

# Development of a Simple LC-HRMS-based Disulfide Mapping Assay Applied to Biopharmaceuticals

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## OVERVIEW

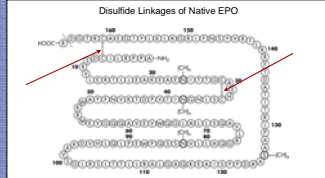
**Purpose:** To develop a simple 3-step reaction LC-HRMS-based disulfide mapping method for common biopharmaceuticals.

**Methods:** Controlled reduction, cyanylation, and cleavage of cyanylated cysteines involved in disulfide linkages monitored by UPLC/ESI-LTQ-Orbitrap MS.

**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.

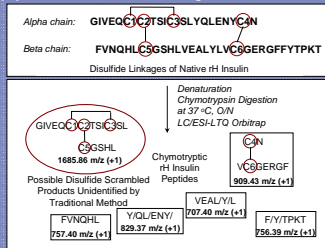
## INTRODUCTION

- Disulfide bond formation in proteins is an important PTM essential for the stabilization of its 3D structure.
- Disulfide scrambling in biopharmaceuticals is a clinical concern since a misfolded protein or one with abnormal linkages may not have the correct structure to function properly.
- A simple assay is presented with no HPLC fractionation involved that can deduce the linkages of closely-spaced cysteine residues.
- Three proteins with different disulfide mapping complexity defined by the number of disulfide linkages, cysteine residues proximity, and protein size were evaluated as test proteins: Ribonuclease A and erythropoietin (easy), insulin (intermediate).
- 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.



Ng J et al. *Postgrad Med J* 2000;79:261-270

- 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 endoproteolase can digest it to produce peptides with one disulfide linkage.

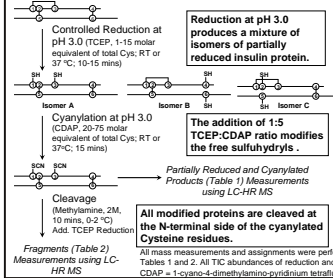


## METHODOLOGY and RESULTS

### Methodology

**Recombinant Human Insulin (Sigma), expressed in yeast--**

Denatured using GuHCl in Citrate Buffer, pH 3.0. Minimal to no disulfide scrambling is expected at this acidic pH.



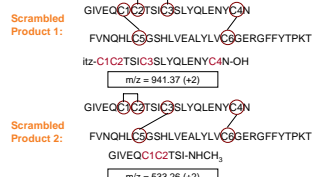
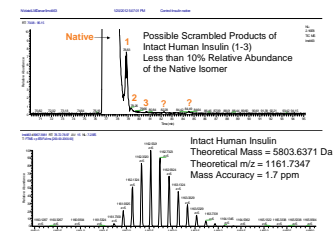
**Thermo ESI-LTQ-Orbitrap XL with Accela 1250 UPLC System**  
Waters Acquity UPLC BEH130 C18 1.7 µm 2.1 x 150 mm Column

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	391.2843
List	413.2662
Scan event 1	FTMS, resolution 60000 Mass Range: 150-2000 Da



### Recombinant Human Insulin



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

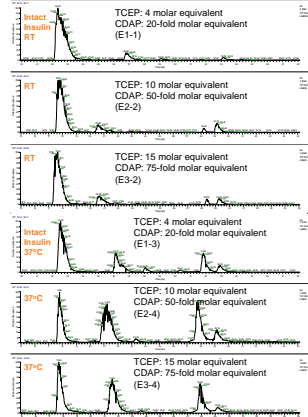
**Experimental Design:**

- Total Number of Cysteines in Protein
- [TCEP]<sub>min</sub> = 4 molar equivalent for every Cys
- [CDAP]<sub>min</sub> = 20 molar equivalent for every Cys
- [CDAP] should be minimum 5x of [TCEP]
- Both reactions performed at pH 3.

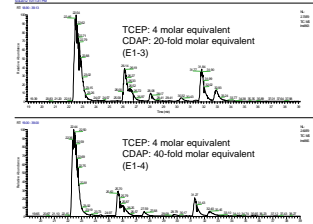
Table 1. Mass-to-charge Values of Insulin Partial Reduction and Cyanylation Products

