.5174	497.4154	414.6807	355.5645

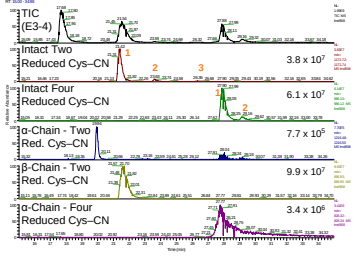
Incubation at 37ºC Temperature Produced More Abundant Reduction and Cyanylation Products



TCEP-to-CDAP Ratio was Optimal at 1:5 Minimum



Identification of Reduction and Cyanylation Products

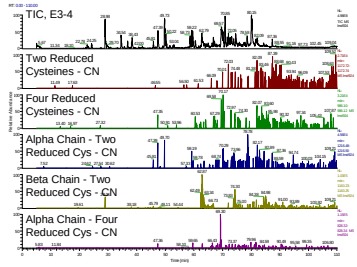


Chemical Cleavage Products

Objective: Complete N-terminal Cleavage of Cyanylated Cysteines with Minimal Side-Rxn Products

Experimental Conditions Tested:
- **Cleavage using 1 M NH_4OH, RT, 1 hour**
  - **Various forms of carbamylated insulin were the dominant product of the reaction.**
- **Cleavage using 2 M Methyl Amine, 0-2 ºC, 10 mins.**

2 M CH_3NH_2 Showed Efficient Conversion of Cyanylation Products to Truncated Peptides



All Cleavage Products in Table 2 Identified and Confirmed with High Confidence

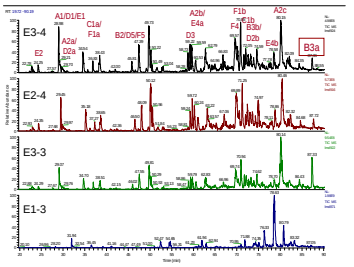


Table 2. Mass-to-charge Values of all Cleavage Products from all Partially Reduced and Cyanylated 1DB and 2DB Insulin Isomers