	Mass-to-Charge Values, m/z											
	B to A	B to C	B to D	B to E	B to F	B to G	B to H	B to I	B to J	B to K	B to L	B to M
Insulin Non-Reduced H m/z	5803.6371	5803.6444	2902.6201	1335.0353	1411.8116	1111.7317	586.2622	583.2633	580.2644	577.2655	574.2666	571.2677
Controlled Reduction Products												
Two Cys - A Chain m/z	5803.617	5803.6243	2902.6001	1335.0153	1411.7916	1111.7117	586.2422	583.2433	580.2444	577.2455	574.2466	571.2477
Two Cys - B Chain m/z	5807.5663	5807.5736	2906.5459	1339.0656	1437.8219	1115.7620	590.2925	587.2936	584.2947	581.2958	578.2969	575.2980
Two Cys - A Chain m/z	2576.862	2576.8691	1189.8964	79.2534	586.0584	477.0541	367.0513	364.0524	361.0535	358.0546	355.0557	352.0568
Two Cys - B Chain m/z	1687.944	1687.9511	174.8616	114.0166	87.0634	60.0684	33.0734	30.0745	27.0756	24.0767	21.0778	18.0789
Two Cys - A Chain m/z	2580.862	2580.8691	1193.8964	79.2533	588.0573	479.0534	368.0501	365.0512	362.0523	359.0534	356.0545	353.0556
Cyanylation Products												
Two Cys - A Chain m/z	5803.617	5808.6176	2929.5933	1345.0357	1422.8120	1121.7321	587.2626	584.2637	581.2648	578.2659	575.2670	572.2681
Two Cys - B Chain m/z	5807.5667	5812.5672	2933.5675	1349.0361	1426.8124	1125.7325	591.2631	588.2642	585.2653	582.2664	579.2675	576.2686
Two Cys - A Chain m/z	2580.864	2585.8645	1197.8967	79.2535	590.0575	481.0536	369.0503	366.0514	363.0525	360.0536	357.0547	354.0558
Two Cys - B Chain m/z	1687.946	1692.9461	175.0619	114.0168	87.0636	60.0686	33.0736	30.0747	27.0758	24.0769	21.0780	18.0791

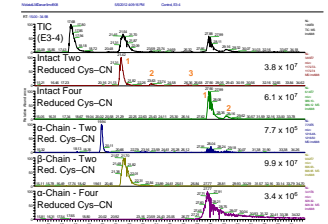
### Incubation at 37°C Temperature Produced More Abundant Reduction and Cyanylation Products



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



### Identification of Reduction and Cyanylation Products

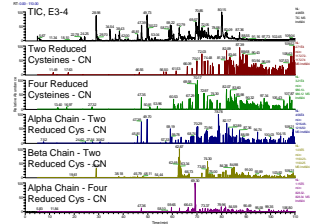


### Chemical Cleavage Products

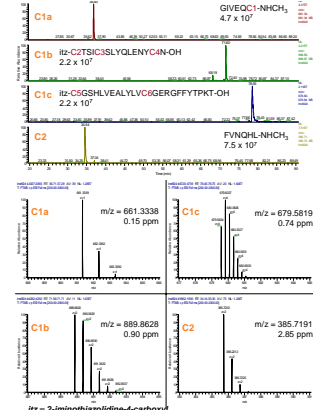
**Objective:** Complete N-terminal Cleavage of Cyanylated Cysteines with Minimal Side-Reaction Products

**Experimental Conditions Tested:**

- Cleavage using 1 M NH<sub>4</sub>OH, RT, 1 hour
- Various forms of carbamylated insulin were the dominant product of the reaction.
- Cleavage using 2 M Methyl Amine, 0-2 °C, 10 mins.
- 2 M CH<sub>3</sub>NH<sub>2</sub> Showed Efficient Conversion of Cyanylation Products to Truncated Peptides



### Cleavage Products of Isomer C



### Unique Peptides of Disulfide Scrambled Products Detected and Identified at Low Abundance

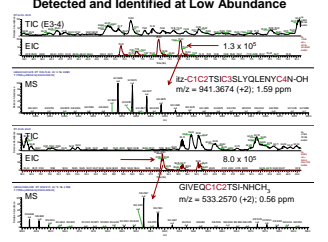


Table 2. Mass-to-charge Values of all Cleavage Products from all Partially Reduced and Cyanylated 10E and 20B Insulin

ID	Sequence	B to A	B to C	B to D	B to E	B to F	B to G	B to H	B to I	B to J	B to K	B to L	B to M	B to N	B to O	B to P	B to Q	B to R	B to S	B to T	B to U	B to V	B to W	B to X	B to Y	B to Z
One Disulfide Linkage Reduced																										
Insulin	A1-E1	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
ADN	DNA	Itz-C1C2CS1SLYQLENYCN-OH	583.2198	584.2269	282.6171	186.1473	141.8102	112.8112																		
ADN	RNA	Itz-C1C2CS1SLYQLENYCN-OH	1273.5300	1274.5341	637.7763	408.8303	304.3019	275.1749																		
Insulin	B	Itz-C1C2CS1SLYQLENYCN-OH	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	C	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	D	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	E	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	F	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	G	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	H	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	I	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	J	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	K	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	L	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	M	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	N	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	O	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	P	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	Q	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	R	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	S	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	T	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	U	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	V	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	W	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	X	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	Y	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Insulin	Z	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Two Disulfide Linkage Reduced																										
Insulin	A1-E1	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
ADN	DNA	Itz-C1C2CS1SLYQLENYCN-OH	583.2198	584.2269	282.6171	186.1473	141.8102	112.8112																		
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Insulin	Z	GIVEQC1NHCH3	587.3173	588.3244	279.8880	186.1707	143.3886	112.4708																		
Bz = 2-aminobenzoyl; 4-Carboxy																										