ID	Sequence	M, Da	Mass-to-Charge Values, m/z [M+H]+	[M+2H]2+	[M+3H]3+	[M+4H]4+	[M+5H]5+
One Disulfide Linkage Reduced							
Isomer A							
A1	D1, E1	GIVEQ-NHCH$_3$	557.3173	558.3246	279.6660	186.7797	140.3366	112.4708
A2		searched	NF	NF	NF	NF	NF	NF
A2(a)	C2(a)	itz-C2TSI-NHCH$_3$	563.2196	564.2269	282.6171	188.7472	141.8122	113.6512
A2(b)	E4(a)	itz-C5SLYQLENYC4N-OH	1373.5380	1374.5453	687.7763	458.8533	344.3918	275.7149
A2(c)	UNIQ	Fully reduced Beta chain	3427.6845	3428.6918	1714.8496	1143.5688	857.9284	686.5442
Isomer B								
B1	D4, F2	itz-C4N-OH	261.0657	NF	NF	NF	NF	NF
B2	D5, F5	C6GERGFFYTPKT-OH	1429.6448	1430.6521	715.8297	477.5556	358.4185	286.9363
B3		searched	4226.0087	NF	NF	NF	NF	NF
B3(a)	UNIQ	GIVEQC1C2TSIC3SLYQLENYC4N-NHCH$_3$	2177.9795	2178.9868	1089.9971	727.0005	545.5022	436.6032
B3(b)	D2(b)	FVNQHLC6GSHLVEALYLVC6GERGFFYTPKT-NHCH$_3$	3054.0771	2055.0844	1028.0459	685.6997	514.5266	411.8227
Isomer C								
C1		searched	NF	NF	NF	NF	NF	NF
C1(a)	F1(a)	GIVEQC1-NHCH$_3$	660.3265	661.3338	331.1706	221.1161	166.0869	133.0726
C1(b)	UNIQ	itz-C2TSIC3SLYQLENYC4N-OH	1777.7110	1778.7183	889.8628	593.5776	445.4351	356.5495
C1(c)	E4(b)	C5GSHLVEALYLVC6GERGFFYTPKT-OH	2714.2985	2715.3058	1358.1566	905.7735	679.5819	543.8670
C2	E5, F3	H-FVNQHL-NHCH$_3$	769.4235	770.4308	385.7191	257.4818	193.3632	154.8920
Two Disulfide Linkages Reduced								
Isomer D								
D1	A1,E1	GIVEQ-NHCH$_3$	557.3173	558.3246	279.6660	186.7797	140.3366	112.4708
D2		searched	2615.2811	NF	NF	NF	NF	NF
D2(a)	A2(a)	itz-C2TSI-NHCH$_3$	563.2196	564.2269	282.6171	188.7472	141.8122	113.6512
D2(b)	B3(b)	FVNQHL-C6GSHLVEALYLVC6GERGFFYTPKT-NHCH$_3$	2054.0771	2055.0844	1028.0459	685.6997	514.5266	411.8227
D3	UNIQ	itz-C5SLYQLENYC4N-NHCH$_3$	1169.5175	1170.5248	585.7661	390.8465	293.3867	234.9108
D4	B1, F2	itz-C4N-OH	NF	NF	NF	NF	NF	NF
D5	B2, F5	itz-C6GERGFFYTPKT-OH	1429.6448	1430.6521	715.8297	477.5556	358.4185	286.9363
Isomer E								
E1	A1, D1	GIVEQ-NHCH$_3$	557.3173	558.3246	279.6660	186.7797	140.3366	112.4708
E2		itz-C-NHCH$_3$	2615.2811	NF	NF	NF	NF	NF
E3	UNIQ	itz-C2TSI-NHCH$_3$	460.2154	461.2177	231.1125	154.4108	116.0599	93.0494
E4		searched	4085.8209	NF	NF	NF	NF	NF
E4(a)	A2(b)	itz-C5SLYQLENYC4N-OH	1373.5380	1374.5453	687.7763	458.8533	344.3918	275.7149
E4(b)	C1(c)	C5GSHLVEALYLVC6GERGFFYTPKT-OH	2714.2985	2715.3058	1358.1566	905.7735	679.5819	543.8670
E5	C2, F3	H-FVNQHL-NHCH$_3$	769.4235	770.4308	385.7191	257.4818	193.3632	154.8920
Isomer F								
F1		searched	NF	NF	NF	NF	NF	NF
F1(a)	C1(a)	GIVEQC1-NHCH$_3$	660.3265	661.3338	331.1706	221.1161	166.0869	133.0726
F1(b)	UNIQ	itz-C5TSIC3SLYQLENYC4N-NHCH$_3$	1573.6905	1574.6978	787.8526	525.5706	394.4299	315.7454
F2	B1, D4	itz-C4N-OH	261.0657	NF	NF	NF	NF	NF
F3	C2, E5	H-FVNQHL-NHCH$_3$	769.4235	770.4308	385.7191	257.4818	193.3632	154.8920
F4	UNIQ	itz-C5GSHLVEALYLVC6GERGFFYTPKT-OH	1340.6911	1341.6984	671.3529	447.9043	336.1801	269.1455
F5	B2, D5	itz-C6GERGFFYTPKT-OH	1429.6448	1430.6521	715.8297	477.5556	358.4185	286.9363

All six partially reduced isomers have overlapping cleavage products

CONCLUSIONS

- **A simple method has been demonstrated using Insulin to correctly assign disulfide linkages in a protein containing closely spaced or adjacent cysteine residues.**
- **The method is rapid, sensitive, and does not require fraction collection, but instead relies on the accurate mass measurement capabilities of the LTQ-Orbitrap MS.**
- **This method can be applied to monoclonal antibody based therapeutics with consecutive cysteines involved in disulfide linkages (e.g. hinge region of mAbs). An alternate technique - after digestion with trypsin or any protease, the identified non-reduced peptides containing the target regions for characterization can be fractionated for enrichment purposes. Apart from performing a systematic optimization of controlled reduction and complete cyanylation steps, the rest of the method can be applied accordingly.**